

Differences of Protein Fractions Among Fresh, Frozen and Powdered Donkey Milk

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Received: September 16, 2009; Accepted: November 2, 2009; Revised: November 3, 2009

Abstract: Recently donkey milk has been the focus of several studies because of its special nutritional properties and composition, which is very close to human milk. When a mother cannot breastfeed, or chooses not to breastfeed, the use of a milk substitute must provide the best option to meet the nutritional and health needs of the infant. Donkey milk has been widely used in the past to replace human milk, because chemical composition and protein content are close to that of human milk, and also because the allergenicity of donkey milk is low. The recent studies of the paediatric scientists have demonstrated that infant formulae, which are based on dairy cows milk, are less adapted than donkey milk. In fact, donkey's milk digestibility is higher than cows' milk and similar to human milk, because of the high whey proteins content and the few casein content. Since donkey milk supply is related to its seasonal availability during the year, in this study were evaluated the effects of a specific technological treatment (spray-dryer) and a particular storage temperature (-20°C) on the protein fractions of donkey milk. The results obtained in fresh, frozen and powdered donkey milk showed different values in total proteins, caseins, whey proteins and lysozyme content. The article presents some promising patents on protein fractions among fresh, frozen and powdered donkey milk.

Keywords: Donkey milk, protein fractions, powdered milk, frozen milk, lysozyme.

INTRODUCTION

Milk is a highly nutritious food for both children and adults since it is a source of minerals, vitamins and proteins that possess specific biological properties which make them potential ingredients for human nutrition and promotion of human health. Some of them are antimicrobial factors, such as lactoferrin, lactoperoxidase, lysozyme and N-acetyl- β -D-glucosaminidase: these factors naturally present in milk may have the ability to inhibit a broad spectrum of bacteria [1]. For example, they may function in the digestive tract to reduce the incidence of gastro-intestinal infections or by defending against infections of the mammary gland [2]. A recent invention relates to administration of metal ion-saturated lactoferrin to inhibit tumour formation or growth, maintain or improve white blood cell count and red blood cell count, stimulate the immune system and prevent cancer [3].

The antibacterial properties of these antimicrobial factors make them suitable for use in different applications in the animal productions, dairy and health industries. The amount and the distribution of these various antimicrobial agent is different in milks of different species. Lactoferrin is an iron

binding glycoprotein that exerts its antibacterial effect by two different mechanisms involving two separate domains of the protein [4, 5]. Furthermore lactoferrin may act as a potent antiinflammatory protein at local sites of inflammation, including the respiratory and gastrointestinal tracts [6,7]. Use of lactoferrin for stimulation of skeletal growth, chondrocyte and osteoblast proliferation and inhibition of bone resorption and osteoclast development has been recently patented [8].

Human milk is particularly rich in lactoferrin: the concentration ranges from 1 to 16g/l in the colostrum while in mature milk it is about 1g/l [9, 10]. In bovine colostrum the amount of lactoferrin is from 0.2 to 5g/l and in the mature milk it is 0.1g/l [11]. Peroxidase enzymes can exert their antibacterial effect by oxidative mechanism. The lactoperoxidase in the presence of hydrogen peroxide and thiocyanate catalyzes the synthesis of hypothiocyanate, compound responsible of the antimicrobial activity because it inhibits by oxidation the bacterial metabolic enzymes [12]. Because of its antimicrobial activity, lactoperoxidase has been used as a preservative agent in reducing microflora in bovine milk [12, 13]. Bovine milk lactoperoxidase concentration is about 0.03-0.1g/l [14], while in human milk the amount of this enzyme is 0.77mg/l [15].

Lysozyme (EC 3.2.1.17, muramidase) exerts its antibacterial activity against a number of bacteria catalyzing the hydrolysis of β 1 $\rightarrow$ 4 glycosidic bonds between N-acetylmuramic acid (Mur2Ac) and N-acetylglucosamine (GlcNAc) in the bacterial cell walls polysaccharides [16]. This enzyme functions in association with lactoferrin or immunoglobulin A. Furthermore lysozyme may limit the migration of neutrophils into a damaged tissue and therefore it might

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function as an anti-inflammatory agent. In human milk the concentration of lysozyme is 0.12g/l, and it is highest in human colostrum and pre-colostral milk. In bovine and in goat milk the amount of lysozyme is nearly undetectable, whereas in mare's milk is 1.1g/l [17]. In donkey milk, a previous study [18] revealed a high lysozyme content (1mg/ml), varying during the different stages of lactation, which may be responsible for the low bacterial concentration noted [19]. Furthermore in the literature it is reported that the presence of two variants of lysozyme in donkey milk: LYS A and LYS B that differ for three aminoacid substitutions at position 48, 52 and 61 [20].

