Donkey Milk for Manufacture of Novel Functional Fermented Beverages

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Abstract: The aim of this work was to investigate on the functional features of a donkey milk probiotic berevage as a novel food. Particularly, it was to study the decrease of lactose content and the antioxidant activity of standard yogurt (YC) and probiotic yogurt (YP; *Lactobacillus acidophilus*, *Lactobacillus casei*) from donkey milk during the storage up to 30 d at 4 °C. The evolution of lactose content using enzymatic-spectrophotometric kits was analyzed. Antioxidant activity of yogurt was measured using 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), ferric reducing antioxidant power (FRAP), and thiol assays. Parallel consumer sensory studies were carried out as consumer test in order to gain information about the impact of these novel fermented beverages on sensory perceptions. The statistical analysis has shown significant effect of studied factors. The results showed that the lactose content gradually decreased during storage in both yogurts, reaching values of 2.36% and 2.10% in YC and YP, respectively, at 30 d ($P < 0.05$). During storage of both yogurt types, the antioxidant activity increased, but YP showed a higher antioxidant activity than YC. The results suggest that the antioxidant activity of yogurt samples was affected by cultures of lactic acid bacteria (LAB). We conclude that the fermented donkey milk could be configured as health and nutraceutical food, which aims to meet nutritional requirements of certain consumers groups with lactose or cow milk protein intolerance.

Keywords: antioxidant activity, donkey fermented beverages, lactose content, sensory quality

Practical Application: Donkey milk is now configured as "pharmafood" for its nutritional, nutraceutical, and functional properties. For its biochemistry very close to human milk, it may be considered an alternative food source in children with cow milk protein allergy. However, it has a high lactose content, resulting inadequate for people suffering from lactose intolerance. Therefore, the development and prospects of fermented donkey milk were introduced. LAB are able to hydrolyze lactose, casein, and whey protein with release of a large number of organic acids, peptides, and amino acids that are protective agents to help human body to reduce oxidative damage. This work highlights that the fermented probiotic donkey milk could allow development of novel foods useful to meet the needs of consumers with lactose or cow milk protein intolerance.

Introduction

Functional foods are a large and heterogeneous group of food that provide a health benefit beyond basic nutrition (Health Canada 1998). Donkey milk is now configured as "pharmafood" for its nutritional, nutraceutical, and functional properties. Recently, there has been increasing interest in donkey milk due to health-promoting properties (Tafaro and others 2007). For its biochemistry very close to human milk (Carroccio and others 2000), it may be considered an alternative food source in children with cow's milk protein allergy. The low allergenicity of donkey milk is mainly due to the low casein content and to the low casein/whey protein ratio (Lara-Villoslada and others 2005). Compared with ruminant's milk, donkey milk presents high lactose content, therefore resulting as inadequate for people suffering from lactose intolerance (10% to 60% of the population). Also, the donkey milk has a low fat content and, hence, a low energetic value which suggests its potential use in the hypocaloric human diets. Many authors (Heyman 2006; Lomer and others 2008) have been shown that fermented milk products, such as yogurt, can be tolerated by lactose-intolerant people because they contain live bacteria that help to convert the lactose into lactic acid. Chiavari and others (2005) underlined the benefic effect of donkey milk consumption, mainly fermented milks for the treatment of intolerance to cow milk in young children. Coppola and others (2002) investigated the fermentative properties of donkey milk suggesting the possibility of using this milk for such purposes. Therefore, the biological and nutritional value of donkey milk urges a deep knowledge of this milk, which could be used advantageously for the production of fermented products that can be considered as "novel foods." The novels foods are regulated by EU Food Law Regulation (EC) 258/97, and defined as the foods that have not been used for human consumption to a significant degree within the community before the entry into force of Regulation (15 May 1997). Today, different types of fermented food products are emerging following the market's demands, and the nutritional and organoleptic qualities of fermented products depend on the starting milk, on the microorganisms used, and on the production processes. According to the Codex Alimentarius (2003), the classic yogurt culture is characterized by a protosymbiosis between *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. Often, however these 2 are both cocultured with other lactic acid bacteria (LAB) with probiotic properties. The probiotics (that is, *Lactobacillus acidophilus*, *Lactobacillus casei*, and

