EFFECT OF HIGH HYDROSTATIC PRESSURE AND HEAT TREATMENT ON ANTIMICROBIAL PROTEIN STABILITY, SHELF-LIFE AND RHEOLOGICAL CHARACTERISTICS OF DONKEY MILK

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EFFECT OF HIGH HYDROSTATIC PRESSURE AND HEAT TREATMENT ON ANTIMICROBIAL PROTEIN STABILITY, SHELF-LIFE, AND RHEOLOGICAL CHARACTERISTICS OF DONKEY MILK

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ABSTRACT

EFFECT OF HIGH HYDROSTATIC PRESSURE AND HEAT TREATMENT ON ANTIMICROBIAL PROTEIN STABILITY, SHELF-LIFE, AND RHEOLOGICAL CHARACTERISTICS OF DONKEY MILK

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Donkey milk has been gaining popularity due to its beneficial properties and similarity to human milk, however, since heat treatment lead to phase seperation and nutritional losses, it is consumed raw, which may cause health risks and limit its shelf life. High hydrostatic pressure (HHP) is a non-thermal food processing technique in which microbial inactivation can be obtained with minimum effects on fresh like properties of food product. This study aimed to investigate the effects of HHP, and heat treatment on physicochemical and rheological properties, stability of proteins, microbial load, and shelf-life of donkey milk. Increasing processing pressure (200, 400, and 500 MPa) decreased microbial load, lysozyme, and lactoferrin contents significantly (p<0.05). Although HHP application temperatures of 25 °C and 35 °C had no significant effect (p>0.05) on microbial load, lysozyme, and lactoferrin loss, while at 45 °C, decreased these parameters significantly (p<0.05). By using different processing times similar values were obtained (p>0.05) in terms of microbial inactivation, lysozyme, and lactoferrin content. Even though heat treatment of donkey milk samples at 75 °C for 1 and 2 min resulted in sufficient microbial

inactivation, high lysozyme and lactoferrin inactivation were observed. Total aerobic mesophilic bacteria (TAMB) counts of HHP-treated and heat-treated milk samples stored at 4 °C exceeded 5 log CFU/mL after 21 days of storage, in contrast to untreated milk stored at 4 °C in which TAMB count increased to 5.82 log CFU/mL after 7 days of storage. pH values were decreased and titratable acidity values increased within the storage period for all samples. Significantly higher flow consistency index (K) and significantly lower flow behavior index (n) were observed in heat-treated samples comparing with untreated and HHP-treated samples (p<0.05). The results suggest that HHP is a more suitable process than heat treatment for treatment of donkey milk since lower loss of valuable antimicrobial proteins with longer shelf-life is obtained by HHP.

Keywords: Donkey Milk, High Hydrostatic Pressure (HHP), Antimicrobial Proteins, Microbial Inactivation, Shelf-life

ÖZ

YÜKSEK HİDROSTATİK BASINÇ VE ISIL İŞLEMİNİN ANTİMİKROBİYAL PROTEİN KARARLILIĞI, RAF ÖMRÜ VE REOLOJİK ÖZELLİKLERİ ÜZERİNE ETKİSİ

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Eşek sütü, faydalı özellikleri ve insan sütüne benzerliği nedeniyle popülerlik kazanmaktadır, ancak ısıl işlem faz ayrımına ve besin kayıplarına yol açtığından çiğ olarak tüketilmektedir ve bu yüzden raf ömrü sınırlıdır ve sağlık risklerine neden olabilir. Yüksek hidrostatik basınç (YHB), gıda ürününün taze benzeri özellikleri üzerinde minimum etki ile mikrobiyal inaktivasyonun elde edilebildiği, termal olmayan bir gıda işleme tekniğidir. Bu çalışma, YHB ve ısıl işlemin eşek sütünün fizikokimyasal ve reolojik özellikleri, proteinlerin stabilitesi, mikrobiyal yük ve raf ömrü üzerine etkilerini araştırmayı amaçlamıştır. Artan işleme basıncının (200, 400 ve 500 MPa) mikrobiyal yükü, lizozim ve laktoferrin içeriğini önemli ölçüde azalttığı gözlemlendi ($p<0.05$). 25 °C ve 35 °C YHB uygulama sıcaklıklarının mikrobiyal yük, lizozim ve laktoferrin kaybı üzerinde önemli bir etkisi olmamasına rağmen ($p>0.05$), 45 °C'de bu parametreleri önemli ölçüde azalttığı gözlemlenmiştir ($p<0.05$). Farklı işlem süreleri kullanılarak mikrobiyal inaktivasyon, lizozim ve laktoferrin içeriği açısından benzer değerler elde edilmiştir ($p>0.05$). Eşek sütü örneklerinin 75 °C'de 1 ve 2 dakika ısıl işlemi yeterli mikrobiyal inaktivasyon ile sonuçlanmasına rağmen, yüksek lizozim ve laktoferrin inaktivasyonu gözlemlenmiştir. 4 °C'de saklanan YHB ile muamele edilmiş ve ısıl işlem görmüş süt numunelerinin toplam aerobik mezofilik bakteri (TAMB) sayısı 21 günlük depolama süresi sonrasında 5 log CFU/mL'yi aşarken çiğ sütte TAMB sayısı 7 günlük depolama süresi sonrasında 5,82 log CFU/mL olarak tespit edilmiştir. Depolama süresince tüm numunelerde pH değerleri düşmüş ve titre edilebilir asitlik değerleri yükselmiştir. Isıl işlem uygulanmış numunelerde, işlem görmemiş ve YHB uygulanmış numunelere kıyasla önemli ölçüde daha yüksek akış tutarlılık indeksi (K) ve önemli ölçüde daha düşük akış davranış indeksi (n) gözlendi ($p<0.05$). YHB işlemi ile daha düşük değerli protein kaybı ve daha uzun raf ömrü gözlemlendiği için bu sonuçlar YHB işleminin eşek sütünün işlenmesi için ısıl işlemden daha uygun bir proses olduğunu göstermektedir.

Anahtar Kelimeler: Eşek Sütü, Yüksek Hidrostatik Basınç (YHB), Antimikrobiyal Proteinler, Mikrobiyal İnaktivasyon, Raf Ömrü

To my family

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INTRODUCTION

1.1 Milk

The primary function of the mammary glands in mammals is secreting milk as the nutrition source of the mammalian neonates. Milk and dairy products are highly important parts of the human diet due to their nutritional values and health benefits. Throughout history, humans domesticated various dairy breeds, e.g. cows, sheep, goat, and buffalo, and milk from different dairy breeds has different nutritional properties and values. In nature, mammals secrete limited amount of milk which is required for the nourishment of their offspring, thus, higher amount of milk production is targeted by humans in order to retrieve adequate amount for the nutritional needs of humans. Cows provide 81% of milk produced in the world, and buffalo milk, goat milk, and sheep milk follow cow milk with 15%, 2.2%, and 1.1% market share, respectively (FAOstat, 2019). Although cow milk is predominant in the dairy market, buffalo, sheep, goat, yak, camel, horse, and donkey milk are consumed in regions around the world due to nutritional and therapeutical properties, and environmental conditions to which cows are not suited.

1.1.1 Donkey Milk

Donkey milk is reported to be nutritionally similar to human milk (Guo et al., 2007; Malissiova et al., 2016; Vincenzetti et al., 2011). In recent years, donkey milk has gaining popularity due to its beneficial and therapeutic effects such as anti-microbial (Zhang, Zhao, Jiang, Dong, & Ren, 2008), anti-inflammatory (Jirillo & Magrone, 2014), anti-carcinogenic (Mao et al., 2009). Monti et al. (2012) reported that donkey milk is a good alternative for children (age range from 7.5 months to 121.5 months) with cow milk protein allergy and multiple food allergies.

High lysozyme and lactoferrin contents are the major characteristic of donkey milk that supports health benefitting properties (Cunsolo, Saletti, Muccilli, & Foti, 2007; Ozturkoglu-Budak, 2018; Polidori & Vincenzetti, 2010). Lysozyme is an antibacterial enzyme that can be found in bodily secretions and inhibits the growth of bacteria by catalyzing the hydrolysis of glycosidic bonds of mucopolysaccharides in the bacterial cell wall (Polidori & Vincenzetti, 2010). Lysozyme was found in donkey milk at a high concentration of 1300 – 1400 mg/L (Soto del Rio, Dalmasso, Civera, & Bottero, 2017), which is reported as 40-200 mg/L in human milk and 0.09 mg/L in cow milk (Carminati et al., 2014; Chiavari, Coloretti, Nanni, Sorrentino, & Grazia, 2005). Lactoferrin is an iron-binding beneficial protein, which can be found in mucus, tears, saliva, and milk, found in donkey milk at high concentrations, and inhibits a broad spectrum of bacteria, some yeast species, and fungi species (Sanchez, Calvo, & Brock, 1992). As a result of high concentrations of lysozyme and lactoferrin in donkey milk (Cunsolo et al., 2017; Uniacke-Lowe, Huppertz, & Fox, 2010) microbial count was reported to be lower than the cow, goat, and ewe milk, and foodborne pathogens were not detected (Ivanković, Ramljak, & Štulina, 2009; Malissiova et al., 2016).

Effects of different treatments on donkey milk were examined. Addo & Ferragut, (2015) examined the effects of ultra-high pressure homogenization and pasteurization, Ozturkoglu-Budak (2018) investigated heat treatments at different temperatures and freezing. Effect of freezing and spray drying (Polidori & Vincenzetti, 2010; Vincenzetti et al., 2018), and freeze-drying (Vincenzetti et al., 2011). Thermal treatments at high temperatures were reported to be causing irreversible damage in heat-labile proteins in donkey milk (Addo & Ferragut, 2015). Therefore, high-temperature treatment is not applicable for the preservation of functional, nutritional, rheological, and sensorial properties of donkey milk (Ozturkoglu-Budak, 2018; Vincenzetti et al., 2018). Therefore, it is important to sustain components in donkey milk that have nutritional values and functional benefits.

1.2 High Hydrostatic Pressure (HHP)

Heat temperature application is the main treatment of processed foods to kill microorganisms. Although heat treatment is effective against microorganisms, several negative effects develop, such as degradation of proteins, enzymes, and vitamins, and changes in sensorial properties. Furthermore, consumers prefer foods that have fresh-like properties with a longer shelf-life. With the demand of obtaining food products that are safer to consume and have longer shelf life without negatively affecting sensorial and nutritional quality parameters, novel food processing methods such as high hydrostatic pressure (HHP), pulsed electric field (PEF), ultrasound, cold plasma, and pulsed UV-light, has been under the scope of the food industry and researchers (Santhirasegaram et al., 2016). Different novel food processing technologies have different mechanisms that affect microbial load and physicochemical characteristics of foods, and the effects of novel techniques depend on processing parameters, microbial load, microbial flora, and physicochemical properties of food.

Among novel food processing methods, HHP is one of the foremost non-thermal food processing applications. The first usage of HHP in food science was done by Hite (1899) and showed that the shelf-life of raw bovine milk increased with pressure application of 600 MPa for 1 hour at room temperature. HHP gained popularity in the last decades by using in the processing of many food products, e.g. jams, fruit juices, meat, poultry, oysters, and salad dressings, (Chawla et al., 2011). In Figure 1.1 some of the HHP treated commercial products were given, which are guacamole, oven-roasted deli turkey, baby food, and stir-fried shrimp.

Figure 1.1 Examples of HHP treated commercial products

In the HHP process, pressure is transmitted to sample with incompressible pressure transmitting fluid and kept constant for a suitable time at desirable temperature (Koker, Okur, Ozturkoglu-Budak, & Alpas, 2020). A high hydrostatic pressure system consists of a pressure generating device, pressure intensifier, high-pressure vessel, and pressure and temperature control device (Figure 1.2). High pressure generated in the system between 200 MPa and 800 MPa is transmitted through a pressure transmitting medium to sample isostatically. The most common pressure transmitting fluid is water, and other pressure transmitting fluids are sodium benzoate solution, glycol, ethanol, silicone oil, or castor oil. According to Buzrul et al. (2007), the temperature of the water in the high-pressure vessel increases between 1.4 °C and 3.4 °C for every 100 MPa applied. The pressure generated in the system applied to the sample in the high-pressure vessel instantaneous and uniformly from all directions, which is explained by Pascal's principles of transmission of fluid pressure. According to Le Chatelier's principle, any reaction, phase transition or

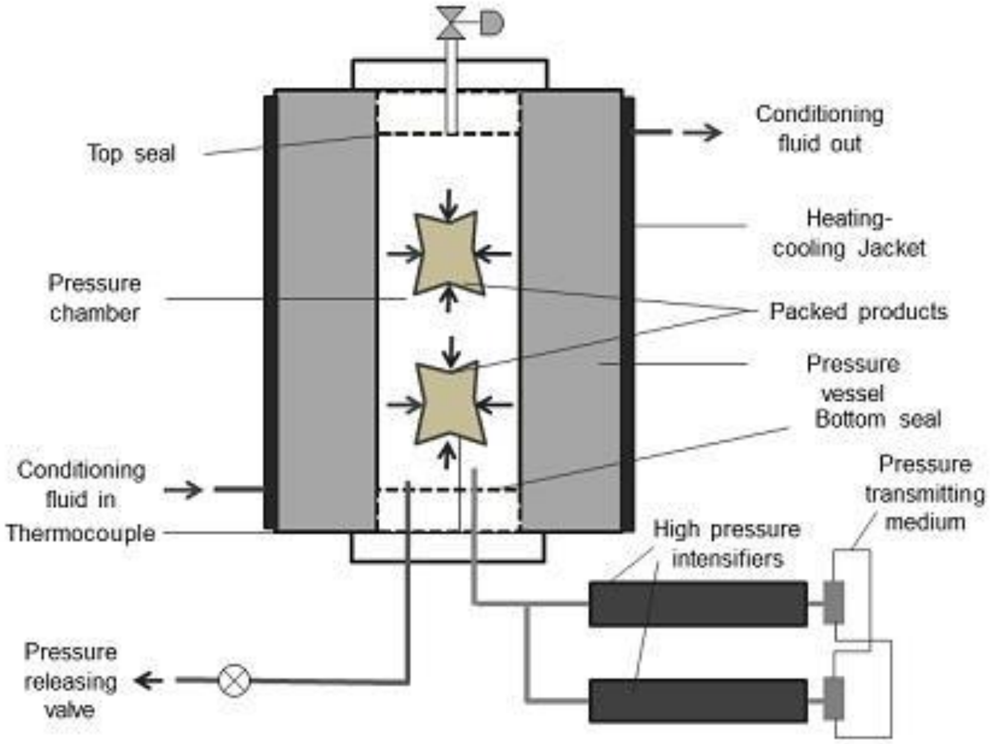

Figure 1.2 Schematic drawing of HHP (Chawla, Patil, & Singh, 2011)

conformational change occurs as a result of volume decrease caused by the high-pressure application.

1.2.1 Effect of HHP on milk and dairy products

The first application of HHP in food science was done by Hite (1988) in order to determine the effects of high pressure on the shelf-life of bovine milk. Up to this day, the effect of HHP on microorganisms present in milk and dairy products and the physicochemical properties of milk and dairy products have been studied comprehensively.

Elimination of pathogenic and spoilage microorganisms in food products is one of the most important functions of food processing, and the effects of HHP on pathogenic and spoilage microorganisms in milk were studied thoroughly by researchers. Giacometti et al. (2016) reported that 30-day stability and less than 10 CFU/mL observation of *Pseudomonas* spp., *Enterobacteriaceae,* and *Bacillus cereus*, in pasteurized donkey milk pressurized at 400 MPa for 180 s during 30-day storage at 4 °C. During 22 days of storage at 8 °C, no increase in coliform bacteria, *B. cereus*, yeast, and molds were observed in goat milk HHP treated at 600 MPa for 7 min at 15 °C (Tan et al., 2020). Bovine milk inoculated with *E. coli* subjected to HHP between 250 MPa and 400 MPa for a range of holding time from 0 min to 80 min at 3 °C and 21 °C resulted in higher microbial inactivation with higher pressure values, longer holding times, and lower temperature values (Pandey, Ramaswamy, & Idziak, 2003). 8 log reduction of *E. coli, L. monocytogenes, and S. aureus* were observed with HHP application of 345 MPa for 5 min at 50 °C, whereas 5.33 log reduction was observed on *S. aureus* (Alpas, Kalchayanand, Bozoglu, & Ray, 2000).

The effects of HHP application on milk components, particularly proteins and enzymes also investigated comprehensively. HHP application can affect the interaction between proteins and protein dynamics (Ohmae, Murakami, Gekko, & Kato, 2007). Pressure treatment affects the conformation of enzymes and protein solubility, which can lead to inactivation or activation of enzymes depending on enzyme type and processing conditions. Non-covalent bonds, e.g. hydrophobic interactions and hydrogen bonds, in molecular structures are affected by high hydrostatic pressure application (Messens, Van Camp, & Huyghebaert, 1997). High-pressure application might also strengthen hydrogen bonds according to Kunugi (1992) by decreasing the length of bonds and molecular size. Secondary, tertiary, and quaternary structures of proteins may be affected by HHP application reversible and irreversible (Yang & Powers, 2016).

