GLOBAL ACADEMIC RESEARCH INSTITUTE

COLOMBO, SRI LANKA

GARI International Journal of Multidisciplinary Research

ISSN 2659-2193

Volume: 02 | Issue: 01

On 31st March 2016

http://www.research.lk

Author: Fathima Nafla Mohamed, Dr. Mathivathani Kandiah

School of Science, BMS, Sri Lanka

GARI Publisher | Nutrition | Volume: 02 | Issue: 01

Article ID: IN/GARI/04ICHM/2016/155 | Pages: 42-64 (22)

ISSN 2659-2193 | Edit: GARI Editorial Team

Received: 15.01.2016 | Publish: 31.03.2016

EFFECT OF BOILING ON THE ANTIOXIDANT ACTIVITY AND ANTI-MICROBIAL ACTIVITY OF MILK NATIVE TO SRI LANKA

[1] Fathima Nafla Mohamed ,[2] Dr. Mathivathani Kandiah

[1,2] School of Science. BMS, No. 591, Galle Road, Colombo 06, Sri Lanka

[1]mkandiah@gmail.com

ABSTRACT

Milk is a fluid rich in nutritional value possessing immunological and anti-inflammatory properties. It also contains various antioxidant compounds and antimicrobial effects. Compounds with recognized antioxidant activity include phenols, flavonoids and carotenoids. Major role of antioxidants is to scavenge free radicals and reactive oxygen species (ROS) which cause damage of cellular macromolecules such as, lipids, proteins and DNA resulting in apoptosis and tissue damage. Antimicrobial property of the milk will either inhibit the growth of microorganism or kills the pathogenic microorganism. In this research, antioxidant activity and antimicrobial property of milk before boiling and after boiling was measured for five different varieties (raw, pasteurized short expiry date, pasteurized long expiry date, sterilized, sterilized fat free) of milk of the same company (native to Sri Lanka). To assess the antioxidant ferric reducing antioxidant power (FRAP), 2,2-azino-bis 3 ehtylbenzothiazoline-6-sulphoric acid (ABTS), total phenolic content (TPC), total flavonoid content (TFC) and total antioxidant content (TAC) tests were performed. Antimicrobial property against Staphylococcus aureus (S. aureus) and Escherichia coli (E. coli) was conducted through well diffusion method. All-inconclusive milk samples after boiling indicated considerably high levels of antioxidant ($P<0.05$ for total antioxidant content), raw boiled milk indicating the highest concentration. In addition to it, boiled milk samples revealed antimicrobial effect for S. aureus and E. coli. Whereas, effect of boiling is significantly proven in S. aureus. *Key words: Phenolic content, ABTS, Anti-microbial, Antioxidant activity, Public health, Nutrition*

INTRODUCTION

Majority of the milk's constituent is water (87.2%) and the rest of the 12.8% consist of milk solids such as fat (4.0%), protein (3.4%), lactose (4.5%) and minerals (0.9%) (Pandey and Voskuil 2011). Milk is rich in physiologically functional elements such as proteins, vitamins (vitamin A and E), minerals, carotenoids, phenols and flavonoids. Elements present in the milk are known as biologically active, which are bioactive proteins, peptides, vitamins, oligosaccharide, hormones and immunological agents. These properties of milk had led to its use in the daily diet especially for the young and the old all over the world. In addition, it also contains antioxidant, antimicrobial and immunomodulatory biological properties which would thereby protect the host which consume it (Simos et al. 2011).

Cells require oxygen to generate energy, in this process they release by products such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). They have both beneficial and harmful effects in the body. They have favorable effects in cellular maturation and immune function when they are present in average levels. These species can be divided into either free radical [hydroxyl ($OH^{\bullet}$), superoxide ($O_2^{\bullet}-$), nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$), peroxyl ($ROO^{\bullet}$) and lipid peroxyl ($LOO^{\bullet}$)] or non-free radicals also known as the oxidants [(H_2O_2), ozone (O_3), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), nitrous acid (HNO_2), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), lipid peroxide (LOOH)] (Pham-Huy et al. 2008).

Free radicals are unstable molecules with unpaired electron in their outer shell. Free radicals can be both exogenous and endogenous. Cellular metabolism or exogenous free radicals are caused by cigarette smoke, ozone exposure, hyperoxia, ionizing radiation and heavy metal ions. Whereas, endogenous radicals are caused immune cell activation, inflammation, mental stress, excessive exercise, ischemia, infection, cancer and aging. These radicals become stable by contributing or receiving an electron from species such as reductants or antioxidants. Antioxidants act as free radical scavengers neutralizing them thereby stopping and reconstructing the damage caused by them. Unbalance in the levels of antioxidants and free radical causes a situation known as oxidative stress. This is when the levels of free radicals increase abundantly than the antioxidants. This is a process which changes or damages the cellular macromolecules such as proteins, DNA, RNA, lipids and lipoproteins. This is one of the main cause of chronic and degenerative disorders such as, cancer, autoimmune condition, aging, cataract, cardiovascular and neurodegenerative diseases (figure 1) (Parkinson's and Alzheimer's) (Lobo et al. 2010)

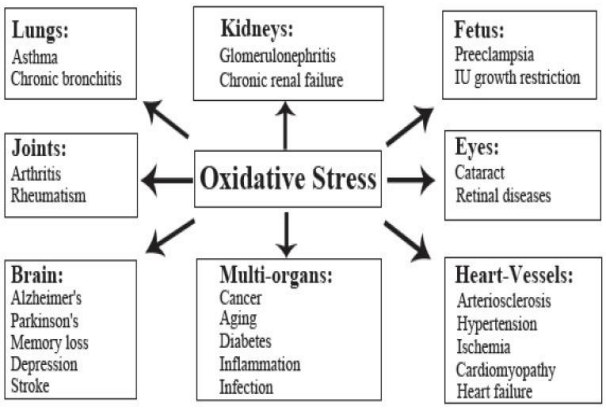

Figure 1. Diseases caused due to oxidative stress in various organs (Pham-Huy et al. 2008).

To demolish the buildup of free radicals and to protect them against oxidative stress body involves in the production of antioxidants, antioxidants that are produced inside the body is known as endogenous antioxidants. This is produced in small quantity and is not sufficient to perform the task of free radicle clearance therefore, antioxidants are taken from external sources such as supplements and foods which are known as exogenous antioxidants. Endogenous antioxidants are classified into enzymatic and non-enzymatic antioxidants. In the process of neutralizing the free radicles antioxidants get oxidized therefore, they should be restored continuously. This is mainly done by the nutritional antioxidants that we take in our diets (Birben et al. 2012).

Milk exhibit both lipophilic and hydrophilic nature as they are emulsion of lipid droplet spread in aqueous phase. Antioxidants that are derived from plant product are hydrophilic and they are functionally active against oxidative stress in the hydrophilic environment of the cell. Whereas, when considering the natural product milk from mammals, this consist of both hydrophilic and lyophilic antioxidants therefore they can counterbalance oxidative stress in both the environments of the cell overall enhancing the effect. Antioxidants present in the milk fat are conjugated linoleic acid (CLA), vitamins (A and E), coenzyme Q_{10}, β-carotene, vitamin D_3, phospholipids, ether lipids and 13-methyl-tetradecanoic acid. Enzymatic antioxidants in milk include SOD, catalase, glutathione peroxidase (GSHPx), lactoperoxidase (LPx) and catalase (CAT). Non-enzymatic antioxidants include lactoferrin, vitamin C, vitamin E and carotenoids. Though the lipophilic and hydrophilic antioxidants are present in different they form a network by connecting with each other to provide a synergetic effect (Grazyna et al. 2017). Milk also contains proteins such as casein and whey proteins. Casein exhibit antioxidant activity by inhibiting lipid peroxidation, they mostly exist in micelles. Whey proteins contain serum albumin and amino acids including tyrosine and cysteine which exhibit antioxidative property by chelation of transition metals and free radicle scavenging activity respectively (Tong et al. 2000).

Some of the milk constituents displaying antioxidant property also reveal antimicrobial properties. Compounds such as lactoferrin, LPx, lipids (triglycerides), lysozyme, N-acetyl-β-D-glucosaminidase and Xanthine oxidase (XO) present antimicrobial activity. They exist in different concentrations in distinct species. LPx is found in higher titer in cow milk than lactoferrin and lysozyme. Whereas, human breast has larger concentration of lactoferrin and lysozyme and small amount of LPx (O'Flaherty *et al*., 2005, Losnedahl et al. 1996). In addition, research work also proves that peptides derived from hydrolysis of proteins in milk through gastrointestinal digestion, microbial fermentation and enzymatic activity present antimicrobial activity against numerous gram positive and gram negative bacteria (Mohanty et al. 2015).

XO is an enzyme found in milk, has antimicrobial property on its own. Recent study reveals that in anaerobic conditions this catalyzes the reaction which produce nitric oxide. Which in aerobic conditions reacts with superoxide giving rise to peroxynitrite. This is known to possess a strong bactericidal effect (Hancock et al. 2002). Lactoferrin (LF) has both bactericidal effect and bacteriostatic effect.

This is achieved by its property to bind to iron. Bacteria require iron for growth, iron is not available for them to survive when it's in a complex with LF. Other than that, it also can bind directly to the microbe's cell wall and membrane affecting their metabolism and stability which will eventually collapse them. Further, it also capable to trigger immune response against the pathogen. Lactoperoxidase is known for its properties such as bactericidal, bacteriostatic, antiviral, tumoricidal and defend us from peroxidation caused by hydrogen peroxide and SCN^- (Hoojidonk et al. 2000). Moreover, Gutiérrez-Larraínzar et al. 2012 and Cushnie and Lamb, 2005 proves that phenolic and flavonoid constituents present in the food samples also possess antimicrobial activity. Equol is a poly phenol present in the milk which is generated by the gut microbial action to the plant constituents, display a great role in free radicle scavenging.

Both production and consumption of milk has increased in Sri Lanka over the years (figure 2). Increasing the consumption rate from 45.16 L/year in 2014 to 48.56 L/year in 2015. And elders (79 %) more than the children (56.5 %) like to consume milk. In which more than half of the population prefer milk powders as they are cheaper and yield high quantity (Livestock Statistical Bulletin, 2015; Thampoe, 2010).

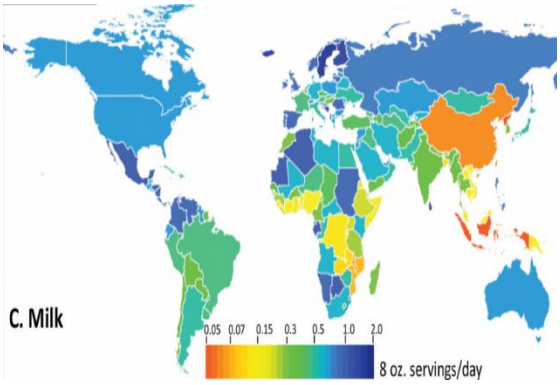

Figure 2. Statistics of milk drinking population. Sri Lankan adults drink around 10 ounces per day (Ferdman, 2015).

Mainly raw milk undergoes two types of processing pasteurization (figure 3) and sterilization (figure 4). Other than that ultra-heat treatment (UHT) (138°C for 2–4 seconds) is performed which increases the shelf life. Highland brand uses the conditions such as, Pasteurization - 72°C, 15-20 seconds, UHT - 140°C, 5 seconds, Sterilization - 118°C, 20 minutes

Temperature	Time
63°C (145°F)	30 min
72°C (161°F)	15 s
89°C (191°F)	1.0 s
90°C (194°F)	0.5 s
94°C (201°F)	0.1 s
96°C (204°F)	0.05 s
100°C (212°F)	0.01 s

[a]For milks with high fat (≥10%) or high solids (≥18%) content, the specified temperature is increased by 3°C.

