J. Dairy Sci. 97:6662–6670
http://dx.doi.org/10.3168/jds.2013-7843

Antioxidant activity of yogurt made from milk characterized by different casein haplotypes and fortified with chestnut and sulla honeys

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ABSTRACT

The aim of this work was to evaluate the antioxidant activity of yogurt made from milk characterized by different casein (CN) haplotypes (α_{s1}-, β-, κ-CN) and fortified with chestnut and sulla honeys. The CN haplotype was determined by isoelectric focusing, whereas antioxidant activity of yogurt was measured using 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid and ferric-reducing antioxidant power. The statistical analysis showed a significant effect of the studied factors. The results showed that chestnut honey presented the highest phenolic acid and flavonoid contents, which are closely associated with its high antioxidant activity. The antioxidant activity of fortified yogurt samples was affected both by different CN haplotypes and by type of honey added. Yogurts fortified with chestnut honey showed higher antioxidant activity than those fortified with sulla honey. The different behavior observed among the fortified yogurts led us to hypothesize that the effects of protein-polyphenol complex on antioxidant activity are interactive. The results suggest that milk proteins polymorphism and polyphenols play different roles in affecting the bioavailability and the antioxidant activity of yogurt.

Key words: yogurt, honey, casein haplotype, antioxidant activity

INTRODUCTION

Yogurt's popularity is linked to both health benefits and texture resulting from the product's preparation, in line with consumer tastes and needs. Known since ancient times, yogurt is a fermented product, generally obtained from cow milk, and it represents the final result of milk protein coagulation due to the lactic acid produced by both *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (Robinson, 2003). Yogurt with no added flavor is predominantly sour; therefore, to make it more palatable, fruit, flavorings, and sweeteners are added to improve flavor balance (Kagan, 1985) and to partly mask acetaldehyde flavor (Bills et al., 1972).

As a frequently used sweetener in fermented dairy products, honey (Chick et al., 2001) can be considered a natural syrup, containing primarily fructose (38.5%) and glucose (31.3; Ustunol and Gandhi, 2001), with flavorings arising from flower essences. Many authors have reported the inhibitory effects of honey against lactic acid bacteria (**LAB**; Čurda and Plocková, 1995; Roumyan et al., 1996), which are due to the high sugar concentration, relatively high acidity, and presence of both organic acids and low concentrations of hydrogen peroxide (Roumyan et al., 1996; Mundo et al., 2004). Recently, other authors (Sanz et al., 2005; Ezz El-Arab et al., 2006) showed that, when used at suitable levels, honey does not inhibit the growth of common bacteria, and could be used as a sweetener and a useful preservative agent in dairy products (Chick et al., 2001; Varga, 2006).

Yogurt and honey are now considered functional foods. In fact, it has been amply demonstrated that their consumption has beneficial effects on health. Previous research showed that some qualities of yogurt, such as its antioxidative, antithrombotic, antimicrobial, immunomodulatory, ion binding, opioid antagonistic activities, or angiotensin-converting enzyme inhibitory qualities, have beneficial effects on bodily functions in humans (Pattorn et al., 2012). In particular, the antioxidant activity of whey and casein proteins in yogurt could be related to their high tendency to chelate metals (Tong et al., 2000; Rival et al., 2001) and to their ability to donate electrons and atoms (Colbert and Decker, 1991). The antioxidant activity of yogurt is influenced by bacterial fermentation that leads to the release of several of bioactive peptides and the relationship between antioxidant activity and concentration of low-molecular weight peptides has been reported in many studies (Kudoh et al., 2001; Virtanen et al., 2007; Gomez-Ruiz et al., 2008). Galleher et al. (2005) reported that the antioxidant capacity is conditioned also by the heat treatment undergone by the milk for the manufacture of yogurt (95°C for 15 min) because the denaturation of proteins exposed initially

Received December 17, 2013.
Accepted August 4, 2014.
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buried reactive sites. Moreover, the antioxidant activity is strongly influenced by strain-specific characteristics of LAB (Kudoh et al., 2001; Ryhanen et al., 2001; Hernández-Ledesma et al., 2005; Virtanen et al., 2007; Gupta et al., 2009). In a recent report, Perna et al. (2013a) observed that the antioxidant activity of yogurt made from cow milk was significantly influenced by the casein haplotype. This could be due to specific amino acid sequence of the milk protein variants. In support of this, Minervini et al. (2003), in sodium caseinates of milk from different species, reported that the degree of heterogeneity of CN may influence the released of peptides formed during proteolysis.

