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Short communication: Effect of genetic type on antioxidant activity of Caciocavallo cheese during ripening

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ABSTRACT

The aim of this work was to investigate the antioxidant activity of Caciocavallo cheese made from the milk of 2 breeds, Italian Brown and Italian Holstein, and ripened for 1, 30, 60, 90, and 150 d. The antioxidant activity of cheese was measured using the 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), ferric-reducing antioxidant power (FRAP), and thiol assays. Statistical analysis showed a significant effect of the studied factors. Italian Brown cheese had higher antioxidant activity than Italian Holstein cheese, and antioxidant activity increased during ripening of both cheeses types. Moreover, antioxidant activity varied during ripening depending on the rate of formation of soluble peptides. To date, few studies have evaluated the effect of genetic type on antioxidant capacity of the *pasta filata* cheeses; thus, this study forms the basis of new knowledge that could lead to the production of a *pasta filata* cheese with specific nutraceutical characteristics.
Key words: Caciocavallo cheese, genetic type, ripening, antioxidant activity

Short Communication

Cheese was and remains a staple in Western diets, representing a large part of total milk product consumption in many countries, including France, Greece, and Italy. Although cheese has had an adverse nutritional image due to the association of saturated fatty acids, cholesterol, and salt content with cardiovascular diseases, it is a rich source of proteins, vitamins, and minerals (especially calcium in a highly bioavailable form), and of short-chain fatty acids and certain *trans* fatty acids that can be considered part of a healthy diet.

During cheese ripening, casein is hydrolyzed into a large variety of peptides by an enzymatic system that consists of proteases of different origins (predominantly pepsin, trypsin, and chymotrypsin), fermentation of milk with proteolytic starter cultures, or proteolysis by enzymes derived from microorganisms or plants (Korhonen and Pihlanto, 2006). Many authors have reported that the proteolytic system plays an important role in the release of various bioactive peptides from the precursor protein where they are encrypted; these peptides influence different biological functions, such as antioxidant activity (Addeo et al., 1992; Smacchi and Gobbetti, 1998; Saito et al., 2000; Donkor et al., 2007). The domains within the milk proteins responsible for antioxidant activity have been identified (Kudoh et al., 2001; Rival et al., 2001; Hernandez-Ledesma et al., 2005a,b).

The biopeptides generated during ripening can be beneficial for oxidative defense by preventing the formation of free radicals or scavenging free radicals and active oxygen species that induce oxidative damage to biomolecules and cause aging, cancer, heart diseases, strokes, and arteriosclerosis (Fridovich, 1999). Previous research has reported that casein represents the primary substrate for proteolysis by bacterial starter and is a reserve for a wide variety of bioactive peptides (Meisel, 1998). Many studies have reported the relationship between antioxidant activity and concentration of low-molecular-weight peptides (Kudoh et al., 2001; Virtanen et al., 2007; Gómez-Ruiz et al., 2008). Moreover, many authors have shown that the antioxidant activity of CN could be related to its high tendency to chelate metals (Rival et al., 2001) and ability to donate electrons and atoms (Colbert and Decker, 1991).

Caciocavallo cheese is a typical product of southern Italy. It is a *pasta filata* cheese of medium or long proteolytic-lipolytic ripening, produced exclusively from raw cow milk, and coagulated with lamb rennet paste. *Pasta filata* cheese is made by stretching the acidified curd in hot water to about 80°C. This stretching leads to alignment of the paracasein matrix and coalescence of fat and moisture into elongated pools parallel to the protein fibers (Battistotti and Corradini, 1993); stretching also influences proteolysis (De Angelis and Gobbetti, 2011). The physicochemical, nutritional, and organoleptic characteristics of Caciocavallo cheese are closely related to milk quality, which can change ac-

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cording to genetic and physiological factors of the cow (De Marchi et al., 2008). Many reports described the influence of addition of starter and nonstarter cultures on the antioxidant activity of cheese (Gupta et al., 2009), whereas the effect of livestock factors, such as genetic type, have not yet been well established.

The objective of this study was to assess the effect of genetic type (**GT**; Italian Holstein or Italian Brown) on antioxidant activity of water-soluble extracts of Caciocavallo cheese at different stages of ripening (1, 30, 60, 90, and 150 d) using the 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (**ABTS**), ferric-reducing antioxidant power (**FRAP**), and thiol assays.

