

Variability of lysozyme and lactoferrin bioactive protein concentrations in equine milk in relation to *LYZ* and *LTF* gene polymorphisms and expression

Running title: Variability of lysozyme and lactoferrin concentration in mare's milk

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Abstract

BACKGROUND: Equine milk is considered to be an interesting product for human nutrition, mainly due to its low allergenicity and significant amounts of bioactive proteins, including lysozyme (LYZ) and lactoferrin (LTF). In the present study we assessed the effect of genetic factors on LYZ and LTF concentration variability in mare's milk.

RESULTS: Significant effects of horse breed and lactation stage on milk LYZ and LTF contents were observed. The highest level of LTF and the lowest concentration of LYZ were recorded for the Polish Warmblood Horse breed. The highest amounts of both proteins were found for the earliest investigated time point of lactation (5th week). Altogether 13 (9 novel) polymorphisms were found in the 5'-flanking regions of both genes, but they showed no significant relationship with milk LYZ and LTF contents. Several associations were found between selected SNPs and the *LYZ* gene relative transcript level.

CONCLUSION: While the present study indicated the existence of an intra- and interbreed variability of LYZ and LTF contents in mare's milk, this variation is rather unrelated to the 5'-flanking variants of genes encoding both proteins. Our study is a good introduction for broader investigations focused on the genetic background for variability of bioactive protein contents in mare's milk.

Keywords: equine milk, lysozyme, lactoferrin, polymorphism, gene expression

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INTRODUCTION

Consumption of mare's milk is probably one of the oldest forms of the domestic horse utilization.¹ Currently horse milk is collected on a larger scale in countries of central Asia (e.g. Mongolia, Kazakhstan), where the tradition of Koumiss production is cultivated. Since the unique composition of mare's milk has been better recognized, increasing interest in this product has been observed in the world. In terms of major components, equids' milk is much closer to human milk than milk of ruminants is. Therefore, its potential wider application (e.g. for infants with allergy to cows' milk) is now extensively studied.² Much attention is focused on whey proteins, which percentage amount in equine milk is higher than in ruminants' milk.³ Among them an interesting group is composed of bioactive proteins, e.g. lysozyme (LYZ) and lactoferrin (LTF), which are considered to be major health-promoting components of horse's milk. Both proteins are known predominantly for their antimicrobial activity, but based on current knowledge they are classified to the multifunctional protein family.⁴

Lysozyme (LYZ) is a small (e.g. molecular mass of mare's milk LYZ is 14.4 kDa) enzyme capable of hydrolysing the β -1,4 glycosidic bonds between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine, building the bacterial cell wall peptidoglycan.⁵ Expression of LYZ is observed in many cells and tissue types and the LYZ protein is present in almost all body fluids (with the highest concentrations observed in tears, gastric juice and milk). Equine milk LYZ (c-type, also known as calcium binding lysozyme) is a protein consisting of 129 amino acids. Analysis of its structure provided grounds for a hypothesis that equine lysozyme (EL) is an evolutionary link between lysozymes and α -lactalbumins, because it presents some structural and functional features of both protein families.^{6,7} The average concentration of EL in milk varies between different studies (0.8-1.3g L⁻¹), but it is generally higher than in human or cow's milk.^{8,9}

Lactoferrin (LTF) is about 80 kDa glycoprotein belonging to the transferrin family. Neutrophils and secretory epithelia are the most important cell types, in which LTF is expressed. The LTF protein is present in various body fluids, such as milk, colostrum, saliva, semen and the vaginal fluid. The most known biochemical property of LTF is its capability of iron (Fe³⁺) ion binding and transporting. Similarly to LYZ, LTF is considered to be a part of the immune system due to its involvement in defense reactions against various pathological agents, including bacteria, viruses, fungi and protozoa.¹⁰ The intracellular LTF isoform (delta-lactoferrin) presents an ability of DNA binding and is considered as a transcription factor involved e.g. in cell cycle regulation. Due to its antiproliferative activity delta-lactoferrin is

probably an important anti-tumoral agent.¹¹ Concentration of LTF in mare's milk is around 1 g L⁻¹, but some differences between independent investigations are noticed (within the range of 0.6-1.3 g L⁻¹). Generally, the LTF content in the equine milk is placed between an average concentration of lactoferrin observed for human breast milk and for cow's milk.^{9,12}