Honey is a natural inert sugar dissolved in around 14 to 20% of water, with minor amounts of organic acids, along with traces of minerals, vitamins, flavonoids, and phenolic acids. These components define its role as a nutritional source of natural antioxidants responsible for protecting human health (Gheldof and Engeseth, 2002; Gheldof et al., 2002). Honey's therapeutic importance as a known antibacterial agent has been revalorized, as well as its antioxidant, anti-inflammatory, and antitumoral properties were demonstrated (Tonks et al., 2001; Orsolic et al., 2005). The antioxidant activity of honey depends largely on its chemical composition, such as flavonoids, some enzymes (glucose oxidase, catalase and peroxidase), ascorbic acid, Maillard reaction products, organic acids, amino acids, and proteins (Gheldof and Engeseth, 2002; Al-Mamary et al., 2002; Aljadi and Kamaruddin, 2004). Many authors found a strong correlation between antioxidant capacity and phenol content (Gheldof and Engeseth, 2002; Beretta et al., 2005; Meda et al., 2005; Blasa et al., 2006). Phenolic compounds are synthesized by plants as secondary metabolites which, in many cases, serve in plant defense mechanisms to counteract reactive oxygen species (Peterson and Dwyer, 1998; Robards et al., 1999; Wollgast and Anklam, 2000). The phenolic content in honey depends on the floral source which markedly influences the antioxidant activity (Perna et al., 2012).

Few researchers have focused on the effect of fortifying yogurt with honey (Varga, 2006; El-Baz and Zommara, 2007; Abd El-Rahman and Salama, 2008). However, in the scientific literature, no reports are available on the antioxidant capacity of yogurt made from milk with different casein haplotype and fortified with honey. The aim of the present work was to evaluate the antioxidant activity of yogurt made from milk characterized by different CN haplotypes (α_{s1}-, β-, κ-CN) and fortified with chestnut and sulla honey. Sulla (*Hedysarum* spp.) and chestnut (*Castanea sativa*) honeys are produced widely in southern Italy and represent a large portion of the annual honey production in this area.

MATERIALS AND METHODS

Chemicals and Apparatus

The 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (**ABTS**), 2,4,6-tripyridyl-s-triazine (**TPTZ**), potassium persulfate, hydrochloric acid, ferric chloride, iron(II) sulfate heptahydrate, sodium phosphate, sodium hydroxide, phosphoric acid, acetic acid, 2-mercaptoethanol, urea, N,N,N′,N′-tetramethylethylenediamine, ammonium persulfate, and sodium acetate were purchased from Sigma-Aldrich (Milan, Italy). Acrylamide, bis-acrylamide, and ampholine buffer were purchased from GE Healthcare Amersham Bioscience (Buckinghamshire, UK). Coomassie Brilliant blue G250 was purchased from Bio-Rad (Richmond, CA). *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Strep. thermophilus* were purchased from Insao s.r.l. (Liscate, Milan, Italy). The spectrophotometer UV-VIS Spectrophotometer 1204 (Shimadzu, Japan) was used. The apparatus for isoelectric focusing Multiphor II Electrophoresis System (Pharmacia LKB, Uppsala, Sweden) was used.

Milk Sample

This study was conducted on an intensive farm, consisting of more than 350 Italian Holstein and Italian Brown cattle, in the countryside of Potenza, in southern Italy. Before starting the test, about 200 animals in lactation were identified by isoelectric focusing (**IEF**) to define their haplotypes. Haplotypes were formed by the combination to the individual allelic loci aggregated by α_{s1}-, β-, or κ-CN. After the definition of individual phenotypes, the cows were grouped by haplotype to obtain more consistent milk, which is needed to manufacture yogurt. Overall, the average of the milk total solids and protein was 13.11 and 3.51%, respectively. Yogurt was obtained with a specific yogurt starter culture consisting of a mixture of 2 species of LAB, *Strep. thermophilus*, and *Lb. delbrueckii* ssp. *bulgaricus* and incubated at 4°C for 24 h.