Thirty Caciocavallo cheeses produced with milk of 2 breeds (IB and IH) reared indoors on the same farm in the province of Potenza (southern Italy) were used in this experiment. All cows received the same feed ration, which was calculated according to their daily requirements for maintenance and production. Feeding was based on the use of silage and dried alfalfa hay as the main forage sources and concentrates based on ground corn and soybean meal. The cows were in good clinical condition, and the results obtained from analysis of individual milk showed a mean SCC of 4.89 $\log_{10}$ cells/mL, a value below the legislative limit established by the European Union (2004). For each group (15 animals), all milk from the morning milking was collected to make a composite sample, for a total of 2 composite samples (2 GT). On the same dairy farm, 15 Caciocavallo cheeses (average weight of 2.2 kg each) were produced from each composite sample at the same time and under the same conditions, which were then ripened for different periods: 1, 30, 60, 90, and 150 d.

After milking, the milk was filtered to remove foreign substances, heated to 37 to 38°C, coagulated by using lamb rennet paste (177 international milk clotting units/mL; 40.0 mg/kg), and inoculated (3%, vol/vol) with a natural whey starter (pH 3.8). The natural whey starter was obtained by incubating, at 42°C for about 24 h, fresh whey derived from the Caciocavallo cheese manufacture of the previous day. Lactic acid bacteria load of the natural whey starter, measured on de Man, Rogosa, and Sharpe and M17 media at 37°C, ranged from 10^6 to 10^7 cfu/mL. The coagulum was first cut coarsely, heated under whey at 45°C for 2 h, milled into pieces of about 1.5 cm, and held at room temperature until the pH reached approximately 5.3. When the acidified curd was ready, it was manually stretched in hot water (70–80°C). Caciocavallo cheeses were dipped in brine (27–30% NaCl) for 24 h and then ripened at 10 to 12°C and 75 to 80% relative humidity.

Sixty milliliters of distilled water was added to 20 g of grated Caciocavallo cheese and the mixture was placed in an ultrasonic water bath (Elma Transsonic 460/H, Singen, Germany) for 10 min. The homogenates were centrifuged (CR 4.12, fixed-angle rotor; Jouan, Saint Herblain, France) at 5,000 × *g* at 4°C for 20 min. The upper fat layer was discarded and the water extract was retained. The water-soluble extracts (**WSE**) were filtered on paper filter and, to remove any further impurities, were then filtered through a membrane filter (0.45 μm) and used to measure the antioxidant activity.

A modification of the original method of Re et al. (1999) was applied to assess the scavenging capacity of WSE in a reaction with the ABTS radical cation ($\mathbf{ABTS^{\bullet+}}$), generated by oxidation of ABTS 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 $ABTS^{\bullet+}$ 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. To evaluate antioxidant capacity, the $ABTS^{\bullet+}$ solution was diluted with ethanol (96%) to obtain absorbance of 0.700 ± 0.020 at 734 nm. Two milliliters of $ABTS^{\bullet+}$ solution was mixed with 100 μL of the WSE of samples 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. A calibration curve was constructed, using ascorbic acid (2.2–0.25 μ*M*) as standard, and the results were expressed as milligrams of ascorbic acid equivalents (**AAE**) per 100 g of cheese.

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* 2,4,6-tripyridal-s-triazine (TPTZ) in 40 m*M* HCl, and 1 mL of 20 m*M* $FeCl_3$ (in the ratio 10:1:1 vol/vol/vol). The reagent was prepared daily and warmed to 37°C before use. Aliquots of 100 μL of WSE 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). 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 milligrams of AAE per 100 g of cheese.

The number of free thiol groups was determined according to Ellman's method 155 (Ellman, 1959), with some modifications. Two hundred fifty microliters of WSE of sample was 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 [5,5'-dithiobis-(2-nitrobenzoic acid)] reagent solution (4 mg in 1 mL of sodium phosphate buffer). After the solution was mixed

Table 1. Antioxidant activity[1] of Italian Brown (IB) and Italian Holstein (IH) Caciocavallo cheeses during ripening

	ABTS				FRAP				Thiols			
	IB		IH		IB		IH		IB		IH	
Ripening, d	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	$33.55^{A,a}$	4.54	$27.93^{A,b}$	8.58	$2.11^{A,a}$	0.19	$1.98^{A,a}$	0.41	$316.72^{A,a}$	40.19	$338.58^{A,a}$	35.06
30	$38.34^{B,a}$	5.59	$37.18^{B,a}$	6.61	$2.80^{B,a}$	0.48	$2.61^{B,a}$	0.45	$368.88^{B,a}$	33.99	$347.57^{A,a}$	28.00
60	$40.52^{BC,a}$	6.22	$39.83^{B,a}$	3.60	$3.74^{C,a}$	0.70	$3.20^{C,b}$	0.36	$399.48^{C,a}$	41.97	$367.62^{AC,b}$	22.97
90	$42.97^{CD,a}$	4.00	$38.50^{B,b}$	3.34	$3.94^{CD,a}$	0.55	$3.42^{C,b}$	0.19	$428.21^{C,a}$	38.14	$393.03^{BC,b}$	38.47
150	$44.70^{D,a}$	4.34	$44.08^{C,a}$	2.23	$4.35^{D,a}$	0.43	$4.02^{D,a}$	1.22	$462.58^{D,a}$	67.27	$401.88^{B,b}$	15.02
Overall	40.02	6.22	37.50	7.49	3.39	0.95	3.05	0.94	395.17	67.10	369.73	37.56

[A–D] For each parameter, different uppercase superscripts within a column indicate a statistical difference ($P < 0.05$) between means for the same genetic type at different ripening times.