Casein is one of the main whey proteins present in milk and HHP treatment at pressure values higher than 300 MPa results in interference with the micelle structure of caseins, one of the main whey proteins in milk, by affecting hydrophobic and electrostatic structure (Schrader, Buchheim, & Morr, 1997). Rocha-Pimienta et al. (2020) reported that no Immunoglobulin loss in human milk with HHP application of 400 MPa for 5 min and 593.96 MPa for 233 s. HHP treatment was observed to increase α-lactoalbumin and β-lactoglobulin loss with increasing treatment time and pH (Trujillo, Ferragut, Juan, Roig-Sagués, & Guamis, 2016). García-Risco et al. (2000) reported that HHP treatment of 400 MPa for 15 min at 20 °C and 40 °C reduced β-lactoglobulin concentration in bovine milk by 76% at 95%, respectively. They also reported that α-lactoalbumin was more pressure resisting comparing with β-lactoglobulin, and denaturation of both proteins increased with increasing processing temperature.

It is important to know the effects of HHP on milk enzymes for the determination of optimum process conditions without affecting product quality negatively and selecting indicator enzymes for process efficiency. Indicator enzyme in the heat treatment of bovine milk is alkaline phosphatase and there are several studies on the effects of HHP on alkaline phosphatase. Rademacher et al. (1998) stated that at HHP application of 400, 500, and 600 MPa at 20 °C for 8 min initiates the deactivation of phosphohexose isomerase, γ-glutamyltransferase, and alkaline phosphatase, respectively.

In terms of the effects of HHP on shelf-life, HHP application of 600 MPa for 7 min at 15 °C increased the shelf-life of goat milk to 22 days at 8 °C (Tan et al., 2020). Mussa & Ramaswamy (1997) reported that an increase in shelf-life of raw bovine milk of 12 days at 10 °C and 18 days at 5 °C were observed with pressure application of 350 MPa for 32 min. In another study, HHP-treated raw bovine milk with process conditions of 300 and 500 MPa for 30 min and 5 min, respectively, at 20 °C achieved 10 days of shelf-life at a storage temperature of 10 °C. (Rademacher, Pfeiffer, & Kessler, 1998). HHP application of 400 MPa for 30 min at 25 °C to raw bovine milk that was stored at 7 °C for 45 days reported to have lower microbial count comparing with raw milk stored at 7 °C for 15 days (García-Risco, Cortés, Carrascosa, & López-fandiño, 1998).

Some characteristics of milk were also found to be affected by HHP treatment. Renes et al. (2020) reported that storage and loss modulus of HHP treated kefir at 200 and 400 MPa for 5 min increased with frequency, and also lightness, greenness, and yellowness decreased with the application of 400 MPa. The effect of HHP on reconstituted micellar casein concentrate was examined and no rheological and multiple light scattering measurements were detected (Iturmendi et al., 2020). Gaucheron et al. (1997) reported that solubility of calcium and phosphorus increases slightly and average particle size and lightness of bovine milk decreases with combined treatment of temperature and HHP. Changes in casein micelle size were reported to decrease turbidity of milk with HHP treatment up to 220 MPa with processing time increase from 10 min to 20 min (Altuner, Alpas, Erdem, & Bozoglu, 2006). HHP treatment increases creaming properties of milk by 70% at pressures lower than 250 MPa and decreases 40% at pressures higher than 400 MPa (Huppertz, Fox, & Kelly, 2003). Nakai & Li-chan (1988) reported that with HHP treatment exposure of hydrophobic groups in bovine milk were increased which lead to changes in water binding, emulsifying, gelling, and foaming properties of milk. Decrease of syneresis and enhancement of elastic modulus and yield stress were observed in low-fat yogurt that was processed by the combination heat treatment and HHP treatment (Harte, Luedecke, Swanson, & Barbosa-Cánovas, 2010).

1.3 Objectives of the Study

Donkey milk has gained more interest in recent years due to its similarity to human milk. The high content of lysozyme and lactoferrin in donkey milk inhibits growth of microorganisms and have nutraceutical effects. Due to its low allergenicity and similarity to human milk, donkey milk is suitable for feeding of infants with cow milk protein allergy.

In the scope of this study, different pressure-temperature-time application of HHP investigated in order to obtain potential treatment method for donkey milk that preserve physical, chemical, and therapeutic properties of fresh donkey milk. Chemical properties, lysozyme concentration, lactoferrin concentration, and microbial load of the samples were examined in different HHP treatment conditions and compared with heat-treated and untreated samples. Shelf-life analysis of most suitable HHP treatment condition performed for 28 days and compared with heat-treated and untreated samples. For shelf-life analysis microbial load, pH, titratable acidity, color, and rheological properties examined.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Donkey milk samples were supplied by Koruköy Donkey Farm, Kırklareli. After milking, fresh milk samples were transported by keeping their temperature at 4 °C, and before the application of HHP treatment, heat treatment, and raw milk analyses, milk samples were stored at 4 °C.

2.2 High Hydrostatic Pressure (HHP) Treatment

High hydrostatic pressure (HHP) treatment was performed with a laboratory-scale HHP unit (Type-760.0118, SITEC, Zürich, Switzerland) with a built-in heating-cooling system (Huber Circulation Thermostat, Offenburg, Germany) given in Figure 2.1. The pressure vessel had an inner diameter and length of 153 and 24 mm, and a volume of 100 mL. Distilled water was used as a pressure transmitting medium. The temperature in the pressure vessel before and during HHP treatment was monitored by using a type K thermocouple. Samples were filled into 25 mL sterile high-density polyethylene vials (LP Italiana SPA). During HHP treatment, three different pressures were applied to donkey milk samples as 200, 400, and 500 MPa; at three different temperatures, 25, 35, and 45 °C; for three different times, 5, 10, 15 minutes. Pressurization rates were 150 MPa/min for 200 MPa, 250 MPa/min for 400 MPa, and 300 MPa/min for 500 MPa. Pressure release times were less than 20 s for each pressure value. Pressurization and pressure release times did not include in processing times.

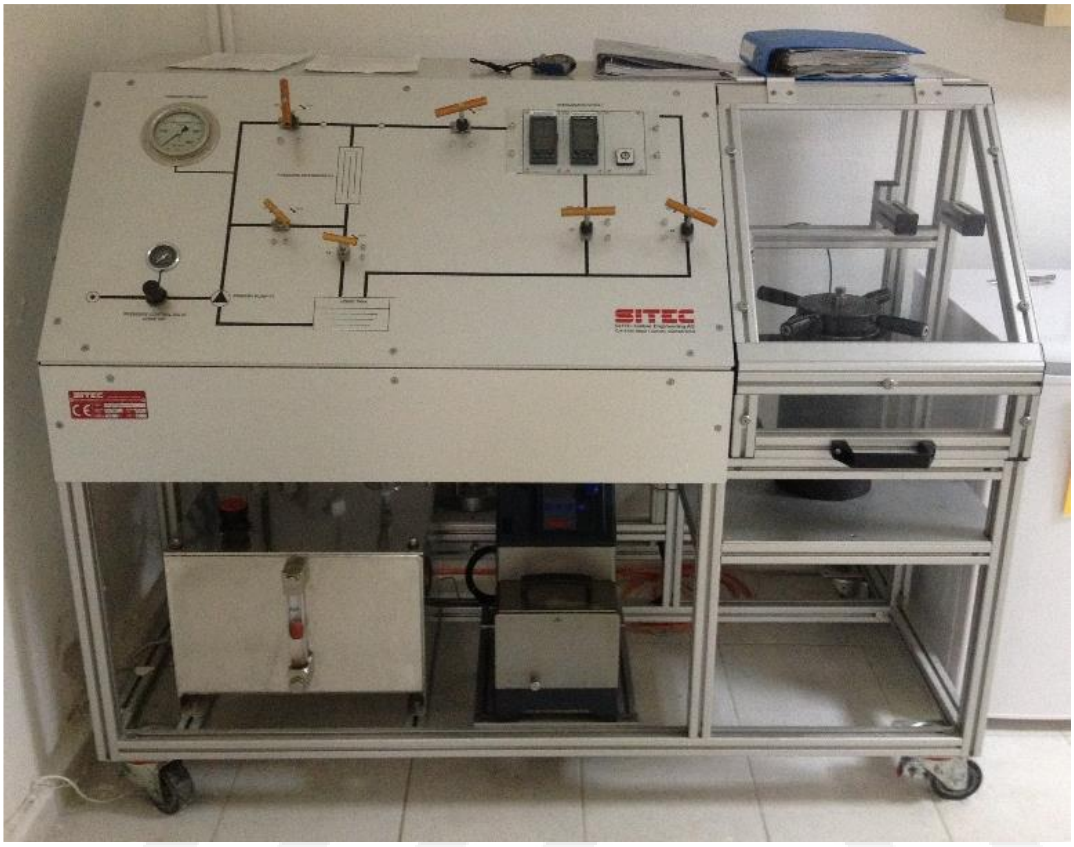

Figure 2.1 HHP equipment

2.3 Heat Treatment

Donkey milk samples were filled into 25 mL sterile high-density polyethylene vials. Heat treatments were carried out in a water bath (WiseCirCu®, Germany) at 75 °C for 1 min and 2 min. During heat treatment temperature values of donkey milk samples were monitored by a thermal probe. After heat treatment, donkey milk samples were immediately cooled in a water bath filled with cold water.

2.4 Chemical Composition Analysis

The composition of raw donkey milk and HHP treated donkey milk were analyzed by identifying the total nitrogen content, fat content, and dry matter content of the samples.

2.4.1 Total Nitrogen Content Analysis

The total nitrogen contents of samples were analyzed by the Kjeldahl method in order to obtain the total protein content of donkey milk samples. The Kjeldahl method consists of three main steps, which are digestion of samples by addition of an oxidizing agent, a boiling point raising agent and a catalyst at temperatures higher than 400 °C, distillation of the digested samples by presence of alkali and steam, and titration of distilled samples with an acid solution. For the digestion part of the Kjeldahl method, 5 ml of donkey milk samples, Kjeldahl tablets (Sigma, Germany), antifoaming agent tablets (Sigma, Germany), and 25 ml of sulfuric acid (Sigma, Germany), were put into digestion tubes. After the digestion process samples were collected for the distillation step. At the distillation part of the procedure, boric acid solution (Sigma, Germany) and 3 drops of methyl red (Sigma, Germany) were mixed in a conical flask and placed into a distillation unit. In the distillation unit, distillation takes place with sodium hydroxide solution (Sigma, Germany) and pale yellow colored solutions were obtained from distilled samples that contain ammonium borate solution. In the final part of the Kjeldahl method, the total nitrogen contents of donkey milk samples were obtained by titrating the solutions that were obtained from the distillation unit with a hydrochloric acid solution (Sigma, Germany) until the desired purple color was obtained. Total nitrogen contents of the samples were calculated and the total protein contents of the samples were calculated by using a conversion factor of 6.38 (James, 1995).

2.4.2 Fat Content Analysis

Gerber method, which is a common method for the estimation of fat contents of milk and dairy products (James, 1995), was used for the determination of fat contents of donkey milk samples. The addition of concentrated sulfuric acid (Sigma, Germany) was done in order to increase the temperature of the solution to liquefying temperature of milk fat and the addition of amyl alcohol (Sigma, Germany) was done in order to obtain a clear separation between aqueous and fat phases of milk sample. Separations of fat and aqueous phases of milk samples were done in a Gerber centrifuge. After centrifugation, the fat contents of donkey milk samples were obtained by the readings from Gerber butyrometers.

2.4.3 Dry Matter Content Analysis

Dry matter contents of donkey milk samples were determined gravimetrically at 103 °C according to the method that was described by the Turkish Standards Institution (2002). Water in donkey milk samples was removed in a two-step. Firstly, samples were put into glass Petri dishes and a part of water evaporated in the water bath in order to prevent film formation which may lead to inaccurate results. The rest of the water was evaporated in the oven at 103 °C.

2.4.4 pH and Titratable Acidity Analyses

The pH values of the samples were determined by a pH meter (Mettler-Toledo MP 220, Schwerzenbach, Switzerland) with direct insertion of the electrode in the samples. The titratable acidities of samples were performed according to the method reported by Bradley et al. (1993) and expressed as the lactic acid percentage (LA%).

2.5 Microbial Analyses

Total aerobic mesophilic bacteria (TAMB) counts of raw donkey milk, HHP-treated milk samples, and heat-treated milk samples were analyzed by using the pour plate technique. 1 mL of milk was transferred to a tube with a screw cap containing 9 mL of sterile Ringer solution (Merck, Darmstadt, Germany), vortexed, and serially diluted. Bacterial counts were enumerated on Plate Count Agar (Merck, Darmstadt, Germany) and incubated at 37 °C for 24 h. Petri dishes that contain 30 to 300 colony-forming units were counted. All bacterial analyses were conducted in duplicate.

2.6 Determination of Lysozyme and Lactoferrin Content with RP-HPLC

Determination of lysozyme and lactoferrin was performed according to the method described by (Billakanti, Fee, Lane, Kash, & Fredericks, 2010). Following the application of HHP treatment, temperatures of donkey milk samples were increased to 45 °C and the pH values of the samples were adjusted to 4.6 with 1 M HCl. This pH provided the precipitation of casein molecules and the separation of precipitated caseins was done by centrifuging the samples at 17.500 x *g* for 15 minutes (Sigma K 3-18, Sartorius AG, Germany) and the pH values of obtained supernatants were adjusted to 7 with 1N NaOH. The supernatants were filtered through 0.45 µm cellulose membrane filters, before injection into the high-performance liquid chromatography (HPLC).

The quantification of lysozyme and lactoferrin in milk samples was performed using HPLC (Agilent 1100 HPLC system, CA, USA) equipped with a UV detector at 214 nm and a C18 column (4.6 cm x 250 mm x 5 µm, 300 Å pore size). The column temperature was 45 °C. Gradient separation was conducted by the mobile phases of 0.1% (v/v) trifluoroacetic acid in deionized water and 0.1% (v/v) trifluoroacetic acid in acetonitrile with a flow rate of 0.8 mL/min, according to the method of Elgar et al. (2000). The injection volume was 50 µL and the quantitative determination of whey proteins was carried out using a calibration curve was prepared in NaCl at the concentration of 10, 25, 50, 75, 150 μg/mL. Standard solution of lysozyme from egg white and lactoferrin from human milk (Sigma-Aldrich, St. Louis, MO, USA) were used.

2.7 Rheology Analyses

Rheology analyses were performed using the Kinexus Pro+ rheometer (Malvern Panalytical, Malvern, UK). Sample temperatures of frequency sweep measurements set to 4 °C and 25 °C according to storage temperature in duplicate. Experiments were performed using 2-mm gap with stainless-steel 4° conical geometry probe. Storage (G') modulus, loss (G") modulus shear stress, and shear rate values were recorded, and flow curves were described by the power-law model.

$$\sigma = K(\dot{\gamma})^n$$

where σ is shear stress (Pa), $\dot{\gamma}$ is shear rate (s^{-1}), K is consistency index (Pa s^n), and n is flow behavior index.

2.8 Color Analysis

Lightness ($L*$), red-green ($a*$), and blue-yellow ($b*$) properties of samples were measured in the CIELAB color scale. Color measurements were done by using DATACOLOR 110® dual-beam d/8° spectrophotometer (Lawrenceville, NJ, USA) in triplicate.

2.9 Evaluation of Shelf-Life

Shelf-life analyses were done at day 0 as raw milk and immediately after HHP and heat treatment, day 3, day 7, day 14, day 21, and day 28. pH values, titratable acidities, TAMB counts, rheology analyses, and color analyses of raw milk, HHP-treated milk, and heat-treated milk were performed for samples stored at 4 °C (refrigeration temperature) and 25 °C (room temperature) for shelf-life assessment.

2.10 Statistical Analysis

Analyses of the results were done by using Minitab Statistical Software (16.1.1, State College, PA). Significant differences between samples subjected to different HHP-treatment and heat-treatment parameters were determined by three-way ANOVA using p-values less than 0.05. One-way ANOVA was used for the effects of storage time on shelf-life analyses. Tukey's multiple range test was used to identify the statistical differences between samples by the comparison of performed analyses.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Chemical Composition Analysis

Protein, fat, dry matter contents, titratable acidity, and pH values of untreated milk, HHP-treated milk, and heat-treated milk were shown in Table 3.1. With HHP applications, no significant difference was observed in terms of all chemical composition parameters between the HHP-treated samples at different pressure-temperature-time levels ($p > 0.05$).

The mean composition of total protein, fat, and dry matter contents of untreated donkey milk samples were determined as 2.15% (w/w), 1.00% (w/w), and 8.40% (w/w), respectively. Higher total protein content was observed in this study comparing with several other studies (Addo & Ferragut, 2015; Ivanković et al., 2009; Malissiova et al., 2016; Martini et al., 2018). However, Ozturkoglu-Budak (2018) reported the total protein content of donkey milk as 2.08%, similarly. Although fat content obtained in this study was in the range reported by Salimei et al. (2004) and Malissiova et al. (2016), it is higher than the values reported in other studies (Ivanković et al., 2009; Ozturkoglu-Budak, 2018). Determined dry matter content was slightly lower than the data demonstrated by Salimei et al. (2004), Addo & Ferragut (2015), and Ozturkoglu-Budak (2018). The mean pH value of untreated donkey milk was 6.95 which was lower than the pH value obtained by Addo and Ferragut (2015) and Martini et al (2018). LA% of untreated donkey milk was 0.036%. Cosentino et al. (2012) and Nayak et al. (2020) reported LA% of untreated donkey milk as 0.056% and 0.052%, respectively, which were higher than the values obtained in this study. Breeding conditions, lactation stages, and seasonal changes were reported causing variance in milk composition (Ivanković et al., 2009; Salimei et al., 2004).