Honey Samples

Chestnut and sulla honey samples from southern Italy were collected directly from beekeepers during the 2010 harvest (250 g each). The honey purity was carefully checked by pollen analysis carried out according to DIN 10760 (DIN, 2002; Von der Ohe et al., 2004). On the basis of this analysis, the predominant pollen type was *Hedysarum* spp. (frequency = >50%) and *Castanea sativa* (frequency = 75–90%) for sulla and chestnut honeys, respectively. Honey samples were stored at

4°C in the dark until analyzed. The experiments were performed using freshly prepared 10% honey solutions in distilled water. A sugar analog (80% sugar, wt/vol), serving as a blank, was prepared by dissolving 0.2 g of sucrose, 0.8 g of maltose, 4 g of fructose, and 3 g of glucose in distilled water to make a solution with 10 mL of final volume (White, 1979). The antioxidant activity was calculated by subtracting the obtained values for the blank from that of each sample. All tests were performed in triplicate.

Sample Preparation for IEF

Individual milk samples, kept at 4°C, and defatted by centrifugation (3,000 × *g* for 30 min at 4°C); the fat layer was solidified at −20°C for 20 min and removed. Casein was prepared by isoelectric precipitation at pH 4.6 with 10% (vol/vol) acid acetic and 1 *M* sodium acetate at room temperature. After centrifugation at 3,000 × *g* for 10 min at 4°C, the CN pellet was washed twice with distilled water and stored at −20°C. The whole CN was dissolved in 9 *M* urea and 1% 2-mercaptoethanol for IEF analysis, according to Aschaffenburg and Drewry (1959).

Genetic Variants of CN by IEF

The genetic variants of the different CN by IEF were determined according to the method of Trieu-Cuot and Gripon (1981). The IEF analysis was performed on polyacrylamide gel (5% acrylamide and 0.15% bisacrylamide) with a thickness of 1 mm and 2% carrier ampholytes to create a gradient of pH 2.5 to 10.0. Gel was prefocused at a constant value of 0.35 W/mL of gel and at the maximum limit of 1,200 V. The gel was stained in Coomassie blue G-250 according to Blakesley and Boezi (1977). Haplotype frequencies were determined by the ratio of the number of each haplotype to the total number of haplotypes [$\% = (n_{i,\ \text{haplotype}}/n_{tot,\ \text{haplotype}}) \times 100$]. Haplotypes are presented as α_{s1}-, β-, or κ-CN.

Yogurt Manufacture

Yogurt samples with added sulla and chestnut honey, as well as the control (without any addition), were prepared. After being heat treated at 95°C for 15 min followed by cooling to 45°C, all whole milk samples were inoculated at the same time with 1% (vol/vol) *Strep. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus*. Fermentation was carried out at 45°C. Each fermentation process was monitored by continuous recording of pH values to measure the acidification rates during fermentation until the pH value reached 4.6 ± 0.1. Once the desired pH was reached, the sulla and chestnut honey (30%, wt/vol) were added and incorporated by mechanical stirring; consequently, the prepared product was a stirred type yogurt. Finally, yogurts were cooled at 4°C and stored for 24 h before analysis.

Preparation of Water-Soluble Extracts of Control and Fortified Yogurt

Yogurt samples were centrifuged at 5,000 × *g* at 4°C for 20 min. The supernatant was separately filtered through a membrane filter (0.45 nm) and was used to measure the antioxidant activity.