Figure 3. Pasteurization is heat treatment of the milk. Any of the combination listed can be performed (Lucey, 2015).

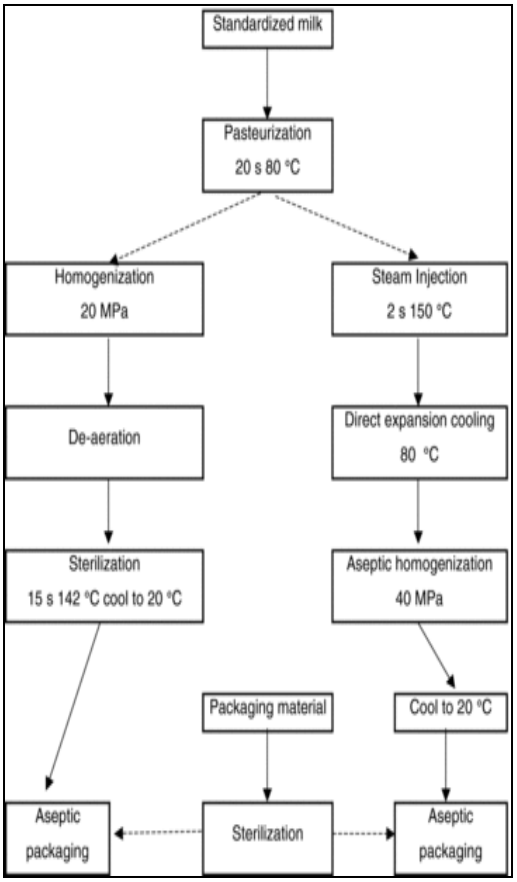

Figure 4. sterilization process with aseptic packaging (Chavan et al. 2011)

As most of the people prefer to drink milk after boiling whether its raw, pasteurized or sterilized as it could be contaminated with harmful disease causing pathogens in the farm or post pasteurization period. Boiling kills microorganisms present and make them healthier to be used (Tremonte et al. 2014; Leedom, 2006). It also affects the nutritional value of the milk by modifying the functional properties of bio active proteins through the process of denaturation and agglutination. milk consist of many proteins in which whey proteins and casein (80%) are present abundantly than the others. Casein is a heat stable protein whereas, whey protein is a heat liable protein. In the process of boiling proteins changes their shapes in order to express their thiol groups which behaves as a hydrogen donor (Bajwa, 2013 and Aloglu, 2012; Shukla). Further expression of sulfhydryl's group also increased the antioxidant activity of the boiled milk. Over all boiling does not cause much changes in the nutritional values and digestibility properties of the in milk (Tong et al. 2000).

The aim of this project is to determine the antioxidant and antimicrobial effect on different cow milk samples before boiling and after boiling. Public can also made aware of the best type of milk to consume there can decrease in the disease conditions.

MATERIALS

Reagents

2, 2'-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) ($C_{18}H_{18}N_4O_6S_4$), acetic acid ($C_2H_4O_2$), aluminum chloride ($AlCl_3$),ammonium molybdate ($[NH_4]_6Mo_7O_{24}.4H_2O$), ammonium persulphate $(NH_4)_2S_2O_8$), ascorbic acid ($C_6H_8O_6$), barium chloride ($BaCl_2$), sulphuric acid (H_2SO_4), Folin-Ciocalteu phenol reagent, gallic acid ($C_7H_6O_5$), methanol (CH_3OH), quercetin ($C_{15}H_{10}O_7.2H_2O$), sodium carbonate (Na_2CO_3), ethanol (C_2H_5OH), hydrochloric acid (HCl), sodium carbonate (Na_2CO_3), sodium acetate ($C2H_3NaO_2$), sodium sulphate (Na_2SO_4), , nutrient agar, DMSO and Muller Hinton agar

Instrumentation

Analytical weighing scale, fume hood (BIOBASE FH1000), hot air oven (meditry DHA-9053A), micropipettes, spectrophotometer (JENWAY 6305),

water bath (GEMMYCO YCW-010E), autoclave, incubator and weighing Scale

Glass ware

Beakers (50 mL, 100 mL and 250 mL), conical flasks (500 mL, 150 mL, 1L), cuvettes, falcon tubes (50 mL and 15 mL), filter funnels, filter papers, spatulas, test tubes, volumetric flasks (100 mL), Petri plates, and watch glasses.

Sample collection

All the cow milk samples except the raw milk was collected from the local supermarket around Maradhana Colombo – 09 (Highland brand). Raw milk sample was collected from the highland milk factory situated in Nawala road Colombo.

Sample preparation

The milk samples of 10 g each was boiled in a container by using a Bunsen burner. The sample extraction was done using the methodology described by Alyaqoubi *et al.*, 2014, with small modifications. The solvent for the extraction was prepared by mixing 1M HCl and 95% ethanol in 15:85 (v/v ratio). 4 g of milk samples (before boiling and after boiling for each sample) were added to 40 mL of solvent mixture each in a 50 mL falcon tubes. The tubes were then subjected to shaking using a roller shaker for one hour at room temperature. Thereafter the samples were incubated at 5 ºC for half an hour. Followed by, centrifuged at 3800 rpm for 25 minutes. The supernatant was stored in 2 ºC in dark. The samples were named according to the code listed in table 1

Table 1. Different types of sample and the symbol used.

Name of the sample	Symbol used
Highland pasteurized short expiry	HP(SE)
Highland pasteurized boiled short expiry	HPB(SE)
Highland raw milk	HR
Highland raw boiled	HRB
Highland pasteurized long expiry	HP(LE)
Highland pasteurized boiled long expiry	HPB(LE)
Highland sterilized	HS
Highland sterilized boiled	HSB
Highland sterilized fat free	HS(FF)
Highland sterilized boiled fat free	HSB(FF)

DETERMINATION OF ANTIOXIDANT ACTIVITY

Total phenolic content (TPC)

The methodology was adopted from Lu *et al.*, 2011 with slight modifications. 1.5 mL of 10-fold diluted Folin-Ciocalteu reagent was mixed with 0.2 mL of milk extract and incubated for 10 minutes at room temperature in dark. Then, 1.5 mL of 2% sodium carbonate (w/v) solution was added to the mixture and incubated for 45 minutes in dark. The absorbance was measured at

765 nm the UV- visible spectrophotometer with water used as the blank. The absorbance was measured in triplicates to obtain mean absorbance. Calibration was then achieved with aqueous gallic acid solution. The TPC values were expresses as gallic acid equivalents (GAE) based on the calibration curve (calibration curve with concentration between 5-40 g/dm^3 in figure 20 under appendix III).

Total flavonoid content (TFC)

The methodology was adopted from Akhtar *et al.*, 2015 with slight modifications. 0.5 mL of milk extract was added to a test tube containing 1.5 mL of 75% ethanol, 0.1 ml of 10% aluminum chloride, 0.1 mL of potassium acetate and 3.8 mL of water. Test tubes were then incubated for 30 minutes at room temperature. The absorbance was measured at 415 nm against the water blank. The readings were taken as triplicates to determine the mean absorbance and calibration was achieved with quercetin solution, where concentration of TPC was expressed in quercetin equivalents (QE) (calibration cure with concentration between 10-100µg/mL in figure 22 under appendix III).

Total antioxidant concentration (TAC)

TAC was determined by using the method of Kumara and Karunakaran, 2005 with small modifications. TAC reagent was prepared by mixing 0.6 M sulphuric acid with 28 mM sodium phosphate and 4 mM ammonium molybdate in a ratio of 1:1:1. 3 mL of the working solution was added to 0.3 mL of extract in test tube. The tubes were then incubated at 90 ºC for 90 minutes, absorbance was measured at 695 nm after the samples were cooled to the room temperature against water as a blank. The readings were taken as triplicates to determine the mean absorbance. Ascorbic acid was used as the standard (calibration curve with concentration between 2-14 g/dm^3 in figure 21 under appendix III) and the TAC was expressed as equivalents of ascorbic acid (AAE).

ABTS Free Radical Scavenging Assay

The methodology was developed with the aid of the methodology used by of Okoh *et al.*, 2014 with slight modifications. The ABTS solution was made by mixing 7 mM of ABTS with 2.45 mM potassium persulfate in 1:1 ratio. The solution was incubated for 12-16 hours in dark at room temperature. A 3 mL of ABTS stock solution was then diluted in 100 mL of methanol in a volumetric flask (working solution). Initial absorbance of the working solution was then measured at 734 nm (A-control). A 2 mL of working solution was added to 2 mL of extract and absorbance was measured with time (A-sample). Percentage inhibition was calculated with time.

% Inhibition = (A-control) – (A-sample)/ (A-control)

Ferric Reducing Antioxidant Power (FRAP)

Determination of FRAP was carried out according to method of Lu *et al.*, 2011. FRAP reagent was made by combining 300 mM acetate buffer pH 3.6, 10 mM TPTZ (2,4,6-tris (2-pyridyl) -5- triazine) in 40 mM HCl and 20 mM of $FeCl_3.6H_2O$ in a ratio of 10:1:1. A 600 µl of milk extract was added to 6mL of FRAP reagent, absorbance was measured at 595 nm in time intervals until the absorbance value dropped. Ascorbic acid was used as the standard (calibration curve with concentration between 2-14 g/dm^3 in figure 21 under

appendix III) and the FRAP concentrations were expressed as equivalents of ascorbic acid (AAE).

DETERMINATION OF ANTIMICROBIAL ACTIVITY

Subculture of test organism

2.8g of nutrient agar was mixed in 100 mL of water and autoclaved, this was transferred into two petri plates and allowed to solidify. The pure culture of bacteria (E. coli and S. aureus) was strike on each plate to subculture. This was incubated for 24 hours at 36 ºC in aerobic conditions.

Antimicrobial Assay (Well diffusion)

Methodology was adapted from Balouriri *et al.*, 2016 and Valgas *et al.*, 2007 with small modifications. Muller Hinton agar (MHA) plates were made (see appendix III) which were divided into 4 quadrants and labelled positive control, negative control and samples (duplicate). Bacterial inoculum (*S. aureus* and *E. coli*) was prepared to 0.5% turbidity which was compared with 0.5% McFarland (see appendix III for preparation). Bacterial inoculum of *S. aureus* was evenly spread to whole plate for 10 plates using a cotton swab. Bacterial inoculum of *E. coli* was evenly spread to another 10 plates. Thereafter, three wells of approximately 5mm in diameter were made on the negative control, and 2 sample quadrants. Sample extracts of 0.2-0.3 mL was added to the sample wells, ethanol for the negative control and Gentamicin disc on the positive quadrant. The zone of inhibition was measured after 24 hours of incubation at 36 ºC in aerobic conditions.

Statistical analysis

The experimental data were expressed as mean absorbance ± SD of three replicates of each sample, as equivalents of the respective standards that were used where appropriate. The data were statistically analyzed using a single factor ANOVA (Analysis of Variance) using Microsoft Office Excel 2016, in which the significance was considered at a 95% confidence level ($P<0.05$). The correlation coefficients and scatter plot was analyzed using the IBM SPSS statistic 21, in which the significance was considered at $P<0.01$ at 95% confidence level.

RESULTS

TAC absorbance and concentration graphs for different types of milk extracts are illustrated in figure 5 and 6.