In these recent years, milk from domesticated equids (*Equus Caballus* and *Equus asinus*, *Perissodactyla*) has been the focus of several studies because of their nutritional properties and their composition (especially considering proteins and fatty acids contents) which is very close to human milk [19,21]. In particular a previous work [18] evidenced the presence of α S1- and β -caseins, in donkey milk while it was not possible to determine the presence of other types of caseins, such as α S2-, κ - and γ - that may be present in donkey milk but in minute, and therefore undetectable quantities. The high lysozyme content found in donkey milk could be useful to prevent intestine infections in infants [18]. Because of its special nutritional properties donkey milk could be considered suitable for feeding young children affected by severe Ig-E mediated cow's milk allergy and has been proposed as an alternative to cow's milk for children affected by cow's milk protein intolerance [22].

Nevertheless, one of the most important problems in donkey milk supply is related to its seasonal availability during the year. Fertility of donkey female is strictly connected with photoperiod, and the delivering period for donkey females is normally limited in a range of a few months, basically from February to July. The aim of this work was to evaluate the effects on the protein fractions of donkey milk of a particular storage temperature (-20°C), and of a specific technological process "spray-dryer" in which milk is subjected to a strong thermal treatment necessary to produce powdered milk that may affect the activity of some antimicrobial factors, such as lysozyme and lactoperoxidase. Another patent discloses an antimicrobially active peptide comprising a dermcidin (DCD) protein, preferably derived from the C-terminal region for protecting and/or treating human skin against microorganisms [23].

For this purpose the protein profile of donkey milk was investigated in the fresh milk, in the frozen milk and in the powdered milk, furthermore the content and the enzymatic activity of lysozyme was determined in order to verify if this important antimicrobial factor was preserved in different storage conditions or after a strong thermal treatment. Finally a study on the stability of donkey milk lysozyme toward temperatures has been performed.

MATERIALS AND METHODS

Bulk milk samples obtained from twenty Martina Franca breed pluriparous asses in mid-stage of lactation (60 days after foaling) were used, all the asses delivered during March 2007. The animals were machine milked once a month (from

June 2007 to October 2007) following the protocol previously described by [19]. Skimmed milk was prepared from 20ml of fresh milk by centrifugation at 3000g for 30min at 15°C. One aliquot of fresh skimmed milk (10ml) was immediately analyzed; another aliquot (10 ml) was frozen at -20°C and after a period of 3 months analyzed as frozen skimmed milk. The fresh milk was powdered using the technique of two-stage drying system: skimmed milk was firstly treated at 90°C for 25 sec, than dried at 170°C, the volume of milk in the tank was around 8 L/hour.

Before to be analyzed, 1.0g of powdered milk was resuspended in 10ml of distilled water, the reconstituted milk was analyzed as powdered skimmed milk.

Protein concentration was determined by the Bradford protein assay [24].

Fresh, frozen and powdered skimmed milk analysis and lysozyme content determination was performed by a reversed-phase chromatography on HPLC (RP-HPLC) as previously described [18].

For the quantitative determination of lysozyme, α -lactalbumin and β -lactoglobulin milk samples were taken at different stages of lactation: 60, 90, 120, 160, and 190 days after parturition. Donkey's milk lysozyme and whey proteins content was determined by an HPLC method by using a reversed-phase column. Each standard solution of egg white lysozyme (2.0, 1.0, 0.5, 0.25, 0.15mg/ml), bovine milk β -lactoglobulin (0.17, 0.30, 0.75, 1.0, 1.5mg/ml) and bovine milk α -lactalbumin (0.25, 0.35, 0.50, 0.75, 1.0, 1.5mg/ml) was prepared in CL buffer. A 100- μ l solution of each standard was separately loaded on the RP-HPLC column. The area of each standard peak was measured by using the valley-to-valley integration mode and quantification was achieved by a calibration curve obtained relating the concentration in micrograms of each standard loaded in the column to the peak area corresponding to each concentration.