Table 3.1 Chemical composition and pH of untreated, HHP-treated, and heat-treated donkey milk.

	Untreated Milk	HHP-Treated Milk	Heat-Treated Milk
Total Protein Content (w/w%)	2.15±0.15 [a]	2.17±0.11 [a]	2.10±0.13 [a]
Fat Content (w/w%)	1.00±0.01 [a]	1.00±0.01 [a]	1.00±0.01 [a]
Dry Matter Content (w/w%)	8.40±0.41 [a]	8.46±0.39 [a]	8.37±0.49 [a]
pH	7.00±0.09 [a]	7.00±0.13 [a]	7.21±0.18 [a]
Titratable Acidity (LA%)	0.036±0.002 [a]	0.036±0.004 [a]	0.027±0.002 [a]

Results expressed as the mean ± standard deviation. Lowercase letters in the same row indicate significant differences ($P<0.05$) 400 MPa-25 °C-5 min and 75 °C-2 min were given as HHP-treated milk and heat-treated milk, respectively, because there were no significant differences ($p>0.05$) within groups.

3.2 Microbiological Analysis

The effects of HHP treatment and heat treatment on the microbial population of donkey milk were shown in Figure 3.1. The initial total bacteria count of untreated donkey milk was found as 4.04 log CFU/ml. Low microbial counts found in this study are in accordance with the data reported in the literature (Addo & Ferragut, 2015; Ivanković et al., 2009; Zhang et al., 2008). The initial microbial count of donkey milk was found to be less than 5 log CFU/ml (Chambers, 2002) due to antimicrobial proteins present in donkey milk (Zhang et al., 2008).

HHP treatments of 200, 400, and 500 MPa at all temperature and time values caused a reduction in TAMB count as 0.85, 1.58, and 4.04 log CFU/ml, respectively. Heat treatment of 75 °C for 1 and 2 min resulted in 0.72 and 1.34 log reduction of TAMB. 3 log reduction of initial microbial load reported in heat-treated donkey milk at 63 °C for 30 min in the literature (Giacometti et al., 2016). Charfi et al. (2019) reported 1 log CFU/mL and 2 log reduction of total bacterial count in donkey milk samples treated at 68 °C for 2.5 and 75 °C for 10 min, respectively.

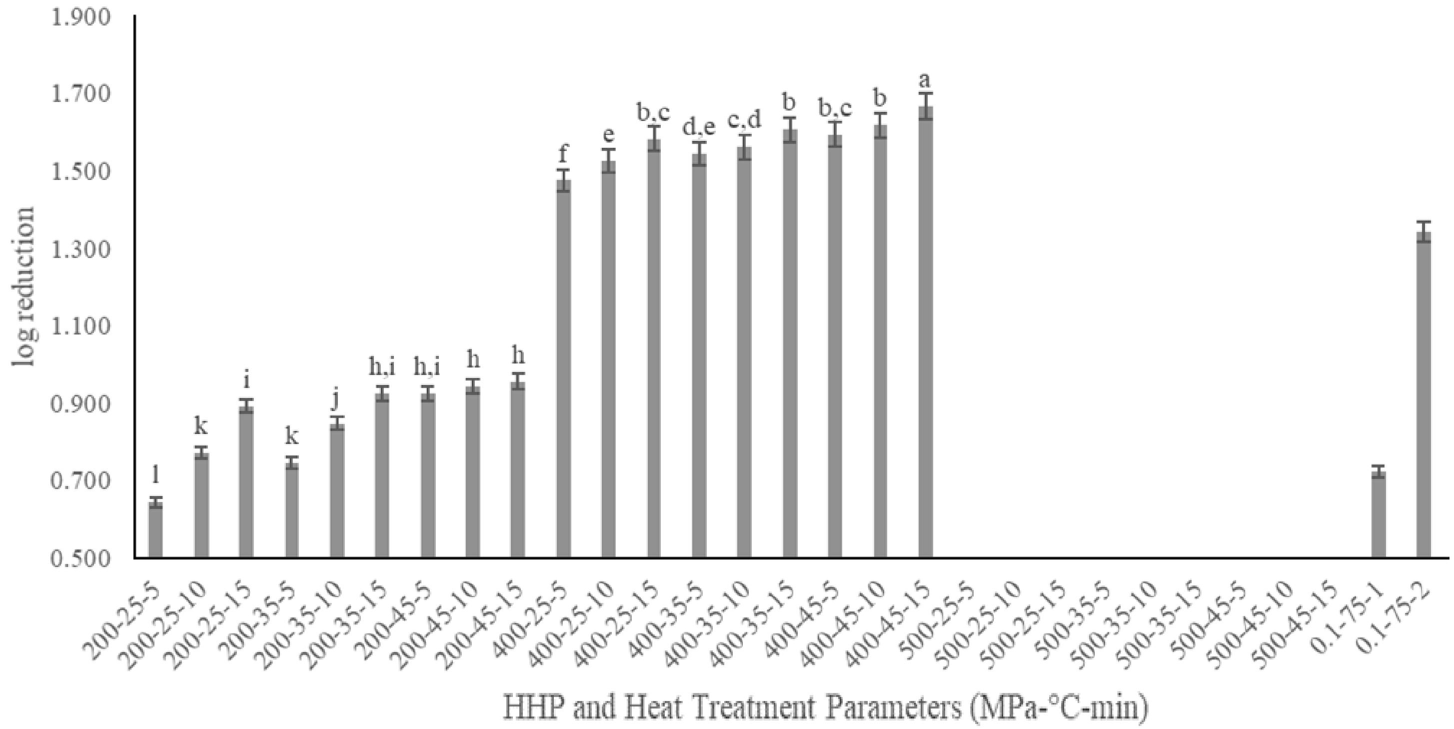

Figure 3.1 Effect of HHP and heat treatment on log reduction initially from 4.04 log CFU/mL in donkey milk. nd: none detected. Lowercase letters indicate significant differences between samples (p<0.05).

3.3 Lysozyme and Lactoferrin Determination

The effects of HHP and heat treatment on lysozyme and lactoferrin concentration of donkey milk are given in Figure 3.2 and Figure 3.3. With increasing pressure values, lysozyme and lactoferrin loss increased significantly (p<0.05). Although a significant decrease of lysozyme and lactoferrin concentration did not observed (p>0.05) as processing temperature increased from 25 °C to 35 °C, and 45 °C, a significant loss of stability is observed for each protein (p<0.05). Processing time is observed to have no significant effect on both lysozyme and lactoferrin concentration (p>0.05).

HHP treatment of donkey milk with increasing pressure values leads to a significant reduction of total lysozyme content as 16.4% at 200 MPa, 21.6% at 400 MPa, and 28.1% at 500 MPa (p<0.05). Considering processing temperature although there is a significant loss of lysozyme stability at 45 °C (p<0.05), no significant decrease was observed in lysozyme content at 25 °C and 35 °C (p>0.05). HHP processing time had no significant effect on the lysozyme content of donkey milk by itself (p>0.05). Viazis, Farkas, and Allen (2007) reported that during 400 MPa pressure application on human milk processing time range from 30 to 120 min resulted in 106.9% to 95.8% retention of lysozyme activity. No significant reduction of lysozyme activity in human milk was reported with HHP application of 500 MPa for 8 min (Pitino et al., 2019). The lysozyme concentration of heat-treated donkey milk samples decreased 71.6% and 93% with application of 75 °C for 1 min and 2 min, respectively. Lactoferrin has been reported to be a heat-labile component in donkey milk, with complete loss reported at 75 °C for 2 min process (Ozturkoglu-Budak, 2018).

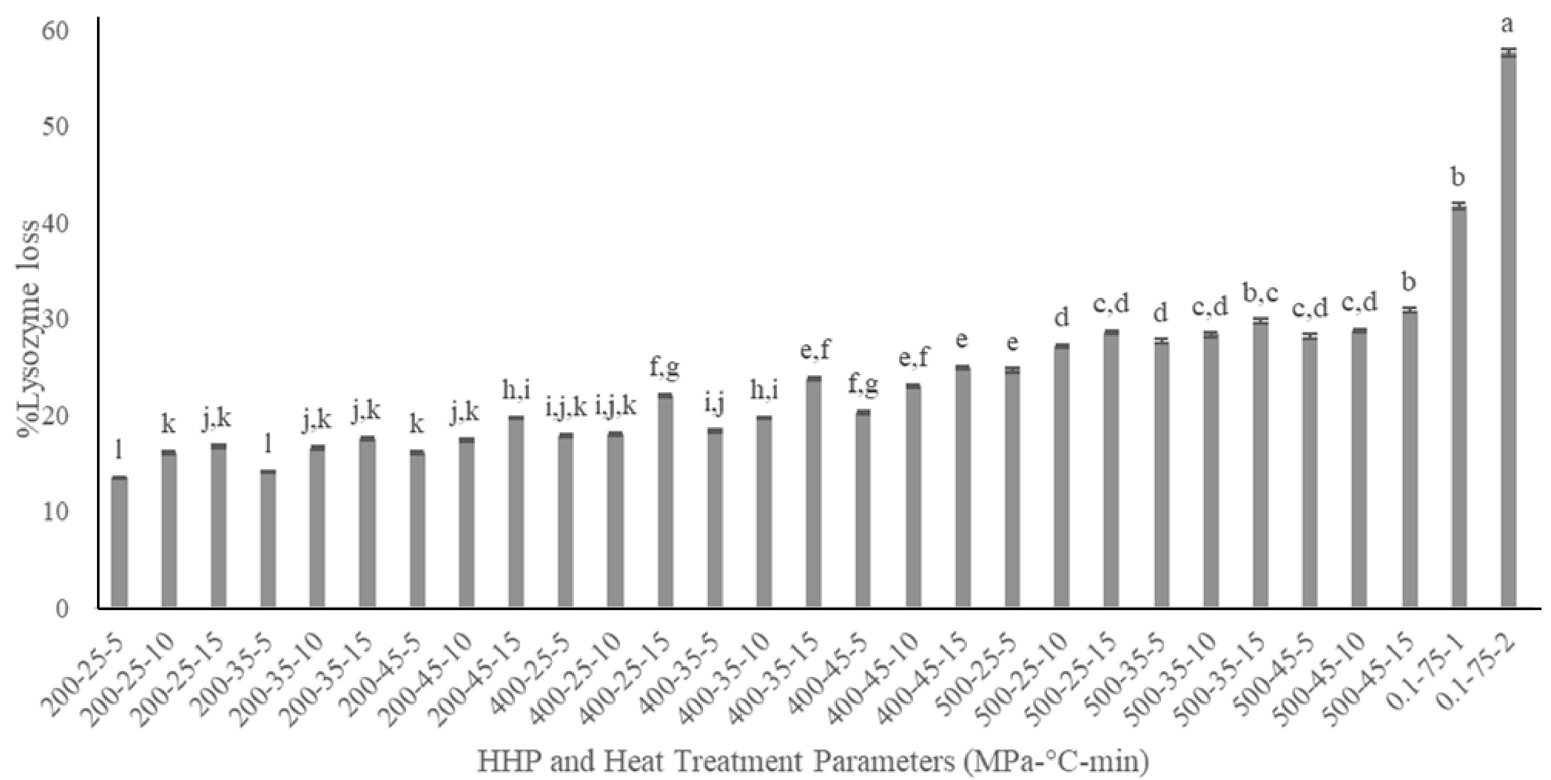

Figure 3.2 Effects of HHP and heat treatment on lysozyme loss % (w/w%). Lowercase letters indicate significant differences between samples (p<0.05).

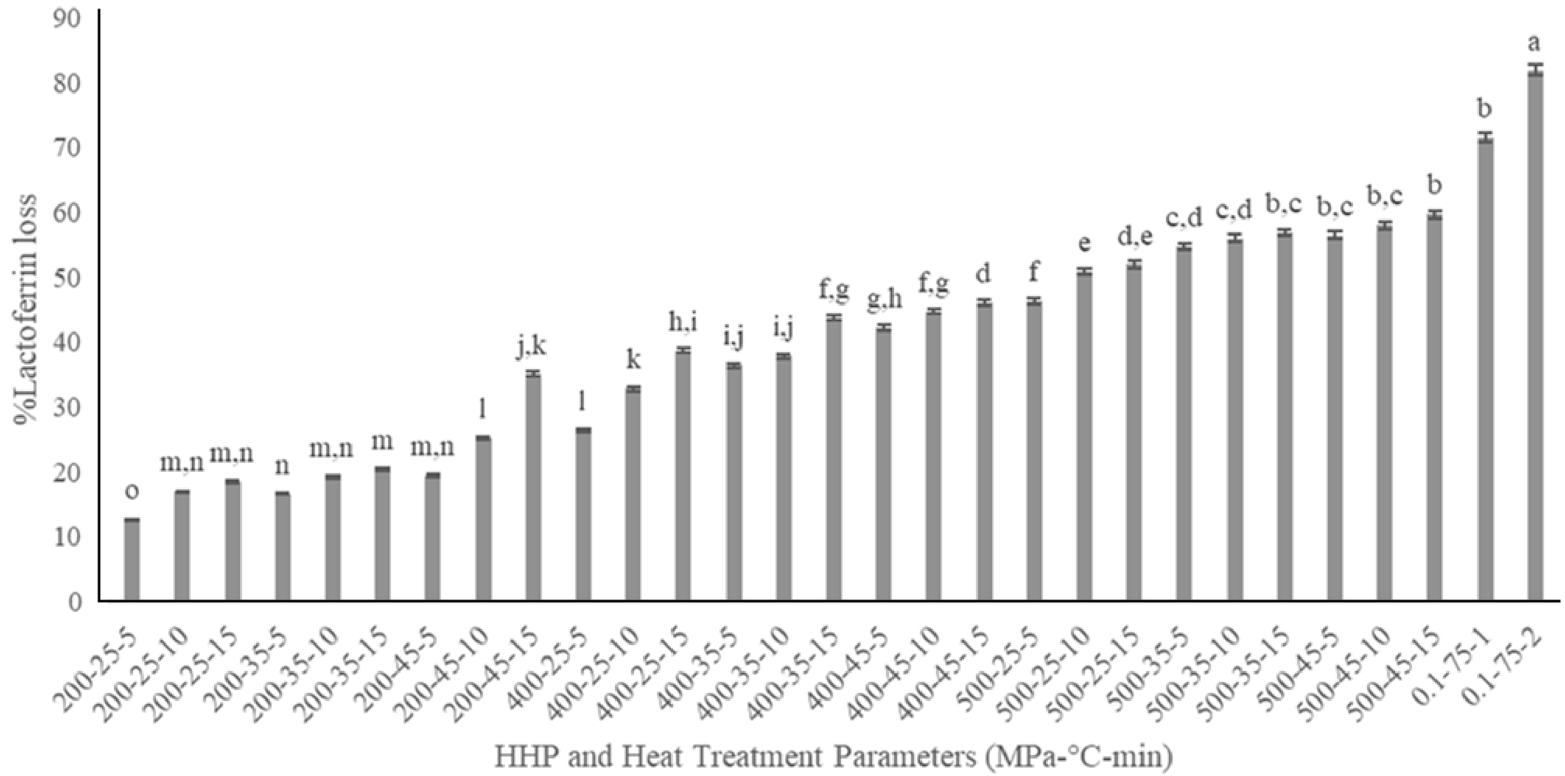

Figure 3.3 Effects of HHP and heat treatment on lactoferrin loss % (w/w%). Lowercase letters indicate significant differences between samples (p<0.05).