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Bifidobacterium bifidum) as "live micro-organisms" which, when administered in adequate amounts, confer health benefits on the host" (Araya and others 2002). These microorganisms are added to the classic yogurt culture to achieve some "functional" health benefits to humans because the yogurt bacteria do not survive the gastrointestinal conditions or colonize the human gut (Shah 2000; Schrezenmeir and de Vrese 2001). Recent studies have shown that probiotics are able to provide several health benefits, including improved lactose digestion, diarrhea prevention, immune system modulation, serum cholesterol reduction, prevention of urogenital infections, colon cancer, maintaining remission in patients with Crohn's disease, and antioxidant activity (Hekmat and others 2009). It has been widely shown that reactive oxygen species (ROS) and free radicals have been implicated in many degenerative diseases, such as Alzheimer, Parkinson, emphysema, cirrhosis, and diabetes (Beckman and Ames 1998). Most living species have an efficient defense system to protect themselves against the oxidative stress induced by ROS (Hazra and others 2010). It involves a variety of components, both endogenous and exogenous in origin, that function interactively and synergistically to neutralize free radicals (Percival 1998). These components include antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase) and nonenzymatic antioxidant compounds with low molecular mass (glutathione, ubiquinol, and uric acid). However, antioxidant supplement may be used to help the human body to reduce the oxidative damage (Kullisaar and others 2003). Many authors have demonstrated the ability of LAB to release certain compounds with antioxidant activity during fermentation of the milk (Suetsuna and others 2000; Kudoh and others 2001; Pena-Ramos and Xiong 2001; Virtanen and others 2007; Gomez-Ruiz and others 2008). The antioxidative effect of fermented milk products is well documented in animal and human studies (Korhonen and Pihlanto 2006). The objectives of this study were to evaluate the antioxidant characteristics and the decrease of lactose content in donkey's yogurts containing *Lactobacillus acidophilus* and *Lactobacillus casei* (YP) and standard yogurt (YC) during the storage. Parallel consumer sensory studies were carried out as consumers test in order to gain information about the impact of these novel fermented beverages on sensory perceptions.

Materials and Methods

Chemicals and apparatus

The chemical compound 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate, sodium phosphate, 2,4,6-tripyridyl-s-triazine (TPTZ), Iron(II) sulfate heptahydrate, hydrochloric acid, ferric chloride, acetic acid, sodium acetate, ethylenediaminetetraacetic acid (EDTA), 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's Reagent), and peptone water were purchased from Sigma-Aldrich (Milan, Italy). Bacteriological media were purchased from Merck Pty. Ltd. (Kilsyth, Vic., Australia). Glucose, lactose, and galactose were determined using enzymatic-spectrophotometric kits, from R-Biopharm Gmbh (Darmstadt, Germany). The LAB, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Streptococcus thermophilus* were purchased from Bionova (Villanova sull'Arda, Piacenza, Italy).

Samples

Donkey milk (Martina Franca breed) was taken in a breeding situated in Basilicata region (Southern Italy). The milk was tested for pH (model PHM 92, Radiometer, Copenhagen, Denmark), DM, ash (IDF 1962), total protein (TP; total N × 6.38), non-nitrogen protein (NPN × 6.38), casein (casein N × 6.38), whey protein (whey protein N × 6.38; all by Kjeldahl method; AOAC International 2000), fat (Rose-Gottlieb method; IDF 1996), and lactose (IDF 1974).

Yogurt manufacture

Two sets of yogurt were produced with donkey milk. A standard yogurt (YC) was obtained by fermentation of milk with the traditional yogurt cultures *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*. A probiotic yogurt (YP) was prepared with the same yogurt starters plus *Lactobacillus acidophilus* and *Lactobacillus casei*. The milk (3 L) was heated at 95 °C for 15 min and subsequently was divided into equal aliquots (3 per YC and 3 per YP, respectively) for yogurt's manufacture. The milk aliquots were cooled to 45 °C and inoculated at the same time, with traditional yogurt culture (at rates of 1% w/v) and *Lactobacillus acidophilus* and *Lactobacillus casei* (at rates of 1% v/v). After inoculation with the appropriate inoculum type, milk was distributed to 250 mL in plastic containers, sealed, incubated at 37 to 42 °C until pH reached 4.6. Then, each yogurt was immediately cooled at 4 °C and stored in a refrigerator for 1, 3, 6, 9, 15, 22, and 30 d.

Microbiological analyses

The colony counts of *L. delbruekii* ssp. *bulgaricus*, *S. thermophilus*, *L. acidophilus*, and *L. casei* were determined using the pour plate technique (Dave and Shah 1996). Briefly, one gram of yogurt sample was diluted with 9 mL of 0.1% peptone water and the sequence of decimal dilutions was prepared. The cell counts of the YC and YP were enumerated after 1, 3, 6, 9, 15, 22, and 30 d storage. The experiments were carried out in triplicate.

Appropriate dilutions were plated using the following bacteriological media:

- For S. thermophilus, M17 agar, aerobic incubation at 37 °C for 24 h (IDF 1981);
- For L. delbrueckii ssp. bulgaricus, MRS Agar (pH 5.4), anaerobic incubation at 37 °C for 72 h (Dave and Shah 1996);
- For L. acidophilus, MRS-maltose agar, anaerobic incubation at 37 °C for 72 h (IDF 1995);
- For L. casei, MRS-vancomycin (1%, w/v), anaerobic incubation at 37 °C for 72 h (Ravula and Shah 1998).