Lysozyme activity was performed by turbidimetric assay according to the method of [25, 26]. The turbidimetric assays were performed by using a Beckman DU 640 UV-VIS spectrophotometer. The effect of temperature on lysozyme activity was investigated in the range +4°C to +70°C. The enzymatic reaction was started by adding the sample to the assay medium adjusted to the specific temperature. The effect of temperature on lysozyme stability was checked by incubating the enzyme at different temperatures (from +4 to +70°C) for 5min, rapidly cooled in ice, centrifuged by a microfuge at high speed and finally assayed at +37°C.

RESULTS

In Table 1, it is shown that the content of total caseins and the total whey proteins in the three kinds of milk determined by the method of [4]. The total whey proteins content, was almost similar in the three types of milk, whereas substantial differences were found in total caseins content, with a value 3 fold and 2 fold lower in the powdered milk and in the frozen milk, respectively with respect to the fresh milk.

The concentration of the total whey proteins was 7.5 mg/ml. The lysozyme, β -lactoglobulin and α -lactalbumin

Table 1. Determination of Total Caseins, Total whey Proteins and Lysozyme Content in Fresh, Frozen and Powdered Donkey Milk

	Fresh (mg/ml)	Frozen (mg/ml)	Powdered (mg/ml)
Total whey proteins	7.50	7.20	6.70
Total caseins	6.60	3.12	2.30
Lysozyme	1.00	1.00	0.94

concentrations (in mg/ml) at different stages of lactation (60, 90, 120, 160, and 190 days after parturition) are shown in Table 2. The lysozyme content was variable during the different stages of lactation with a marked decrease of 160 days after parturition. The mean content of lysozyme in donkey's milk was about 1.0mg/ml. The β -lactoglobulin content proved to be stable during the different stages of lactation with a mean value of about 3.75mg/ml. Finally, the donkey's milk α -lactalbumin content 60 days after parturition was 0.81mg/ml and increased two-fold 90 days after parturition, remaining relatively stable with a mean of about 1.8mg/ml until the last stage of lactation.

The lysozyme activity, in fresh as well as in frozen milk, resulted to be exactly the same: U/ml = 0.035 in the fresh milk and U/ml = 0.035 in the frozen milk, indicating that the freezing and thawing out process in donkey's milk did not affect the activity of this important enzyme. Furthermore fresh donkey's milk lysozyme retained completely its activity when the milk was stored for 1 month at 4°C.

In powdered milk, lysozyme showed a 30% residual activity with respect to fresh and frozen milk, indicating that the spray-dry process utilized in the powderization of milk strongly affected the activity of the enzyme.

Furthermore, in this work the optimum temperature and the thermostability of lysozyme in fresh milk was checked. As shown in Fig. (1) donkey's milk lysozyme started to be active at temperature of about 15°C showing an optimum of temperature of 35-40°C, at 50°C retained 80% of activity and finally it showed a 50% of residual activity, when temperature increased until 70°C. The thermostability of lysozyme was also checked in the range from +5°C to

+70°C. In this experiment the milk sample was incubated for 5min at the desired temperature, and then assayed at 37°C.

The results are shown in Fig. (2): the enzyme is thermostable (about 100% of residual activity) from 4°C to 50°C, when the temperature increases the enzyme activity started to decrease and at 70°C lysozyme showed a 50% of residual activity indicating that an irreversible denaturation process of the enzyme start from this temperature.

DISCUSSION AND CONCLUSION

Fresh donkey milk showed a content in total caseins and whey proteins higher with respect to the frozen milk and powdered milk, furthermore powdered milk showed the lowest total casein content if compared to the fresh and frozen milk. The protein profile evidenced on RP-HPLC and SDS-PAGE resulted to be almost similar in the three different kinds of milk, indicating that the freezing and thawing out process does not affect significantly the quality of milk under this point of view as well as the thermal treatment used to produce powdered milk. In this study a particular attention was directed on the lysozyme content and its enzymatic activity determined in the three different kinds of donkey's milk. In fresh, frozen and powdered milk a very similar lysozyme content was found, whereas the enzymatic activity of lysozyme decreased of about 70% in the powdered milk with respect to fresh and frozen milk because of the effect of the spray-dry process. This latest observation may be important in the choice of a valid method of storage for donkey's milk since the presence of an active lysozyme in the milk may be useful to prevent or to reduce intestinal infections in infants.