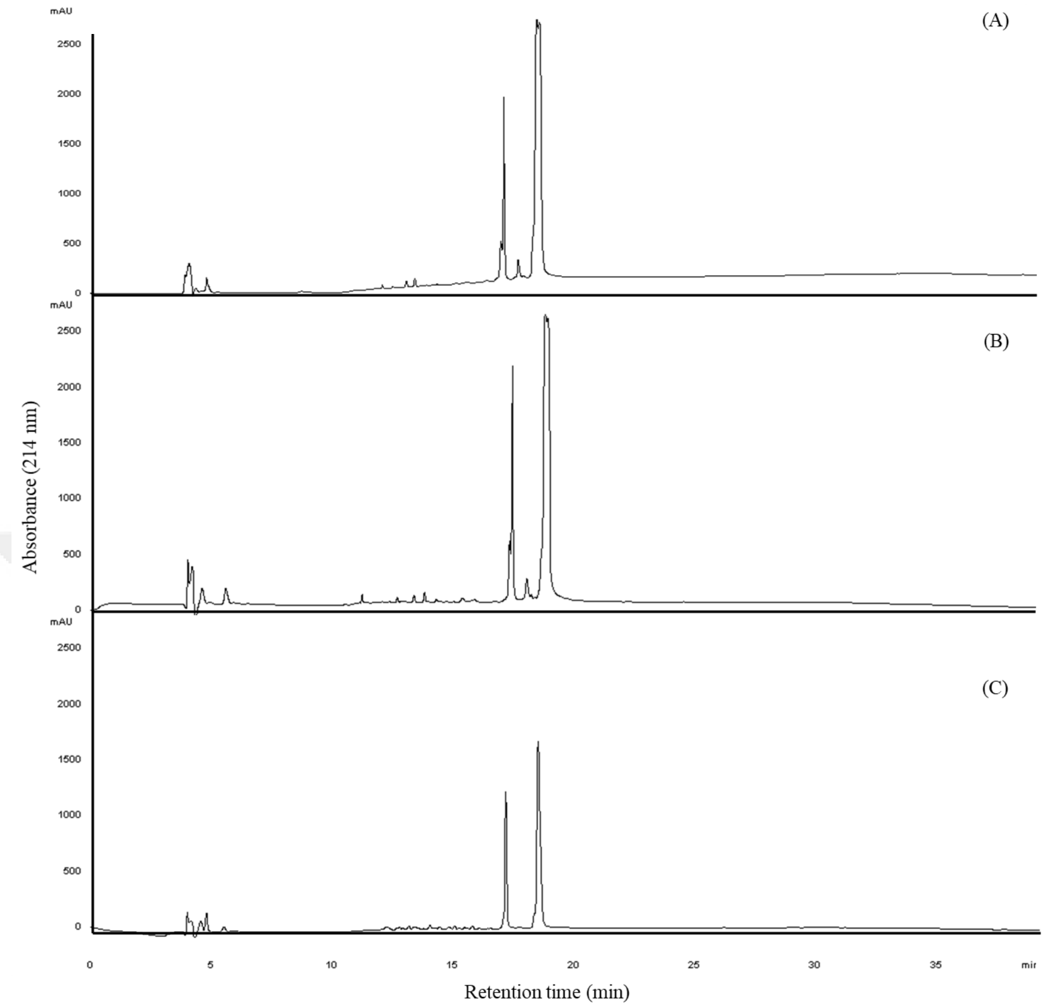

Figure 3.4 Reversed-phase high-pressure liquid chromatography elution profiles of (A) untreated, (B) HHP-treated (400 MPa-25 °C-5 min), and (C) heat-treated (75 °C-2 min) donkey milk samples.

Effect of HHP treatment on lactoferrin concentration of donkey milk caused a significant decrease of 20% at 200 MPa, 36.8% at 400 MPa, and 55.5% at 500 MPa (p<0.05). HHP treatment caused a significant loss of lactoferrin when a higher temperature was applied. The loss of stability increased from 35.1% at 25 °C to 40.0% at 45 °C (p<0.05), although there was no significant change from 25 °C to 35 °C, and from 35 °C to 45 °C (p>0.05). Processing time also did not have a significant effect on lactoferrin loss in donkey milk however a significant increase was observed on stability loss of lysozyme (p>0.05). In a study performed on human milk, loss of lactoferrin stability was reported as 9%, 23%, 34%, and 48%, at pressure treatments of 300, 400, 500, and 600 MPa at 20 °C for 15 min, respectively (Mayayo et al., 2014). Pitino et al. (2019) also reported that HHP application of 500 MPa for 8 min decreases 28% lactoferrin stability in human milk. Application of heat treatment led to lactoferrin loss of 35.6% at 75 °C – 1 min and 53.4% at 75 °C – 2 min. Ozturkoglu-Budak (2018) reported heat treatment application of 75 °C and 85 °C cause 10% and 62% lactoferrin loss in donkey milk, respectively. Heat treatment of 75 °C was reported causing 33% loss in bovine milk samples collected from New Zealand and 43% lactoferrin loss in samples collected from China (Liu et al., 2020).

In this study, inspection of lysozyme and lactoferrin content of HHP-treated and heat-treated samples showed that, with pressure application of 200, 400, and 500 MPa at 25, 35, and 45 °C for 5, 10, and 15 min lead to higher retention of lysozyme and lactoferrin activity comparing with heat treatment of 75 °C for 1 and 2 min.

3.4 Shelf-life Analysis

Effects of different pressure-temperature-time conditions of HHP application on donkey milk samples were evaluated in terms of shelf-life analyses. Treatment at 400 MPa – 25 °C – 5 min was determined as the most suitable HHP processing condition with regard to lower lysozyme and lactoferrin loss ratio and pressure applied at 500 MPa cause precipitation at different temperature and time parameters Shelf-life analysis was performed with the comparison of pressure and heat application conditions. In consideration of obtained values, similar TAMB reduction was observed at 75 °C – 2 min heat treatment process when compared to 400 MPa – 25 °C – 5 min HHP application.

3.4.1 Microbiological Analysis

TAMB counts during the shelf-life analysis of untreated, HHP-treated, and heat-treated donkey milk samples are given in Figure 3.4. The initial microbial count of untreated donkey milk was 4.04 log CFU/mL. TAMB count of untreated milk samples showed an increase to 5.82 log CFU/mL during 7-day storage at 4 °C and 5.10 log CFU/mL after storage for 3 days at 25 °C. A significant reduction was seen in the TAMB count of HHP-treated and heat-treated samples as 1.48 log CFU/mL and 1.34 log CFU/mL, respectively ($p<0.05$). The microbial load of HHP-treated samples increased to 5.05 log CFU/mL during 21 days of storage at 4 °C. A similar mean TAMB count as 5.20 log CFU/mL was obtained on day 14 when HHP-treated samples were stored at 25 °C. Heat-treated samples stored at 4 °C showed an increase in microbial load to 5.15 log CFU/mL during 21 days. After 7 days of storage at 25 °C resulted in an increase of TAMB count of heat-treated samples to 5.14 log CFU/mL. After 28 days lowest TAMB count was observed in pressure-treated samples stored at 4 °C and the highest TAMB count was observed in untreated samples stored at 25 °C. For all samples, TAMB counts were increased during 28 days of storage.

Stratakos et al. (2019) reported that HHP treatment of 600 MPa at 18 °C for 3 min led to 3.95 log CFU/mL of TAMB counts in cow milk to 2.05 log CFU/mL, and after 28 days of storage at 4 °C, TAMB count of HHP-treated cow milk increased to 7.05 log CFU/mL. Although at the start of the shelf life analysis similar TAMB counts were observed, the TAMB count of HHP-treated donkey milk samples stored at 4 °C was lower than Stratakos et al. (2019) reported due to higher concentrations of lysozyme and lactoferrin in donkey milk comparing with cow milk.

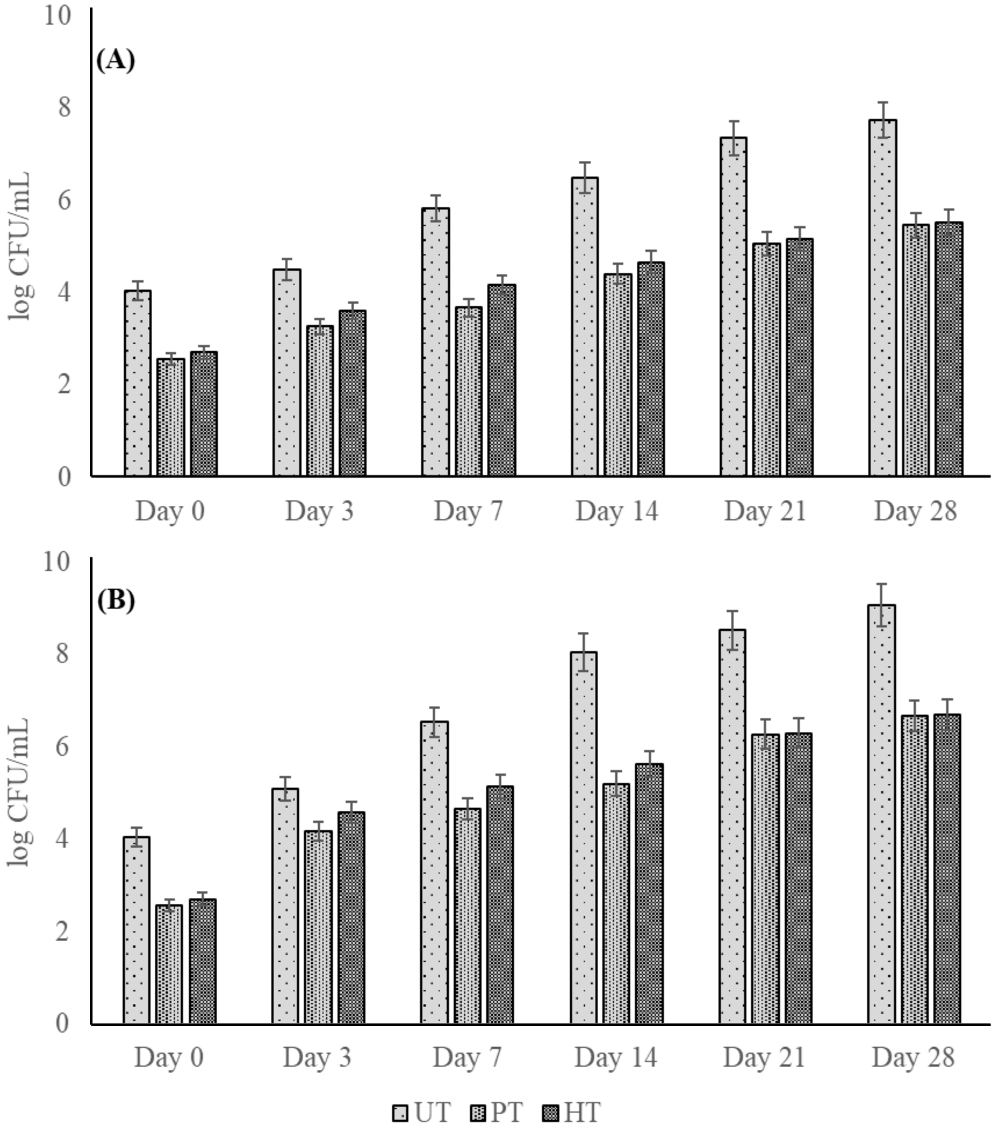

Figure 3.5 Microbial load values of donkey milk samples during **(A)** 4 °C and **(B)** 25 °C storage. UT, untreated milk; PT, HHP-treated milk; HT, heat-treated milk during shelf-life analysis.

3.4.2 pH and Titratable Acidity Analyses

The pH and titratable acidity values of untreated, HHP-treated, and heat-treated donkey milk samples during 28 days of storage were given in Table 3.2 and Table 3.3. pH and LA% of untreated donkey milk samples were found as 7.00 and 0.036%, respectively. No significant changes were observed at pH and titratable acidity values on the day of HHP treatment performed (day 0) (p>0.05). However, for heat-treated samples, pH increased to 7.21 and titratable acidity decreased to 0.027%. During storage at 4 °C, pH and LA% of untreated donkey milk samples did not significantly change for 7 days, while after 14 days, a decrease in pH and an increase in LA% were observed. pH values of HHP-treated and heat-treated samples stored at 4 °C remained substantially unchanged for 28 days of storage, however titratable acidity values increased after 14 days of storage. Storage of untreated milk at 25 °C cause a decrease in pH value and an increase was determined in titration acidity during 7 days of storage. A Decrease in pH value and an increase in LA% were observed in HHP-treated samples during 14 days and 7 days of storage at 25 °C, respectively. The change was observed in pH and titration acidity of heat-treated samples stored at 25 °C on day 7.

Tan et al. (2020) reported that during 22 days of storage pH values decreased significantly and titratable acidity values increased significantly for both cow milk and goat milk. Similar results were obtained by Brodziak et al. (2017) for 7 days of storage of cow milk under refrigeration conditions. Decrease in pH and increase in titratable acidity are due to lactic acid bacteria growth during storage.

Table 3.2 pH values of untreated, HHP-treated, and heat-treated donkey milk samples during shelf-life

	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
4 °C						
Untreated	$7.00 \pm 0.09^{a,A,B}$	$7.16 \pm 0.15^{a,A}$	$7.09 \pm 0.08^{a,A,B}$	$6.25 \pm 0.06^{b,F,G}$	$6.13 \pm 0.06^{b,G}$	$5.23 \pm 0.04^{c,I,J}$
Heat-Treated	$7.21 \pm 0.18^{a,A}$	$7.19 \pm 0.16^{a,A}$	$7.00 \pm 0.08^{a,A,B}$	$7.00 \pm 0.06^{a,A,B}$	$7.00 \pm 0.10^{a,A,B}$	$6.64 \pm 0.07^{a,D,E}$
HHP-Treated	$7.00 \pm 0.13^{a,A,B}$	$7.06 \pm 0.08^{a,A,B}$	$7.08 \pm 0.11^{a,A,B}$	$6.94 \pm 0.07^{a,A,B,C}$	$7.00 \pm 0.08^{a,A,B}$	$6.66 \pm 0.11^{a,C,D,E}$
25 °C						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
Untreated	$7.00 \pm 0.09^{a,A,B}$	$7.08 \pm 0.10^{a,A,B}$	$5.37 \pm 0.05^{a,H,I}$	$4.97 \pm 0.06^{b,J}$	$5.01 \pm 0.09^{b,J}$	$4.51 \pm 0.06^{c,K}$
Heat-Treated	$7.21 \pm 0.18^{a,A}$	$6.98 \pm 0.11^{a,A,B}$	$6.48 \pm 0.06^{a,b,E,F}$	$6.10 \pm 0.10^{b,c,G}$	$5.48 \pm 0.07^{e,H,I}$	$5.62 \pm 0.05^{d,e,H}$
HHP-Treated	$7.00 \pm 0.13^{a,A,B}$	$7.00 \pm 0.07^{a,A,B}$	$6.83 \pm 0.08^{a,B,C,D}$	$4.57 \pm 0.10^{a,K}$	$4.43 \pm 0.07^{b,K,L}$	$4.21 \pm 0.05^{b,L}$

Results expressed as the mean ± standard deviation. Lowercase letters in the same row indicate significant differences ($p<0.05$). Uppercase letters indicate significant differences between samples ($p<0.05$).

Table 3.3 Titratable acidity values (LA%) of untreated, HHP-treated, and heat-treated donkey milk samples during shelf-life analysis.

	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
4 °C						
Untreated	$0.036 \pm 0.002^{d,O,P}$	$0.036 \pm 0.004^{d,O,P}$	$0.036 \pm 0.002^{d,O,P}$	$0.126 \pm 0.002^{c,K}$	$0.189 \pm 0.003^{b,I}$	$0.219 \pm 0.005^{a,H}$
Heat-Treated	$0.027 \pm 0.002^{b,P}$	$0.027 \pm 0.001^{b,P}$	$0.027 \pm 0.001^{b,P}$	$0.036 \pm 0.002^{b,O,P}$	$0.063 \pm 0.004^{b,M}$	$0.081 \pm 0.005^{a,L}$
HHP-Treated	$0.036 \pm 0.004^{c,O,P}$	$0.036 \pm 0.001^{c,O,P}$	$0.036 \pm 0.005^{c,O,P}$	$0.045 \pm 0.004^{c,N,O}$	$0.045 \pm 0.004^{b,N,O}$	$0.075 \pm 0.003^{a,L}$
25 °C						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
Untreated	$0.036 \pm 0.002^{d,O,P}$	$0.045 \pm 0.003^{d,N,O}$	$0.306 \pm 0.002^{c,F}$	$0.387 \pm 0.005^{b,C}$	$0.369 \pm 0.003^{b,D}$	$0.421 \pm 0.004^{a,A}$
Heat-Treated	$0.027 \pm 0.002^{e,P}$	$0.054 \pm 0.001^{e,M,N}$	$0.153 \pm 0.004^{d,J}$	$0.180 \pm 0.003^{c,I}$	$0.261 \pm 0.003^{b,G}$	$0.351 \pm 0.006^{a,E}$
HHP-Treated	$0.036 \pm 0.004^{f,O,P}$	$0.045 \pm 0.003^{e,N,O}$	$0.252 \pm 0.003^{d,G}$	$0.306 \pm 0.002^{c,F}$	$0.342 \pm 0.005^{b,E}$	$0.398 \pm 0.006^{a,B}$

Results expressed as the mean ± standard deviation. Lowercase letters in the same row indicate significant differences ($p<0.05$). Uppercase letters indicate significant differences between samples ($p<0.05$).

3.4.3 Color Analysis

Color data of untreated, HHP-treated, and heat-treated samples were given in Figure 3.5. White tile selected as reference for color analyses (L*=95.81, a*=-0.16, and b*=1.30). Comparing with untreated milk and heat-treated milk, HHP-treated milk showed lower L* values. With increasing storage time, L* values decreased in all samples. a* and b* values decreased in HHP-treated and heat-treated milk samples, lower values were observed in HHP-treated samples. Harte et al. (2007) reported that HHP-treated and HHP treated after heat-treated cow milk loses its white color due to casein micelles size reduction. However when initial treatment was done with HHP, and then heat treatment was done, the initial color of cow milk was regained. Gervilla et al. (2001) reported a similar color change in sheep milk. In this study, the lowest ΔL^*, Δa^*, and Δb^* values were obtained in HHP-treated donkey milk samples which shows that HHP treatment affected casein micelle sizes.