Anaerobic conditions were created using AnaeroGen (Oxoid, Basingstoke, UK). After incubation, plates containing 25 to 250 colonies were enumerated and recorded as colony forming units (CFU) per gram of the product.

Preparation of water-soluble extracts of yogurt for antioxidant activity

Yogurt samples were centrifuged at 5000 × *g* at 4 °C for 20 min. The supernatant was separately filtered through a 0.45 μm membrane filter and was used to measure the antioxidant activity.

ABTS radical scavenging activity

A modification of the original method of Re and others (1999) was applied to assess the scavenging capacity of yogurt samples in a reaction with the ABTS radical cation (ABTS•+), generated by oxidation of ABTS diammonium salt stock solution with potassium persulfate (K2S2O8). Stock solutions of ABTS (7 m*M*) and potassium persulfate (140 m*M*) were prepared in water, and ABTS•+ radical solution was produced by reacting 10 mL of the

ABTS stock solution with 175 μL of potassium persulfate solution. The mixture was left in the dark at room temperature for 12 to 16 h before use. For the evaluation of antioxidant capacity, the ABTS•+ solution was diluted with ethanol (96%) to obtain the absorbance of 0.700 ± 0.020 at 734 nm. Two milliliters of ABTS•+ 150 solution was mixed with 100 μL of the water-soluble extracts of samples in a cuvette and the decrease in the absorbance was measured after 30 min, using a UV-VIS Spectrophotometer 1204 (Shimadzu, Kyoto, Japan). The reagent blank was prepared by adding 100 μL of ethanol instead of the sample. Antioxidant activity was expressed as a percentage inhibition (*I*) of ABTS•+ radical and calculated by the equation:

$$\%I = \left(\frac{\text{A734Control} - \text{A734Extract}}{\text{A734Control}}\right) * 100.$$

FRAP—ferric reducing antioxidant power

The FRAP assay was performed according to the procedure described by Benzie and Strain (1996), with some modifications. The FRAP reagent was prepared by mixing 10 mL of 300 m*M* acetate buffer (pH 3.6), 1 mL of 10 m*M* TPTZ in 40 m*M* HCl, and 1 mL of 20 m*M* FeCl3 (in the ratio 10:1:1, v/v/v). It was daily prepared and warmed to 37 °C before use. Aliquots of 100 μL of water-soluble extracts of samples were mixed with 2.9 mL of FRAP reagent, and incubated at 37 °C for 30 min. The increase in absorbance was measured at 593 nm against acetate buffer (pH 3.6), using a UV-VIS Spectrophotometer 1204 (Shimadzu, Kyoto, Japan). The blank reagent was prepared by adding distilled water instead of the sample. Aqueous solutions of FeSO4 7H2O (100 to 1000 μM) 166 were used for the calibration and the results were expressed as FRAP value (μM Fe (II)) of the sample. Each determination and measurement was made in triplicate.

Determination of free thiol groups

The number of free thiol groups was determined according to Ellman's method (1959), with some modifications. Two hundred fifty μL of water-soluble extract of samples were mixed with 2.5 mL of 0.1 *M* sodium phosphate buffer (containing 1 m*M* EDTA; pH 8.0, reaction buffer) and 50 μL of DTNB reagent solution (4 mg in 1 mL of sodium phosphate buffer). After the solution was mixed and allowed to stand at room temperature (25 °C) for 30 min, absorbance was read at 412 nm, using UV-VIS Spectrophotometer 1204 (Shimadzu, Kyoto, Japan). Reaction buffer was used instead of sample, as a reagent blank. A molar extinction coefficient of 14.150 $M^{-1}cm^{-1}$ was used to calculate moles of thiol groups. Each determination and measurement was made in triplicate.

Sensory analysis

An affective method was used to evaluate consumer acceptability. The test consisted of 310 untrained consumers who had been selected based on their regular consumption of yogurt as well as their sex and age, attempting to represent the distribution of the population as closely as possible. In particular, 163 females and 147 males between the ages of 21 and 60 were selected. The samples were tasted 1 d after the yogurt production. The test was conducted on 10 d with one session per day carried out between 11:00 and 13:00. Each consumer participated in one session and tasted the 2 yogurt samples. Ten milliliter of each yogurt sample were presented in random order, at room temperature, to each consumer in 40 mL glass vials sealed with a twist-off cap coded with 3-digit numbers. The design was balanced for order and carry over effects. Consumers were asked to evaluate the samples, visually (appearance, and color) and then organoleptically (taste and odor), finally expressing a judgment on overall acceptability. The judgments were expressed individually, assigning a numerical value, on a hedonic scale, between 1 (dislike extremely) and 9 (like extremely) (Peryam and Pilgrim 1957). The consumers were isolated in individual booths to reduce collaboration, and oligomineral water and unsalted crackers were provided for the consumers mouth-rinsing between samples. All assessments were carried out in a sensory laboratory equipped according to UNI-ISO 8589 recommendations (International Organization for Standardization 1988).