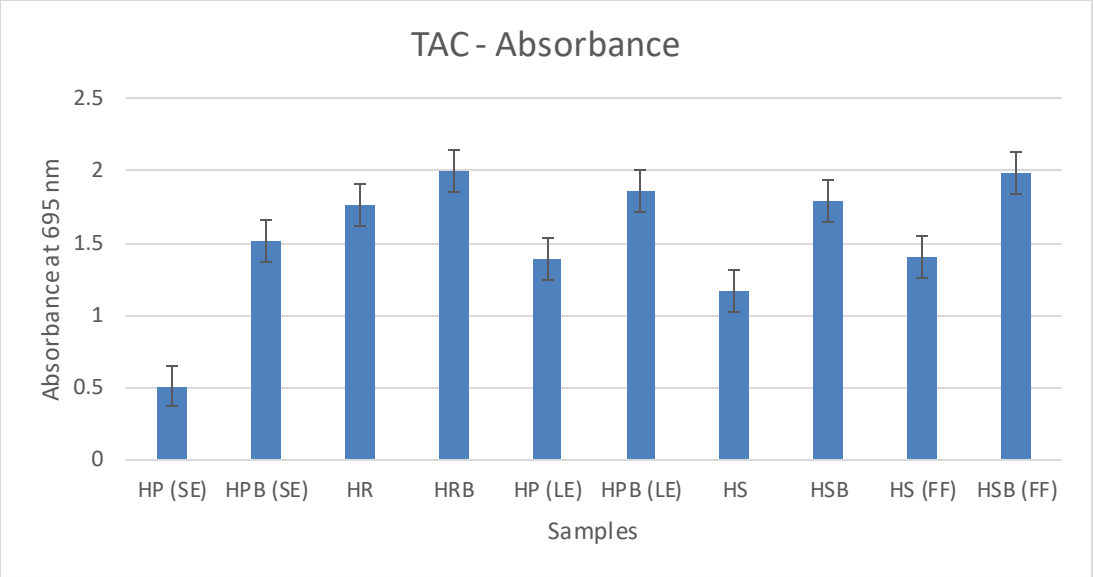

Figure 5. TAC absorbance of different milk samples.

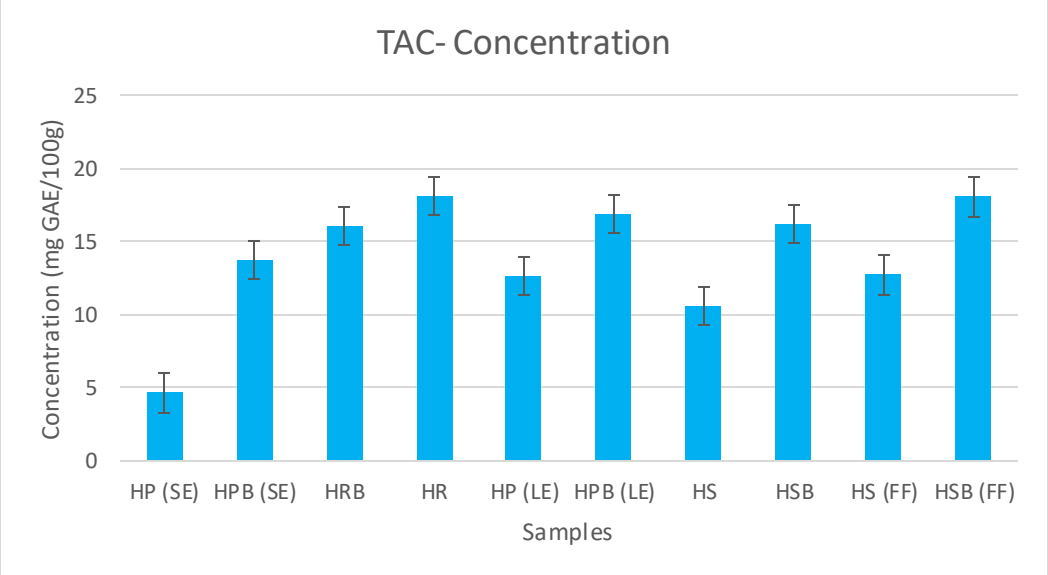

Figure 6. TAC concentration of milk samples. Boiled milk samples show higher TAC absorbance than the non-boiled sample

Table 2. ANOVA: single factor for TAC of before boiling and after boiling milk extracts

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*

Before boiling	5	56.34821	11.26964	17.76707
After boiling	5	83.1636	16.63272	3.253159

ANOVA

	Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
	Between Groups	71.90651409	1	71.90651	6.84165	0.030858	5.317655
	Within Groups	84.08090233	8	10.51011			
	Total	155.9874164	9				

Absorbance and concentration of TPC for different milk extract are illustrated in figure 7 and figure 8. Table 3 indicates the one-way ANOVA for TPC concentration for milk samples before boiling and after boiling.

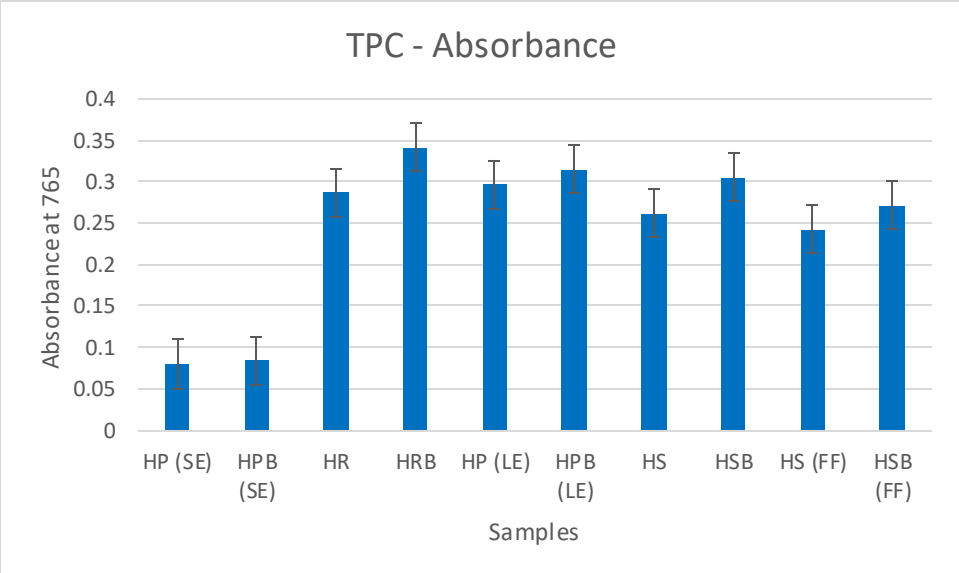

Figure 7. Absorbance of TPC of different milk samples

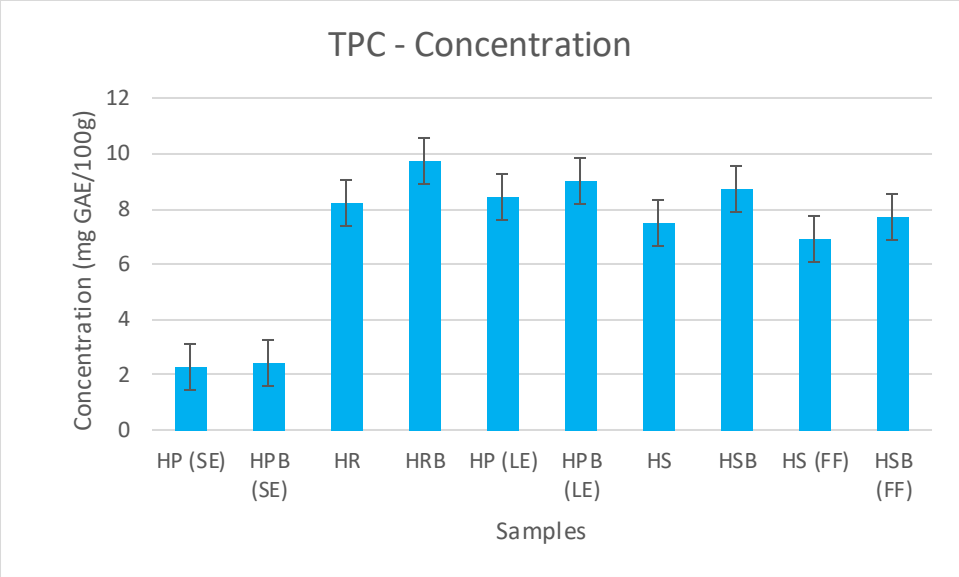

Figure 8. Concentration of TPC of the milk samples. Boiled milk samples show higher TPC concentration compared to the before boiling milk samples

Table 3. ANOVA: single factor for TPC of before boil and after boil milk extracts

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Before boiling	5	33.3619	6.672381	6.386286
After boiling	5	37.61905	7.52381	8.691701

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.812327	1	1.812327	0.240394	0.637085	5.317655
Within Groups	60.31195	8	7.538993			
Total	62.12427	9				

Both absorbance and concentrations of TFC with the different samples are plotted in figure 9 and 10. Table 4 provide the one-way ANOVA for TFC concentration for before boiling samples and after boiling

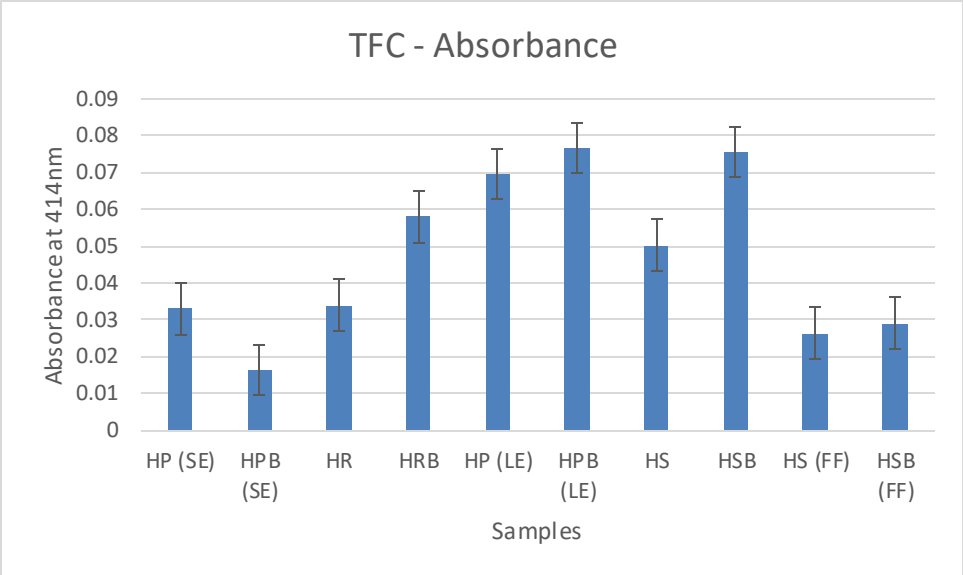

Figure 9. Absorbance of TFC of different milk extracts.

