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Effect of g.9476869G>A myeloperoxidase (MPO) gene polymorphism on the antioxidant activity of milk from Polish Holstein-Friesian cows of the Black-and-White variety (HO)

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Myeloperoxidase (MPO) is an important enzyme, which is one of the components of the antibacterial system in neutrophils and monocytes. MPO participates in the inflammatory response in multiple locations in the body, including the mammary glands. As a result of the activity of MPO, many oxidising compounds as well as reactive oxygen species are generated. It seems that myeloperoxidase may be a marker linking inflammation processes and oxidative stress. So far, there are no literature data on the association between the MPO gene polymorphism and the antioxidant properties of milk. The aim of the study was to analyse the effect of g.9476869G > A polymorphism of myeloperoxidase (MPO) gene and age of cows on the antioxidant activity of milk and other milk traits in Polish Holstein-Friesian cows. Polymorphism of MPO gene was identified by the PCR-RFLP method using the HphI endonuclease. The total antioxidant capacity of milk samples was measured by the Trolox Equivalent Antioxidant Capacity (TEAC) method. It was found that the GG genotype was the most frequent (0·606). The genotype at the tested MPO locus and the age of the animals affected the antioxidant activity of milk. Milk from cows with the GA genotype was characterised by a significantly higher antioxidant activity than milk from cows with the GG genotype ($P < 0·0001$). The analysis of interaction showed that cows with the GA genotype and older than 6·5 years produced milk with a significantly higher antioxidant activity compared with younger cows with the same genotype ($P < 0·0001$), as well as cows with the GG genotype of all ages ($P < 0·0001$).

Keywords: Myeloperoxidase, milk antioxidant activity, gene polymorphism.

Disorders of pro- and antioxidant homeostasis in the body provide the basis for many diseases both in humans and animals (Čipak Gašparović et al. 2010). Long-term oxidative stress (due to the presence of reactive oxygen species, ROS) causes permanent changes in the structure of many active molecules, including DNA, proteins, carbohydrates and lipids, which in turn disrupt biological functions in cells (Rahal et al. 2014). Free radicals formed as by-products of oxygen metabolism include, first of all: the superoxide anion radical ($O_2\bullet$), singlet oxygen ($^1O_2$), hydroxyl radical ($\bullet OH$), hydroperoxyl radical ($HO_2\bullet$), as well as non-radical oxygen derivatives: hypochlorous acid (HClO) and hydrogen peroxide (H_2O_2). Along with consumers' growing awareness, an increased interest in high health-value food products rich in natural antioxidants has been observed. Some of the richest sources of biologically active compounds with antioxidant properties include milk and dairy products. Antioxidants present in milk include mainly vitamins A, C and E, carotenoids, enzymes (such as glutathione peroxidase, superoxide, dismutase, glutathione reductase), selected proteins (e.g. lactoferrin (Lf), lysozyme (Lys), casein, lactoperoxidase) and fatty acids (e.g. C18 : 2 cis -9 trans-11 (CLA9), C18:2 trans -10 cis-12 (CLA10), butyric acid (C4:0), and *n*-3 and *n*-6 fatty acids) (Kuczyńska et al. 2013). Antioxidants limit the negative effects of oxidative stress as a result of the neutralisation of free radicals and their transformation into less active derivatives.

Myeloperoxidase (MPO, EC 1·11·2·2) is an important enzyme, which can affect the antioxidant activity of milk. Myeloperoxidase belongs to the oxidoreductase family which can oxidise various types of chemical compounds in the presence of hydrogen peroxide. MPO is one of the components of the antibacterial system in neutrophils and

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monocytes, which participates in the inflammatory response in multiple locations in the body, e.g. in the mammary glands. Myeloperoxidase catalyses the oxidation of halide ions (mostly chlorides) to respective acids in the presence of hydrogen peroxide. On the one hand, this enzyme uses H_2O_2, preventing the formation of hydroxyl radicals, while on the other hand, causing the formation of the hypochlorous acid which is a strong oxidising agent. As a result of its activity, multiple compounds with oxidising properties are generated, as well as reactive oxygen species, in particular singlet oxygen, hydroxyl radicals and ozone (Klebanoff, 2005). It has been determined that in humans, a partial or total lack of myeloperoxidase causes a greater susceptibility to infection and increases the risk of cancer (Marchetti et al. 2004). Additionally, the lower quantity or the lack of MPO causes an increase in oxygen metabolism, and thus a greater than normal production of reactive oxygen species (Locksley et al. 1983).