Statistical analysis

Data were analyzed according to the following linear model (SAS Institute 1996):

$$y_{ij} = \mu + \alpha i + \beta_j + \varepsilon ij$$

where y_{ij} is the observation, μ is the overall mean, αi is the fixed effect of the *i*th yogurts ($i = 1, 2$), βj is the fixed effect of the *j*th storage time ($j = 1, 2, 3, 4, 5, 6, 7$), and εij is the random error.

A mono-factorial model was used for sensory analysis:

$$yij = \mu + \alpha i + \varepsilon ij$$

where y_{ij} is the observation, μ is the overall mean, αi is the fixed effect of the *i*th yogurts ($i = 1, 2$),and εij is the random error.

Before setting the values, expressed as a percentage, they were subjected to angular transformation. Student's *t*-test was used to compare all the variables. Differences between means at the 95% ($P < 0.05$) confidence level were considered statistically significant.

Results and Discussion

Chemical composition of donkey's milk and fermented products

The chemical composition (g/100 g) of composite milk samples, used for yogurt's manufacture, are following: 9.16 dry matter, 0.44 fat, 1.43 TP, 0.18 NPN, 0.45 ash, and 7.02 lactose. The pH of milk was of 7.03. The protein fraction (g/100 g of crude protein) was composed of 0.69 casein and 0.56 whey proteins. Thus, the casein/whey protein ratio was 1.23. Our results were in agreement with the data reported in the literature for donkey milk (Salimei and others 2004; Polidori and others 2009; Martini and others 2014). Compared with cow milk, donkey milk has a higher concentration of lactose and lower levels of fat and protein. As regards the nitrogenous fractions, casein and whey protein content was of 48.25% and 39.16%, respectively, in agreement with Salimei and others (2004) and Guo and others (2007). The casein/whey protein ratio was similar to the values reported by Tidona and others (2011). The low casein content and casein/whey protein ratio play an important role in the sensitization capacity of the milk, as showed by Lara-Villoslada and others (2005).

Microbial viability of starter and probiotic cultures during storage

The count of cultures is indispensable to evaluate if fermented milk contains a sufficient number of viable probiotics useful in health enhancing. Lactobacilli and streptococci were investigated at 1, 3, 6, 9, 15, 22, and 30 d of cold storage at 4 °C. Viable counts of *L. bulgaricus* slightly decreased in both probiotic

Table 1–Lactose content (%) of yogurts during storage at 4 °C for up to 30 d.

	YC		YP	
Days	Mean	±SD	Mean	± SD
1	4.54^{a,A}	0.01	4.74^{a,A}	0.01
3	3.53^{b,A}	0.02	3.46^{b,A}	0.01
6	3.03^{c,A}	0.04	2.84^{c,B}	0.06
9	2.93^{d,A}	0.07	2.61^{c,B}	0.08
15	2.68^{e,A}	0.01	2.48^{d,B}	0.01
22	2.50^{f,A}	0.01	2.27^{e,B}	0.01
30	2.36^{g,A}	0.03	2.10^{f,B}	0.04

$^{A-C}$Different capital letter superscripts depict the statistical difference within a row ($P < 0.05$) between means for different yogurt batches ($P < 0.05$).
$^{a-g}$Different small letter superscripts depict the statistical difference within a column ($P < 0.05$) between means for the same yogurt batches ($P < 0.05$).

($P = 0.094$) and control yogurts ($P = 0.080$) during 30 d of storage at 4 °C. *L. bulgaricus* counts in YP (6.33 log cfu/mL) were greater than YC (5.98 log cfu/mL) at 30 d; however, there were no significant differences between yogurts. This could be attributed to the synergetic effect of probiotic bacteria with *L. bulgaricus*, and improved proteolytic activity that could have provided more amino acids required for sustaining the viability of *L. bulgaricus* (Mortazavian and others 2006). Viable counts of *S. thermophilus* in both probiotic and control yogurts during 15 d of storage at 4 °C were the same as on the 1st day without any significant difference (9.45 cfu/mL; $P > 0.05$), afterwards *S. thermophilus* counts decline about of 18%, maintaining, however, values $> 10^7$ cfu/mL. These findings are in agreement with the results of Mani-Lopez and others (2014), who reported also that *S. Thermophilus* counts in probiotic yogurt were not affected by the presence of probiotic bacteria. *L. acidophilus* counts decreased significantly throughout the storage from 8.80 log cfu/mL (on day 1) to 6.75 log cfu/mL (on day 30). *L. casei* counts in YP during storage decreased from day 1 (8.28 log cfu/mL) through day 30 (6.56 log cfu/mL). In general, the concentration of starter cultures in all samples was above the lowest recommended therapeutic level of 6 log cfu/mL (Kurmann and Rasic 1991) at the end of storage.