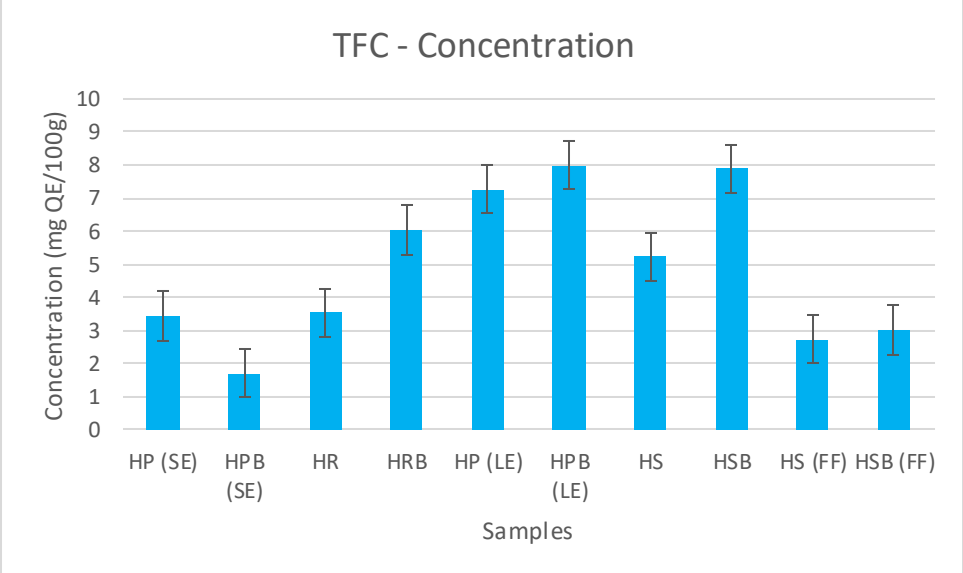

Figure 10. Concentration of TFC of different milk extracts. Overall boiled samples showing a higher concentration compared to fresh samples.

Table 4. ANOVA: single factor for TFC concentration in milk extracts before boiling and after boiling.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Before boiling	5	22.22222	4.44444444	3.317901235
After boiling	5	26.63194	5.32638889	8.143205054

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.944565	1	1.94456501	0.339332863	0.57626	5.317655
Within Groups	45.84443	8	5.73055314			
Total	47.78899	9				

Figure 11 illustrates the ABTS radical scavenging activity absorbance over a time period for all 5 samples before boiling and after boiling. Whereas, figure 12 illustrates the percentage inhibition of each sample with time. Most of the samples show a higher % inhibition

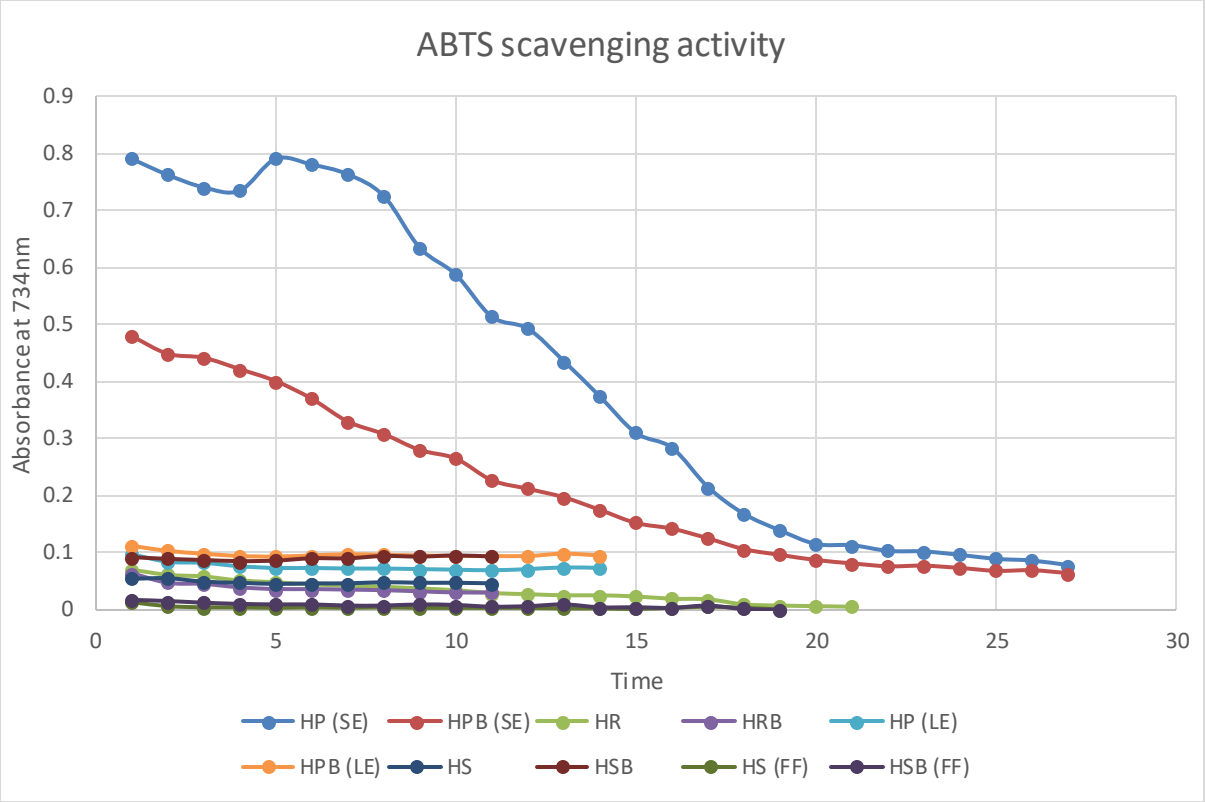

Figure 11. ABTS absorbance measured in time intervals for different samples.

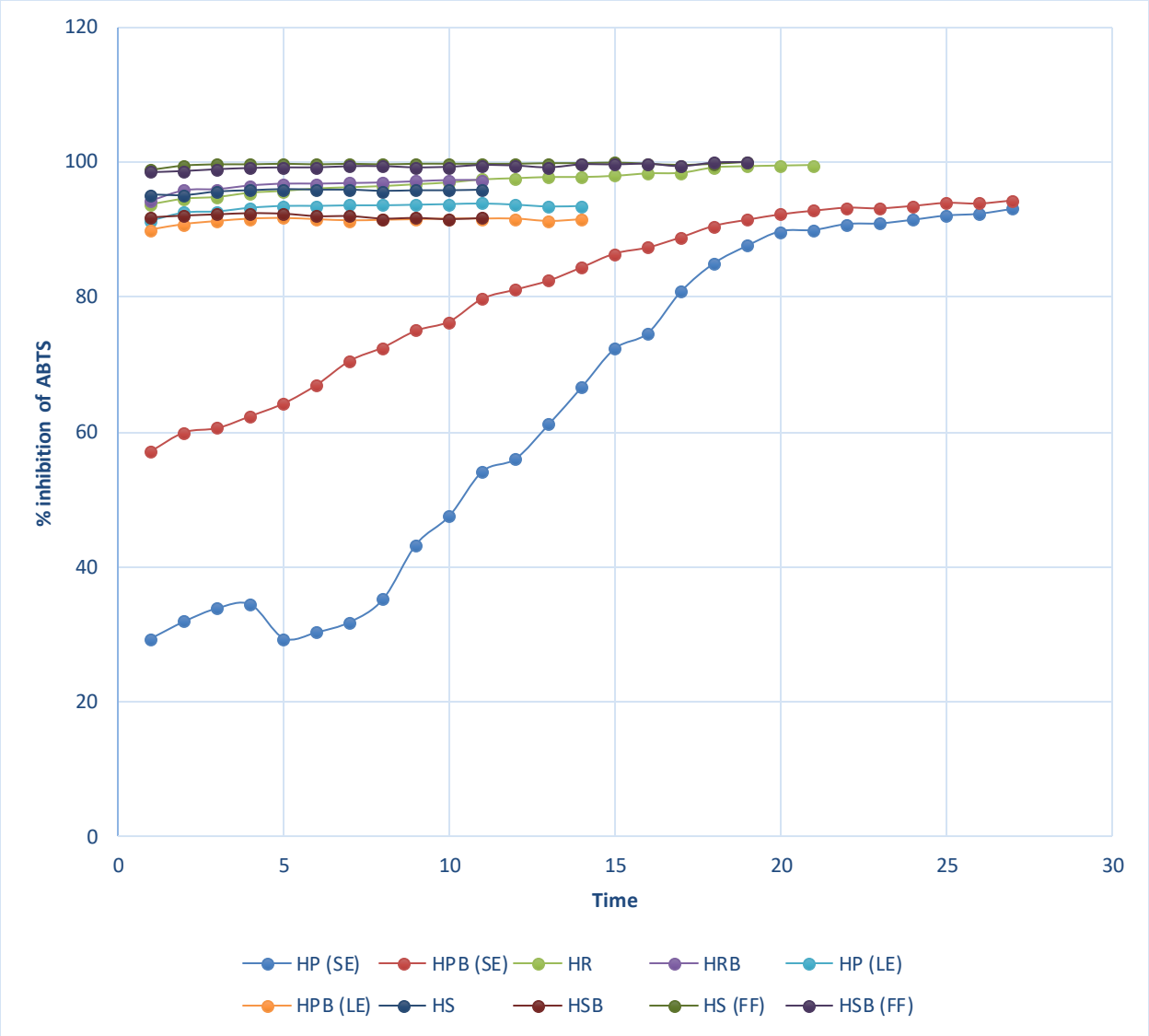

Figure 12. Percentage inhibition for different milk extracts with time. HP(SE) and HPB(SE) has a slower scavenging activity than the rest of the samples.

FRAP activity for different samples of milk are illustrated in figure 13 and 14.

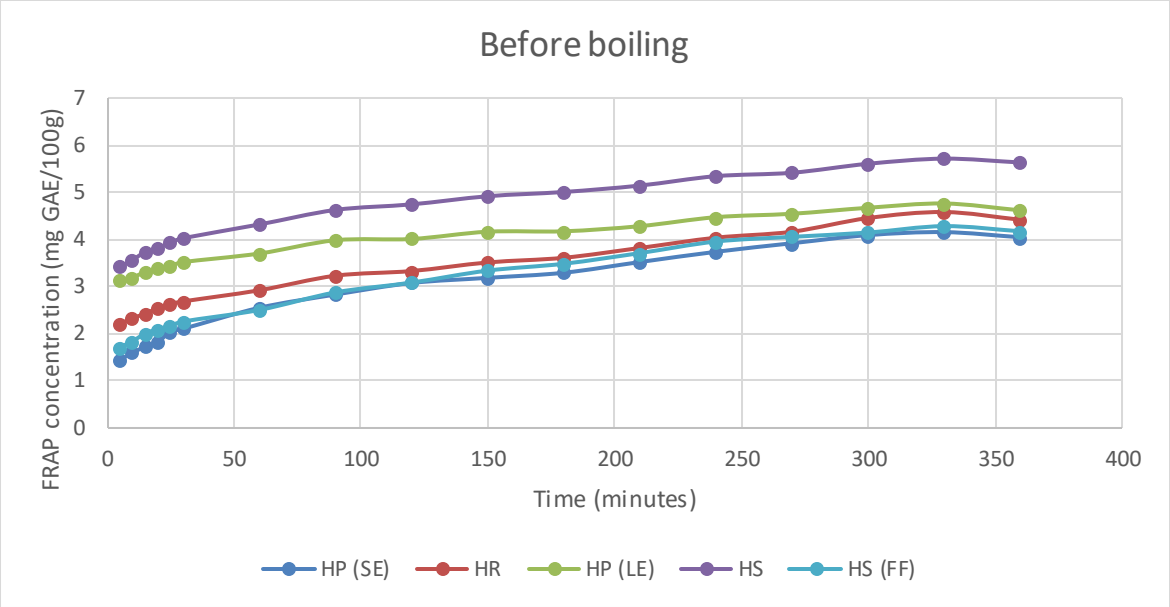

Figure 13. Ferric reducing assay concentration with time for samples of milk extract before boiling.