Antioxidant Activity of ABTS Radical Scavenging Assay

A modification of the original method of Re et al. (1999) was applied to assess the scavenging capacity of yogurt samples in a reaction with the ABTS radical. The ABTS radical solution was generated by oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt stock solution with potassium persulfate ($K_2S_2O_8$). 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 to stand 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 solution were mixed with 100 μL of water-soluble extracts of yogurt in a cuvette and the decrease in the absorbance was measured after 30 min. The reagent blank was prepared by adding 100 μL of ethanol instead of the sample. The calibration curve was constructed using ascorbic acid (2.2–0.25 μ*M*) and the results were expressed as micrograms of ascorbic acid equivalents (**AAE**) per milliliter of extract.

Determination of Antioxidant Activity by Ferric-Reducing Antioxidant Power

The ferric-reducing antioxidant power (**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* $FeCl_3$ (in the ratio 10:1:1 vol/vol/vol). It was prepared daily 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

Table 1. Frequencies of the α_{S1}-, β-, and κ-CN haplotypes in cow milk

Haplotype			Frequency
α_{s1}-CN	β-CN	κ-CN	(%)
BB	A^2A^1	AA	20.83
BB	A^2A^2	BB	20.83
BB	A^2B	AB	16.67
BB	A^2A^1	AB	12.50
CC	A^2A^2	BB	12.50
BB	A^2A^2	AB	8.33
BB	A^2A^2	AA	8.33

in absorbance was measured at 593 nm against acetate buffer (pH 3.6). The blank reagent was prepared by adding distilled water instead of the sample. The calibration curve was constructed using ascorbic acid (2.2–0.25 μ*M*) and the results were expressed as micrograms of AAE per milliliter of extract.

The percentage change of antioxidant activity of fortified yogurt was determined by the formula

$$\%\Delta = (AA_C - AA_{YF}/AA_C) \times 100,$$

where %Δ is the percentage change of antioxidant activity of fortified yogurt; AA_C is the antioxidant activity value of control yogurt; and AA_{YF} is the antioxidant activity value of fortified yogurt.

Determination of Total Phenolic and Flavonoid Contents of Honey Samples

The total phenolic content of honey was estimated according to the Folin-Ciocalteu method as modified by Beretta et al. (2005). Gallic acid (0–200 mg/L) was used as standard to derive the calibration curve and the results were expressed as milligrams of gallic acid equivalents per 100 g of honey. Total flavonoid content was determined using the Dowd method as adapted by Arvouet-Grand et al. (1994). Quercetin (0–200 mg/L) was used as standard to derive the calibration curve and the results were expressed as mg of quercetin equivalents per 100 g of honey.

Statistical Analysis

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

$$y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk},$$

where y_{ijk} is the observation; μ is the overall mean; α_i is the fixed effect of the ith haplotype (i = 1, 2, 3, 4, 5, 6, 7); β_j is the fixed effect of the jth honey (j = 1, 2); $(\alpha\beta)_{ij}$ is the interaction of haplotype × honey; and ε_{ijk} is the random error. Before setting the values, expressed as percentages, they were subjected to angular transformation. Student's t-test was used for all variable comparisons. Correlation between parameters was determined by Pearson correlation analysis.

RESULTS

Haplotype Frequencies

Seven different CN haplotypes were identified by isoelectric focusing. The different allelic combinations of loci α_{S1}-, β-, and κ-CN and their frequencies are reported in Table 1. Haplotypes BB-A^2A^1-AA, BB-A^2A^2-BB (20.83%), and BB-A^2B-AB (16.67%) were frequent, whereas BB-A^2A^2-AA, BB-A^2A^2-AB, and BB-A^2A^2-BB showed the lowest frequency (8.33%).

Antioxidant Activity

The ABTS and FRAP values of yogurt with different CN haplotypes, with and without (control) added honey, are reported in Tables 2 and 3. Statistical analysis showed a significant effect of the added honey and CN

Table 2. Radical-scavenging activity of yogurts with different haplotypes, with and without added chestnut or sulla honey[1]