Besides the above-mentioned variability in the levels of LYZ and LTF proteins in mare's milk, observed between independent experiments or even between individual animals^{9,13}, there are no studies focused on the putative genetic background of this variation. Therefore, we decided to perform this complex investigation focused on the presence of potential inter- and intra-breed variation in LYZ and LTF concentrations in mare's milk and its relationship to polymorphism and expression of *LYZ* and *LTF* genes. The detailed aim of this study was to measure the LYZ and LTF contents in milk of mares representing three different breeds (Polish Primitive Horse, Polish Coldblood Horse and Polish Warmblood Horse) across lactation. Moreover, relative transcript levels of both genes were assessed based on RNA extracted from mare's milk somatic cells. Finally, an association study of polymorphisms found in the 5'-flanking regions of the *LTF* and *LYZ* genes and their expression levels was conducted.

EXPERIMENTAL

Animals

Horse milk samples from three breeds: (Polish Primitive Horse, PPH, n=20), (Polish Warmblood Horse, PWH, n=27) and (Polish Coldblood Horse, PCH, n=27) were collected at three different time points of lactation (5th, 10th and 15th week postpartum). Milked horses came from four Polish national studs (Kobylniki, Sieraków, Racot and Nowe Jankowice), where they were kept under similar environmental conditions. Mares were milked manually in the morning (7-9 a.m.). During milking, mares and foals remained in visual contact. Immediately, milk samples (c.a. 100 ml each) were partly (15 ml) frozen in liquid nitrogen (for gene expression studies). The remaining part was stored at -20°C (for milk composition analyses). The study was approved by the National Commission for Ethics of Animal Experimentation, the Local Ethics Committee for Animal Research (Poznan, Poland; permission number: 39/2012).

Screening for polymorphism was performed on the basis of 96 DNA samples derived from 12 horse breeds (Arabian, n=8; Thoroughbred, n=8; Welsh Pony, n=8; Shetland Pony, n=8; Haflinger, n=8; Percheron, n=8; Polish Primitive Horse, n=8; Polish Coldblood Horse,

n=8; Polish Warmblood Horse, n=8; Fiording, n=8; Silesian, n=8 and Hucul, n=8). Furthermore, in the case of the above-mentioned breeds, for which milk samples were collected, a broader animal set was genotyped (Polish Primitive Horse, n=95; Polish Coldblood Horse, n=89 and Polish Warmblood Horse, n=90) in order to calculate the detailed genotype frequencies. All genomic DNA samples used for screening for polymorphism and animals genotyping were derived from the collection of the Horse Genetic Markers Laboratory (Poznan University of Life Sciences, Poland).

Assessment of LYZ and LTF concentrations in mare's milk

A volume of 10 ml of milk was centrifuged for 15 min at 4500 g, then the fat layer was removed. The rest of the sample was heated at 40°C in a water bath and 10% solution of acetic acid was added, in order to precipitate the casein fraction. Afterwards, each sample was centrifuged for 5 min at 3000 g. The supernatant was filtered through a nylon filter and used in further analyses.

Concentrations of LYZ and LTF in milk samples were measured in triplicate using an Agilent 1100 Series reverse phase high-performance liquid chromatograph (Agilent Technologies, Germany) according to the methodology described previously for bovine milk LYZ, LTF and beta-lactoglobulin.¹⁴

Relative transcript abundance measurements

The total RNA was extracted from milk somatic cells using the Tripure Isolation Reagent (Roche, USA). The detailed procedure of RNA isolation, as well as its quantity and quality control were described previously.¹⁵