Lactose content

Results of lactose content for control and probiotic yogurts during storage up to 30 d are showed in Table 1. At the start of storage, the lactose content was 4.54% and 4.74% in YC and YP, respectively. The lactose content was observed to gradually decrease during storage in both yogurts. The lactose hydrolysis continued throughout storage, reaching lactose levels of 2.36% and 2.10% in YC and YP, respectively, at 30 d. As can be seen, YP showed a higher decrease in the lactose content with statistically significant differences ($P < 0.05$), except in the 1st and 2nd interval compared to YC. This could be due to greater activity of traditional yogurt cultures at typical yogurt pH value. Similar data were found by Batista and others (2008) and Martins and others (2012). In general, there was a significant decrease in lactose content as a function of storage time linked to ability by starter bacteria to produce lactic acid. Postacidification of yogurt occurred during refrigeration resulted in a pH decrease. In fact, during the storage, the pH values decreased linearly and significantly ($P < 0.05$), but the differences between YP and YC were not significant (Figure 1).

YP showed a much more marked variation in all intervals compared to YC ($P < 0.05$). As well known, the acid lactic bacteria can produce high amounts of lactic acid by fermenting lactose (Vedamuthu 2006). Shah (2000) reported that *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*, the traditional cultures used in making yogurt, contain substantial quantities of β-d-galactosidase and so both yogurt and probiotic yogurt can help alleviate symptoms of lactose intolerance.

Antioxidant activity

The antioxidant activity of yogurt samples was assessed by 3 different tests: the ABTS, FRAP, and thiols assays during storage at 4 °C and up to 30 d (Table 2). In the last years, several methods have been developed to assess the total antioxidant capacity because of the lack of standard quantification methods and the interactions among different antioxidant components (Schlesier and others 2002). The ABTS assay is one of the most widely used methods for the screening of antioxidant activity as it measures the scavenging activity of several natural products and is applicable to both hydrophilic and lipophilic antioxidant systems (Re and others 1999). FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method, employing an easily reduced oxidant system present in stoichiometric excess. The thiols assay measure the number of thiol groups (SH-), such as glutathione and protein thiol groups, which play an essential role as antioxidants. In particular, the thiols assay measures sulfhydryl groups with the thiol reagent 5-5'-dithiobis(2-nitrobenzoic acid) (DTNB), which forms the 5-thionitrobenzoic acid and a mixed disulfide. Under conditions of oxidative stress, free sulfhydryl decreases and disulfides increase (Stapelfeldt and others 1997). This assay reflects the ability to detoxify lipid and other peroxides in biological samples. At the start of storage, all yogurt samples showed antioxidant activity, which is a desirable characteristic that enhance the therapeutic values of both fermented milk (Table 2). The average values of antioxidant activity were above the 50% for ABTS assay, to 380 μMFe(II) for FRAP assay, and to 380 μM-SH for thiols assay. As well known, the fermented milk products themselves have a large antioxidant capacity related to the presence of different bioactive peptides from milk proteins through proteolysis by LAB (Kudoh and others 2001; Virtanen and others 2007; Gomez-Ruiz and others 2008). Milk proteins are considered the most important source of bioactive peptides. The donkey milk presents a lower protein percentage, but its casein/whey protein ratio is clearly in favor of whey proteins respect cow milk (Salimei and others 2004). Furthermore, it has been found that the donkey milk proteins are characterized by high concentrations of peptide-bound AA, particularly essential amino acids (Taha and Kielwein 1990). Compared to Fresian cow milk, donkey milk is characterized by higher levels of Val and Lys (Abd-EISalam and others 1992). The composition of amino acids, their sequence, and configuration influence the antioxidant properties of peptides (Pena-Ramos and Xiong 2001). Several authors reported that the antioxidant activity of peptides containing methionine, glutamine, tyrosine, lysine, histidine, cysteine, valine, and proline is very strong (Rajapakse and others 2005). The antioxidant capacity is also conditioned by the heat treatment undergone by the milk for the manufacture of the yogurt (Galleher and others 2005), by the fermentation and postacidification during storage that determine production of organic acids (Correia and others 2004). The antioxidant activity increased significantly during refrigerated storage ($P < 0.01$). Comparing the yogurts, the differences between the average FRAP and thiols values were statistically significant ($P < 0.05$) in all studied levels, while for the average ABTS values, significant differences at 3 d, 6 d, 9 d, and 30 d were found.