The bovine myeloperoxidase gene is located on chromosome 19. It is composed of 14 exons, but only 12 of them are coding regions. Within the bovine MPO gene, multiple single nucleotide polymorphisms (SNP) were identified (http://www.ncbi.nlm.nih.gov). These include both coding sequences and introns. So far, there are no literature data on the association between the MPO gene polymorphism and the antioxidant properties of milk, and therefore the main objective of this study was to analyse the g.9476869G > A polymorphism within intron 8 of the myeloperoxidase gene and to determine its effects on the composition and antioxidant activity of milk of Polish Holstein-Friesian cows.

Material and methods

This study was performed using milk samples obtained from 127 Polish Holstein-Friesian cows of the Black-and-White variety (HO) (1sample/animal). The cows were maintained in one dairy farm, in a free-stall housing system and fed a total mixed ration (TMR). Cows were between the ages of 3·5 and 10 years (between 2 and 7 lactations). The animals analysed were daughters of 61 sires, with sire halb-sib family sizes varying between 1 and 14. Milk samples were collected on the same day from all cows in the same stage of lactation (above 200 d) into 100 ml plastic tubes and kept at a temperature of 4 °C for further analysis. All cows at the day of sampling were healthy. The data on milk performance (daily milk yield, fat and protein percentage and SCC) were obtained from the reports of monthly milk recording carried out in the studied herd.

Determination of antioxidant activity of milk using the TEAC method

The Trolox Equivalent Antioxidant Capacity (TEAC) method is used for measuring the total antioxidant capacity of biological and food samples. One of its main advantages is the possibility to determine the antioxidant properties of both hydrophilic and hydrophobic samples. The method involves a spectrophotometric measurement of the quenching of ABTS● radical cations by antioxidants contained in the sample at 734 nm. Radicals are formed due to the presence of potassium persulphate, and the resulting free radicals add green and blue colour to the sample. Antioxidants present in the solution cause a reduction in the number of ABTS● radicals, thus reducing the colour intensity of the solution which in turn decreases its absorbance at 734 nm. The decrease in the colour intensity of the solution is proportional to the level of antioxidants in the sample (Re et al. 1999).

Preparation of extracts

The milk samples (2 ml) were homogenised in the presence of methanol (Dorchem, Cracow, Poland) at a ratio of 1 : 10 for 2·5 min at 8600 rpm using the homogeniser X120 (Danlab, Białysok, Poland). The resulting homogenate was centrifuged for 20 min at 4000 rpm using the centrifuge MPW 223E (MPW Med. Instruments, Warsaw, Poland). Afterwards, the supernatant liquid was carefully transferred to test tubes. The resulting extract was set aside to determine the antioxidant capacity by the TEAC method (Plust et al. 2011).

Preparation of reagents and performance of tests

The basic reagent was obtained by dissolving ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid, Sigma-Aldrich, Saint Louis,MO USA) in distilled water at a concentration of 7 mM, and then persulphate potassium was added to achieve the final concentration of 2·45 mM. The resulting reagent was left for 16 h in darkness to generate free radicals. After 16 h, the basic reagent was diluted with 96% ethanol so that the absorbance of the solution at 734 nm was 0·7 ± 0·2. The resulting solution was used as the test reagent. To properly perform the tests, 30 µl of methanol was mixed with 3 ml of the test reagent and after 30 min absorbance was measured at 734 nm using the spectrophotometer UV-VIS Genesys 20 (Thermo ScientificTM, Waltham, MA USA), thus eliminating the effects of the solvent on the end results. Next, 30 µl of the extract was mixed with 3 ml of the test solution, and after 30 min absorbance was measured at 734 nm. The antioxidant activity tests were performed in two replicates. The results are presented as antioxidant activity in µM TE (Trolox Equivalent)/ml (Van den Berg et al. 1999; Plust et al. 2011).

Genomic DNA isolation

The DNA was isolated from the milk somatic cells according to the method of Pokorska et al. (2016). The quantitative and qualitative analysis of the isolated DNA was verified spectrophotometrically using NanoDrop 2000 (Thermo Scientific, Waltham, MA USA). The isolated DNA was stored at a temperature of −25 °C for further analysis.