Haplotype			Control		Chestnut yogurt		Sulla yogurt	
α_{s1}-CN	β-CN	κ-CN	Mean	SD	Mean	SD	Mean	SD
BB	A^2A^2	BB	$199.30^{a,A}$	11.09	$339.38^{a,c,B}$	15.82	$260.10^{a,b,C}$	23.83
BB	A^2A^1	AA	$236.48^{b,A}$	18.81	$325.26^{a,d,B}$	14.82	$252.08^{a,A}$	19.14
BB	A^2A^2	AA	$118.39^{c,A}$	17.46	$287.58^{b,B}$	19.87	$253.17^{a,C}$	18.26
CC	A^2A^2	BB	$169.72^{d,A}$	19.73	$280.03^{b,B}$	34.63	$213.50^{d,C}$	10.52
BB	A^2A^1	AB	$251.35^{e,A}$	12.93	$329.16^{a,B}$	10.74	$294.55^{c,C}$	18.5
BB	A^2A^2	AB	$196.96^{a,A}$	6.08	$342.63^{c,B}$	2.84	$270.94^{b,C}$	11.76
BB	A^2B	AB	$263.92^{e,A}$	20.20	$312.38^{d,B}$	14.02	$211.73^{d,C}$	6.56

[A–C]Different uppercase superscripts depict the statistical difference within a row ($P < 0.05$) between means for different yogurt batches.

[a–e]Different lowercase letter superscripts depict the statistical difference within a column ($P < 0.05$) between means for the same yogurt batches at different casein haplotype.

[1]Measured using 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) assay and expressed as micrograms of ascorbic acid equivalents per milliliter.

Table 3. Ferric-reducing antioxidant power of yogurts with different haplotypes, with and without added chestnut or sulla honey[1]

Haplotype			Control		Chestnut yogurt		Sulla yogurt	
α_{s1}-CN	β-CN	κ-CN	Mean	SD	Mean	SD	Mean	SD
BB	A^2A^2	BB	113.40^{a,A}	32.27	231.01^{a,c,B}	19.55	156.78^{a,b,C}	34.44
BB	A^2A^1	AA	143.99^{b,A}	35.74	211.35^{a,B}	46.59	162.52^{a,b,C}	47.09
BB	A^2A^2	AA	91.63^{a,A}	7.59	292.98^{b,B}	32.08	178.20^{a,b,C}	31.67
CC	A^2A^2	BB	95.87^{a,A}	3.71	246.18^{c,e,B}	21.47	145.14^{a,C}	15.77
BB	A^2A^1	AB	141.78^{b,A}	24.31	130.58^{d,B}	3.48	180.87^{b,C}	10.38
BB	A^2A^2	AB	117.13^{a,A}	3.44	268.72^{b,e,B}	51.88	186.16^{b,C}	13,19
BB	A^2B	AB	116.72^{a,A}	31.6	264.91^{b,e,B}	34.87	171.56^{a,b,C}	21.34

$^{A–C}$Different uppercase superscripts depict the statistical difference within a row ($P < 0.05$) between means for different yogurt batches.

$^{a–e}$Different lowercase superscripts depict the statistical difference within a column ($P < 0.05$) between means for the same yogurt batches at different casein haplotype.

[1]The values are expressed as micrograms of ascorbic acid equivalents per milliliter.

haplotype on the antioxidant activity of yogurt ($P < 0.001$). The antioxidant activity of the control sample showed different values as a function of the CN haplotype. The average ABTS value was 204.43 ± 57.81μg of AAE/mL, which increased in the order of BB-A^2A^2-AA < CC-A^2A^2-BB < BB-A^2A^2-AB < BB-A^2A^2-BB < BB-A^2A^1-AA < BB-A^2A^1-AB < BB-A^2B-AB (Table 2). The average FRAP value was 116.74 ± 29.3 μg of AAE/mL and the increasing sequence of radical scavenging was BB-A^2A^2-AA < CC-A^2A^2-BB < BB-A^2A^2-BB < BB-A^2B-AB < BB-A^2A^2-AB < BB-A^2A^1-AB < BB-A^2A^1-AA (Table 3). In particular, the control sample with the haplotype BB-A^2A^2-AA showed the lowest radical scavenging activity, whereas the control sample with the haplotype BB-A^2A^1-AA, BB-A^2A^1-AB and BB-A^2B-AB showed the highest value in both assays.