Assessment of the *LYZ* and *LTF* gene transcripts levels in equine milk somatic cells was performed using the LightCycler 480 instrument (Roche, USA), based on the TaqMan® probe signal detection. Primers and probes were designed and synthesized by TIB Molbiol (nucleotide sequences are shown in Table 1). Analyses of each sample were conducted in duplicate using the following cycling conditions: initial denaturation (95°C, 5 min), 45 cycles of: denaturation (95°C, 10 s); primer and probe annealing (60°C, 30 s) and DNA synthesis (72°C, 1 s). Afterwards samples were cooled (40°C, 30 s). Obtained results were normalized to the geometric mean of four internal reference genes: *ACTB*, *GAPDH*, *TOP2B* (housekeeping genes) and *KRT8* (marker of the mammary epithelial cells number). Selection

of the above-mentioned reference genes was made on the basis of the geNorm algorithm, as it was described in detail in our previous article.¹⁶

Screening for polymorphism and animal genotyping

The direct sequencing technique was applied to screen for polymorphic sites in the *LYZ* and *LTF* gene 5'-flanking regions. Primer sequences were designed using the Primer3 tool.¹⁷ Afterwards, obtained oligonucleotides were synthesized by Sigma-Aldrich (Germany). PCR amplification was conducted for two overlapping fragments of each gene using a Bio-Rad T100 thermocycler (Bio-Rad, USA). In total, analyses included 1132 bp and 942 bp of *LYZ* and *LTF* gene fragments, respectively. The most important details of the PCR are shown in Table 1. Obtained PCR products were cleaned from unused primers and nucleotides by FastAP Thermosensitive Alkaline Phosphatase and Exonuclease I (Thermo Fisher Scientific, USA) digestion (37°C, 30 min; 80°C, 15 min). The sequencing reaction (based on the BigDye® Terminator v1.1 Cycle Sequencing Kit, Thermo Fisher Scientific, USA) was conducted in the above-mentioned thermocycler using the following conditions: initial denaturation (95°C, 5min); 25 cycles: of denaturation (95°C, 30s), primer annealing (50°C, 10s) and DNA synthesis (60°C, 4 min). Afterwards, samples were filtered through a 96-well plate with Sephadex (Sigma Aldrich, Germany) by centrifugation (3180g, 3 min) and separated electrophoretically using an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, USA). Obtained electropherograms were analyzed in the Lasergene SeqMan Pro (version 12.2.0) software (DNASTAR, USA).

Statistical analysis

The unknown variance components were estimated using the general mixed model, based on REML (restricted maximum likelihood). The significance of each factor included in the model was previously examined by non-parametric analysis of variance using Kruskal-Wallis and Friedman tests to check their impact separately. To verify whether the mRNA and protein levels depend on genotype, the models including the fixed effect of breed and sampling time (5th, 10th or 15th week postpartum) as a repeated-measure factor was applied. The analysis for each breed separately were also carried out. Hypotheses were tested using the *F*-test and multiple comparison procedure based on least significant differences (LSD) with the Tukey-Kramer adjustment. Genotype groups consisting of fewer than 5 individuals (15

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measurements) were excluded from the calculations. All statistical analyses were conducted with the application of the SAS 9.3 package (SAS Institute Inc., USA).

RESULTS AND DISCUSSION

From the biological point of view, the amount of bioactive proteins in mammals' milk is considered important due to their role in the proper development of infants. A major function of LYZ and LTF, investigated in the current study, is to strengthen the not fully developed immune system of newborns. Moreover, their antimicrobial activities may be useful also for the food industry, e.g. it is well known that LYZ helps to control the growth of butyric fermentation bacteria during cheese making.¹⁸ Unfortunately, in ruminants, which remain the most important source of milk for human consumption, the natural level of the above-mentioned bioactive proteins is low or almost undetectable. Therefore, the main aim of several independent research groups was to create transgenic ruminants (cattle or goats) producing significant amounts of human LYZ in milk.¹⁹⁻²¹

As it was repeatedly indicated in the previous investigations, the composition of equids' milk (including significant amounts of LYZ and LTF bioactive proteins) show a much closer resemblance to human breast milk than milk of the ruminants. Thus, an increased interest has been observed in horse's and donkey's milk as valuable products for human nutrition.² On the other hand, it should be noticed that concentrations of LYZ and LTF in mare's milk can vary strongly, e.g. between different farms but also between samples collected from mares representing one breed, investigated at the same lactation time point and kept under similar environmental conditions.^{9,13} This situation, as well as the initial results of LYZ and LTF milk concentrations obtained in the present study, convinced us to make an attempt at verifying the hypothesis that the observed variability may be related to the genetic background.