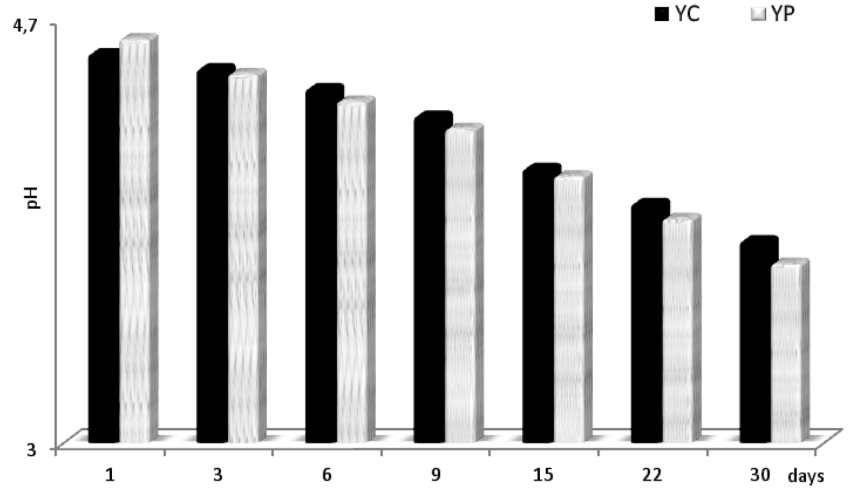

Figure 1–The pH values of YC and YP during the refrigerated storage.

Table 2–Antioxidant activity of yogurts during storage at 4 °C for up to 30 d.

	ABTS (I%)				FRAP μMFe(II)				THIOLS (μM-SH)			
	YC		YC		YP		YC		YC		YC	
Days	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD
1	$50.40^{a,A}$	0.56	$50.69^{a,A}$	0.42	$382.15^{a,B}$	0.85	$388.72^{a,B}$	0.14	$381.21^{a,A}$	0.05	$391.50^{a,B}$	0.02
3	$55.50^{b,A}$	0.09	$56.39^{b,B}$	0.23	$408.73^{b,A}$	7.07	$423.54^{b,B}$	6.36	$406.62^{b,A}$	0.05	$403.06^{b,B}$	0.04
6	$57.63^{c,A}$	0.28	$58.89^{c,B}$	0.38	$440.84^{c,A}$	7.07	$446.40^{c,B}$	3.53	$427.80^{c,A}$	0.02	$412.97^{c,B}$	0.03
9	$58.22^{c,d,A}$	0.19	$59.75^{c,d,B}$	0.28	$482.92^{d,A}$	2.83	$467.09^{d,B}$	3.54	$432.89^{d,A}$	0.01	$436.10^{d,B}$	0.04
15	$59.02^{d,A}$	0.38	$59.81^{c,d,A}$	0.38	$542.71^{e,A}$	3.54	$491.04^{e,B}$	1.41	$464.23^{e,A}$	0.02	$469.97^{e,B}$	0.01
22	$60.34^{e,A}$	0.19	$60.34^{d,A}$	0.38	$578.15^{f,A}$	7.07	$549.82^{f,B}$	3.54	$478.63^{f,A}$	0.06	$498.05^{f,B}$	0.01
30	$63.38^{f,A}$	0.4	$66.21^{e,B}$	0.47	$629.09^{g,A}$	0.28	$679.35^{g,B}$	0.45	$505.74^{g,A}$	0.05	$510.31^{g,B}$	0.07

$^{A-B}$Different capital letters in the same row, for each assay, with different superscripts were significantly different ($P < 0.05$).
$^{a-g}$Different small letters in the same column with different superscripts were significantly different ($P < 0.05$).

At 30 d, YP showed the highest antioxidant activity compared to YC ($P < 0.05$). The relationship between antioxidant activity and proteolysis was previously reported in several studies (Kudoh and others 2001; Ryhanen and others 2001; Hernandez-Ledesma and others 2005; Gupta and others 2009). Gupta and others (2009), in Cheddar cheese, have found that the antioxidant activity varied depending on the rate of formation of soluble peptides (proteolysis). The same authors, in a later work (2013), have shown that inhibitory ACE activity increased with the increase in the protein content of the WSE of Cheddar cheeses. In support of this, Igoshi and others (2008) have found significant correlation between the antioxidant activity and the amount of peptides generated during cheese ripening. Contrary, Bottesini and others (2013), in Parmigiano Reggiano cheese, have reported that the antioxidant activity remained quite constant during ripening time suggesting that the peptides/proteins are not particularly affected by the biochemical processes during the aging time.