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Identification of g.9476869G > A myeloperoxidase gene polymorphism by the PCR-RFLP method

Based on the analysis of DNA sequenced of 6 cows selected at random, the g.9476869G > A polymorphism in intron 7 of the MPO gene was determined. This mutation was later identified in other animals by the PCR–RFLP method using the HphI (Thermo Scientific, Waltham, MA USA) endonuclease.

The 403 bp of the MPO gene was amplified with the use of starters (5′-TCAGGAGCAGGTGAAGAATC-3′ and 5′-TA GCTCCCTGTGACCAAGTC-3′) designed using Primer3Plus (Untergasser et al. 2007) based on a reference sequence (GenBank accession number NW_003104488·1). PCRs were carried out with a C1000 Thermal Cycler (Bio-Rad, Hercules, CA USA) in PCR reaction mixture (1× Taq Bufor + KCl (Thermal Scientific, Waltham, MA USA), 2·14 mM $MgCl_2$, 0·33 μM each primers, 0·12% formamide, 200 μM of each dNTP, 80–100 ng of DNA template and 0·35 unit of Taq polymerase (Thermal Scientific, Waltham, MA USA) in a final volume of 20 μl. The cycling conditions were: initial denaturation 95 °C for 5 min, 34 cycles: 95 °C for 35 s, 60 °C for 35 s, 72 °C for 30 s, and a final elongation 72 °C for 6 min. The amplicons were digested in a reaction mixture containing 10 μl PCR products, 1× buffer (BioLabs, Hitchin, UK), 0,25 U HphI (BioLabs, Hitchin, UK) and water in a final volume of 20 μl. The reactions were incubated at 37 °C for 1 h. The digested products were detected by 12% polyacrylamide gel electrophoresis. The polyacrylamide gels were prepared by mixing 9·0 ml 40% polyacrylamide solution (29 : 1), 3 ml 5× TBE buffer, 1·5 ml glycerol, 0·3 ml 10% ammonium persulfate, 30 μl TEMED and 17·67 ml water. Electrophoresis was performed using Mini-PROTEAN (Bio-Rad, Hercules, CA USA) at 100 V for 90 min in the presence of the pUC19 DNA/MspI marker (Thermo Scientific, Waltham, MA USA). RFLP bands were stained with silver nitrate and photographed for further analysis.

Statistical analysis

The allele and genotype frequencies of MPO gene were calculated using PROC FREQ of SAS® 9.4 software (SAS Institute, Cary NC, USA). The χ^2 test for Hardy-Weinberg equilibrium (H–W) was used to compare the observed and expected MPO genotype frequencies.

In order to estimate the effects of MPO polymorphism and cow age on the antioxidant capacity of milk and other milk traits estimated as the averages for the lactation in which the antioxidant capacity of milk was measured, two-way analysis of variance with interaction was performed. The data were analysed by PROC GLM of SAS® 9.4 software (SAS Institute, Cary NC, USA), according to the following model:

$$Y_{ijk} = \mu + a_i + b_j + (ab)_{ij} + \varepsilon_{ijk}$$

where: Y_{ijk} – milk traits (antioxidant capacity, daily yield, fat percentage, protein percentage, SCC); μ – overall mean; a_i – effect *i*th MPO genotype (GG, GA), b_j – effect of j^{th} cow age (≤5 year, 5·1–6·5 year, >6·5 year), $(ab)_{ij}$ – genotype by cow age interaction, ε_{ijk} – random error.

The effects of daily milk yield, milk fat and protein percentage and SCC (measured on the day of milk sampling) on the antioxidant capacity of milk were analysed by means of PROC GLM of SAS® 9.4 software (SAS Institute, Cary NC, USA), according to the following model:

$$Y_{ijklm} = \mu + c_i + d_j + e_k + f_l + \varepsilon_{ijklm}$$

Y_{ijklm} – antioxidant capacity of milk; μ – overall mean, c_i – effect of i^{th} daily milk yield (kg) (≤20·0, 20·1–30·0, >30·0), d_j – effect of *j*th milk fat percentage (≤3·20, 3·21–4·00, >4·00); e_k – effect of k^{th} milk protein percentage (≤3·50, 3·51–4·00, >4·00); f_l – effect of *l*th SCC ($\times 10^3$ cells/ml) (<200, 200–400, >400).