[a,b]For each parameter, different lowercase superscripts within a row indicate a statistical difference ($P < 0.05$) between means for different genetic types.

[1]ABTS = 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid radical scavenging assay (values expressed as milligrams of ascorbic acid equivalents per 100 g); FRAP = ferric-reducing antioxidant power assay (values expressed as milligrams of ascorbic acid equivalents per 100 g); Thiols = thiol assay [values expressed as μM of thiol groups (SH)].

and allowed to stand at room temperature (25°C) for 30 min, absorbance was read at 412 nm. 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 (**SH**). The results were expressed as concentration (μM) of SH. Each determination and measurement was made in triplicate.

Statistical analysis was performed using the general linear model (GLM) procedure of SAS software (SAS Institute, 1996), using a 2-factor model without interactions:

$$y_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij},$$

where y_{ij} is the observation; μ is the overall mean; α_i is the fixed effect of the ith GT ($i = 1, 2$); β_j is the fixed effect of the jth ripening time ($j = 1, 2, 3, 4, 5$); and ε_{ij} is the random error. Before setting the values, expressed as a percentage, they were subjected to angular transformation. Student's t-test was used for all variable comparisons. Differences between means at the 95% ($P < 0.05$) confidence level were considered statistically significant.

The antioxidant activity of WSE of Caciocavallo cheeses was assessed by ABTS, FRAP, and thiol assays during ripening for 150 d (Table 1). In the last years, several methods have been developed to assess total antioxidant capacity because of the lack of standard quantification methods and the interactions among different antioxidant components (Schlesier et al., 2002). Several studies have reported that antioxidant activity depends on the system used (Janaszewska and Bartosz, 2002; Bauzaite et al., 2003) and, therefore, it recommended to base any conclusions on at least 2 different test systems (Moon and Shinamoto, 2009). The ABTS assay is a method to screen antioxidant activity that is applicable to both lipophilic and hydrophilic antioxidants (Re et al., 1999). The FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method, using an easily reduced oxidant system present in stoichiometric excess. The thiol assay measures the number of thiol groups (SH), such as glutathione and protein thiol groups, which play an essential role as antioxidants. These compounds can act as free radical scavengers and chelators of metal ions.

In general, the results showed that all tested samples exhibited antioxidant activity at 1 d of ripening: the average ABTS value was 30.73 mg of AAE/100 g, the FRAP value was 2.04 mg of AAE/100 g, and the thiol value was 327.65 μM SH. Antioxidant activity increased significantly during ripening ($P < 0.05$). In particular, the average values varied from 33.55 (1 d) to 44.70 mg of AAE/100 g (150 d) in the ABTS assay, from 2.11 to 4.35 mg of AAE/100 g in the FRAP assay, and from 316.72 to 462.58 μM SH in the thiol assay. This increase is strictly connected to the increasing concentration and bioavailability of antioxidant compounds with beneficial effects on the consumer's health. In this regard, Higurashi et al. (2007) suggested that cheese antioxidant peptides might have a beneficial suppressive effect on abdominal adipose accumulation and could prevent the development of metabolic syndrome. Furthermore, the increase in antioxidant activity could further prevent oxidation reactions during storage of cheese and increase its nutritional value (Gómez-Ruiz et al. 2008). Our results are in agreement with those reported by Gupta et al. (2009), who observed an increase in antioxidant activity as ripening proceeded in Cheddar cheese samples, reaching a maximum value during the fourth month. In contrast, Bottesini et al.

(2013) reported that antioxidant activity in Parmigiano Reggiano cheese remained constant during ripening, suggesting that the peptides and proteins are not particularly affected by biochemical processes during aging. During ripening of cheese, hydrolysis of casein by coagulants, plasmin, and starter and nonstarter bacterial proteases and peptidases lead to the formation of water-soluble peptides and free amino acids (Fox et al., 1994). The presence of small peptides and free amino acids is attributed to the role played by bacterial proteinases and peptidases in the degeneration of primary proteolytic products from α_{S1}-CN and β-CN produced by chymosin and plasmin, respectively (Singh et al., 1997). Casein solubilization is also influenced by the technique of stretching used during Caciocavallo cheesemaking. Gagnaire et al. (2011) observed, in cheese from Ragusa, that casein degradation is greatly increased by plasmin action, in agreement with the stretching used during cheesemaking at 70 to 80°C and the presence of residual chymosin, which is known to be active during long periods of aging in other types of hard cheeses. The heat treatment of *pasta filata* cheese, in addition, exposes reactive thiol groups, which can form disulfide links with other reactive thiol groups and disulfide bridges through thiol group-exchange reactions.