The chestnut and sulla honeys were used to fortify yogurt in our study. Total phenol, total flavonoid, ABTS, and FRAP values of the studied honey are reported in Table 4. Darker honey, such as chestnut honey, tends to have higher antioxidant properties than lighter ones (Gheldof et al., 2002). Total phenolic and flavonoid contents were higher in chestnut honey, around 2.5 times more than those found in sulla honey (18.6 vs. 7.23 mg of gallic acid equivalents/100 g and 5.15 vs. 1.95 mg of quercetin equivalents/100 g for total phenolic and flavonoid contents, respectively). The values found in studied samples match the results reported by other authors for chestnut and sulla honey (Bertoncelj et al., 2007; Pichichero et al., 2009; Perna et al., 2012). Chestnut honey presented the greater antioxidant activity in both assays. The findings confirm what has been found in previous studies by Perna et al. (2012), which highlighted a close correlation between antioxidant activity and polyphenol content.

Yogurts with added honey showed an increase of the antioxidant activity compared with control (Table 2 and 3). In particular, yogurts with added chestnut honey showed higher ABTS and FRAP values than the yogurts with added sulla honey. Among the yogurt with added chestnut honey, the one with the haplotype BB-A^2A^2-AB showed the highest radical scavenging activity (342.63 μg of AAE/mL), whereas that with the haplotype CC-A^2A^2-BB showed the lowest value (280.03 μg of AAE/mL; Table 3); chestnut yogurt with the haplotype BB-A^2A^2-AA showed the highest FRAP value (292.98 μg of AAE/mL), whereas that with the haplotype BB-A^2A^1-AB showed the lowest value (130.58 ug of AAE/mL; $P < 0.05$). Among the yogurt with added sulla honey, the one with the haplotype BB-A^2A^1-AB showed the highest ABTS value, whereas that made with the haplotype BB-A^2B-AB showed the lowest value (211.73 μg of AAE/mL); sulla yogurt with the haplotype CC-A^2A^2-BB showed the lowest FRAP value (145.14 μg of AAE/mL) and that with the haplotype BB-A^2A^2-AB showed the highest value (186.16 μg of AAE/mL).

The percentage change of antioxidant activity (Δ%) highlights the effect of the added depending on the haplotype and type of honey and is reported in Fig-

Table 4. Total phenolic and flavonoid contents and antioxidant activity of honey samples from different botanical origins

	Honey	
Item	Chestnut	Sulla
Total phenolic (mg of gallic acid equivalents/100 g)	18.6	7.23
Total flavonoid (mg of quercetin equivalents/100 g)	5.15	1.95
2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (μg of ascorbic acid equivalents/mL)	359.55	252.31
Ferric-reducing antioxidant power (μg of ascorbic acid equivalents/mL)	253.29	92.88

ures 1 and 2. The addition of chestnut honey resulted in an increase of the antioxidant activity of yogurt, evaluated by ABTS assay, showing a mean increase of 54.33%, with a variation from 18.36% (yogurt with the haplotype BB-A^2B-AB) to 142.90% (yogurt with the haplotype BB-A^2A^2-AA). Considering the FRAP assay, the effect of the addition of chestnut honey showed a significant increase in antioxidant activity in almost all yogurts. The average increase of antioxidant activity was 100.6%, with a variation from 46.79% (yogurt with the haplotype BB-A^2A^1-AA) to 219.76% (yogurt with the haplotype BB-A^2A^2-AA). Chestnut yogurt with the haplotype BB-A^2A^1-AB showed a decrease in antioxidant activity, about of −7.90% compared to the control. The antioxidant activity of yogurt with added sulla honey, measured by ABTS assay, showed a mean increase of 22%, with values that ranged from 6.60% (yogurt with the haplotype BB-A^2A^1-AA) to 113.84% (yogurt with the haplotype BB-A^2A^1-AA). The yogurt with the haplotype BB-A^2B-AB showed a decrease of −19.77% compared to the control. The mean percentage increase of antioxidant activity detected in the yogurt with added sulla honey, evaluated by FRAP assay, was 44%, with values that ranged between 94.48% (yogurt with the haplotype BB-A^2A^2-AA) and 12.87% (yogurt with the haplotype BB-A^2A^1-AA).