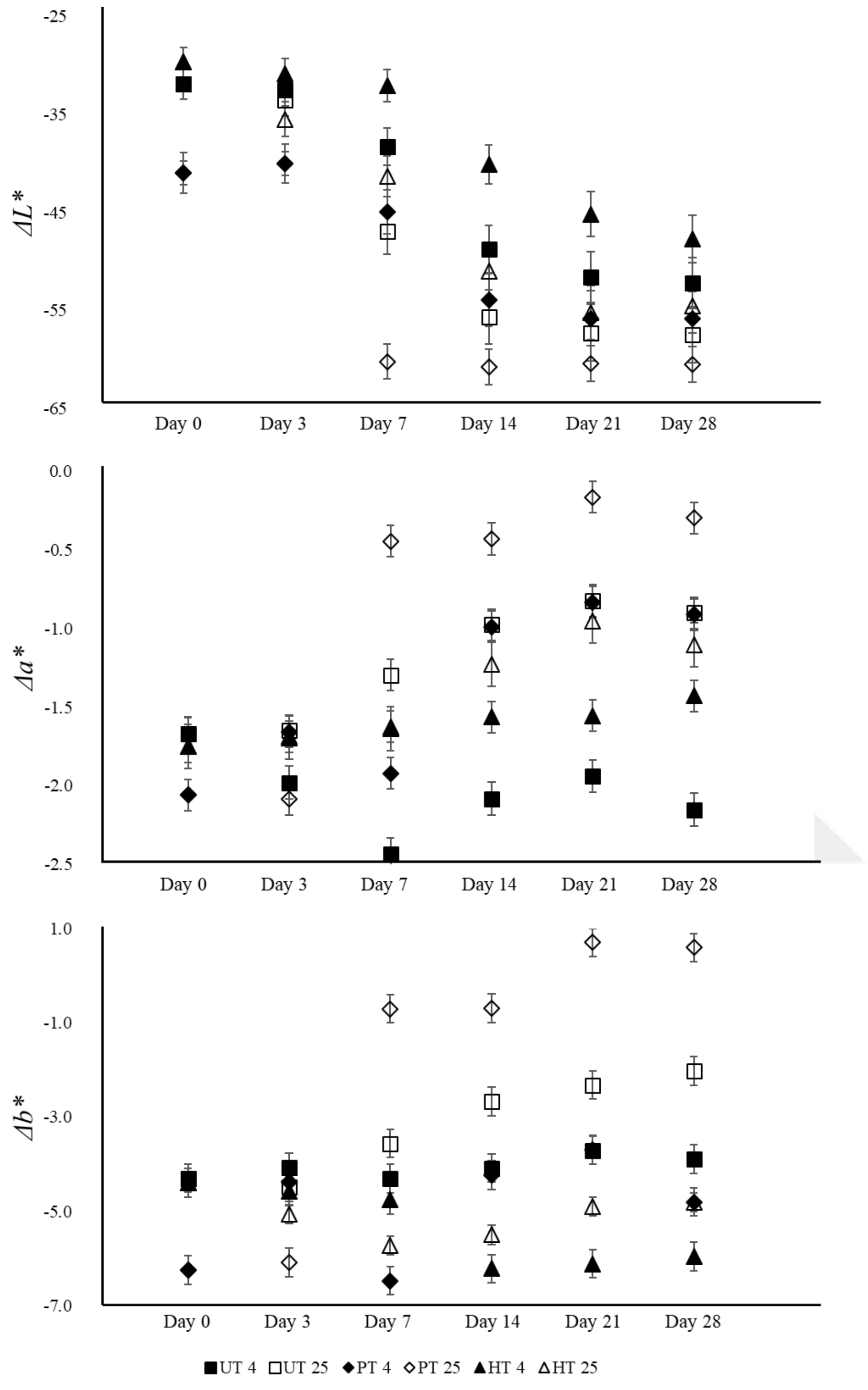

Figure 3.6 *ΔL**, *Δa**, and *Δb** values during 28 days of storage. UT 4, untreated milk stored at 4 °C; UT 25, untreated milk stored at 25 °C; PT 4, HHP-treated milk stored at 4 °C; PT 25, HHP-treated milk stored at 25 °C; HT 4, heat-treated milk stored at 4 °C; HT 25, heat-treated milk stored at 25 °C.

3.4.4 Rheological Analysis

Rheological analyses of untreated, heat-treated, and HHP-treated donkey milk are shown in Table 3.4 and Table 3.5. At the beginning of the shelf-life analysis highest flow consistency index was observed at heat-treated samples and lowest flow consistency index was observed at untreated samples. Xiang, Simpson, Ngadi, & Simpson (2011) reported that protein denaturation leads to an increase in consistency index. Consistency index values of untreated, HHP-treated, and heat-treated milk samples were analyzed during storage conditions of 4 °C and 25 °C for 28 days. The consistency index all samples stored at 4 °C and 25 °C increased during 28 days of storage.

Flow behavior is pseudoplastic for flow behavior index (n) is higher than 1, dilatant for n is lower than 1, and Newtonian fluid if n is equal to 1. At the beginning of the shelf-life analysis, n values were observed to be closer to 1 for all donkey milk samples (Table 3.5). During 28 days of storage, significant decrease in flow behaviour index were observed in all samples, although remained closer to 1, except heat-treated samples.

Shear-thinning flow behavior depends on particle size, larger particle size leads to lower n-values, which leads to reduced flow rates and high pressure drops (Bienvenue, Jiménez-Flores, & Singh, 2003; Warncke & Kulozik, 2020). Ding et al. (2020) reported that the flow behavior of donkey milk was shear-thinning and highly dependent on storage temperature. Debon, Prudêncio, & Cunha Petrus (2010) reported that the storage temperature of prebiotic fermented milk affects the mobility of macromolecules and intermolecular interactions. The apparent viscosity of fermented milk was reported to be dependent on storage time (Debon et al., 2010).

Table 3.4 Flow consistency index values (K) of untreated, HHP-treated, and heat-treated donkey milk samples during shelf-life analysis

	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
4 °C						
Untreated	2.866E-05±4.243E-8 ^{f,X}	3.360E-05±6.364E-8 ^{e,X}	4.038E-05±5.657E-8 ^{d,X}	6.021E-05±6.364E-8 ^{c,V,W,X}	8.178E-05±5.657E-8 ^{b,V,W}	1.233E-04±7.254E-7 ^{a,U}
Heat-Treated	8.188E-04±5.749E-7 ^{f,Q}	9.318E-04±5.703E-7 ^{e,P}	1.232E-03±5.660E-6 ^{d,N}	2.204E-03±2.831E-6 ^{c,I}	4.042E-03±6.588E-6 ^{b,E}	7.473E-03±9.231E-6 ^{a,C}
HHP-Treated	4.552E-04±6.437E-7 ^{f,T}	5.237E-04±4.918E-7 ^{e,S}	5.886E-04±4.035E-7 ^{d,R}	8.952E-04±5.932E-7 ^{c,P}	1.395E-03±3.674E-6 ^{b,M}	3.035E-03±6.081E-6 ^{a,H}
25 °C						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
Untreated	2.866E-05±4.243E-8 ^{f,X}	5.150E-05±7.135E-8 ^{e,W,X}	6.190E-05±7.148E-8 ^{d,V,W,X}	9.230E-05±7.019E-8 ^{c,U,W}	1.254E-04±2.540E-7 ^{b,U}	4.201E-04±4.261E-7 ^{a,T}
Heat-Treated	8.188E-04±5.749E-7 ^{f,Q}	1.471E-03±7.073E-6 ^{e,L}	2.034E-03±2.431E-6 ^{d,J}	3.867E-03±4.593E-6 ^{c,F}	7.615E-03±3.511E-6 ^{b,B}	1.049E-02±8.310E-5 ^{a,A}
HHP-Treated	4.552E-04±6.437E-7 ^{f,T}	8.179E-04±6.147E-7 ^{e,Q}	1.029E-03±6.142E-6 ^{d,O}	1.819E-03±6.240E-6 ^{c,K}	3.403E-03±4.884E-6 ^{b,G}	6.145E-03±7.504E-6 ^{a,D}

Results expressed as the mean ± standard deviation. Lowercase letters in the same row indicate significant differences ($p<0.05$). Uppercase letters indicate significant differences between samples ($p<0.05$).

Table 3.1 Flow behavior index values (n) of untreated, HHP-treated, and heat-treated donkey milk samples during shelf-life analysis.

	4 °C					
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
Untreated	1.141±0.006 ^{a,A}	1.102±0.006 ^{b,B}	1.057±0.005 ^{c,E,F}	0.960±0.007 ^{d,I,J}	0.901±0.006 ^{e,K,L}	0.882±0.008 ^{e,M}
Heat-Treated	0.989±0.006 ^{a,H,I}	0.893±0.002 ^{b,L,M}	0.823±0.002 ^{c,O,P}	0.812±0.001 ^{c,d,PQ}	0.792±0.005 ^{d,Q,R}	0.759±0.006 ^{e,S}
HHP-Treated	1.089±0.006 ^{a,B,C}	1.083±0.002 ^{a,b,C,D}	1.080±0.007 ^{a,b,C,D,E}	1.065±0.003 ^{b,D,E,F}	1.019±0.007 ^{c,G}	0.970±0.005 ^{d,I}
	25 °C					
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
Untreated	1.141±0.006 ^{a,A}	1.069±0.006 ^{b,C,D,E,F}	0.998±0.005 ^{c,G,H}	0.912±0.006 ^{d,K}	0.822±0.005 ^{e,O,P}	0.775±0.006 ^{f,R,S}
Heat-Treated	0.989±0.006 ^{a,H,I}	0.859±0.006 ^{b,,N}	0.825±0.004 ^{c,O,P}	0.803±0.005 ^{c,P,Q}	0.779±0.007 ^{d,R,S}	0.732±0.004 ^{e,T}
HHP-Treated	1.089±0.006 ^{a,B,C}	1.056±0.004 ^{b,F}	0.983±0.005 ^{c,H,I}	0.949±0.006 ^{d,J}	0.924±0.003 ^{e,K}	0.840±0.005 ^{e,N,O}

Results expressed as the mean ± standard deviation. Lowercase letters in the same row indicate significant differences (p<0.05). Uppercase letters indicate significant differences between samples (p<0.05).

CHAPTER 4

CONCLUSION

To the best of our knowledge, this study is the first study to evaluate the effect of HHP on microbial and physicochemical properties of donkey milk compared to the heat treatment.

The results obtained in this study demonstrated that with HHP treatment, the microbial load of donkey milk decreases significantly with smaller losses of lysozyme and lactoferrin in comparison with heat treatment. Although similar microbial inactivation was obtained with heat treatment, higher losses of lysozyme and lactoferrin were observed when compared to HHP application, which is undesirable for donkey milk.

The shelf-life of donkey milk samples increased with HHP and heat treatment. Microbial load of HHP-treated donkey milk samples stored at 4 °C increased higher than 5 log CFU/mL after 21 days of storage. The pH values of untreated, HHP-treated, and heat-treated donkey milk samples decreased significantly during storage. Shelf-life analyses showed that the lightness values of the samples decreased during the storage. Effect of HHP treatment and heat treatment on flow behaviour of donkey milk showed that both treatments increased flow consistency index values significantly and highest values were obtained with heat treatment. Flow behaviour index values of the samples significantly decreased with HHP treatment and heat treatment, and lowest n values were observed at the heat-treated samples. During 28 days of storage, the K values of samples increased significantly, and the n values of the samples decreased significantly.

In brief, the results of this study highlight HHP technology has potential use in the treatment of donkey milk for extending shelf-life without high losses of lysozyme and lactoferrin. As future work, sensory analysis of untreated, HHP-treated, and heat-treated donkey milk samples may be suggested in order to determine effects of HHP treatment and heat treatment on the acceptance of sensory attributes of donkey milk.

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APPENDICES

A. Analysis of Variance Tables

General Linear Model: % Lysozyme Loss versus Pressure, Temperature, Time

Factor	Type	Levels	Values
Pressure	fixed	3	200, 400, 500
Temperature	fixed	3	25, 35, 45
Time	fixed	3	5, 10, 15

Analysis of Variance for C7, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Pressure	2	1274.320	1274.320	637.160	2813.76	0.000
Temperature	2	66.996	66.996	33.498	147.93	0.000
Time	2	123.962	123.962	61.981	273.71	0.000
Pressure*Temperature	4	5.179	5.179	1.295	5.72	0.002
Pressure*Time	4	9.092	9.092	2.273	10.04	0.000
Temperature*Time	4	0.149	0.149	0.037	0.16	0.955
Pressure*Temperature*Time	8	9.553	9.553	1.194	5.27	0.000
Error	27	6.114	6.114	0.226		
Total	53	1495.364				

S = 0.475862   R-Sq = 99.59%   R-Sq(adj) = 99.20%

Grouping Information Using Tukey Method and 95.0% Confidence

Pressure	N	Mean	Grouping
500	18	28.2	A
400	18	20.8	B
200	18	16.4	C

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Temperature	N	Mean	Grouping
45	18	23.2	A
35	18	21.7	B
25	18	20.5	C

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Time	N	Mean	Grouping
15	18	23.7	A
10	18	21.6	B
5	18	20.0	C

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Pressure	Temperature	N	Mean	Grouping
500	45	6	29.2	A
500	35	6	28.6	A
500	25	6	26.8	B
400	45	6	22.7	C
400	35	6	20.6	D
400	25	6	19.3	E
200	45	6	17.7	F
200	35	6	16.1	G
200	25	6	15.4	G

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Pressure	Time	N	Mean	Grouping
500	15	6	29.7	A
500	10	6	28.0	B
500	5	6	26.8	C
400	15	6	23.5	D
400	10	6	20.2	E
400	5	6	18.8	F
200	15	6	18.0	F
200	10	6	16.7	G
200	5	6	14.5	H

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Temperature	Time	N	Mean	Grouping
45	15	6	25.1	A
35	15	6	23.7	B
45	10	6	23.0	B C
25	15	6	22.4	C D
35	10	6	21.5	D E
45	5	6	21.5	E
25	10	6	20.4	F
35	5	6	20.0	F
25	5	6	18.6	G

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Pressure	Temperature	Time	N	Mean	Grouping
500	45	15	2	30.8	A
500	35	15	2	29.7	A B
500	45	10	2	28.7	B C
500	25	15	2	28.6	B C
500	35	10	2	28.3	B C
500	45	5	2	28.1	B C

500	35	5	2	27.7	C
500	25	10	2	27.1	C
400	45	15	2	24.9	D
500	25	5	2	24.6	D
400	35	15	2	23.8	D E
400	45	10	2	23.0	D E
400	25	15	2	21.9	E F
400	45	5	2	20.2	F G
200	45	15	2	19.7	G H
400	35	10	2	19.7	G H
400	35	5	2	18.3	H I
400	25	10	2	18.0	H I J
400	25	5	2	17.8	H I J
200	35	15	2	17.6	I J
200	45	10	2	17.4	I J
200	25	15	2	16.7	I J
200	35	10	2	16.5	I J
200	25	10	2	16.1	J
200	45	5	2	16.1	J
200	35	5	2	14.1	K
200	25	5	2	13.4	K

Means that do not share a letter are significantly different.

General Linear Model: % Lactoferrin Loss versus Pressure, Temperature, Time

Factor	Type	Levels	Values
Pressure	fixed	3	200, 400, 500
Temperature	fixed	3	25, 35, 45
Time	fixed	3	5, 10, 15

Analysis of Variance for C8, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Pressure	2	10493.65	10493.65	5246.82	7019.61	0.000
Temperature	2	933.00	933.00	466.50	624.12	0.000
Time	2	397.96	397.96	198.98	266.21	0.000
Pressure*Temperature	4	63.90	63.90	15.98	21.37	0.000
Pressure*Time	4	41.92	41.92	10.48	14.02	0.000
Temperature*Time	4	29.61	29.61	7.40	9.90	0.000
Pressure*Temperature*Time	8	113.29	113.29	14.16	18.95	0.000
Error	27	20.18	20.18	0.75		
Total	53	12093.52				

S = 0.864553   R-Sq = 99.83%   R-Sq(adj) = 99.67%

Grouping Information Using Tukey Method and 95.0% Confidence

Pressure	N	Mean	Grouping
500	18	54.5	A
400	18	38.8	B
200	18	20.4	C

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Temperature	N	Mean	Grouping
45	18	43.0	A
35	18	38.0	B
25	18	32.8	C

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Time	N	Mean	Grouping
15	18	41.2	A
10	18	38.0	B
5	18	34.6	C

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Pressure	Temperature	N	Mean	Grouping
500	45	6	58.0	A
500	35	6	55.9	B
500	25	6	49.7	C
400	45	6	44.3	D
400	35	6	39.3	E
400	25	6	32.6	F
200	45	6	26.6	G
200	35	6	18.7	H
200	25	6	16.0	I

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Pressure	Time	N	Mean	Grouping
500	15	6	56.2	A
500	10	6	55.0	A
500	5	6	52.5	B
400	15	6	42.8	C
400	10	6	38.5	D
400	5	6	35.0	E
200	15	6	24.6	F
200	10	6	20.5	G
200	5	6	16.2	H

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Temperature	Time	N	Mean	Grouping
45	15	6	46.9	A
45	10	6	42.7	B
35	15	6	40.3	C
45	5	6	39.3	C D
35	10	6	37.7	D E
25	15	6	36.4	E F

35	5	6	35.9	F
25	10	6	33.6	G
25	5	6	28.5	H

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Pressure	Temperature	Time	N	Mean	Grouping
500	45	15	2	59.7	A
500	45	10	2	58.0	A B
500	35	15	2	56.9	A B
500	45	5	2	56.4	A B
500	35	10	2	56.0	B
500	35	5	2	54.7	B C
500	25	15	2	52.0	C D
500	25	10	2	50.9	D
500	25	5	2	46.3	E
400	45	15	2	46.0	E
400	45	10	2	44.7	E F
400	35	15	2	43.8	E F
400	45	5	2	42.2	F G
400	25	15	2	38.7	G H
400	35	10	2	37.8	H I
400	35	5	2	36.4	H I
200	45	15	2	35.1	I J
400	25	10	2	32.8	J
400	25	5	2	26.4	K
200	45	10	2	25.3	K
200	35	15	2	20.3	L
200	45	5	2	19.4	L M
200	35	10	2	19.1	L M
200	25	15	2	18.5	L M
200	25	10	2	17.0	L M
200	35	5	2	16.6	M
200	25	5	2	12.7	N

Means that do not share a letter are significantly different.