Donkey milk lysozyme resulted to be an enzyme quite thermostable, since its thermal denaturation started when the temperature reached at 70°C.

The results of the present study seems indicate a better nutritious value for fresh and frozen donkey's milk when compared with powdered donkey's milk: further investigations need in order to deepen the knowledge of other components of powdered donkey's milk, such as lipid fractions and vitamins content.

CURRENT & FUTURE DEVELOPMENTS

Considering specifically donkey milk protein content, the average of caseins and whey proteins contents of donkey

Table 2. Quantitative Determination of Lysozyme, β -Lactoglobulin, α -Lactalbumin in Different Stages of Lactation

Days After Parturition	Lysozyme (mg/ml)	β -Lactoglobulin (mg/ml)	α -Lactalbumin (mg/ml)
60	1.34	Not determined	0.81
90	0.94	4.13	1.97
120	1.03	3.60	1.87
160	0.82	3.69	1.74
190	0.76	3.60	1.63

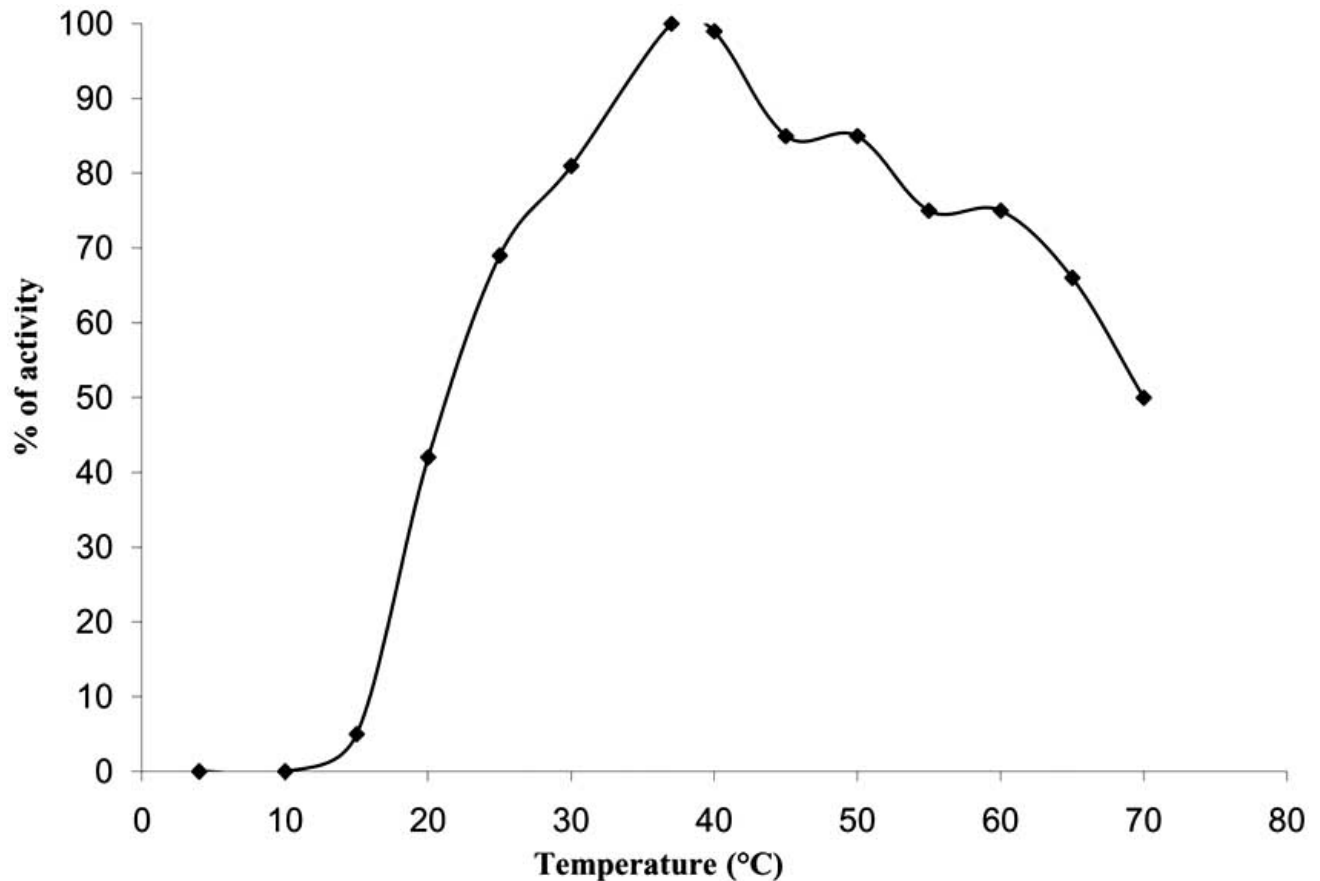


Fig. (1). Effect of temperature on lysozyme activity. The enzymatic activity was measured at different temperatures (from +5 to +70°C).

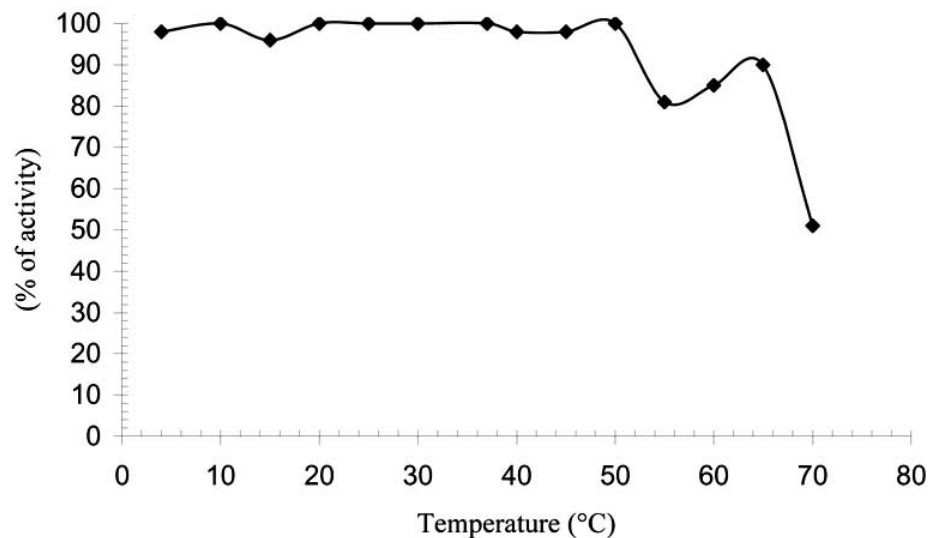


Fig. (2). Effect of temperature on lysozyme thermostability checked by incubating the enzyme at different temperatures (from +5 to +70°C) for 5min and then assaying it at 37°C.

milk were found in many studies similar to the respective levels in human milk, while a low content of allergenic components of milk and significant levels of antimicrobial factors, such as lactoferrin, lactoperoxidase and lysozyme, have also been determined in many studies performed on donkey milk cited in this review. Most of the scientists believe that the positive results from the paediatric trials are due to the particular composition of donkey milk protein

fraction. Besides the traditional nutrients of the diet, bio-active molecules in functional foods, including milk, are clearly important in providing hormonal, antibacterial, probiotic or pharmacological support. In this regard, the properties of donkey milk could be partially due to not only its probiotic activity, related to the high lactose content, but also to the presence of trophic and protective factors, immunoglobulins and peptides with antibacterial, immunomo-

dulating and hormonal activity. Some of these protective factors, often characterized by interspecific activity, are also present in dairy cows milk, but may not survive the technological processing used during the preparation of infant formulas. This could explain the greater susceptibility to infection and development of allergy reported in formula-fed as compared with breast-fed infants. It is also important to consider the high lysozyme content of donkey milk in relation with its probable role in preserving the intestinal ecosystem from potentially harmful microbes. The use in dermatology of donkey milk, well known since ancient Rome, seems as well to be related to its lysozyme content, efficacious in soothing skin and scalp inflammations.

ACKNOWLEDGMENT

This work has been funded by the Italian Ministry of Agriculture, Project “Proteomic Approach to Characterize Donkey Milk Nutritional Quality”, and Principle Investigator Dr. Paolo Polidori.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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