The between-breed comparison of both proteins concentrations in milk (samples from all lactation stages analyzed together) revealed the lowest LYZ and the highest LTF contents in PWH (Table 2). Interestingly, the content of LYZ observed in the present study for PCH is similar to the concentration recorded for the same breed in the investigation by Markiewicz-Kęszycka et al.⁹ ($\sim 1.2 \text{ g L}^{-1}$ vs. 1.0 g L^{-1}) whereas the mean concentration of LTF is significantly lower ($\sim 0.49 \text{ g L}^{-1}$ vs. $\sim 1.3 \text{ g L}^{-1}$). This may be an effect of the non-identical sample sets used in these two experiments (milk samples in the previously published study were derived from the very late lactation only). On the other hand, the concentration of LTF

in our study is very similar to those presented in the paper by Naert et al.,¹³ and in the review article by Uniacke-Lowe et al.³ (0.5 g L⁻¹ and 0.58 g L⁻¹, respectively).

Analogical tendencies as for milk protein concentrations were observed also for *LYZ* and *LTF* genes expression measured at the mRNA level, but the interbreed differences were not statistically significant (Table 2). Therefore, we can confirm the conclusion drawn by other authors multiple times before, that the differences observed between amounts of transcripts cannot be simply extrapolated to the protein level. This is probably related to the complexity of mechanisms regulating gene expression.²²

It should also be stressed here that similarly to previous studies, our investigation confirmed the presence of a significant intra-breed variation in the *LYZ* and *LTF* protein contents in mare's milk. For example, among milk samples derived from PCH, the level of *LYZ* measured at the 5th week postpartum varied from about 0.5 to 2.5 g L⁻¹, whereas the range of *LTF* concentration was 0.2-0.9 g L⁻¹. Markiewicz-Kęszycka et al. reported similar intra-breed variability of the *LYZ* and *LTF* concentrations in PCH milk (0.65 to 1.53 g L⁻¹ and 0.61 to 3.11 g L⁻¹, respectively.)⁹ Since in both experiments the animals were kept in the similar environmental conditions it is likely, that the above mentioned variability has the genetic background.

Analysis performed for different lactation time points indicated the highest concentration of both proteins in mare's milk at the 5th week postpartum, which decreased significantly until the next analyzed stage (10th week) (Table 3). This result is probably related to the increased requirement for immune agents in milk during the first few weeks after birth. It is assumed that anti-microbial proteins obtained from milk passively protect the newborn from potential infections. Similar tendencies were observed before for human breast milk bioactive proteins.^{23,24} Differences observed for *LYZ* and *LTF* relative transcripts levels (mRNA) were not statistically significant between analyzed lactation periods (Table 3).

Altogether 13 SNPs (Single Nucleotide Polymorphisms) were found in the the 5'-flanking regions of the equine *LYZ* (9 SNPs) and *LTF* (4 SNPs) genes (Figure 1). Nine of the described polymorphic sites were previously unknown. Generally, SNPs localized in the *LYZ* gene were common and widely distributed among the analyzed breeds, whereas polymorphisms of the *LTF* gene were much more breed-specific (Table 4). There were two breeds (PCH and PWH), in which all of the 13 polymorphisms were observed, whereas Thoroughbred horses turned out the most monomorphic (the presence of only 5 polymorphic variants was confirmed). Relatively low number of SNPs detected in Thoroughbred horses may be related to the fact that their studbooks are closed since over 200 years and no outside

blood is accepted. Moreover, their original genetic diversity can be partly lost due to the strong, long-term selective pressure for racing related traits, which is conducted in Thoroughbred horses.²⁵