Significance of differences between least squares means was verified using the Sheffe test. Classes of daily milk yield, milk fat and protein percentage and SCC were distinguished based on mean and standard deviation (SD) for these traits. Cow with values of the traits lower than mean – SD were assigned to the first class, the second class was defined for cows with values of the traits equal to mean ± SD, and the third class for cows with values of the traits higher than mean + SD.

Results

Three genotypes (GG, GA and AA) were identified at g.9476869G > A locus of the myeloperoxidase (MPO) gene (Fig. 1). It was determined that the GG genotype was the most frequent (0·606) and the AA genotype was found in only one animal (Table 1). Due to low frequency, the AA genotype was not included in the statistical analysis. The studied herd was not in Hardy-Weinberg equilibrium at g.9476869G > A MPO locus ($P = 0·023$).

It was found that the genotype at the tested MPO locus and the age of cows affected the antioxidant activity of milk. Milk from cows with the GA genotype was characterised by a significantly higher antioxidant activity than milk from cows with the GG genotype ($P < 0·0001$: Table 2). The differences were particularly noticeable in the oldest group of cows aged 6·5 years and above (Table 3). The GA genotype corresponded to a higher percentage of proteins in the milk ($P < 0·05$), but a lower daily milk yield ($P < 0·05$).

When analysing the influence of cow's age on the antioxidant milk capacity, it was found that the oldest cows produced milk with a significantly higher antioxidant activity than cows aged 5 years and younger as well as those from 5·1–6·5 years old ($P < 0·01$ and $P < 0·0001$ respectively) and had a significantly higher protein percentage ($P < 0·001$ and $P < 0·01$ respectively) (Table 2).

The performed analysis of interaction showed that cows with the GA genotype and older than 6·5 years produced milk with a significantly higher antioxidant activity compared with cows of the same genotype but belonging to the first ($P < 0·0001$) and the second ($P < 0·0001$) age

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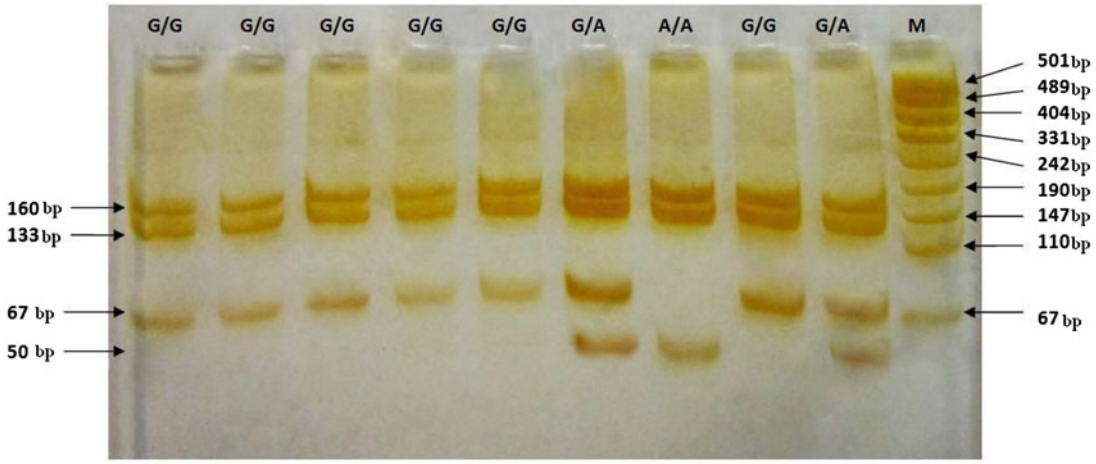

Fig. 1. The MPO gene polymorphism (g.9476869G > A) by electrophoresis in 12% polyacrylamide gels stained with silver nitrate.

Table 1. Frequencies of genotypes and alleles at the MPO g.9476869G > A locus

Genotype	*N*	Frequency
GG	77	0·606
GA	49	0·386
AA	1	0·008
Allele		
G		0·799
A		0·201

group, as well as with cows of the GG genotype in all age groups ($P < 0{\cdot}0001$) (Table 3). However, the age of cows with the GG genotype had no significant impact on the antioxidant activity of their milk.