The ABTS values varied from 50.40% to 63.38% in the YC, from 50.69% to 66.21% in the YP, during the refrigerated storage up to 30 d. The ABTS radical scavenging activity reached its highest value at 30 d, with an increase by about 26% and 31% than to the initial value, in the YC and YP, respectively. The antioxidant activity evaluated by FRAP assay was increased by about 65% and 75% than to the initial value, in the YC and YP, respectively. In particular, YP showed a higher antioxidant activity using FRAP assay at 1, 6, and 30 d of storage, while at 9, 15, and 22 d, YC showed the highest values. The explanation of that behavior could be related to casein and whey protein proteolysis with the formation of a greater proportion of peptides in the molecular mass range of 4 to 20 kDa that showed higher antioxidant activity (Virtanen and others 2007). However, the variation of antioxidant activity is linked to the possible aggregation of peptides that occur during the enzymatic hydrolysis of whey protein and casein, with formation of macro-aggregates that reduce the antioxidant capacity (Adt and others 2011). At the start of storage, the antioxidant activity, using thiols assay, was of 381.21 μMSH in YC and 391.50 μMSH in YP. At the end of storage, YP presented a greater thiols content compared to YC. However, at 3 and 6 d, the thiols content was highest in YC. Thus, the results obtained showed that the probiotic bacteria could help to enhance the antioxidant capacity, according to the results of Sah and others (2014) and Virtanen and others (2007). In support, Donkor and others (2007) and Papadimitriou and others (2007) reported that the inhibitory ACE activity was higher in the probiotic soy yogurt and sheep than the traditional one. The obtained results in this work suggested that the antioxidant activity is closely linked to milk protein degradation, in agreement with Lourens-Hattingh and Viljoen (2001); it was also strongly influenced by strain-specific characteristic of LAB, as reported by many authors (Kudoh and others 2001; Ryhanen and others 2001; Hernandez-Ledesma and others 2005; Virtanen and others 2007; Gupta and others 2009). LAB have a complex proteolytic system including a cell wall bound proteinase, amino acid transport systems, and several intracellular peptidases and proteinases, able to degrade the major milk proteins into small peptides and free amino acids that are subsequently used for their growth (Christensen and others 1999). Generally, *Lactobacillus*

delbrueckii ssp. *bulgaricus* has a higher proteolytic activity than *Streptococcus thermophilus* that produces essential amino acids for establishing their symbiotic relationship (Shihata and Shah 2000). Many authors have reviewed that the proteolytic system has an important role in the release of various bioactive peptides from the precursor protein where they are encrypted, which influence different biological functions, such as antioxidant activity (Donkor and others 2007). It was demonstrated that the antioxidant activity of yogurt is influenced by certain specific proteolytic enzymes of bacterial strain (Virtanen and others 2007; Ramesh and others 2012). It is also observed that caseinolytic activity of LAB is manifested with a significant preference for β-casein, during yogurt fermentation from cow milk (Stefanitsi and Garel 1997). In the case of whey protein, Bertrand-Harb and others (2003) have demonstrated that α-lactalbumin have the higher susceptibility to proteolytic activity compared to β-lactoglobulin, due to the differences in conformations of whey proteins. Zulueta and others (2009) have provided evidence that the major contributors to the total antioxidant capacity in whole milk are the casein fractions. Many authors have showed that the antioxidant activity of caseins and whey proteins could be due to their high tendency to chelate metals (Tong and others 2000; Rival and others 2001) and to the ability to donate electrons and atoms (Colbert and Decker 1991). Kudoh and others (2001) found a k-CN-derived peptide in milk fermented with *L. delbrueckii* ssp. *bulgaricus* and Papadimitriou and others (2007), in traditional and probiotic sheep milk yogurt, have identified peptides derived from β-casein with both antihypertensive and opiate-like activity. Bidasolo and others (2012) in an *in vitro* study simulating gastrointestinal digestion of donkey milk have identified the sequence of β-casein-derived peptide that possess the typical characteristic of ACE-inhibitory peptides. In addition, Tidona and others (2011) reported antimicrobial activity of donkey milk digested *in vitro* with human gastrointestinal enzymes. It has also been demonstrated that the major whey proteins, such as α-lactalbumin and β-lactoglobulin, contain peptides which inhibit ACE (Mullally and others 1997). The whey proteins, in particular the α-lactoglobulin, have a pivotal role in antioxidant defense, likely because of their high sulfur content (1.7%), such as cysteine and methionine, compared to caseins (approximately 0.8%). Marshall (2004) reported that these amino acids enhance immune function upon intracellular conversion to glutathione, a potent antioxidant. Moreover, the whey proteins undergo conformational changes, due the heat treatment by the milk for the manufacture of the yogurt (95 °C for 15 min) that determine the exposure of the reactive thiol groups. This reactive thiol groups can form disulfide links with other reactive thiol groups and through thiol group-disulfide bridge exchange reactions. In the case of yogurt manufacture, both whey proteins and casein micelles are present, and interactions between the 2 groups of protein also occur (Mensink 2006; Oldfield and others 1998). Thiols are an important class of strong antioxidants and their antioxidant properties depend on different mechanisms. These compounds can act as free radical scavengers and chelators of metal ions. The thiol groups are extraordinarily efficient antioxidants protecting cells against consequences of damage induced by ROS due their ability to react with the latter that are converted to a relatively less toxic state (Wardman and von Sonntag 1995). Erel (2004) reported that in human serum samples SH protein groups contribute 52.9% to total antioxidant capacity in healthy subjects. Dias and Weimer (1998), in cheddar cheese, found that the conversion of Met pathways to free thiols are influenced by bacteria used as starter cultures. Additionally, these authors observed that lactococci and lactobacilli contain high levels of enzymes (cystathionine γ-and β-lyases), which produce free thiols; and that the enzymatic activity was dependent on the concentration of sulfur amino acids in the growth medium. However, the variation of antioxidant activity during storage up to 30 d could be due to extensive breakdown of protein by microbial enzymes, either during fermentation or extended refrigeration storage (Meisel 1997). In addition, Hajirostamloo (2010) reported that the concentration of ACE inhibitory peptides depends on a balance between their formation and further breakdown into inactive peptides and amino acids that in turn depends on storage time and conditions. In many studies, it has been reported that LAB possess antioxidative activity, and were able to scavenge ROS. Kim and others (2004) reported that yogurt starter cultures have good antioxidant capacity and, in particular, *L. bulgaricus* showed the highest hydroxy radical scavenging activity and good reducing power. The findings of the current study are consistent with those of Virtanen and others (2007) who found that the milk fermented with mixed cultures of LAB had a higher radical scavenging activity than milk fermented with single bacterial strain.