Association study of selected polymorphisms (which revealed sufficient genotype distribution among the PPH, PCH and PWH breeds) showed no significant effects on LYZ and LTF protein concentrations in mare's milk. Statistically significant associations were found only for several polymorphic variants and the *LYZ* gene relative transcript abundance in PCH and PWH, only if the breeds were analyzed separately (Table 5). Interestingly, in the case of PCH breed all significant SNPs showed the same direction of the relationship (increased relative mRNA level for heterozygous genotype carriers). Similarly, for PWH breed, the significantly elevated level of the *LYZ* transcripts was noticed for rare homozygous genotypes (GG and CC; for -47T>G and c.-701G>C SNPs, respectively). Closer look on genotype frequencies showed in the Table 4 suggests that some of the investigated SNPs may remain in the strong linkage disequilibrium (LD) and therefore, they can manifest the similar effects on the relative *LYZ* mRNA level.

It can be speculated that in the future, finding the genetic variants responsible for the *LYZ* and *LTF* variability may be helpful in animal selection in order to produce equine milk with the possibly highest amounts of bioactive proteins. It should also be underlined once again that since the correlation between gene transcript abundance and final protein concentration is not always strong to provide valuable conclusions, each experiment should be possibly conducted for various gene expression stages. Due to the very low concentrations of *LYZ* and *LTF* in ruminants' milk, it is difficult to find literature data focused on the genetic background of their concentration variability. In a recently published paper Zabolewicz et al. (2014) described an association between the *LTF* gene -28A/C promoter variant and *LTF* amount in cattle milk.²⁶ However, results presented in this study are not fully coherent, because the opposite direction of the relationship was noticed for cows affected and non-affected with mastitis. Additionally, there is a study indicating a relationship between another SNP in the *LTF* gene promoter region (+32G/C) and cow's milk protein level.²⁷ A study by Salehin et al. (2009) has shown a significant impact of SNPs found in the *LYZ* gene and some of the milk traits (milk yield, somatic cell count) in *Bos indicus* x *Bos taurus* cattle.²⁸ Generally we can summarize that due to the anti-inflammatory function of their protein products, until recently polymorphisms of genes encoding *LYZ* and *LTF* were analyzed predominantly within the context of mastitis-related traits in ruminants,²⁹⁻³¹ and relatively little attention was focused to the variation of *LTF* and *LYZ* amounts in milk.

CONCLUSIONS

While we found no spectacular effect of the 5'-flanking variants of the *LYZ* and *LTF* genes and their protein product concentrations in mare's milk, analyses should be continued. We can still speculate that significant differences in *LYZ* and *LTF* milk amounts observed between particular horses are an effect of unknown structural variations at the DNA level. Since it is well known that not only 5'-flanking variants may be associated with gene expression, it seems necessary to extend the investigations to other regions of both genes, including their coding sequences.

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Table 1. PCR primers and amplification conditions.