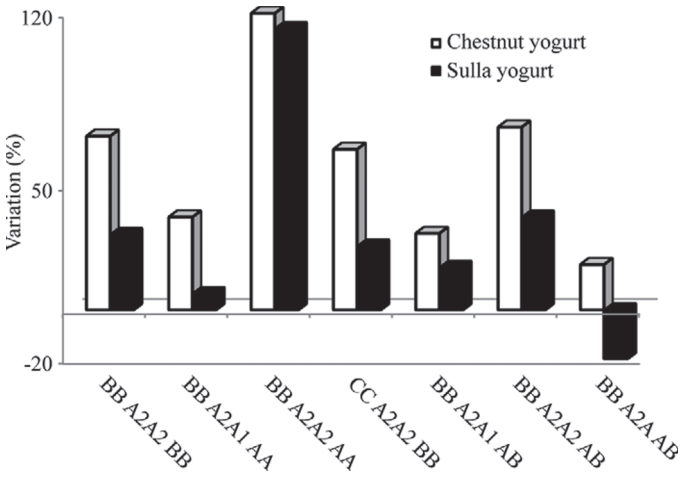

Figure 1. Percentage change of antioxidant activity of yogurt made from milk characterized by different casein haplotypes and fortified with honey than the control sample, measured using 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) assay and expressed as micrograms of ascorbic acid equivalents per milliliter of extract.

DISCUSSION

The lack of a widely accepted standardized method for evaluation of antioxidant properties of foods and the complex reactivity of bioactive compounds are the reason why we employed 2 different antioxidant capacity assays (Schlesier et al., 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 it is applicable to both hydrophilic and lipophilic antioxidant systems (Re et al., 1999). The FRAP assay is considered as a useful indicator of the antioxidant status to counteract the oxidative damage due to reactive oxygen species (Küçük et al., 2007), and it uses antioxidants as reductants in a redox-linked colorimetric method, employing an easily reduced oxidant system present in stoichiometric excess.

Yogurt itself has a large antioxidant capacity, related to the presence of different bioactive peptides from milk proteins through proteolysis by LAB (Kudoh et al., 2001; Virtanen et al., 2007; Gomez-Ruiz et al., 2008). The antioxidant capacity is also conditioned by the heat treatment undergone by the milk for the manufacture of the yogurt (Galleher et al., 2005), by the fermentation and postacidification during storage that determine production of organic acids (Correia et al., 2004), and by possible aggregation of peptide processes that occur during the enzymatic hydrolysis of whey protein and CN (Adt et al., 2011). In a previous study, Perna et al. (2013a) demonstrated that yogurts made from cow milk characterized by different CN haplotypes showed different antioxidant activity due to the specific amino acid sequence of the milk protein variants. Hernández-Ledesma et al. (2005) showed that peptides released from the A variant of β-LG are small (3 kDa) and are mainly responsible for antioxidant activity compared with the AB variant of β-LG.

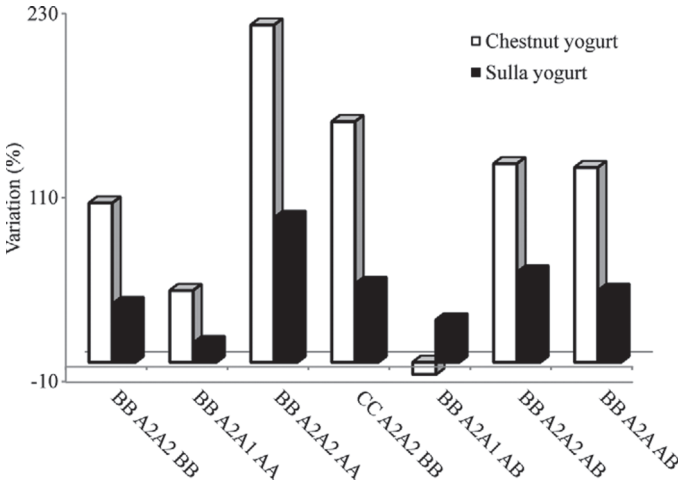

Figure 2. Percentage change of antioxidant activity of yogurt made from milk characterized by different casein haplotypes and fortified with honey than the control sample, measured by ferric-reducing antioxidant power assay and expressed as micrograms of ascorbic acid equivalents per milliliter of extract.