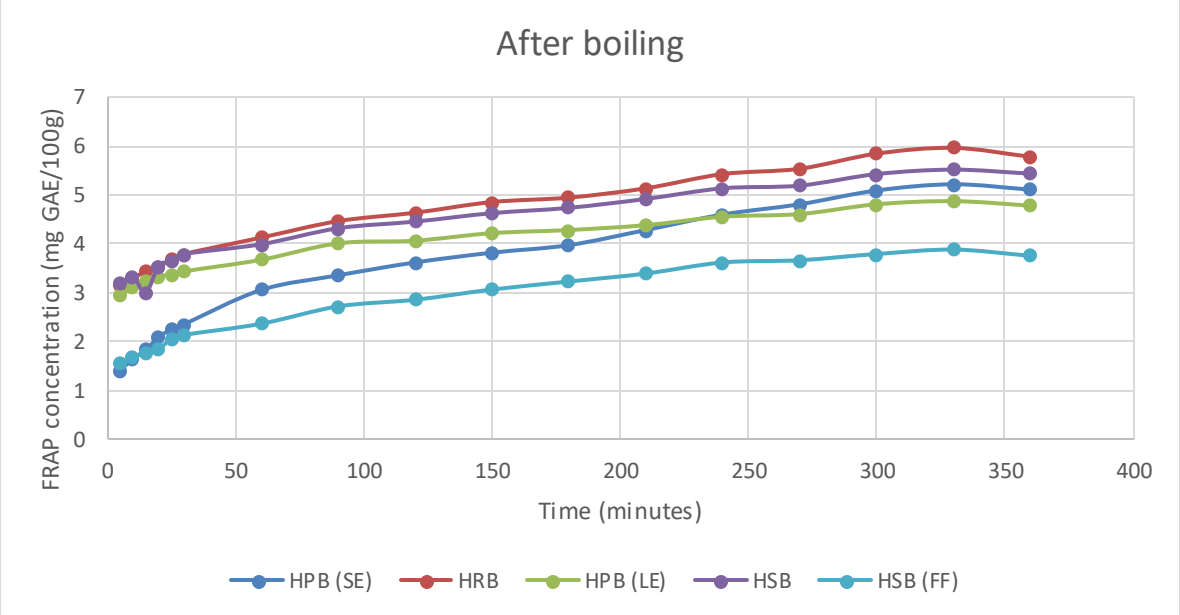

Figure 14. Ferric reducing assay concentration with time for samples of milk extract after boiling.

Correlation

Correlation between assays was determined using SPSS. Pearson correlation was indicated with a scatter plot. Table 5 and figure 15 state the correlation coefficient between TPC and TAC.

Table 5. Correlation between TAC vs TPC at 95 % of confidence interval

Correlations

		TPC	TAC
TPC	Pearson Correlation	1	.604
	Sig. (2-tailed)		.065
	N	10	10
TAC	Pearson Correlation	.604	1
	Sig. (2-tailed)	.065	
	N	10	10

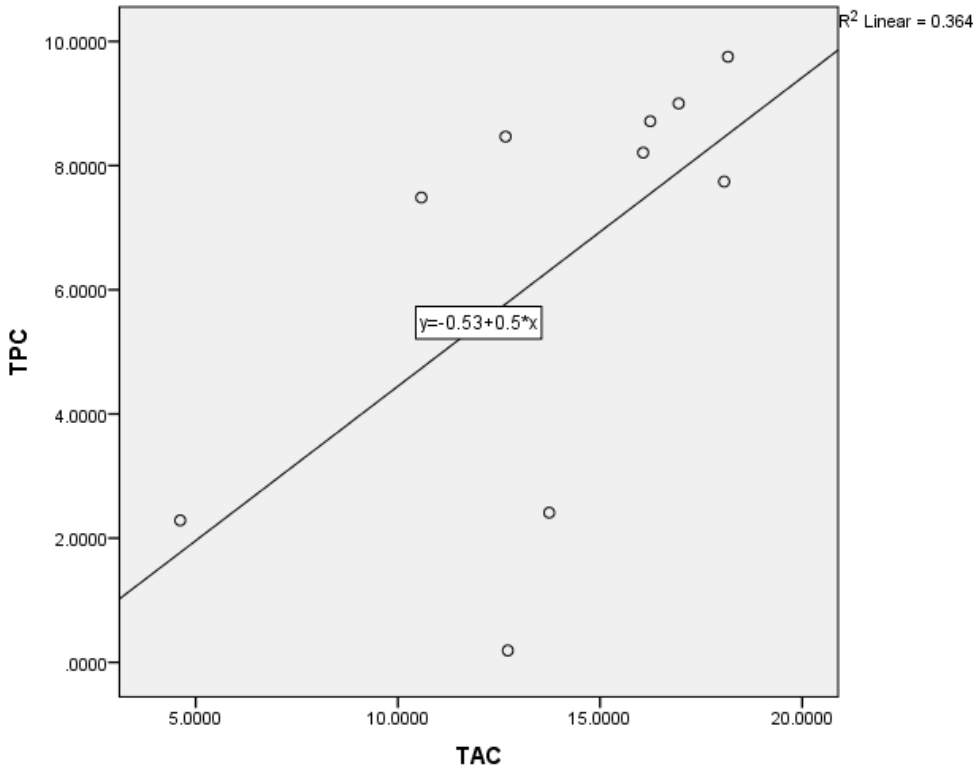

Figure 15. Linear positive moderate correlation between TPC and TAC.

Correlation between ABTS assay and TPC is illustrated in table 6 and figure 16.

Table 6. Correlation between ABTS percentage of inhibition and TPC

Correlations			
		ABTS	TPC
ABTS	Pearson Correlation	1	.814**
	Sig. (2-tailed)		.004
	N	10	10
TPC	Pearson Correlation	.814**	1
	Sig. (2-tailed)	.004	
	N	10	10
****. Correlation is significant at the 0.01 level (2-tailed).**			

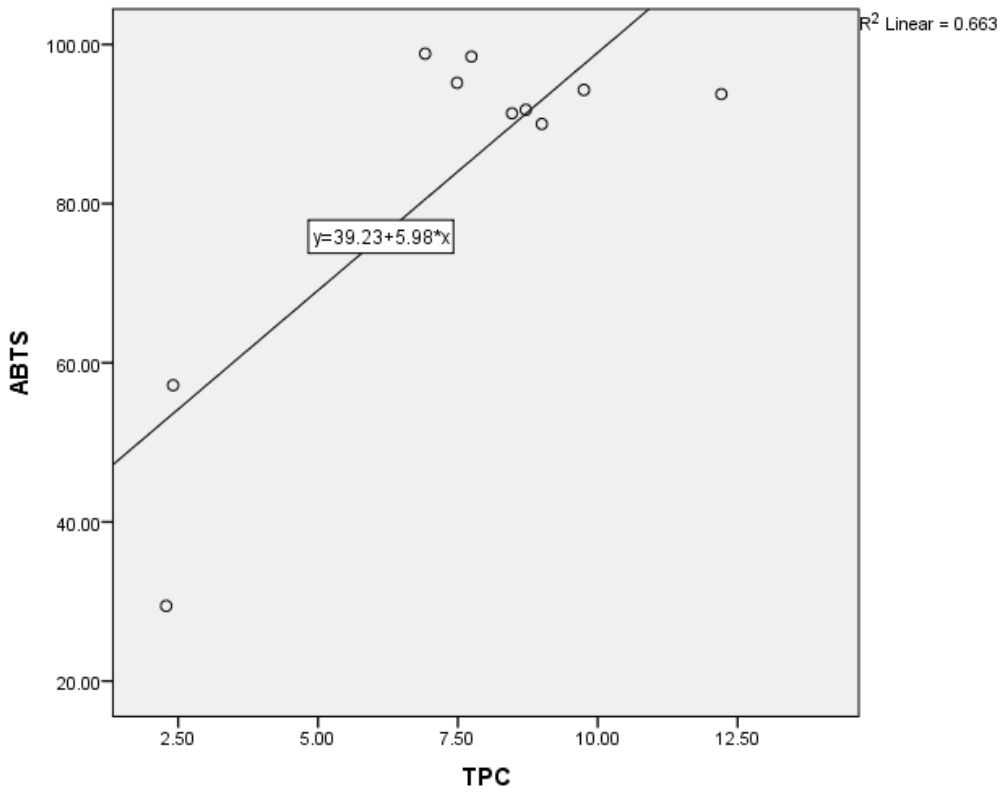

Figure 16. Scatter plot of ABTS % inhibition vs TPC

Table 7 state the Pearson correlation coefficient between FRAP assay and TPC.

Table 7. Correlation factor between FRAP and TPC assay

Correlations		
	FRAP	TPC

FRAP	Pearson Correlation	1	.588
	Sig. (2-tailed)		.074
	N	10	10
TPC	Pearson Correlation	.588	1
	Sig. (2-tailed)	.074	
	N	10	10

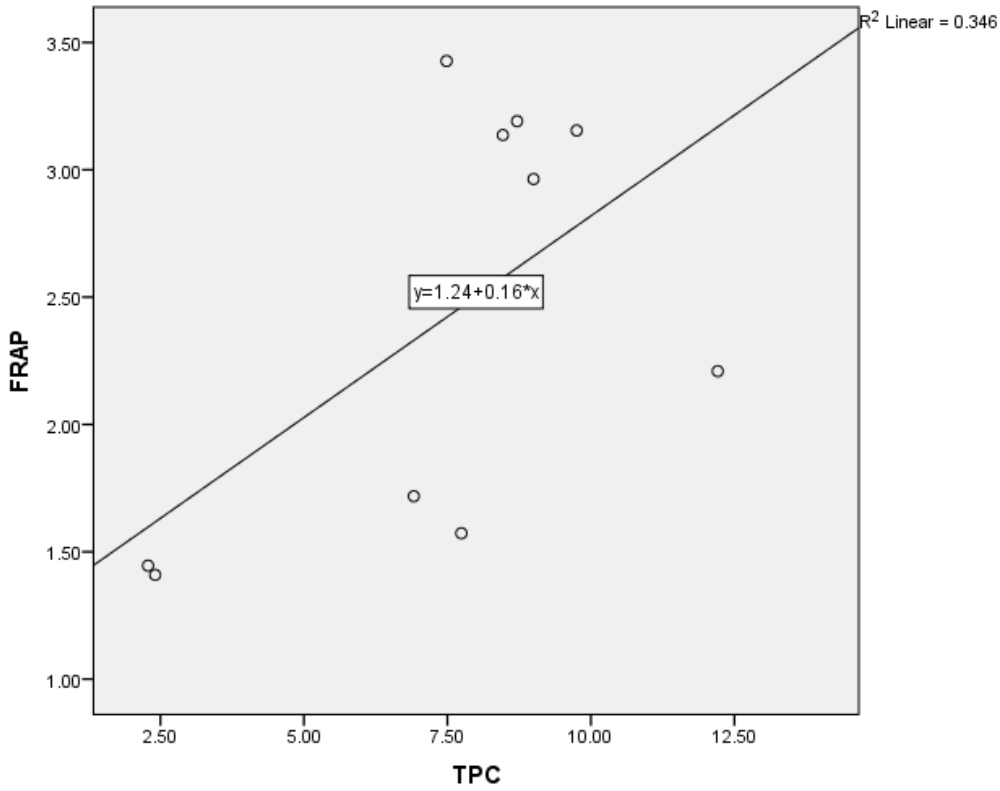

Figure 17. Scatter plot of FRAP and TPC.

Zone of inhibition for two different bacteria are measured for each of the samples are indicated in the figure 18. One way ANOVA was carried out (table 8 and 9).