Primer (probe) sequences	PCR product size (bp)	Annealing temp. (°C)	No. of cycles	Polymerase
Screening for polymorphism and genotyping				
LYZIF: GCTGAGCAACATGGTGAGTG LYZIR: CCTGGACAGTGACAGACAGC	561	60	35	Perpetual Taq (EurX)
LYZIIIF: CATGTCTCCCATGGAAAAGG LYZIIIR: TGACACATTGCCTGTCGTTT	624	65	35	Perpetual Taq (EurX)
LTFIF: TCCTGGGCACTTTAGACACC LTFIR: CTGCTTCTCAACCCAGTTCC	380	62	35	OptiTaq (EurX)
LTFIIF: ACTCGATGTGCCAGAGACG LTFIIR: TCCTAAGGTGAATGTTTGTGGA	642	59	35	Perpetual Taq (EurX)
Gene expression studies				
LYZRTE: CTTGGACTGGACGGCTTTAG LYZRTR: GTAGTTTGTAGCTCGTGTGTTGTA LYZprobe: ACTGGGTCTGTTTGGCCAGATGG	86	60	45	
LTFRTE: GCAACAACGAGAATGAGAACAAGT LTFRTR: GACATCTTTCACAAATGCAACGTC LTFprobe: AGGAGAGATACTATGGCTACACTGGGGCTT	119	60	45	
ACTBRTE: TCCTTCCTGGGGCATGGAATC ACTBRTR: TCCTGTCGGCGATGCCT ACTBprobe: CCGTAAGGACCTGTACGCCAACACAGT	145	60	45	LightCycler® 480 Probes Master (Roche)
GAPDHRTF: GAGGACCAGGTTGTCTCCTGC GAPDHRTR: ATGAGCTTGACAAAGTGGTCGTT GAPDHprobe: ACCCACTCTTCCACCTTCGATGCT	101	60	45	
TOP2BRTE: GCCAGCTGACAATAAACAGAGG TOP2BRTR: TGCCTTTCCATTATTCCAA TOP2Bprobe: TTGATCCTGAATCTAACATTATAAGCA	101	60	45	
KRT8RTF: ACCCAGGAGAAGGAGCAGAT KRT8RTR: GCTCCACTTGGTCTCCAGAA KRT8probe: GCATCTGGAACAGCAGAACA	108	60	45	

Table 2. Interbreed differences in LYZ and LTF expression at mRNA and protein levels. Samples from all lactation stages were analyzed together. Presented values are LS-means and SEM.

Breed	LTF		LYZ	
	Protein (g L ⁻¹)	mRNA (RA)	Protein (g L ⁻¹)	mRNA (RA)
PCH (n=27)*	0.486 ^{ab} (0.02)	5.424 ^{Aa} (0.73)	1.242 ^{ab} (0.06)	0.108 (0.02)
PPH (n=20)*	0.437 ^a (0.02)	12.045 ^b (1.54)	1.272 ^a (0.05)	0.112 (0.04)
PWH (n=27)*	0.538 ^b (0.02)	16.460 ^{Bb} (1.77)	1.057 ^b (0.06)	0.105 (0.04)

Different letters within the same column indicate significant differences (lowercase letters: P<0.05; capital letters: P<0.01, based on non-parametric one-way ANOVA). RA – relative abundance, PCH – Polish Coldblood Horse, PPH – Polish Primitive Horse, PWH – Polish Warmblood Horse. *Each mare was milked 3 times during lactation (altogether 222 milk samples were analyzed).

Table 3. A comparison of LYZ and LTF expression between different lactation stages (samples from all breeds were analyzed together). Presented values are LS-means and SEM.

Week <i>postpartum</i>	LTF		LYZ	
	Protein (g L⁻¹)	mRNA (RA)	Protein (g L⁻¹)	mRNA (RA)
5	0.589 ^A (0.03)	10.250 (1.33)	1.337 ^a (0.06)	0.113 (0.02)
10	0.462 ^{BC} (0.03)	10.466 (1.47)	1.037 ^b (0.05)	0.111 (0.04)
15	0.428 ^C (0.02)	13.417 (1.75)	1.178 ^{ab} (0.05)	0.101 (0.05)

Different letters within the same column indicate significant differences (lowercase letters: P<0.05; capital letters: P<0.01, based on non-parametric one-way ANOVA). RA – relative abundance.

Table 4. SNPs found in the equine *LYZ* and *LTF* genes 5'-flanking regions.