General Linear Model: log reduction versus Pressure, Temperature, Time

Factor	Type	Levels	Values
Pressure	fixed	2	200, 400
Temperature	fixed	3	25, 35, 45
Time	fixed	3	5, 10, 15

Analysis of Variance for log reduction, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Pressure	1	4.73101	4.73101	4.73101	58841.59	0.000
Temperature	2	0.11097	0.11097	0.05549	690.12	0.000
Time	2	0.08217	0.08217	0.04108	510.98	0.000
Pressure*Temperature	2	0.00838	0.00838	0.00419	52.11	0.000
Pressure*Time	2	0.00835	0.00835	0.00418	51.95	0.000
Temperature*Time	4	0.01573	0.01573	0.00393	48.92	0.000
Pressure*Temperature*Time	4	0.01026	0.01026	0.00257	31.91	0.000
Error	18	0.00145	0.00145	0.00008		

```
Total                      35  4.96833


S = 0.00896675   R-Sq = 99.97%   R-Sq(adj) = 99.94%


Grouping Information Using Tukey Method and 95.0% Confidence

Pressure   N  Mean  Grouping
400       18   1.6  A
200       18   0.8    B

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95.0% Confidence

Temperature   N  Mean  Grouping
45           12   1.3  A
35           12   1.2    B
25           12   1.1      C

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95.0% Confidence

Time   N  Mean  Grouping
15    12   1.3  A
10    12   1.2    B
 5    12   1.2      C

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95.0% Confidence

Pressure  Temperature  N  Mean  Grouping
400       45           6   1.6  A
400       35           6   1.6    B
400       25           6   1.5      C
200       45           6   0.9        D
200       35           6   0.8          E
200       25           6   0.8            F

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95.0% Confidence

Pressure  Time  N  Mean  Grouping
400       15    6   1.6  A
400       10    6   1.6    B
400        5    6   1.5      C
200       15    6   0.9        D
200       10    6   0.9          E
200        5    6   0.8            F

Means that do not share a letter are significantly different.
```

Grouping Information Using Tukey Method and 95.0% Confidence

Temperature	Time	N	Mean	Grouping
45	15	4	1.3	A
45	10	4	1.3	B
35	15	4	1.3	B
45	5	4	1.3	B C
25	15	4	1.2	C
35	10	4	1.2	D
25	10	4	1.1	E
35	5	4	1.1	E
25	5	4	1.1	F

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Pressure	Temperature	Time	N	Mean	Grouping
400	45	15	2	1.7	A
400	45	10	2	1.6	B
400	35	15	2	1.6	B
400	45	5	2	1.6	B C
400	25	15	2	1.6	B C
400	35	10	2	1.6	C D
400	35	5	2	1.5	D E
400	25	10	2	1.5	E
400	25	5	2	1.5	F
200	45	15	2	1.0	G
200	45	10	2	0.9	G
200	35	15	2	0.9	G H
200	45	5	2	0.9	G H
200	25	15	2	0.9	H
200	35	10	2	0.8	I
200	25	10	2	0.8	J
200	35	5	2	0.7	J
200	25	5	2	0.6	K

Means that do not share a letter are significantly different.

ANOVA results of pH values during shelf-life analysis

General Linear Model: pH versus Day, Treatment, Temperature

Factor	Type	Levels	Values
Day	fixed	6	0, 3, 7, 14, 21, 28
Treatment	fixed	3	h, p, u
Temperature	fixed	2	4, 25

Analysis of Variance for pH, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Day	5	27.4388	27.4388	5.4878	1181.22	0.000
Treatment	2	4.3512	4.3512	2.1756	468.29	0.000
Temperature	1	15.4939	15.4939	15.4939	3335.01	0.000
Day*Treatment	10	3.2751	3.2751	0.3275	70.50	0.000
Day*Temperature	5	8.2678	8.2678	1.6536	355.93	0.000
Treatment*Temperature	2	1.1741	1.1741	0.5870	126.36	0.000

```
Day*Treatment*Temperature  10   3.9044   3.9044   0.3904    84.04  0.000
Error                      36   0.1673   0.1673   0.0046
Total                      71  64.0726


S = 0.0681604   R-Sq = 99.74%   R-Sq(adj) = 99.49%


Grouping Information Using Tukey Method and 95.0% Confidence

Day   N  Mean  Grouping
 3   12   7.1  A
 0   12   7.1  A
 7   12   6.6    B
14   12   6.0      C
21   12   5.8        D
28   12   5.5          E

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95.0% Confidence

Treatment   N  Mean  Grouping
h          24   6.7  A
p          24   6.3    B
u          24   6.1      C

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95.0% Confidence

Temperature   N  Mean  Grouping
 4           36   6.8  A
25           36   5.9    B

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95.0% Confidence

Day  Treatment  N  Mean  Grouping
 0   h          4   7.2  A
 3   u          4   7.1  A B
 3   h          4   7.1  A B
 3   p          4   7.0  A B
 0   u          4   7.0    B
 0   p          4   7.0    B
 7   p          4   7.0    B
 7   h          4   6.7      C
14   h          4   6.5        D
21   h          4   6.2          E
 7   u          4   6.2          E
28   h          4   6.1          E
14   p          4   5.8            F
21   p          4   5.7            F G
14   u          4   5.6            F G H
21   u          4   5.6              G H
28   p          4   5.4                H
28   u          4   4.9                  I
```

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Day	Temperature	N	Mean	Grouping
3	4	6	7.1	A
0	25	6	7.1	A
0	4	6	7.1	A
7	4	6	7.0	A
3	25	6	7.0	A
14	4	6	6.7	B
21	4	6	6.7	B
7	25	6	6.2	C
28	4	6	6.2	C
14	25	6	5.2	D
21	25	6	5.0	E
28	25	6	4.8	F

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Treatment	Temperature	N	Mean	Grouping
h	4	12	7.0	A
p	4	12	7.0	A
u	4	12	6.5	B
h	25	12	6.3	C
p	25	12	5.7	D
u	25	12	5.7	D

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Day	Treatment	Temperature	N	Mean	Grouping
0	h	25	2	7.2	A
0	h	4	2	7.2	A
3	h	4	2	7.2	A
3	u	4	2	7.2	A
3	u	25	2	7.1	A B
7	p	4	2	7.1	A B
3	p	4	2	7.1	A B
0	u	25	2	7.0	A B
3	p	25	2	7.0	A B
0	p	25	2	7.0	A B
21	h	4	2	7.0	A B
7	h	4	2	7.0	A B
0	u	4	2	7.0	A B
21	p	4	2	7.0	A B
0	p	4	2	7.0	A B
14	h	4	2	7.0	A B
7	u	4	2	7.0	A B
3	h	25	2	7.0	A B
14	p	4	2	6.9	A B C
7	p	25	2	6.8	B C D
28	p	4	2	6.7	C D E
28	h	4	2	6.6	D E

```
 7   h          25            2   6.5          E F
14   u           4            2   6.3            F G
21   u           4            2   6.1              G
14   h          25            2   6.1              G
28   h          25            2   5.6                H
21   h          25            2   5.5                H I
 7   u          25            2   5.4                H I
28   u           4            2   5.2                  I J
21   u          25            2   5.0                    J
14   u          25            2   5.0                    J
14   p          25            2   4.6                      K
28   u          25            2   4.5                      K
21   p          25            2   4.4                      K L
28   p          25            2   4.2                        L

Means that do not share a letter are significantly different.
```

One-way ANOVA of untreated donkey milk samples stored at 4 °C versus days of storage

```
Source  DF      SS      MS      F      P
C1       5  5.4782  1.0956  71.77  0.000
Error    6  0.0916  0.0153
Total   11  5.5698

S = 0.1236   R-Sq = 98.36%   R-Sq(adj) = 96.98%


                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level   N    Mean   StDev  ------+---------+---------+---------+---
Day 0   2  7.0000  0.1273                               (---*--)
Day 14  2  6.2500  0.0849                   (--*---)
Day 21  2  6.1300  0.0849                 (--*---)
Day 28  2  5.2300  0.0566  (--*---)
Day 3   2  7.1600  0.2121                                  (--*---)
Day 7   2  7.0000  0.1131                               (---*--)
                           ------+---------+---------+---------+---
                               5.40      6.00      6.60      7.20

Pooled StDev = 0.1236


Grouping Information Using Tukey Method

C1      N    Mean  Grouping
Day 3   2  7.1600  A
Day 7   2  7.0000  A
Day 0   2  7.0000  A
Day 14  2  6.2500    B
Day 21  2  6.1300    B
Day 28  2  5.2300      C

Means that do not share a letter are significantly different.
```

One-way ANOVA of HHP-treated donkey milk samples stored at 4 °C versus days of storage

```
Source  DF      SS      MS     F      P
C1       5  0.2359  0.0472  2.41  0.158
Error    6  0.1176  0.0196
Total   11  0.3535

S = 0.14   R-Sq = 66.73%   R-Sq(adj) = 39.00%


                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level   N    Mean   StDev  ---+---------+---------+---------+------
Day 0   2  7.0000  0.1838              (---------*---------)
Day 14  2  6.9400  0.0990            (---------*--------)
Day 21  2  7.0000  0.1131              (---------*---------)
Day 28  2  6.6600  0.1556  (--------*---------)
Day 3   2  7.0600  0.1131                 (--------*---------)
Day 7   2  7.0800  0.1556                  (--------*---------)
                           ---+---------+---------+---------+------
                            6.50      6.75      7.00      7.25

Pooled StDev = 0.1400


Grouping Information Using Tukey Method

C1      N    Mean  Grouping
Day 7   2  7.0800  A
Day 3   2  7.0600  A
Day 21  2  7.0000  A
Day 0   2  7.0000  A
Day 14  2  6.9400  A
Day 28  2  6.6600  A

Means that do not share a letter are significantly different.
```

One-way ANOVA of heat-treated donkey milk samples stored at 4 °C versus days of storage

```
Source  DF      SS      MS     F      P
C1       5  0.4191  0.0838  3.03  0.105
Error    6  0.1658  0.0276
Total   11  0.5849

S = 0.1662   R-Sq = 71.65%   R-Sq(adj) = 48.03%


                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level   N    Mean   StDev  --------+---------+---------+---------+-
Day 0   2  7.2100  0.2546                    (--------*---------)
Day 14  2  7.0000  0.0849             (--------*---------)
Day 21  2  7.0000  0.1414             (--------*---------)
Day 28  2  6.6400  0.0990  (--------*---------)
Day 3   2  7.1900  0.2263                   (---------*--------)
Day 7   2  7.0000  0.1131             (--------*---------)
```

```
                                  --------+---------+---------+---------+-
                                        6.60      6.90      7.20      7.50

Pooled StDev = 0.1662


Grouping Information Using Tukey Method

C1       N    Mean  Grouping
Day 0    2  7.2100  A
Day 3    2  7.1900  A
Day 7    2  7.0000  A
Day 21   2  7.0000  A
Day 14   2  7.0000  A
Day 28   2  6.6400  A

Means that do not share a letter are significantly different.
```

One-way ANOVA of untreated donkey milk samples stored at 25 °C versus days of storage

```
Source  DF       SS      MS       F      P
C1       5  12.2343  2.4469  204.47  0.000
Error    6   0.0718  0.0120
Total   11  12.3061

S = 0.1094   R-Sq = 99.42%   R-Sq(adj) = 98.93%


                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level   N    Mean   StDev  ------+---------+---------+---------+---
Day 0   2  7.0000  0.1273                               (--*-)
Day 14  2  4.9700  0.0849        (-*-)
Day 21  2  5.0100  0.1273        (--*-)
Day 28  2  4.5100  0.0849  (-*--)
Day 3   2  7.0800  0.1414                                (--*-)
Day 7   2  5.3700  0.0707             (-*-)
                           ------+---------+---------+---------+---
                               4.80      5.60      6.40      7.20

Pooled StDev = 0.1094


Grouping Information Using Tukey Method

C1       N    Mean  Grouping
Day 3    2  7.0800  A
Day 0    2  7.0000  A
Day 7    2  5.3700    B
Day 21   2  5.0100    B
Day 14   2  4.9700    B
Day 28   2  4.5100      C

Means that do not share a letter are significantly different.
```

One-way ANOVA of HHP-treated donkey milk samples stored at 25 °C versus days of storage

```
Source  DF       SS      MS       F      P
C1       5  19.5251  3.9050  256.91  0.000
Error    6   0.0912  0.0152
Total   11  19.6163

S = 0.1233   R-Sq = 99.54%   R-Sq(adj) = 99.15%


                           Individual 95% CIs For Mean Based on Pooled
StDev
Level   N    Mean   StDev    +---------+---------+---------+---------
Day 0   2  7.0000  0.1838                               (-*-)
Day 14  2  4.5700  0.1414        (-*-)
Day 21  2  4.4300  0.0990      (-*-)
Day 28  2  4.2100  0.0707    (-*-)
Day 3   2  7.0000  0.0990                               (-*-)
Day 7   2  6.8300  0.1131                             (-*-)
                             +---------+---------+---------+---------
                           4.0       5.0       6.0       7.0

Pooled StDev = 0.1233


Grouping Information Using Tukey Method

C1      N    Mean  Grouping
Day 3   2  7.0000  A
Day 0   2  7.0000  A
Day 7   2  6.8300  A
Day 14  2  4.5700    B
Day 21  2  4.4300    B
Day 28  2  4.2100    B

Means that do not share a letter are significantly different.
```

One-way ANOVA of heat-treated donkey milk samples stored at 25 °C versus days of storage

```
Source  DF      SS      MS      F      P
C1       5  4.9938  0.9988  45.74  0.000
Error    6  0.1310  0.0218
Total   11  5.1248

S = 0.1478   R-Sq = 97.44%   R-Sq(adj) = 95.31%


                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level   N    Mean   StDev  ---+---------+---------+---------+------
Day 0   2  7.2100  0.2546                              (---*---)
Day 14  2  6.1000  0.1414            (----*---)
Day 21  2  5.4800  0.0990  (---*----)
Day 28  2  5.6200  0.0707    (----*---)
Day 3   2  6.9800  0.1556                          (---*----)
Day 7   2  6.4800  0.0849                  (---*---)
```

```
   ---+---------+---------+---------+------
    5.40      6.00      6.60      7.20

Pooled StDev = 0.1478


Grouping Information Using Tukey Method

C1      N    Mean  Grouping
Day 0   2  7.2100  A
Day 3   2  6.9800  A B
Day 7   2  6.4800    B C
Day 14  2  6.1000      C D
Day 28  2  5.6200        D E
Day 21  2  5.4800          E

Means that do not share a letter are significantly different.
```

ANOVA results of Titratable acidity% during shelf-life analysis

General Linear Model: LA% versus Day, Treatment, Temperature

```
Factor       Type   Levels  Values
Day          fixed       6  0, 3, 7, 14, 21, 28
Treatment    fixed       3  h, p, u
Temperature  fixed       2  4, 25


Analysis of Variance for LA%, using Adjusted SS for Tests

Source                     DF    Seq SS    Adj SS    Adj MS         F
P
Day                         5  0.502101  0.502101  0.100420  17338.72
0.000
Treatment                   2  0.071372  0.071372  0.035686   6161.64
0.000
Temperature                 1  0.433380  0.433380  0.433380  74828.29
0.000
Day*Treatment              10  0.040278  0.040278  0.004028    695.44
0.000
Day*Temperature             5  0.202545  0.202545  0.040509   6994.34
0.000
Treatment*Temperature       2  0.009710  0.009710  0.004855    838.30
0.000
Day*Treatment*Temperature  10  0.025512  0.025512  0.002551    440.49
0.000
Error                      36  0.000209  0.000209  0.000006
Total                      71  1.285106


S = 0.00240659   R-Sq = 99.98%   R-Sq(adj) = 99.97%


Grouping Information Using Tukey Method and 95.0% Confidence

Day   N  Mean  Grouping
28   12   0.3  A
21   12   0.2    B
```

14	12	0.2	C
7	12	0.1	D
3	12	0.0	E
0	12	0.0	F

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Treatment	N	Mean	Grouping
u	24	0.2	A
p	24	0.1	B
h	24	0.1	C