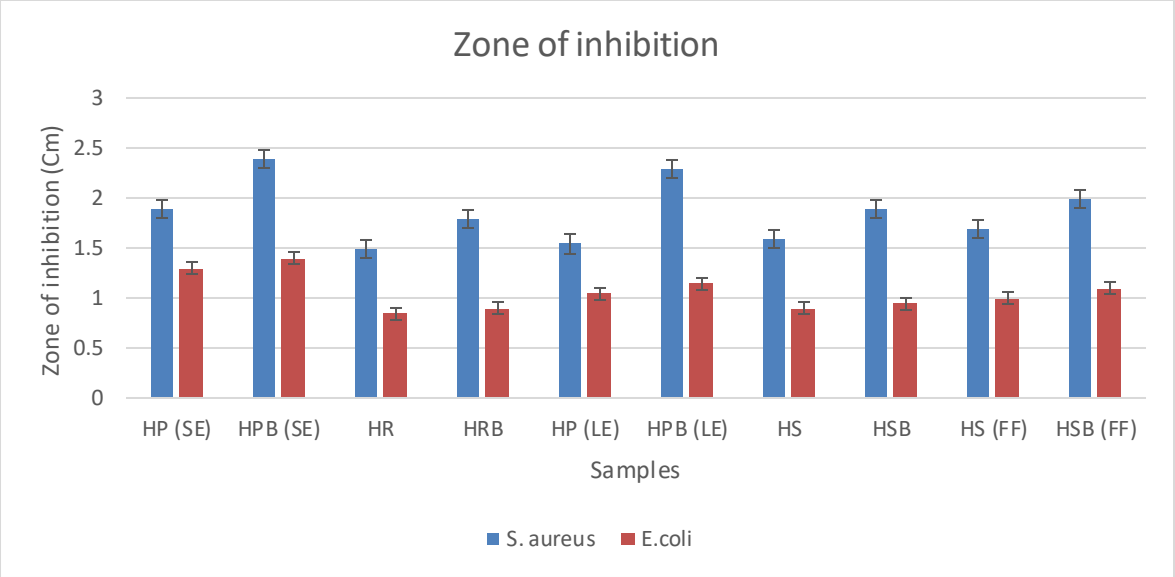

Figure 18. Bar graph of zone of inhibition for both bacteria S. aureus and E. coli with different milk extracts. S. aureus shows greater inhibition zone when compared to E. coli.

Table 8. ANOVA: single factor for zone of inhibition of S. aureus for milk extracts before boiling and after boiling.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	9.75	1.625	0.02375
Column 2	6	12.2	2.033333333	0.066666667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.500208333	1	0.500208333	11.06451613	0.007665078	4.964602744
Within Groups	0.452083333	10	0.045208333			
Total	0.952291667	11				

Table 9. ANOVA: single factor for zone of inhibition of E. coli for milk extracts before boiling and after boiling.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	9.75	1.625	0.02375
Column 2	6	12.2	2.033333333	0.066666667
SUMMARY				
Groups	*Count*	*Sum*	*Average*	*Variance*
Before boiling	5	5.1	1.02	0.03075
After boiling	5	5.5	1.1	0.03875

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.016	1	0.016	0.460432	0.516567	5.317655
Within Groups	0.278	8	0.03475			
Total	0.294	9				

DISCUSSION

Total antioxidant assay determines the total amount of antioxidant present in a sample which could be due to both hydrophilic and lipophilic antioxidants. The principle of the assay is dependent on the conversion of the molybdenum (VI) to molybdenum (V) forming a green color mixture in high temperature with the action of a reducing agent. Antioxidants such as phenols, flavonoids, casein protein, whey protein, and minerals act as reductants, they donate electrons to the oxidants and free radicals and involve in redox reactions (Ahmed *et al.*, 2015). This method is very useful as it is able to asses all the antioxidants together and lower the labor and well as the cost as each antioxidant does not needed to be measured. A main disadvantage of this assay is that it is unable to evaluate the enzymatic antioxidants such as superoxide dismutase, glutathione peroxidase and catalases (Rubio *et al.*, 2016). As depict in Figure 5 and 6 the absorption and concentration graphs represents of TAC vs the samples respectively. Milk samples after boiling show a higher TAC values than the milk samples before boiling. This is proved by the table 2 AVOVA displaying a P value = 0.030858 < 0.05 and Fcrit value lower than the F value (5.317655) which indicates that there is a significant difference between the total antioxidant concentration before boiling the milk and after boiling the milk. This could be due to the production of the Maillard reaction. Maillard reaction occur between the proteins and the sugar, in milk the reaction occur between the lactose and milk proteins. This is influenced by concentration, temperature, pH, time, water

activity and the reactants (Giroux *et al.*, 2008; Morales & Babble, 2002). Certain products of this reaction are studied to have high antioxidant activity possessing radical chain-breaking activity, metal chelating activity, active oxygen species scavenging and hydrogen peroxide destroying activity (Shukla & Bajwa, 2013). As depict highest concentration of TAC is found in HRB followed by the HPB (LE) > HSB > HSB (FF) and HPB (SE) having the lowest. This is supported by Andrei *et al.*, 2015, which indicated that the pasteurization process decreases in the antioxidant concentration by decreasing the enzymatic antioxidants such as lactoperoxidase (LPx) and glutathione peroxidase (GPx) and vitamins such as vitamin A (retinol), vitamin E (alpha-tocopherol) and vitamin C. Vitamin A is sensitive to light and heat, exposure of it to these factors lower their concentrations as they reduce the free radicals generated by heat and light. Further, they form cis isomer which dependent on heat intensities. (Noziere *et al.*, 2006; Whiten *et al.*, 2002). Whereas, vitamin C concentration is influenced by the diet and species. It is affected by light exposure, heat, air oxygen. According to the studies it is revealed that vitamin C loses is greater in sterilized milk compared to the pasteurized milk and it decrease as the self-life increase (Gliguem & Birlouez-Aragon, 2005). Milk does not contain enough vitamin E, but it is important in prevention of autoxidation of milk fats. Its concentration is mainly dependent on the diet containing tocopherol (Grega *et al.*, 2005). As illustrated in the figure 6 the concentration of the milk samples after boiling shows elevated concentrations compared to the non-boiled samples. The reason for this could be due to the exposure of the reactive groups of thiols that does not participate in forming bonds and further the presence of other antioxidants that are heat stable and Maillard products (Cosio *et al.*, 2000).

TPC uses the Follin Ciocalteu reagent which forms a blue color mixture when reacted with phenols/ polyphenols when electron transfer occurs from phenols to phosphomolybdic/ phoshotungstic acid in the low pH levels (Lallianrawna *et al.*, 2013). Figure 7 and 8 illustrates the absorbance and concentration of the TPC in the different samples. The results show that boiled samples have higher phenolic concentration compared to the before boiling samples but the difference is not statistically significant as the P> 0.05 and F value (0.240394) greater than Fcrit 5.317655 (table 3). HRB possessing the highest concentration followed by the HPB(LE) > HSB > HSB(FF) > HPB. The major type of polyphenol present in milk is equol which is formed in the gut of bovine using the daidzein (flavone) which is obtained from the cow's diet. Concentration of eqoul present is affected by many factors such as the plant food, action of microbes and the lipid concentration. It is observed in figure 8 HSB(FF) has low concentration as observed in Tsen *et al.*, 2014 compared to the rest except HPB(SE). Usually phenols stay in combined state as esters, glycosides or polymers. These phenols, flavones, flavanols are bound to beta-lactoglobulins and the beta-lactoglobulin act as the transporter and carrier of them. Therefore, in fat free sterilized milk after boiling has less phenolic components (Riihimaki et al., 2008).

Total flavonoid content of the milk samples is illustrated in figure 9 (absorbance vs sample) and figure 10 (concentration in quercetin equivalents) at 415 nm. It is determined by using aluminum chloride

which will form bonds with the carboxyl or hydroxyl groups of flavones and flavanols which give rise to a yellow color mixture (Pontis *et al.*, 2014). TFC concentration also depict the pattern observed in both phenols and total antioxidant capacity except for the sample highland pasteurized short expiry, which elicit high levels of flavonoid in milk sample before boiling compared to the HPB (SE). The error bars between the before boiling samples and after boiling samples does not overlap indicating that they are not significant except the highland pasteurize long expiry and highland sterilized fat free samples in which the error bars overlap indicating that there is no significant difference between the boil and before boil. The table 4, indicates a higher F-crit value than the F-value (0.3393) and P value = 0.5762 which is P> 0.05 and does not show any significance difference between boil and non-boil samples. Flavonoid content is mainly determined by the diet of the cow as plants have higher phenolic and flavonoid content. Recently cows are given flavonoid supplements as it decreases rancidification of milk and presence of it in milk as an antioxidant source (Cruz & Lizarazo, 2016).

ABTS scavenging activity is an assay used to estimate the antioxidant concentration present in a natural product. This assay can be used in both aqueous and liquid phase and measure both lipophilic and hydrophilic compounds of each sample can be measured. When ABTS is reacted with potassium persulfate forms a ABTS radical cation which possess a blue green color. When the natural product contains antioxidants such as phenolics, flavonoids, carotenoids they scavenge the free radicles thereby decreasing the color of the mixture. The reduction of radical cation is measured as percentage inhibition (Moon & Shibamoto, 2009). The reaction equation of the electron transfer based assay is as follows (Apak *et al.*, 2007)

ABTS/ABTS: ABTS + $(NH_4)_2S_2O_4 \rightarrow ABTS^{\bullet+}$

λ_{max} = 734 nm

$ABTS^{\bullet+} + ArOH \rightarrow ABTS + ArO^{\bullet} + H^{+}$

In figure 11 and 12 indicates the absorbance and % inhibition of the different samples over time. Except sample Highland pasteurized (short expiry) both before boiling and after boiling the rest of the samples have higher % inhibition and rapidly increased to a steady maximum in short period of time. Sample HS(FF) before boiling and after boiling has the highest % inhibition (100 % in 2 hours and 10 minutes) out of all the sample, similar results also been observed in Chen *et al.*, 2003. The observation could be mainly due to the presence of higher amount of casein proteins (heat stable proteins) than the whey proteins (heat unstable) (Vasbinder & Kruif, 2003). Casein has amino acids such as tyrosine, tryptophan, histidine, lysine and methionine which possesses high antioxidative properties (tyrosine a strong proton donor) which inhibit lipid peroxidation. Studies also reveal that they also involve in superoxide anion (DPPH) scavenging activity. Further studies also proved that low fat milk displayed higher total antioxidant activity compared to orange and tomato juice (Chen *et al.*, 2003). Other than that, the hydrophilic antioxidative enzymes in milk also can take part in radical scavenging. Nevertheless, HRB (97.3238 %) and HS (95.8965 %) and HSB (91.7038 %) attain steady maximum state at the shortest time (40 minutes).

FRAP assay is only used to test aqueous antioxidants and uses the principle of reduction of Fe^{3+}-TPTZ complex to Fe^{2+} which generate an intense blue color through the time which is measured at 593 nm. Antioxidants act as reductants which chelate metal ions frim their oxidized state to reduced state (Bordbar *et al.*, 2013). The reaction equation of the assay is as follows (Apak *et al.*, 2007)

FRAP: $Fe(TPTZ)_2^{3+} + ArOH \rightarrow Fe(TPTZ)_2^{2+} + ArO^{\bullet} + H^+$

λ_{max} = 595 nm

As displayed in figure 13 and 14, the reducing power of all the samples increased up to 330 minutes (5 ½ hours). The highest activity was produced by the HRB (5.781818 g (AAE)/100 g) followed by HS > HSB > HPB (SE) > HPB (LE) > HP (LE) > HR > HS (FF) > HP (SE) > HSB (FF). highland sterilized milk has the highest activity (5.645455 AAE) in before boiling samples. This effect is mainly affected by the amino acid arrangements, molecular weight, hydrophobicity and amino acid composition (Zou *et al.*, 2016). Antioxidants present in milk, whey proteins (lactoferrin, serum albumin, β-lactoglobulin), casein and minerals exhibit metal reducing properties due the rich amino acids present such as increased cysteine, methionine, histidine, lysine, arginine, serine and glutamic and glutamine. Further sulfhydryl groups (cysteine groups), carboxylate group and histidine provide binding sites for iron (Jaiswal *et al.*, 2015). Heat treatment such as UHT and sterilization clump whey proteins mainly including β-lactoglobulin and β-lactalbumin. Their thiol groups form disulphide bonds with other thiol groups in proteins which make them inactive which support that the raw milk after boiling poses higher activity than the pasteurized and sterilized milk samples. Further HSB (FF) (3.754545 g (AAE)/100 g) indicates the lowest activity due to the process of sterilization and removal of fat which prevents the hydrophobic antioxidant action (Avan *et al.*, 2016; Considine *et al.*, 2006; Vasbinder & Kruif, 2003).