Gene/SNP (dbSNP number)	Breed Genotype frequencies				Presence in other breeds*
	PPH (n=95)	PCH (n=89)	PWH (n=90)		
<i>LYZ</i>					
c.-6G>C (rs396913128)	GG	0.75	0.66	0.39	FIOR, HAF, HUC, ARAB, PER, SIL, SHET, WELSH, THOR
	GC	0.24	0.31	0.43	
	CC	0.01	0.03	0.18	
c.-47T>G (rs395606103)	TT	0.66	0.29	0.86	FIOR, HAF, HUC, ARAB, PER, SIL, SHET, WELSH, THOR
	TG	0.32	0.53	0.13	
	GG	0.02	0.18	0.01	
c.-78G>C (ss1868862178)	GG	0.81	0.91	0.70	FIOR, HAF, HUC, ARAB, SIL, SHET, WELSH, THOR
	GC	0.16	0.09	0.27	
	CC	0.03	0.00	0.03	
c.-277C>A (ss1868862179)	CC	0.67	0.71	0.73	FIOR, HAF, HUC, ARAB, PER, SIL, SHET, WELSH
	CA	0.30	0.25	0.27	
	AA	0.03	0.04	0.00	
c.-322A>G (rs68714058)	AA	0.03	0.00	0.03	FIOR, HAF, HUC, ARAB, SHET, WELSH, THOR
	AG	0.17	0.09	0.27	
	GG	0.80	0.91	0.70	
c.-639A>C (ss1868862180)	AA	0.67	0.71	0.71	FIOR, HAF, HUC, ARAB, PER, SIL, SHET, WELSH
	AC	0.30	0.25	0.29	
	CC	0.03	0.04	0.00	
c.-701G>C (rs68714057)	GG	0.60	0.27	0.60	FIOR, HAF, HUC, ARAB, PER, SIL, SHET, WELSH
	GC	0.37	0.54	0.39	
	CC	0.03	0.19	0.01	
c.-765T>C (ss1868862181)	TT	0.94	0.72	0.87	FIOR, HAF, HUC, PER, SIL, SHET, WELSH
	TC	0.06	0.24	0.13	
	CC	0.00	0.04	0.00	
c.-776G>C (ss1868862182)	GG	0.00	0.04	0.00	FIOR, HAF, HUC, PER, SHET, WELSH
	GC	0.06	0.24	0.13	
	CC	0.94	0.72	0.87	
<i>LTF</i>		(n=90)	(n=95)	(n=95)	
c.-170T>C (ss1868862174)	TT	1.00	0.98	0.98	SIL
	TC	0.00	0.02	0.02	
	CC	0.00	0.00	0.00	
c.-686G>A (ss1868862175)	GG	1.00	0.75	0.99	PER, SHET, WELSH
	GA	0.00	0.22	0.01	
	AA	0.00	0.03	0.00	
c.-798C>T (ss1868862176)	CC	1.00	0.75	0.99	FIOR, PER, SHET, WELSH
	CT	0.00	0.22	0.01	
	TT	0.00	0.03	0.00	
c.-942T>C (ss1868862177)	TT	0.73	0.93	0.66	FIOR, HUC, ARAB, PER, SIL, THOR
	TC	0.24	0.07	0.32	
	CC	0.03	0.00	0.02	

*Based on multi-breed panel. Due to low numbers of investigated horses (n=8 per breed) the detailed genotype frequencies are not shown. Horse breeds acronyms: FIOR – Fiording, HAF – Haflinger, HUC – Hucul, PPH – Polish Primitive Horse, ARAB – Arabian, PER – Percheron, SIL – Silesian, SHET – Shetland Pony, WELSH – Welsh Pony, PWH – Polish Warmblood Horse, THOR – Thoroughbred, PCH – Polish Coldblood Horse. All SNPs with “ss” (submitted SNP) reference numbers were previously unknown.

Table 5. Association study of *LYZ* and *LTF* SNPs with mRNA and milk rotein levels (presented values are LS-means and SEM) .