Statistical analysis indicated differences in the antioxidant activity of milk depending on the daily milk yield of cows. As the daily yield of cows increased, a decrease in the antioxidant activity of milk was observed. Consequently, the cows with the highest daily yield (>30 kg) produced milk of the lowest antioxidant activity (Table 4).

Discussion

Myeloperoxidase (MPO) enzyme has the ability to oxidise the chemicals compounds and is one of the main antibacterial systems called myeloperoxidase system, occurs in the body fluids (e.g. milk and saliva). The main products of the MPO system are reactive oxygen species (ROS) such as hypochlorous acid, hydroxyl radical, singlet oxygen, superoxide anion which rapidly react with proteins, DNA, and fatty acids (Hawkins et al. 2003). ROS are involved in the aerobic mechanism of cell killing by neutrophils and monocytes (Król & Konopka, 2003), so myeloperoxidase may increase oxidative stress, and thus can affect the antioxidant activity of body fluids.

It can be supposed that the observed limited variety at g.9476869G > A position of MPO gene results from the long-term selection in the population of Holstein-Fresian cattle which was endorsed by H-W equilibrium test. Polymorphism of MPO gene has not been studied in cattle so far and there is no data about it in literature.

In the present study it was found that GG genotype was associated with lower antioxidant activity of milk than GA genotype (Table 2). It seems that analysed polymorphism can be genetic marker of antioxidant activity of milk, but it should be verified on a larger group of animals.

We observed that the percentage of protein in milk increased with the age of cows. Similar results were demonstrated by Gurmessa & Melaku (2012) and Pilarska (2014). Furthermore, we showed that older cows produced milk with higher antioxidant activity. From existing scientific data, the physical ageing process is related to a greater susceptibility to oxidative stress, which in turn may be associated with disorders in the functioning of proteasomes and the excessive accumulation of oxidised proteins in cells due to the reduced performance of repair systems (Friguet, 2006). The higher antioxidant capacity of milk in older cows is probably due to the higher level of antioxidant compounds which are probably formed in the course of cellular breakdown or accompany the ageing processes (Decker et al. 2000). These compounds may include polyamines, amino acids with functional groups on the side chain, enzymes, tocopherols, esters and thiol-delivering compounds (Pisulewski, 2007). Additionally, slower metabolism in older animals may result in antioxidant enzymes being 'depleted' at a slower rate (Michalak et al. 2014). It is difficult to say at this time whether the increased antioxidant capacity of milk from older cows can be considered advantageous for consumers. This issue requires further studies, taking into account, in particular, the type of antioxidants present in the analysed samples.

Moreover, the present study showed that milk from high-producing cows (>30 kg) were characterised by lower antioxidant activity than milk from cows with lower yield (<20 kg). The studies of Lohrke et al. (2004) and

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Table 2. Effects of g.9476869G > A SNP and cow age on the antioxidant capacity and milk traits such as: daily milk yield, milk fat and protein percentage and Somatic Cell Count (SCC)

		TEAC (μM TE)		Daily milk yield (kg)†		Fat (%)†		Protein (%)†		SCC† (×10³ cells/ml)	
Effect	*N*	LSM	SE	LSM	SE	LSM	SE	LSM	SE	LSM	SE
Genotype											
GG	67	$1{\cdot}63^{A}$	0·16	$33{\cdot}07^{D}$	0·72	3·64	0·06	$3{\cdot}44^{D}$	0·03	334·91	46·14
GA	44	$3{\cdot}23^{A}$	0·22	$30{\cdot}20^{D}$	0·97	3·77	0·08	$3{\cdot}55^{D}$	0·04	357·88	62·57
Age (year)											
≤5	42	$1{\cdot}73^{A}$	0·18	31·05	0·81	3·83	0·07	$3{\cdot}44^{C}$	0·04	365·59	52·23
5·1–6·5	50	$2{\cdot}08^{B}$	0·18	32·61	0·81	3·70	0·07	$3{\cdot}37^{B}$	0·04	335·43	51·84
≥6·5	19	$3{\cdot}49^{AB}$	0·32	31·25	1·41	3·60	0·12	$3{\cdot}68^{BC}$	0·06	338·17	90·46

TEAC, The Trolox Equivalent Antioxidant Capacity method; SCC, Somatic Cell Count; LSM, least-squares mean; SE, standard error.
†The average values for the lactation in which the antioxidant capacity of milk was measured.
LSMs with the same superscript letters in the same column within the effect differ significantly at: ^{A} $P < 0{\cdot}0001$; ^{B} $P < 0{\cdot}001$; ^{C} $P < 0{\cdot}01$; ^{D} $P < 0{\cdot}05$.