In the current work, we observed that the antioxidant activity of fortified yogurt is also affected strongly by the type of honey. In fact, the same yogurt sample showed different values as a function of the type of honey added. The addition of chestnut honey to yogurt resulted in a greater increase of the antioxidant activity compared with the addition of sulla honey. This could be due to the higher levels of phenolic acids and flavonoids found in the chestnut honey compared with sulla honey; in fact, these honey showed quantitatively and qualitatively different phenolic profiles (Perna et al., 2013b). Honey from different floral sources possess strong antioxidative activities and are strong reactive oxygen species scavengers (Beretta et al., 2005; Perna et al., 2012). Among the main factors responsible for the biological and nutraceutical activities, phenolic substances of honey have a key role (Al-Mamary et al., 2002; Aljadi and Kamaruddin, 2004). Also, it has been observed that the increase or decrease of antioxidant capacity of yogurts after the addition of honey is closely linked to the different CN haplotype. Prigent et al. (2003) demonstrated that the effect of the interaction between milk protein or peptides and phenolic compounds on antioxidant activity depends on both amino acid composition of proteins and type of phenols. The bond between polyphenol and protein is not identical, and 4 potential types of interactions exist between phenolic metabolites and proteins: hydrogen, hydrophobic, ionic, and covalent bonding (Hagerman et al., 1998; Rawel et al., 2002). The phenolic hydroxyl group is an excellent hydrogen bond donor and forms strong hydrogen bonds with the amide carbonyl of the peptide backbone (Luck et al., 1994; O'Connell and Fox, 2001). Many authors (Poncet-Legrand et al., 2006; Richard et al., 2006; Soares et al., 2007; Frazier et al., 2010) have reported that proline-rich proteins have a particularly high affinity for polyphenols. Dickinson and Mann (2006), using single molecule atomic force microscopy, showed that the protein wraps itself around the polyphenol by forming hydrophobic interactions between aromatic phenolic rings and proline residues. Caseins contain high numbers of proline residues evenly distributed throughout their amino acid sequences, they have relatively open structures, and are avid binders of polyphenols (Jöbstl et al., 2004; Pascal et al., 2008; Yan et al., 2009). Kartsova and Alekseeva (2008) reported that catechins bind strongest to the caseins, according to the order β-CN > α-CN > κ-CN, followed by whey proteins, namely α-LA, β-LG, and BSA. The milk protein polymorphism affects the amino acid composition of protein; the A^2 variant of β-CN differs from the A^1 variant because it has a Pro residue instead of a His residue (Korhonen and Marnila, 2013), which could explain the enhanced ability to interact with phenolic compounds. Likewise, in yogurt, the nitrogen fraction is composed of whole protein and peptides, obtained after proteolysis, which are characterized by reactive sites able to bind with different compounds, such as polyphenols. These protein-polyphenol complexes can reduce or enhance antioxidant activity. The different behavior observed among fortified yogurts led us to hypothesize that effects of protein-polyphenol complex on antioxidant activity are interactive, in agreement with that found by Arts et al. (2002). Those authors reported that the antioxidant capacity of the interaction between polyphenols and proteins is lower than the sum of the antioxidant capacity of individual components. The protein-polyphenol complexes could have effects in terms of bioavailability (Serafini et al., 1996), as the antioxidant activity of polyphenols could be modified by the presence of proteins (Arts et al., 2002).

CONCLUSIONS

This study highlights the complex dynamic actions that occur when foods with different biochemical characteristics interact. We demonstrate that milk protein polymorphisms and polyphenols play different roles in affecting the bioavailability and the antioxidant activity of yogurt. More in-depth mechanisms will require clarification in future investigations to identify the possible combination between casein haplotype and honey that can lead to the manufacture of yogurt formulations with specific nutraceutical properties.

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