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Temperature	N	Mean	Grouping
25	36	0.2	A
4	36	0.1	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Day	Treatment	N	Mean	Grouping
28	u	4	0.3	A
21	u	4	0.3	B
14	u	4	0.3	C
28	p	4	0.2	D
28	h	4	0.2	E
21	p	4	0.2	F
14	p	4	0.2	G
7	u	4	0.2	G
21	h	4	0.2	H
7	p	4	0.1	I
14	h	4	0.1	J
7	h	4	0.1	K
3	h	4	0.0	L
3	p	4	0.0	L
3	u	4	0.0	L
0	u	4	0.0	L
0	p	4	0.0	L
0	h	4	0.0	M

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Day	Temperature	N	Mean	Grouping
28	25	6	0.4	A
21	25	6	0.3	B
14	25	6	0.3	C
7	25	6	0.2	D
28	4	6	0.1	E
21	4	6	0.1	F

14	4	6	0.1	G
3	25	6	0.0	H
7	4	6	0.0	I
3	4	6	0.0	I
0	4	6	0.0	I
0	25	6	0.0	I

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Treatment	Temperature	N	Mean	Grouping
u	25	12	0.3	A
p	25	12	0.2	B
h	25	12	0.2	C
u	4	12	0.1	D
p	4	12	0.0	E
h	4	12	0.0	E

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Day	Treatment	Temperature	N	Mean	Grouping
28	u	25	2	0.4	A
28	p	25	2	0.4	B
14	u	25	2	0.4	C
21	u	25	2	0.4	D
28	h	25	2	0.4	E
21	p	25	2	0.3	E
7	u	25	2	0.3	F
14	p	25	2	0.3	F
21	h	25	2	0.3	G
7	p	25	2	0.3	G
28	u	4	2	0.2	H
21	u	4	2	0.2	I
14	h	25	2	0.2	I
7	h	25	2	0.2	J
14	u	4	2	0.1	K
28	h	4	2	0.1	L
28	p	4	2	0.1	L
21	h	4	2	0.1	M
3	h	25	2	0.1	M N
14	p	4	2	0.0	N O
21	p	4	2	0.0	N O
3	u	25	2	0.0	N O
3	p	25	2	0.0	N O
7	p	4	2	0.0	O P
14	h	4	2	0.0	O P
0	u	4	2	0.0	O P
7	u	4	2	0.0	O P
3	p	4	2	0.0	O P
0	p	4	2	0.0	O P
3	u	4	2	0.0	O P
0	p	25	2	0.0	O P
0	u	25	2	0.0	O P
7	h	4	2	0.0	P
3	h	4	2	0.0	P
0	h	4	2	0.0	P

```
 0   h          25           2   0.0                              P

Means that do not share a letter are significantly different.
```

One-way ANOVA of untreated donkey milk samples stored at 4 °C versus days of storage

```
Source  DF         SS         MS       F      P
C1       5  0.0695040  0.0139008  672.62  0.000
Error    6  0.0001240  0.0000207
Total   11  0.0696280

S = 0.004546   R-Sq = 99.82%   R-Sq(adj) = 99.67%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level   N     Mean    StDev  ----+---------+---------+---------+-----
Day 0   2  0.03600  0.00283  (*-)
Day 14  2  0.12600  0.00283                  (*-)
Day 21  2  0.18900  0.00424                              (-*)
Day 28  2  0.21900  0.00707                                    (-*)
Day 3   2  0.03600  0.00566  (*-)
Day 7   2  0.03600  0.00283  (*-)
                             ----+---------+---------+---------+-----
                               0.050     0.100     0.150     0.200

Pooled StDev = 0.00455


Grouping Information Using Tukey Method

C1      N     Mean  Grouping
Day 28  2  0.21900  A
Day 21  2  0.18900    B
Day 14  2  0.12600      C
Day 7   2  0.03600        D
Day 3   2  0.03600        D
Day 0   2  0.03600        D

Means that do not share a letter are significantly different.
```

One-way ANOVA of HHP-treated donkey milk samples stored at 4 °C versus days of storage

```
Source  DF         SS         MS      F      P
C1       5  0.0022830  0.0004566  16.50  0.002
Error    6  0.0001660  0.0000277
Total   11  0.0024490

S = 0.005260   R-Sq = 93.22%   R-Sq(adj) = 87.57%


                               Individual 95% CIs For Mean Based on
                               Pooled StDev
Level   N      Mean     StDev  --+---------+---------+---------+-------
Day 0   2  0.036000  0.005657  (-----*-----)
Day 14  2  0.045000  0.005657        (-----*-----)
```

```
Day 21  2  0.045000  0.005657        (-----*-----)
Day 28  2  0.075000  0.004243                            (-----*-----)
Day 3   2  0.036000  0.001414  (-----*-----)
Day 7   2  0.036000  0.007071  (-----*-----)
                               --+---------+---------+---------+-------
                               0.030     0.045     0.060     0.075

Pooled StDev = 0.005260


Grouping Information Using Tukey Method

C1      N      Mean  Grouping
Day 28  2  0.075000  A
Day 21  2  0.045000    B
Day 14  2  0.045000    B
Day 7   2  0.036000    B
Day 3   2  0.036000    B
Day 0   2  0.036000    B

Means that do not share a letter are significantly different.
```

One-way ANOVA of heat-treated donkey milk samples stored at 4 °C versus days of storage

```
Source  DF         SS         MS      F      P
C1       5  0.0053190  0.0010638  62.58  0.000
Error    6  0.0001020  0.0000170
Total   11  0.0054210

S = 0.004123   R-Sq = 98.12%   R-Sq(adj) = 96.55%



Level   N      Mean     StDev
Day 0   2  0.027000  0.002828
Day 14  2  0.036000  0.002828
Day 21  2  0.063000  0.005657
Day 28  2  0.081000  0.007071
Day 3   2  0.027000  0.001414
Day 7   2  0.027000  0.001414

        Individual 95% CIs For Mean Based on Pooled StDev
Level     +---------+---------+---------+---------
Day 0     (---*--)
Day 14        (---*---)
Day 21                      (---*--)
Day 28                               (--*---)
Day 3     (---*--)
Day 7     (---*--)
          +---------+---------+---------+---------
        0.020     0.040     0.060     0.080

Pooled StDev = 0.004123
```

```
Grouping Information Using Tukey Method

C1       N      Mean  Grouping
Day 28  2  0.081000  A
Day 21  2  0.063000    B
Day 14  2  0.036000      C
Day 0   2  0.027000      C
Day 7   2  0.027000      C
Day 3   2  0.027000      C

Means that do not share a letter are significantly different.
```

One-way ANOVA of untreated donkey milk samples stored at 25 °C versus days of storage

```
Source  DF         SS         MS        F      P
C1       5  0.3048907  0.0609781  2730.36  0.000
Error    6  0.0001340  0.0000223
Total   11  0.3050247

S = 0.004726   R-Sq = 99.96%   R-Sq(adj) = 99.92%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level   N     Mean    StDev  --------+---------+---------+---------+-
Day 0   2  0.03600  0.00283  (*)
Day 14  2  0.38700  0.00707                               *)
Day 21  2  0.36900  0.00424                             (*
Day 28  2  0.42100  0.00566                                   (*)
Day 3   2  0.04500  0.00424   (*
Day 7   2  0.30600  0.00283                        (*
                             --------+---------+---------+---------+-
                                  0.12      0.24      0.36      0.48

Pooled StDev = 0.00473


Grouping Information Using Tukey Method

C1       N     Mean  Grouping
Day 28  2  0.42100  A
Day 14  2  0.38700    B
Day 21  2  0.36900    B
Day 7   2  0.30600      C
Day 3   2  0.04500        D
Day 0   2  0.03600        D
```

One-way ANOVA of HHP-treated donkey milk samples stored at 25 °C versus days of storage

```
Source  DF         SS         MS        F      P
C1       5  0.2377777  0.0475555  1805.91  0.000
Error    6  0.0001580  0.0000263
Total   11  0.2379357
```

```
S = 0.005132   R-Sq = 99.93%   R-Sq(adj) = 99.88%


                               Individual 95% CIs For Mean Based on
                               Pooled StDev
Level    N     Mean    StDev   -------+---------+---------+---------+--
Day 0    2  0.03600  0.00566   (*
Day 14   2  0.30600  0.00283                           (*
Day 21   2  0.34200  0.00707                              (*)
Day 28   2  0.39800  0.00566                                    (*)
Day 3    2  0.04500  0.00424    *)
Day 7    2  0.25200  0.00424                       (*)
                               -------+---------+---------+---------+--
                                    0.10      0.20      0.30      0.40

Pooled StDev = 0.00513


Grouping Information Using Tukey Method

C1       N     Mean  Grouping
Day 28   2  0.39800  A
Day 21   2  0.34200    B
Day 14   2  0.30600      C
Day 7    2  0.25200        D
Day 3    2  0.04500          E
Day 0    2  0.03600          E

Means that do not share a letter are significantly different.
```

One-way ANOVA of heat-treated donkey milk samples stored at 25 °C versus days of storage

```
Source   DF         SS         MS        F      P
C1        5  0.1506600  0.0301320  1205.28  0.000
Error     6  0.0001500  0.0000250
Total    11  0.1508100

S = 0.005   R-Sq = 99.90%   R-Sq(adj) = 99.82%


                               Individual 95% CIs For Mean Based on
                               Pooled StDev
Level    N     Mean    StDev   --------+---------+---------+---------+-
Day 0    2  0.02700  0.00283   (*)
Day 14   2  0.18000  0.00424                (*)
Day 21   2  0.26100  0.00424                        (*)
Day 28   2  0.35100  0.00849                                 (*)
Day 3    2  0.05400  0.00141     *)
Day 7    2  0.15300  0.00566             (*)
                               --------+---------+---------+---------+-
                                     0.10      0.20      0.30      0.40

Pooled StDev = 0.00500
```

Grouping Information Using Tukey Method

C1	N	Mean	Grouping
Day 28	2	0.35100	A
Day 21	2	0.26100	B
Day 14	2	0.18000	C
Day 7	2	0.15300	D
Day 3	2	0.05400	E
Day 0	2	0.02700	F

Means that do not share a letter are significantly different.

ANOVA results of flow consistency index (K) during shelf-life analysis

General Linear Model: K versus Day, Treatment, Temperature

Factor	Type	Levels	Values
Day	fixed	6	0, 3, 7, 14, 21, 28
Treatment	fixed	3	h, p, u
Temperature	fixed	2	4, 25

Analysis of Variance for K, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F
Day	5	0.0001569	0.0001569	0.0000314	328227.60
Treatment	2	0.0001458	0.0001458	0.0000729	762134.39
Temperature	1	0.0000156	0.0000156	0.0000156	162777.96
Day*Treatment	10	0.0000981	0.0000981	0.0000098	102540.44
Day*Temperature	5	0.0000116	0.0000116	0.0000023	24328.12
Treatment*Temperature	2	0.0000073	0.0000073	0.0000037	38295.81
Day*Treatment*Temperature	10	0.0000058	0.0000058	0.0000006	6014.69
Error	36	0.0000000	0.0000000	0.0000000	
Total	71	0.0004411			

Source	P
Day	0.000
Treatment	0.000
Temperature	0.000
Day*Treatment	0.000
Day*Temperature	0.000
Treatment*Temperature	0.000
Day*Treatment*Temperature	0.000
Error	
Total	

S = 9.779224E-06   R-Sq = 100.00%   R-Sq(adj) = 100.00%

Unusual Observations for K

Obs	K	Fit	SE Fit	Residual	St Resid
67	0.010490	0.010450	0.000007	0.000040	5.78 R
68	0.010410	0.010450	0.000007	-0.000040	-5.78 R

R denotes an observation with a large standardized residual.

```
Grouping Information Using Tukey Method and 95.0% Confidence

Day   N  Mean  Grouping
28   12   0.0  A
21   12   0.0    B
14   12   0.0      C
 7   12   0.0        D
 3   12   0.0          E
 0   12   0.0            F

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95.0% Confidence

Treatment   N  Mean  Grouping
h          24   0.0  A
p          24   0.0    B
u          24   0.0      C

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95.0% Confidence

Temperature   N  Mean  Grouping
25           36   0.0  A
 4           36   0.0    B

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95.0% Confidence

Day  Treatment  N  Mean  Grouping
28   h          4   0.0  A
21   h          4   0.0    B
28   p          4   0.0      C
14   h          4   0.0        D
21   p          4   0.0          E
 7   h          4   0.0            F
14   p          4   0.0              G
 3   h          4   0.0                H
 0   h          4   0.0                  I
 7   p          4   0.0                  I
 3   p          4   0.0                    J
 0   p          4   0.0                      K
28   u          4   0.0                        L
21   u          4   0.0                          M
14   u          4   0.0                            N
 7   u          4   0.0                            N O
 3   u          4   0.0                              O
 0   u          4   0.0                              O

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95.0% Confidence
```

Day	Temperature	N	Mean	Grouping
28	25	6	0.0	A
21	25	6	0.0	B
28	4	6	0.0	C
14	25	6	0.0	D
21	4	6	0.0	E
14	4	6	0.0	F
7	25	6	0.0	F
3	25	6	0.0	G
7	4	6	0.0	H
3	4	6	0.0	I
0	25	6	0.0	J
0	4	6	0.0	J

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Treatment	Temperature	N	Mean	Grouping
h	25	12	0.0	A
h	4	12	0.0	B
p	25	12	0.0	C
p	4	12	0.0	D
u	25	12	0.0	E
u	4	12	0.0	F

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Day	Treatment	Temperature	N	Mean
28	h	25	2	0.0
21	h	25	2	0.0
28	h	4	2	0.0
28	p	25	2	0.0
21	h	4	2	0.0
14	h	25	2	0.0
21	p	25	2	0.0
28	p	4	2	0.0
14	h	4	2	0.0
7	h	25	2	0.0
14	p	25	2	0.0
3	h	25	2	0.0
21	p	4	2	0.0
7	h	4	2	0.0
7	p	25	2	0.0
3	h	4	2	0.0
14	p	4	2	0.0
0	h	25	2	0.0
0	h	4	2	0.0
3	p	25	2	0.0
7	p	4	2	0.0
3	p	4	2	0.0
0	p	4	2	0.0
0	p	25	2	0.0
28	u	25	2	0.0
21	u	25	2	0.0
28	u	4	2	0.0
14	u	25	2	0.0

```
21   u           4            2   0.0
 7   u          25            2   0.0
14   u           4            2   0.0
 3   u          25            2   0.0
 7   u           4            2   0.0
 3   u           4            2   0.0
 0   u           4            2   0.0
 0   u          25            2   0.0

Day  Treatment  Temperature   Grouping
28   h          25            A
21   h          25              B
28   h           4               C
28   p          25                D
21   h           4                 E
14   h          25                  F
21   p          25                   G
28   p           4                    H
14   h           4                     I
 7   h          25                      J
14   p          25                       K
 3   h          25                        L
21   p           4                         M
 7   h           4                          N
 7   p          25                           O
 3   h           4                            P
14   p           4                            P
 0   h          25                             Q
 0   h           4                             Q
 3   p          25                             Q
 7   p           4                              R
 3   p           4                               S
 0   p           4                                T
 0   p          25                                T
28   u          25                                T
21   u          25                                 U
28   u           4                                 U
14   u          25                                 U V
21   u           4                                   V W
 7   u          25                                   V W X
14   u           4                                   V W X
 3   u          25                                     W X
 7   u           4                                       X
 3   u           4                                       X
 0   u           4                                       X
 0   u          25                                       X

Means that do not share a letter are significantly different.
```

One-way ANOVA of untreated donkey milk samples stored at 4 °C versus days of storage

```
Source  DF         SS         MS          F      P
Days     4  0.0000000  0.0000000  295578.31  0.000
Error    5  0.0000000  0.0000000
Total    9  0.0000000

S = 5.709641E-08   R-Sq = 100.00%   R-Sq(adj) = 100.00%
```

```
                                       Individual 95% CIs For Mean Based on
                                       Pooled StDev
Level  N         Mean        StDev    -+---------+---------+---------+-----
---
 0     2  2.86300E-05  4.24264E-08    *
 3     2  3.35500E-05  6.36396E-08       *
 7     2  4.03400E-05  5.65685E-08            *
14     2  6.02550E-05  6.36396E-08                         *
21     2  8.17400E-05  5.65685E-08                                       *)
                                      -+---------+---------+---------+-----
---
                                       0.000030  0.000045  0.000060  0.000075

Pooled StDev = 0.0000000571


Grouping Information Using Tukey Method

Days  N         Mean  Grouping
21    2  8.17400E-05  A
14    2  6.02550E-05    B
 7    2  4.03400E-05      C
 3    2  3.35500E-05        D
 0    2  2.86300E-05          E

Means that do not share a letter are significantly different.
```