Analysis of variance showed a significant effect of GT and ripening time on antioxidant activity of Caciocavallo cheeses ($P < 0.001$). In general, IB cheese had higher antioxidant activity than IH cheese ($P < 0.05$). Chiang and Chang (2005) highlighted the positive correlation between protein content and antioxidant activity. In fact, IB cheese had a higher protein content than IH cheese (45.37 vs. 44.77%, respectively; $P < 0.05$) as reported in our earlier study (Perna et al., 2014). When comparing Caciocavallo cheese made from IB and IH milks at 1 d of ripening, the differences between the average ABTS values were statistically significant ($P < 0.05$), whereas no significant differences were found in FRAP and thiol values.

The ABTS values increased from 33.55 mg of AAE/100 g at 1 d of ripening to 44.70 mg of AAE/100 g at 150 d in IB cheese, and from 27.93 mg of AAE/100 g at d 1 of ripening to 44.08 mg of AAE/100 g at 150 d in IH cheese. The ABTS radical scavenging activity increased by about 33 and 58% over the initial value in IB and IH cheeses, respectively. No significant differences were observed at 30 and 60 d of ripening ($P > 0.05$), whereas, at the end of ripening, IB cheese had an ABTS value significantly higher than that of IH cheese ($P < 0.05$).

Antioxidant activity measured by the FRAP assay increased by more than 106% in IB cheese and by about 103% in IH cheese. Comparing Caciocavallo cheeses, the differences between the average FRAP values were statistically significant at 60 and 90 d of ripening ($P < 0.05$).

The trend of antioxidant activity evaluated by thiol assay confirmed the results obtained by ABTS and FRAP assays: antioxidant activity of IB cheese was more than twice that of IH cheese. The differences between products were statistically significant at the end of ripening (90 and 150 d; $P < 0.05$). Gupta et al. (2009) found that the antioxidant activity in Cheddar cheese varied depending on the rate of formation of soluble peptides (proteolysis). The same authors, in a later work (Gupta et al., 2013), showed that angiotensin-I converting enzyme (ACE) inhibitory activity increased with the increase in the protein content of the WSE of Cheddar cheeses.

Previously, we reported that the trend for proteolysis in Caciocavallo cheese was greatly influenced by GT ($P < 0.05$; Perna et al., 2014). This result was linked to genetic variants of caseins, which are an important differential characteristic because they influence cheese structure and, therefore, the action of proteolytic enzymes during ripening (Mariani et al., 2002). In fact, it was found that proteolysis phenomena occurring during cheese processing are more intense in cheese made from IB milk. In the current study, the IB cheese showed a higher solubilization of casein compared with IH cheese. In particular, IB cheese showed higher proteolysis ($P < 0.05$) of β- and para-κ-CN in each interval of ripening considered. The greater degradation of α_{S1}-CN in IH Caciocavallo cheese is probably due to the greater pH and moisture content that influence its susceptibility to hydrolysis by proteases (Creamer, 1985). Lourens-Hattingh and Viljoen (2001) suggested that antioxidant activity is closely linked to milk protein degradation. Similar results were obtained by Hajirostamloo (2010), who reported that the concentration of ACE-inhibitory peptides depends on a balance between their formation and further breakdown into inactive peptides and AA that, in turn, depends on storage time and conditions. Conversely, variation in antioxidant activity is linked to the possible aggregation of peptide processes that occur during the enzymatic hydrolysis of whey protein and CN, with formation of macro-aggregates that reduce the antioxidant capacity (Pattorn et al., 2012).

In conclusion, cheese is a complex food matrix containing a large number of different peptides, some of which are characterized by bioactivity useful to the consumer's health. The data obtained in this study show that variability in the antioxidant power of the cheeses was a function of genetic type (breed) and ripening time. Cheese from Italian Brown milk had greater antioxidant activity, probably because of its higher protein content compared with cheese made from Italian Hol-

stein milk. Moreover, antioxidant activity varied during ripening, depending on the rate of formation of soluble peptides. To date, few studies have evaluated the effect of genetic type on antioxidant capacity of *pasta filata* cheeses; thus, this study forms the basis of new knowledge that could lead to the production of a *pasta filata* cheese with specific nutraceutical characteristics.

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