Table 5, indicates a Pearson correlation factor of 0.604, and figure 15 illustrates a scatter plot linear positive partial correlation between the total antioxidant capacity determined by the TAC assay and the TPC expressed in GAE. Table 7, depict the Pearson correlation factor of 0.588 with a moderate positive correlation observed in figure 17. This is supported by the literature Alyaqoubi *et al.*, 2014 which shows a strong positive correlation between FRAP and TPC and TPC and TAC (DPPH method). Table 6, illustrates R-value of 0.814 between the ABTS percentage inhibition and TPC. Figure 16 depict a linear positive correlation between the % inhibition of ABTS and TPC indicates that phenols take part in the scavenging activity. Figure 19, illustrates the correlation of other assays, in which ABTS with TAC (0.721) and TPC (0.814) shows higher correlation coefficient. Secondly, TPC-TAC, TPC-FRAP and ABTS-FRAP has moderate correlation.

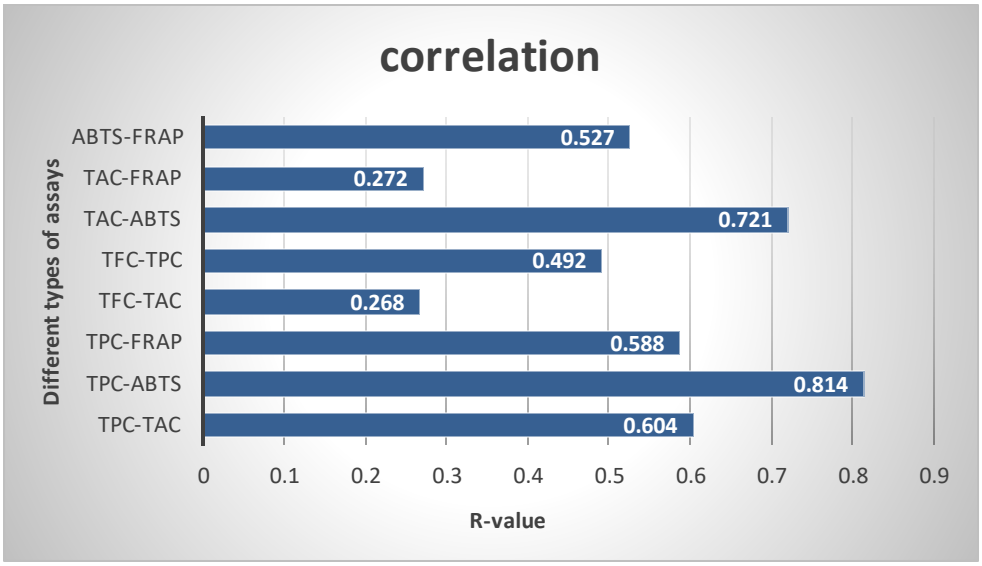

Figure 19. Correlation between different types of assays.

Antioxidants reaction against the free radicles and oxidants can be classified into two methods, hydrogen atom transfer (HAT) and electron transfer (ET). In HAT the antioxidants donate hydrogen and the scavenging activity is determined. Whereas, in ET method the electron transfer ability of the antioxidant to reduce a compound is determined. Assays such as TPC, ABTS, FRAP and DPPH are ET methods which supports the high correlation between them (Huang *et al*., 2005).

Zone of inhibition (ZOI) is measured for milk samples on the test organisms S, aureus and E. coli as depict on the figure 18, in general samples after boiling show a higher zone of inhibition than the samples before boiling. Data reveled a higher ZOI is obtained in pasteurized samples when compared to others with both *E. coli* and *S. aureus*. Samples after boiling significantly inhibited the growth of S. aureus (Table 8) proving that there is a significant difference in between the ZOI in boiled samples and samples before boiling as the Fcrit value (4.9646) is lower than the F-value (11.0645) and $P<0.05$. Milk possess a great amount of antimicrobial agents such as the antioxidants lactoferrin, lactoperoxidase, XO and other compounds such as, lysozyme, immunoglobulin, bacteriocins and oligosaccharides (Claeys *et al*., 2014). Studies reveal that these systems work better in pasteurized milk than in the raw milk and pasteurized milk retain the same amount of nutrient as the raw milk (Lucey, 2015). Studies conducted by Zagorska and Ciprovica 2012, indicate that lysosome are thermally stable and only small percentage of immunoglobulins are lost in different heating treatments. An important compound of milk is lactose, lactose involves in the Maillard reaction thus produce products which possess high antioxidative and antimicrobial properties. Despite, other antioxidants present in milk such as phenols flavonoids and milk proteins also function as antimicrobial agents (Vazquez *et al*., 2015; Claeys *et al*., 2013).

Difference between the types of milk such as raw pasteurized, sterilized and skim does not alter the nutritional value of the milk in

huge ranges. Pasteurized milk almost contains the nutritional value as the raw milk. Some of the heat liable proteins and vitamins are lost in sterilization process, retaining the heat stable proteins and vitamins. Defatted milk consists of all the hydrophilic antioxidants accompanied by enzymatic antioxidants (Zulueta *et al*., 2009). Overall greater levels of antioxidant activity and antimicrobial activity are observed in milk. samples after boiling mainly due to the melanoids formation and expression of thiol (reactive groups) and amino acid sequences which poses antioxidant properties (Claeys, *et al*., 2014). (Words: 2252)

FUTURE WORK

Using different extraction methods and solvents to extract the hydrophilic and

lipophilic antioxidants such as methanol and acetone

Testing the difference antioxidant activity of milk in different species of mammals such as goat milk, donkey milk, buffalo milk, horse and camel

To analyze the scavenging activity using 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) assay

Determining the antioxidant activity of the milks of different brands/ companies that are native to Sri Lanka.

Checking for antioxidant levels between flavored milk and milk subjected to different storing conditions

Quantitative and qualitative test for different antioxidants present in the milk

Investigations to determine other phytochemicals such as saponins, tannins and nitrogen compounds in the milk

Proteolysis and bioactive peptides tends to increase antioxidant activity, therefore, checking the antioxidant activity of milk compounds after proteolysis is essential as the milk undergo gastrointestinal digestion in human gut and plays a n important role in the bioavailability of the antioxidant capacity of the milk.

Major components of protein present in the milk are whey protein and caseins, contribute as major antioxidant activity. Therefore, testing the effects of heat and pH on these constituents of the milk will illustrate a clear picture how these are effected with pasteurization, sterilization, ultra-heat treatment and in the process of producing skimmed milk.

Use of much more efficient methods for extraction such as solid phase extraction, high performance liquid chromatography.

Investigating the process of Maillard reaction with different concentration of sucrose/lactose and temperatures.

ACKNOWLEDGEMENT

I would like to express my sincere thanks to sir Nizam Razaq (director of BMS) to providing such a great opportunity to work on our desired projects. My heartfelt gratitude to my supervisor Dr. Mathi Kandiah for giving all her support and encouragement throughout the project work. Guiding us with her wise knowledge in the subject has enabled us to complete this thesis I would also like to extend my thanks to all the staff members for providing their help and support.

REFERENCE

Ahmed, D., Khan, M. M. & Saeed, R. (2015) *Comparative Analysis of Phenolics, Flavonoids, and Antioxidant and Antibacterial Potential of Methanolic, Hexanic and Aqueous Extracts from Adiantum caudatum Leaves, Antioxidants*, 4, 394-409.

Akhtar, N., Ihsan-ul-Haq, & Mizra, B. (2015) *Phytochemical analysis and comprehensive evaluation of antimicrobial and antioxidant properties of 61 medicinal plant species, Arabian Journal of Chemistry*, http://www.sciencedirect.com/science/article/pii/S1878535215000325

Aloglu, H.S. (2012) *The effect of various heat treatments on the antioxidant capacity of the milk before and after stimulated gastrointestinal digestion, Intentional Journal of Dairy Technology*, 66, 170-174.

Alyaqoubi, S., Abdullah, A. & Addai, Z. R. (2014) *Antioxidant Activity of Goat's Milk from Three Different Locations in Malaysia, API Conference Proceedings*, 1614, 198-201.

Andrei, S., Bunea, A., Pintea, A., Matei, S. & Cozma, A. M. (2015) *Comparative Study of Antioxidant Activity in Fresh and Pasteurized Goat Milk, Bulletin UASVM Veterinary Medicine*, 72, 336-340

Apak, R., Guclu, K., Demirata, B., Ozyurek, M., Celik, S. E., Bektasoglu, B., Berker, K. I. & Ozyurt, D. (2007) *Comparative Evaluation of Various Total Antioxidant Capacity Assays Applied to Phenolic Compounds with the CUPRAC Assay, Molecules*, 12, 1496-1547

Avan, A. N., Cekic, S. D., Uzunboy, S. & Apak, R. (2016) *Spectrophotometric Determination of Phenolic Antioxidants in the Presence of Thiols and Proteins, International Journal of Molecular Sciences*, 17, 1-16.

Bajwa, U. & Shukla, P. (2013) *Effect of Heat Treatments on Antioxidant Activity in Sucrose Milk Protein Model. Systems, International Journal of Computational Engineering Research*, 3, 20-24.

Balouriri, M., Sadiki, M. & Ibnsouda, S. K. (2016) *methods for in vitro evaluating antimicrobial activity: A review, Journal of Pharmaceutical Analysis, Science Direct* http://www.academia.edu/2104 3609/Methods_for_in_vitro_evaluating_antimicrobial_activity_A_review

Birben, E., Sahiner, U. M., Sackesen, C., Erzurum, S. & Kalayci, O. (2012) *Oxidative Stress and Antioxidant Defense, world Allergy Organization Journal*, 5, 9-19.

Bordbar, S., Ebrahimpour, A., Hamid, A. A., Manap, M. Y. A., Anwar, F. & Saari, N. (2013*) The Improvement of The Endogenous Antioxidant Property of Stone Fish (Actinopyga lecanora)*

Tissue Using Enzymatic Proteolysis, BioMed Research International, 2013, 1-9.

Chavan, R. S., Chavan, S. R., Khedkar, C. D. & Jana, A. H. (2011) *UHT Milk Processing and Effect of Plasmin Activity on Shelf Life: A Review, Comprehensive Reviews in Food Science and Food Safety*, 10, 251-268.

Chen, J., Lindmark-Mansson, H., Gorton, L. & Akesson, B. (2003) *Antioxidant capacity of bovine milk as assayed by spectrophotometric and amperometric methods, International Dairy Journal*, 13, 927-935.

Claeys, W. L., Verraes, C., Cardoen, S., Block, J. D., Huyghebaert, A., Raes, k., Dewettinck, K. & Herman, L. (2014) *Consumption of raw or heated milk from different species: An evaluation of the nutritional and potential health benefits, Food Control*, 42, 188-201.