Table 3. Effect of interaction genotype × cow age on the antioxidant capacity of milk

		TEAC (μM TE)	
Genotype × cow age	*N*	LSM	SE
1 × 1	29	$1{\cdot}62^{A1}$	0·21
1 × 2	25	$1{\cdot}61^{A2}$	0·26
1 × 3	13	$1{\cdot}68^{A3}$	0·35
2 × 1	13	$1{\cdot}84^{A4}$	0·30
2 × 2	25	$2{\cdot}55^{B}$	0·26
2 × 3	6	$5{\cdot}30^{A1\ -\ A4,\ B}$	0·52

TEAC, The Trolox Equivalent Antioxidant Capacity method; LSM, least-squares mean, SE, standard error.
Genotype: 1 – GG, 2 – GA, Cow age: 1 – ≤5 year, 2–5·1–6·5 year, 3 – >6·5 year.
LSMs with the same superscript letters differ significantly at: $^{A1\ -\ A4}$ $P < 0{\cdot}0001$; ^{B} $P < 0{\cdot}001$.

Table 4. Effects of milk traits on the antioxidant capacity of milk

			TEAC (μM TE)	
Effect	Classes	*N*	LSM	SE
Daily milk yield (kg)†	≤20·0	30	$2{\cdot}71^{C}$	0·29
	20·1–30·0	49	2·13	0·23
	>30·0	47	$1{\cdot}87^{C}$	0·27
Fat (%)†	≤3·2	30	2·47	0·30
	3·2–4·0	58	2·13	0·21
	>4·0	38	2·10	0·26
Protein (%)†	≤3·5	51	2·15	0·24
	3·5–4·0	54	2·20	0·21
	>4·0	21	2·36	0·36
SCC (×10³ cells/ml)†	<200	61	1·84	0·22
	200–400	27	2·35	0·30
	>400	38	2·51	0·25

TEAC, The Trolox Equivalent Antioxidant Capacity method; LSM, least-squares mean; SE, standard error.
†Measured on the day of milk sampling.
LSMs with the same superscript letter differ significantly at: ^{C} $P < 0{\cdot}01$.

Castillo et al. (2006) show that high-yield cows are exposed to oxidative stress to a greater extent when compared with lower-yield cows, and therefore their milk should be characterised by a higher antioxidant capacity. However, high-yield cows often have a negative energy balance which may cause a reduction of endogenous antioxidant precursors (Celi, 2010), thus reducing the antioxidant activity of milk. Based on the earlier studies carried out in humans (Roberts & Sindhu, 2009), it can be supposed that higher metabolism in high-yield cows may lead to the faster depletion of antioxidant enzymes.

It should be emphasised that to date, this is the first study which attempted to associate MPO gene polymorphism with antioxidant activity and other milk traits. Despite the fact that the analysed polymorphism is located at the gene's intron, it may affect the action of the gene. Some of the most frequently observed intronic polymorphism effects are disorders of mRNA splicing, which in turn may cause improper transcripts. Non-coding sequences may also include loci that are recognised by regulatory factors which, when bound, may affect the transcript level. Additionally, recent studies show that SNPs present in introns of one gene may affect the expression of other genes located at a considerable distance from the former ones (million bp: Cooper, 2010; Patrushev & Kovalenko, 2014).

Summarising, the genotype at g.9476869G > A myeloperoxidase (MPO) locus affected the antioxidant activity of milk. Cows with the GA genotype and older than 6·5 years produced milk with a significantly higher antioxidant activity compared to younger cows with the same genotype, as well as cows with the GG genotype of all ages. The results show that the higher milk antioxidant capacity is associated with the age of cows but only for GA genotype and with lower milk yields regardless of the animal genotypes. Information about genotype at MPO locus can give breeders the opportunity to select cattle producing higher quality of milk. To confirm the above thesis the analyses on higher number of animals should be performed and experience

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should be repeated in different periods of time, including the measure of concentration and activity of MPO enzyme in blood serum or milk.

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