SNP	Genotype	mRNA (RA)			Milk protein concentration (g L ⁻¹)		
		PCH	PWH	PPH	PCH	PWH	PPH
Lysozyme (LYZ)							
c.-6G>C (rs396913128)	GG	0.105 (0.03)	0.250 (0.10)		1.223 (0.08)	0.976 (0.13)	
	GC	0.138 (0.05)	0.048 (0.04)	n/a	1.332 (0.15)	1.106 (0.09)	n/a
	CC	n/a	0.074 (0.07)		n/a	1.048 (0.11)	
c.-47T>G (rs395606103)	TT	0.060 (0.06)	0.052 ^B (0.01)	0.135 (0.07)	1.072 (0.21)	1.073 (0.06)	1.218 (0.07)
	TG	0.169 (0.03)	0.072 ^B (0.03)	0.076 (0.07)	1.264 (0.11)	0.841 (0.14)	1.357 (0.08)
	GG	0.071 (0.03)	1.300 ^A (0.06)	0.141 (0.12)	1.272 (0.10)	1.578 (0.29)	1.260 (0.13)
c.-78G>C (ss1868862178)	GG			0.145 (0.06)			1.336 (0.06)
	GC	n/a	n/a	0.083 (0.07)	n/a	n/a	1.196 (0.08)
	CC			n/a			n/a
c.-322A>G (rs68714058)	AA	n/a		n/a	n/a		n/a
	AG	0.051 (0.07)	n/a	0.084 (0.08)	1.212 (0.20)	n/a	1.196 (0.08)
	GG	0.119 (0.02)		0.144 (0.06)	1.252 (0.07)		1.336 (0.07)
c.-639A>C (ss1868862180)	AA	0.086 ^A (0.02)	0.117 (0.05)	0.131 (0.05)	1.271 (0.08)	1.035 (0.06)	1.243 (0.06)
	AC	0.240 ^B (0.04)	0.023 (0.13)	0.060 (0.05)	1.204 (0.16)	1.183 (0.15)	1.373 (0.10)
	CC	0.022 ^{AB} (0.09)	n/a	n/a	0.991 (0.35)	n/a	n/a
c.-701G>C (rs68714057)	GG	0.069 (0.05)	0.057 ^B (0.01)	0.135 (0.07)	1.236 (0.19)	1.092 (0.07)	1.218 (0.07)

c.-765T>C (ss1868862181)	GC	0.170 (0.03)	0.049 ^B (0.02)	0.079 (0.07)	1.250 (0.11)	0.890 (0.11)	1.356 (0.09)
	CC	0.067 (0.03)	1.300 ^A (0.06)	0.120 (0.10)	1.248 (0.11)	1.578 (0.29)	1.285 (0.11)
	TT	0.086 ^{abC} (0.02)	0.109 (0.05)		1.271 (0.08)	1.128 (0.06)	
	TC	0.254 ^{bB} (0.04)	0.027 (0.18)	n/a	1.242 (0.17)	1.417 (0.20)	n/a
	CC	0.031 ^a (0.07)	n/a		0.935 (0.29)	n/a	
	GG	n/a			0.991 (0.35)		
c.-777G>C (ss1868862182)	GC	0.240 ^b (0.04)	n/a	n/a	1.204 (0.16)	n/a	n/a
	CC	0.086 ^a (0.02)			1.271 (0.08)		
Lactoferrin (LTF)							
c.-686G>A (ss1868862175)	GG	5.375 (1.16)			0.532 (0.06)		
	GA	5.665 (1.60)	n/a	n/a	0.406 (0.05)	n/a	n/a
	AA	n/a			n/a		
c.-798C>T (ss1868862176)	CC	5.376 (1.15)			0.532 (0.04)		
	TC	5.666 (1.60)	n/a	n/a	0.406 (0.05)	n/a	n/a
	TT	n/a			n/a		
c.-942T>C (ss1868862177)	TT		17.129 (2.73)			0.519 (0.03)	
	TC	n/a	14.873 (4.20)	n/a	n/a	0.574 (0.04)	n/a

Different letters within the same column (for each SNP separately) indicate significant differences (lowercase letters: P<0.05; capital letters: P<0.01). RA – relative abundance, PCH – Polish Coldblood Horse, PWH – Polish Warmblood Horse, n/a – not analyzed. Two SNPs (LYZ c.-277C>A and LTF c.-170T>C are not presented due insufficient distribution of genotypes to perform analyses).

Figure 1. Localization of investigated SNPs in the equine *LYZ* and *LTF* genes. SNPs marked with asterisks were previously unknown.