Claeys, W., L., Cardoen, S., Daube, G., Block, J. D., Dewettinck, K., Dierick, K., Zutter, L. D., Huyghebaert, A., Imberechts, H., Thiange, P., Vandenplas, Y. & Herman, L. (2013) *Raw or heated cow milk consumption: Review of risks and benefits, Food Control*, 31, 251-262.

Considine, T., Patel, H. A., Anema, S. G., Singh, H. & Creamer, L. K. (2007) *Interactions of milk proteins during heat and high hydrostatic pressure treatments —A Review, Innovative Food Science and Emerging Technologies*, 8, 1-23.

Cosio, M. S., Mannino, S., & Buratti, S. (2000). *Electrochemical sensor detecting free sulfhydryl groups: Evaluation of milk heat treatment. Journal of Dairy Science*, 83, 1933–1938.

Cruz, C. A. & Lizarazo, C. C. S. (2016) *Effects of diets rich in flavonoids on the quality of bovine milk, Revista de Medicina Veterinari*, 31, 137-150.

Cushnie, T. P. T. & Lamb, A. J. (2005) *Antimicrobial activity of flavonoids, International Journal of Antimicrobial Agents*, 26, 343-356.

Ferdman, R. A. (2015) What people around the world drink when they aren't drinking water, The Washington Post, at https://www.washingtonpost.com/news/wonk/wp/2015/08/21/maps-sugary-drink-fruit-juice-and-milk-consumption-in-187-countries-around-the-world/?utm_term=.40fb6f8b20bd visited 7th May 2017

Giroux, H. J., Acteau, G., Sabik, H. & Britten, M., (2008) *Influence of dissolved gases and heat treatments on the oxidative degradation of polyunsaturated fatty acids enriched dairy beverages, Journal of Agricultural and Food Chemistry*, 56, 5710-16.

Gliguem, H. & Birlouez-Aragon, I. (2005) *Effects of Sterilization, Packaging, and Storage on Vitamin C Degradation, Protein Denaturation,*

and Glycation in Fortified Milks, Dairy Science, 88, 891-899.

Grazyna, C., Hanna, C., Adam, A. & Magdalena, B. M. (2017) *Natural antioxidants in milk and dairy products, International Journal of Dairy Technology*, 17, 1-14.

Grega, T., Sady, M., Najgebauer, D., Domagała, J., Pustkowiak, H. & Faber, B. (2005) *Factors affecting the level of conjugated linoleic acid (CLA) in milk form different cow's breeds, Biotechnology in Animal Husbandry*, 21, 241–244.

Gutiérrez-Larraínzara, M., Rúaa, J., Carob, I., de Castroa C., de Arriagaa, D., García-Armestob, M. R. & del Vallea, P. (2012) *Evaluation of antimicrobial and antioxidant activities of natural phenolic compounds against foodborne pathogens and spoilage bacteria, Food Control*, 26, 555-563.

Hancock, J. T., Salisbury, V., Ovejero-Boqlione, M. C., Cherry, R., Hoare, C., Eisenthal, R. & Harrison, R. (2002) *Antimicrobial properties of milk: Dependence on presence of xanthine oxidase and nitrite, Antimicrobial Agents and Chemotherapy*, 46, 3308-3310.

Hooijdonk, A. C. M. V., Kussendrager, K. D. & Steijns, J. M. (2000) *antimicrobial and antiviral activity of components in bovine milk and colostrum involved in non-specific defense, British Journal of Nutrition*, 84, 121-134.

Huang, D., Ou, B. & Prior, R. L. (2005) *The Chemistry behind Antioxidant Capacity Assays, Journal of Agricultural and Food Chemistry*, 53, 1841-1856.

Jaiswal, A., Bajaj, R., Mann, B. & Lata, K. (2015) *Iron (II)-chelating activity of buffalo αS-casein hydrolysed by corolase PP, alcalase and flavourzyme, Journal of Food Science and Technology*, 52, 3911-3918.

Kumaran, A. & Karunakaran, R. J. (2007) *In vitro antioxidant activities of methanol extracts of five Phyllanthus species from India, LWT-Food Science and Technology*, 40, 344-352.

Lallianrawna, S., Muthukumaran, R., Ralte, V., Gurusubramanian, G. & Kumar, N. S. (2013) *Determination of total phenolic content, total flavonoid content and total antioxidant capacity of Ageratina adenophora (Spreng.) King & H. Rob, Science Vision*, 13, 149-156.

Leedom, J. M. (2006) *Milk of nonhuman origin and infectious disease in humans, Clinical Infectious Disease, An official publication of the infectious disease society of America*, 43, 610-615.

LIVESTOCK STATISTICAL BULLETIN (2015) Department of Animal Production and Health Peradeniya Sri Lank at http://www.daph.gov.lk/web/images/content_image/publications/other_publications/stat_bulletin_2015.PDF visited 7th May 2017

Lobo, V., Patil, A., Phatak, A. & Chandra, N. (2010) *Free radicals, antioxidant and*

functional foods: impact on human health, Pharmacognosy Review, 4, 118-126.

Losnedahl, K. J., Wang, H., Aslam, M., Zou, S. & Hurley, W. L. (1996) *Antimicrobial factors in milk, ResearchGate*, 4, 1-4.

Lu, X., Wang, J., Al-Qadir, H. M., Ross, C. F., Powers, J. R., Tang, J. & Rasco, B. A. (2011) *Determination of total phenolic content and antioxidant capacity of onion (Allium cepa) and shallot (Allium oschaninii) using infrared spectroscopy, Food Chemistry*, 129, 637-644.

Luecy, J. A. (2015) *Raw milk Consumption, Nutrition Today*, 50, 189-193.

Mohantany, D. P., Mohapatra, S., Misra, S. & Sahu, P. S. (2015) *Milk derived bioactive peptide and their impact on human health – A review, Saudi Journal of Biological Science*, 23, 577-583.

Moon, J. & Shibamoto, T. (2009) *Antioxidant Assay for Plant and Food Components, journal of Agricultural and Food Chemistry*, 57, 1655-1666.

Morales, F. J. & Babbel, M. B. (2002) *Antiradical efficiency of Maillard reaction mixtures in a hydrophilic media, Journal of Agriculture and Food Chemistry*, 50, 2788-92.

Noziere, P., Graulet, B., Lucas, A., Martin, B., Grolier, P. & Doreau, M. (2006) *Carotenoids for ruminants: From forages to dairy products, Animal Feed Science and Technology*, 131, 418– 450.

O'Flaherty, S., Coffey, A., Meaney, W. J., Fitzgerald, G. F. & Ross, R. P. (2005) *Inhibition of bacteriophage K proliferation on Staphyloccocus aureus in raw bovine milk, Letters in Applied Microbiology*, 41, 274-279.

Okoh, S. O., Asekun, O. T., Familoni, O. B. & Afolayan, A. J (2014) *Antioxidant and Free Radical Scavenging Capacity of Seed and Shell Essential Oils Extracted from Abrus precatorius (L), Antioxidants*, 3, 278-287

Pandey, G. S. & Voskuil, G. C. J. (2011) *Manual on milk safety, quality and hygiene, golden Valley Agricultural Research Trust, Zambia*. 1-52.

Pham-Huy, L. A., HE, H. & Pham-Huy, C. (2008) *Free Radicals, Antioxidants in Disease and Health, International Journal of Biomedical Science*, 4, 89-96.

Pontis, J. A., Mendonca, L. A., Costa, A. D., Silva, S. J. R. D. & Flach, A. (2014) *Color, phenolic and flavonoid content, and antioxidant activity of honey from Roraima, Brazil, Food Science and Technology (Campinas)*, 34, 69-73.

Riihimaki, L.H., Vainio, M. J., Heikura, J. M. S., Valkonen, K. H., Virtaneen, V. T. & Vuorela, P. M. (2008) *Binding of Phenolic Compounds and Their Derivatives to Bovine and Reindeer β-Lactoglobulin, Journal of Food and Agricultural and Food chemistry*, 56, 7721-7729.

Rubio, C. P., Hernandez-Ruiz, J., Martinez-Subiela, S., Tvarijonaviciute, A., & Ceron, J. J. (2016) *Spectrophotometric*

assays for total antioxidant capacity (TAC) in dog serum: an update, BioMed Central Veterinary Research, 12, 166

Simos, Y., Metsios, A., Verginadis, I., D'Alessandro, A. G., Loiudice, P., Jirillo, E., Charalampidis, P., Kouimains, V., Boulaka, A., Martemucci, G. & Karkabounas, S. (2011) *Antioxidant and anti-platelet properties of milk from goat, donkey and cow: An in vitro, ex vivo and in vivo study, International Dairy Journal*, 21, 901-906.

Thampoe, M., Ganaseelan, J. & Morais, N. (2010) *Dairy market trends in Vauniya: identifying potentials and challenges in a post-conflict setting, University of Kelaniya, Sri Lanka*, 1-21

Tong, L. M., Sasaki, S., McClements, J. & Decker, E. A. (2000*) Mechanisms of the Antioxidant Activity of a High Molecular Weight Fraction of Whey, journal of Agricultural and Food Chemistry*, 48, 1473-1478.

Tremonte, P., Tipaldi, l., Succi, M., Pannella, G., Faisaca, L., Capilongo, V., Coppola, R. & Sorrentino, E. (2014) *Raw milk from vending machines: Effects of boiling, microwave treatment and refrigeration on microbiology quality, Journal of Dairy Science*, 97, 3314-3320.

Tsen, S., Siew, J., Lau, E., Roslee, F. A. B., Chan, H. & Loke, W. (2014) *Cow's milk as a dietary source of equol and phenolic antioxidants: differential distribution in the milk aqueous and lipid fractions, Dairy Science and Technology*, 94, 625-632.

Valgas, C., Souza, S. M. D. S., Smania, E. F. A. & Smania, A. J. (2007) *Screening methods to determine antibacterial activity of natural products, Brazilian journal of Microbiology*, 38, 369-380.

Vasbinder, A. J. & Kruif, C. G. D. (2003) *Casein–whey protein interactions in heated milk: the influence of pH, International Dairy Journal*, 13, 669-677.

Vazquez, C. V., Rojas, M. G. V., Ramirez, C. A., Chavez-Servin, J. L., Garcia-Gasca, T., Martinez, R. A. F., Garcia, O. P., Rosado, J. L., Lopez-Sabter, C. M., Castellote, A. I., Montemayor, H. M. A. & Carot, k. D. L. T. (2015*) Total phenolic compounds in milk from different species. Design of an extraction technique for quantification using the Folin–Ciocalteu method, Food Chemistry*, 176, 480-486

Whited, L. J., Hammond, B. H., Chapman, K. W. & Boor, K. (2002). *Vitamin A Degradation and Light-Oxidized Flavor Defects in Milk, Journal of Dairy Science*, 85, 351–354

Zagorska, J. & Ciprovica, I. (2012) *The Influence of Heat Treatment on Antimicrobial Proteins in Milk, International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering*, 6, 231-235.

Zou, T., He, T., Li, H., Tang, H. & Xia, E. (2016) *The Structure-Activity*

Relationship of the Antioxidant Peptides from Natural Proteins, Molecules, 21, 1-14. Iron (II)-chelating activity of buffalo αS-casein hydrolysed by corolase PP, alcalase and flavourzyme, Journal of Food Science and Technology, 52, 3911-3918.

Zulueta, A., Maurizi, A., Frigola, A., Esteve, M. J., Coli, R. & Burini, G. (2009) *Antioxidant capacity of cow milk, whey and deproteinized milk, international Dairy Journal*, 19, 380-385.