One-way ANOVA of heat-treated donkey milk samples stored at 4 °C versus days of storage

```
Source  DF         SS         MS          F      P
Days     4  0.0000145  0.0000036  215402.78  0.000
Error    5  0.0000000  0.0000000
Total    9  0.0000145

S = 0.000004098   R-Sq = 100.00%   R-Sq(adj) = 100.00%

                                     Individual 95% CIs For Mean Based on
                                     Pooled StDev
Level  N        Mean       StDev  --+---------+---------+---------+-------
 0     2  0.00081843  0.00000057  *
 3     2  0.00093142  0.00000057   *
 7     2  0.00123602  0.00000566      *
14     2  0.00220245  0.00000283                *
21     2  0.00404680  0.00000658                                 *)
                                  --+---------+---------+---------+-------
                                  0.0010    0.0020    0.0030    0.0040
Pooled StDev = 0.00000410

Grouping Information Using Tukey Method

Days  N        Mean  Grouping
21    2  0.00404680  A
14    2  0.00220245    B
 7    2  0.00123602      C
 3    2  0.00093142        D
 0    2  0.00081843          E

Means that do not share a letter are significantly different.
```

One-way ANOVA of HHP-treated donkey milk samples stored at 4 °C versus days of storage

```
Source  DF         SS         MS          F      P
Days     4  0.0000012  0.0000003  102064.53  0.000
Error    5  0.0000000  0.0000000
Total    9  0.0000012

S = 0.000001709   R-Sq = 100.00%   R-Sq(adj) = 100.00%


                                  Individual 95% CIs For Mean Based on
                                  Pooled StDev
Level  N        Mean       StDev  --+---------+---------+---------+-------
 0     2  0.00045560  0.00000064  *
 3     2  0.00052337  0.00000049     *
 7     2  0.00058829  0.00000040       (*
14     2  0.00089559  0.00000059                    *
21     2  0.00139261  0.00000367                                        *
                                  --+---------+---------+---------+-------
                                  0.00050   0.00075   0.00100   0.00125

Pooled StDev = 0.00000171


Grouping Information Using Tukey Method

Days  N        Mean  Grouping
21    2  0.00139261  A
14    2  0.00089559    B
 7    2  0.00058829      C
 3    2  0.00052337        D
 0    2  0.00045560          E

Means that do not share a letter are significantly different.
```

One-way ANOVA of untreated donkey milk samples stored at 25 °C versus days of storage

```
Source  DF         SS         MS          F      P
Days     4  0.0000000  0.0000000  173607.38  0.000
Error    5  0.0000000  0.0000000
Total    9  0.0000000

S = 0.0000001275   R-Sq = 100.00%   R-Sq(adj) = 100.00%


Level  N         Mean        StDev
 0     2  0.000028630  0.000000042
 3     2  0.000051550  0.000000071
 7     2  0.000061851  0.000000071
14     2  0.000092250  0.000000070
21     2  0.000125186  0.000000254
```

```
       Individual 95% CIs For Mean Based on Pooled StDev
Level  ---------+---------+---------+---------+
 0     *)
 3               *
 7                   *
14                               *
21                                            *
       ---------+---------+---------+---------+
              0.000050  0.000075  0.000100  0.000125

Pooled StDev = 0.000000127


Grouping Information Using Tukey Method

Days  N         Mean  Grouping
21    2  0.000125186  A
14    2  0.000092250    B
 7    2  0.000061851      C
 3    2  0.000051550        D
 0    2  0.000028630          E

Means that do not share a letter are significantly different.
```

One-way ANOVA of heat-treated donkey milk samples stored at 25 °C versus days of storage

```
Source  DF         SS         MS          F      P
Days     4  0.0000598  0.0000150  835084.60  0.000
Error    5  0.0000000  0.0000000
Total    9  0.0000598

S = 0.000004232   R-Sq = 100.00%   R-Sq(adj) = 100.00%


                                  Individual 95% CIs For Mean Based on
                                  Pooled StDev
Level  N        Mean       StDev  ------+---------+---------+---------+---
 0     2  0.00081845  0.00000053  *
 3     2  0.00147637  0.00000707     *
 7     2  0.00203210  0.00000243        *
14     2  0.00386331  0.00000459                *
21     2  0.00761253  0.00000351                                   *
                                  ------+---------+---------+---------+---
                                      0.0020    0.0040    0.0060    0.0080

Pooled StDev = 0.00000423


Grouping Information Using Tukey Method

Days  N        Mean  Grouping
21    2  0.00761253  A
14    2  0.00386331    B
 7    2  0.00203210      C
 3    2  0.00147637        D
 0    2  0.00081845          E

Means that do not share a letter are significantly different.
```

One-way ANOVA of HHP-treated donkey milk samples stored at 25 °C versus days of storage

```
Source   DF         SS         MS          F      P
Days      4  0.0000110  0.0000028  136370.37  0.000
Error     5  0.0000000  0.0000000
Total     9  0.0000110

S = 0.000004497   R-Sq = 100.00%   R-Sq(adj) = 100.00%


                                  Individual 95% CIs For Mean Based on
                                  Pooled StDev
Level  N        Mean       StDev  ----+---------+---------+---------+-----
 0     2  0.00045558  0.00000061  *
 3     2  0.00081744  0.00000061      *
 7     2  0.00102443  0.00000614         *
14     2  0.00181450  0.00000624                   *
21     2  0.00340661  0.00000488                                    (*
                                  ----+---------+---------+---------+-----
                                    0.00080   0.00160   0.00240   0.00320

Pooled StDev = 0.00000450


Grouping Information Using Tukey Method

Days  N        Mean  Grouping
21    2  0.00340661  A
14    2  0.00181450    B
 7    2  0.00102443      C
 3    2  0.00081744        D
 0    2  0.00045558          E

Means that do not share a letter are significantly different.
```

ANOVA results of flow behavior index (n) during shelf-life analysis

General Linear Model: n versus Day, Treatment, Temperature

```
Factor       Type   Levels  Values
Day          fixed       6  0, 3, 7, 14, 21, 28
Treatment    fixed       3  h, p, u
Temperature  fixed       2  4, 25


Analysis of Variance for n, using Adjusted SS for Tests

Source                     DF    Seq SS    Adj SS    Adj MS        F
P
Day                         5  0.463565  0.463565  0.092713  3463.27
0.000
Treatment                   2  0.415688  0.415688  0.207844  7763.96
0.000
Temperature                 1  0.043157  0.043157  0.043157  1612.13
0.000
```

```
Day*Treatment              10  0.068589  0.068589  0.006859   256.21
0.000
Day*Temperature             5  0.017443  0.017443  0.003489   130.31
0.000
Treatment*Temperature       2  0.011846  0.011846  0.005923   221.25
0.000
Day*Treatment*Temperature  10  0.011233  0.011233  0.001123    41.96
0.000
Error                      36  0.000964  0.000964  0.000027
Total                      71  1.032485


S = 0.00517401   R-Sq = 99.91%   R-Sq(adj) = 99.82%


Grouping Information Using Tukey Method and 95.0% Confidence

Day   N  Mean  Grouping
 0   12   1.1  A
 3   12   1.0    B
 7   12   0.9      C
14   12   0.9        D
21   12   0.9          E
28   12   0.8            F

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95.0% Confidence

Treatment   N  Mean  Grouping
p          24   1.0  A
u          24   1.0    B
h          24   0.8      C

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95.0% Confidence

Temperature   N  Mean  Grouping
 4           36   1.0  A
25           36   0.9    B

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95.0% Confidence

Day  Treatment  N  Mean  Grouping
 0   u          4   1.1  A
 3   u          4   1.1    B
 0   p          4   1.1    B
 3   p          4   1.1      C
 7   u          4   1.0        D
14   p          4   1.0          E
 7   p          4   1.0          E
21   p          4   1.0          E
 0   h          4   1.0            F
14   u          4   0.9              G
28   p          4   0.9                H
```

3	h	4	0.9	I
21	u	4	0.9	I
28	u	4	0.8	J
7	h	4	0.8	J K
14	h	4	0.8	K
21	h	4	0.8	L
28	h	4	0.7	M

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Day	Temperature	N	Mean	Grouping
0	25	6	1.1	A
0	4	6	1.1	A
3	4	6	1.0	B
3	25	6	1.0	C
7	4	6	1.0	D
14	4	6	0.9	E
7	25	6	0.9	F
21	4	6	0.9	F
14	25	6	0.9	G
28	4	6	0.9	H
21	25	6	0.8	I
28	25	6	0.8	J

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Treatment	Temperature	N	Mean	Grouping
p	4	12	1.0	A
u	4	12	1.0	B
p	25	12	1.0	C
u	25	12	1.0	D
h	4	12	0.8	E
h	25	12	0.8	F

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Day	Treatment	Temperature	N	Mean	Grouping
0	u	4	2	1.1	A
0	u	25	2	1.1	A
3	u	4	2	1.1	B
0	p	25	2	1.1	B C
0	p	4	2	1.1	B C
3	p	4	2	1.1	C D
21	p	4	2	1.1	C D E
3	u	25	2	1.1	C D E F
14	p	4	2	1.1	D E F
7	u	4	2	1.1	E F
3	p	25	2	1.1	F
7	p	4	2	1.0	G
7	u	25	2	1.0	G H
7	p	25	2	1.0	H I
0	h	25	2	1.0	H I

```
 0   h          4           2   1.0              H I
28   p          4           2   1.0                I
14   u          4           2   1.0                I J
14   p         25           2   0.9                  J
21   p         25           2   0.9                   K
14   u         25           2   0.9                   K
21   u          4           2   0.9                 K L
 3   h          4           2   0.9                   L M
28   u          4           2   0.9                     M
 3   h         25           2   0.9                      N
28   p         25           2   0.8                      N O
21   u         25           2   0.8                        O P
 7   h         25           2   0.8                        O P
 7   h          4           2   0.8                        O P
14   h          4           2   0.8                          P Q
14   h         25           2   0.8                          P Q
21   h          4           2   0.8                            Q R
28   u         25           2   0.8                              R S
21   h         25           2   0.8                              R S
28   h          4           2   0.8                                S
28   h         25           2   0.7                                 T

Means that do not share a letter are significantly different.
```

One-way ANOVA of untreated donkey milk samples stored at 4 °C versus days of storage

```
Source  DF         SS         MS       F      P
Days     4  0.0784383  0.0196096  520.84  0.000
Error    5  0.0001883  0.0000377
Total    9  0.0786265

S = 0.006136   R-Sq = 99.76%   R-Sq(adj) = 99.57%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  --+---------+---------+---------+-------
 0     2  1.14550  0.00636                                  (-*)
 3     2  1.10575  0.00601                            (-*-)
 7     2  1.05325  0.00460                     (*-)
14     2  0.96500  0.00707         (-*)
21     2  0.90550  0.00636  (*-)
                            --+---------+---------+---------+-------
                            0.910     0.980     1.050     1.120

Pooled StDev = 0.00614

Grouping Information Using Tukey Method

Days  N     Mean  Grouping
 0    2  1.14550  A
 3    2  1.10575    B
 7    2  1.05325      C
14    2  0.96500        D
21    2  0.90550          E

Means that do not share a letter are significantly different.
```

One-way ANOVA of heat-treated donkey milk samples stored at 4 °C versus days of storage

```
Source  DF         SS         MS       F      P
Days     4  0.0491275  0.0122819  809.35  0.000
Error    5  0.0000759  0.0000152
Total    9  0.0492034

S = 0.003896   R-Sq = 99.85%   R-Sq(adj) = 99.72%


                            Individual 95% CIs For Mean Based on Pooled
StDev
Level  N     Mean    StDev  ---------+---------+---------+---------+
 0     2  0.98450  0.00636                                 (*)
 3     2  0.89138  0.00194                 (-*)
 7     2  0.82137  0.00194       (*)
14     2  0.81088  0.00124     (*)
21     2  0.79537  0.00513  (-*)
                            ---------+---------+---------+---------+
                                 0.840     0.900     0.960     1.020

Pooled StDev = 0.00390


Grouping Information Using Tukey Method

Days  N     Mean  Grouping
 0    2  0.98450  A
 3    2  0.89138    B
 7    2  0.82137      C
14    2  0.81088      C D
21    2  0.79537        D

Means that do not share a letter are significantly different.
```

One-way ANOVA of HHP-treated donkey milk samples stored at 4 °C versus days of storage

```
Source  DF         SS         MS      F      P
Days     4  0.0065570  0.0016393  57.36  0.000
Error    5  0.0001429  0.0000286
Total    9  0.0066999

S = 0.005346   R-Sq = 97.87%   R-Sq(adj) = 96.16%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  --------+---------+---------+---------+-
 0     2  1.08450  0.00636                            (---*---)
 3     2  1.08150  0.00212                           (---*--)
 7     2  1.01465  0.00658  (---*---)
14     2  1.06267  0.00309                    (---*---)
21     2  1.07475  0.00672                         (---*---)
                            --------+---------+---------+---------+-
                                1.025     1.050     1.075     1.100
```

```
Pooled StDev = 0.00535


Grouping Information Using Tukey Method

Days  N     Mean  Grouping
 0    2  1.08450  A
 3    2  1.08150  A B
21    2  1.07475  A B
14    2  1.06267    B
 7    2  1.01465      C

Means that do not share a letter are significantly different.
```

One-way ANOVA of untreated donkey milk samples stored at 25 °C versus days of storage

```
Source  DF         SS         MS       F      P
Days     4  0.1241481  0.0310370  920.45  0.000
Error    5  0.0001686  0.0000337
Total    9  0.1243167

S = 0.005807   R-Sq = 99.86%   R-Sq(adj) = 99.76%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  --------+---------+---------+---------+-
 0     2  1.14545  0.00629                                (-*)
 3     2  1.06454  0.00630                        (*-)
 7     2  0.99380  0.00537                 (*)
14     2  0.91596  0.00560          (*)
21     2  0.82611  0.00539  (*)
                            --------+---------+---------+---------+-
                                 0.90      1.00      1.10      1.20

Pooled StDev = 0.00581


Grouping Information Using Tukey Method

Days  N     Mean  Grouping
 0    2  1.14545  A
 3    2  1.06454    B
 7    2  0.99380      C
14    2  0.91596        D
21    2  0.82611          E

Means that do not share a letter are significantly different.
```

One-way ANOVA of heat-treated donkey milk samples stored at 25 °C versus days of storage

```
Source  DF         SS         MS       F      P
Days     4  0.0528457  0.0132114  429.80  0.000
Error    5  0.0001537  0.0000307
```

```
Total     9  0.0529994

S = 0.005544   R-Sq = 99.71%   R-Sq(adj) = 99.48%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  ---+---------+---------+---------+------
 0     2  0.98455  0.00630                                    (-*-)
 3     2  0.85436  0.00605                (*-)
 7     2  0.82252  0.00350          (-*-)
14     2  0.80679  0.00500        (*-)
21     2  0.77457  0.00634  (-*-)
                            ---+---------+---------+---------+------
                             0.780     0.840     0.900     0.960

Pooled StDev = 0.00554


Grouping Information Using Tukey Method

Days  N     Mean  Grouping
 0    2  0.98455  A
 3    2  0.85436    B
 7    2  0.82252      C
14    2  0.80679      C
21    2  0.77457        D

Means that do not share a letter are significantly different.
```

One-way ANOVA of HHP-treated donkey milk samples stored at 25 °C versus days of storage

```
Source  DF         SS         MS       F      P
Days     4  0.0385171  0.0096293  360.86  0.000
Error    5  0.0001334  0.0000267
Total    9  0.0386505

S = 0.005166   R-Sq = 99.65%   R-Sq(adj) = 99.38%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  -------+---------+---------+---------+--
 0     2  1.08454  0.00630                                 (-*-)
 3     2  1.05303  0.00420                           (-*)
 7     2  0.98621  0.00525               (-*-)
14     2  0.94449  0.00623       (-*-)
21     2  0.92225  0.00312  (*-)
                            -------+---------+---------+---------+--
                                 0.950     1.000     1.050     1.100


Pooled StDev = 0.00517
```

```
Grouping Information Using Tukey Method

Days  N      Mean  Grouping
 0    2  1.08454  A
 3    2  1.05303    B
 7    2  0.98621      C
14    2  0.94449        D
21    2  0.92225          E

Means that do not share a letter are significantly different.
```

B. Experimental Results

Processing Conditions (MPa-°C-min)	Lysozyme content (ppm)	Lactoferrin content (ppm)	TAMB Count
Untreated Sample	900.72	179.52	4.04
200-25-5	779.61	156.74	3.40
200-25-10	755.61	149.05	3.27
200-25-15	749.88	146.40	3.15
200-35-5	773.79	149.72	3.29
200-35-10	751.77	145.16	3.19
200-35-15	742.55	143.06	3.11
200-45-5	755.75	144.74	3.12
200-45-10	744.32	134.16	3.10
200-45-15	723.44	116.53	3.08
400-25-5	740.41	132.19	2.56
400-25-10	738.51	120.63	2.51
400-25-15	703.06	110.05	2.46
400-35-5	736.26	114.17	2.50
400-35-10	723.63	111.62	2.48
400-35-15	686.44	100.94	2.43
400-45-5	718.45	103.77	2.45
400-45-10	693.58	99.23	2.42
400-45-15	676.87	96.89	2.37

500-25-5	678.81	96.40	0.00
500-25-10	656.63	88.07	0.00
500-25-15	643.36	86.25	0.00
500-35-5	651.63	81.30	0.00
500-35-10	645.65	78.97	0.00
500-35-15	633.13	77.40	0.00
500-45-5	647.24	78.24	0.00
500-45-10	642.07	75.40	0.00
500-45-15	623.23	72.33	0.00
0.1-75-1	525.43	51.02	3.32
0.1-75-2	381.73	32.49	2.70