Table 3–Sensory profile of YP and YC samples.

Descriptor	YC		YP	
	Mean	± S.D.	Mean	± S.D.
Overall acceptability	7.11	1.01[a]	7.02	1.12[a]
Appearance	6.93	0.86[a]	6.86	0.91[a]
Color	6.98	1.23[a]	7.07	0.76[a]
Odor	7.35	1.21[a]	7.31	1.02[a]
Taste	6.51	0.81[a]	7.32	0.69[b]

[a–b]Different small letter in the same row with different superscripts were significantly different ($P < 0.05$).

Sensory analysis

For functional foods, such as probiotic yogurts, the sensory quality of the products is essential for its effect on consumer acceptability. To investigate the degree of acceptance of the 2 different yogurt types, 310 consumers were invited to take part in a hedonic test: overall acceptability, appearance, color, odor, and taste descriptors were assessed and the results (mean scores and standard error values) are shown in Table 3. Generally, it was observed that the donkey yogurts showed a good overall acceptability score (range 7.01 to 7.12 for YC and YP, respectively). This result is extremely encouraging for its placing on the market, because, regardless by health benefit, the most important marker in choosing a functional food is flavor. The statistical analysis showed no significant differences ($P > 0.05$) on acceptance level of the 2 yogurt types, except for taste, significantly higher in YP ($P < 0.05$). This finding could be explained by the high capacity of probiotics to increase organic acid concentrations (lactic and acetic acid) and proteolysis, which involves the progressive hydrolysis of the caseins to polypeptides, peptides, and amino acids during fermentation and refrigerate storage of yogurts, resulting influence on yogurt flavor, as reported by many authors (Donkor and others 2007; Allgeyer and others 2010). Conversely, Mani-Lopez and others (2014) reported that yogurt containing *Lactobacillus casei* was better perceived because it was less acidic than the control yogurt; while Batish and others (1997) reported that the addition of some LAB can spoil milk and yogurt resulting in unpleasant flavor and odor The appearance, color, and odor of YP were comparable and similar to that of YC ($P > 0.05$), in agreement with other authors (Atunes and others 2005; Hekmat and Reid 2006), who reported that the probiotics do not alter the sensory properties of yogurt.

These results are very interesting because the connection between functional probiotic food industry and consumer acceptability is one of the most important aspects for success of functional food.

Conclusion

The possibility of using donkey milk for the production of a fermented probiotic beverage can allow development of industry for nutraceutical foods. The findings highlighted that YP, compared to YC, presented a lower lactose content and a higher antioxidant activity, representing a health and nutraceutical food, which aims to meet nutritional requirements of certain consumers groups with lactose or cow milk protein intolerance. In addition, sensory analysis pointed out that the donkey's yogurt was well accepted by consumers, in particular, the appearance, flavor, texture, and overall quality of YP were comparable to the YC. This result suggested that the novel products could be successfully introduced commercially.

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