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5'-flanking variants of the equine α-lactalbumin (*LALBA*) gene – relationship with gene expression and mare's milk composition

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ABSTRACT. α-Lactalbumin (α-LA) is one of the most abundant milk whey proteins among different mammalian species including domestic horse. The aim of this study was to screen for polymorphism in the 5'-flanking region of the equine α-LA (*LALBA*) gene and to assess the potential relationship of particular genotypes with *LALBA* gene expression variability (measured at the mRNA and protein levels) and with basic milk composition traits. Initial screening for *LALBA* gene 5'-flanking variants was conducted using direct sequencing of DNA derived from 96 horses representing 12 breeds (Polish Primitive Horse, Polish Coldblood Horse, Polish Warmblood Horse, Silesian, Hucul, Fiording, Haflinger, Shetland Pony, Welsh Pony, Arabian, Thoroughbred and Percheron). Association analysis of detected polymorphisms, gene expression and milk composition traits was carried out for 74 horses (Polish Primitive Horse, Polish Coldblood Horse and Polish Warmblood Horse breeds). Altogether 4 single nucleotide polymorphisms (SNPs) (c.−165G>C, c.−222A>G, c.−357C>A and c.−928C>T) were found in the *LALBA* gene 5'-flanking region (NC_009149.3 GenBank sequence, gene coordinates on ECA6: 67372475–67375877). Although bioinformatic prediction suggested that 3 of them may alter the consensus sequences for transcription factors, no significant associations between genotypes and *LALBA* gene expression were recorded. However, a significant relationship ($P < 0.05$) was noticed for c.−928C>T SNP genotypes and basic milk composition (fat and protein contents) of Polish Primitive Horse mares. Additionally, in our study the significant impact of horse breed and lactation period on *LALBA* gene expression and basic milk composition traits was revealed.

Introduction

Currently, mare's milk is considered to be not only the main source of food for the newborn foal, but it also seems to be an interesting and valuable product for human nutrition (Salimei and Fantuz, 2012). Due to numerous similarities between mare's milk and human breast milk composition, the equine milk is seen as a potential nutritional substitute for human infants (Martin et al., 2016). It should be also

stressed that horse's milk has a significantly lower allergenic potential when compared to that of ruminants' (goat, sheep or cow) (Docena et al., 2002), which seems to be extremely important in the era of the common hypersensitivity to cow's milk proteins (El-Agamy, 2007). Moreover, due to its high concentration of bioactive components e.g., lysozyme and lactoferrin (Cieslak et al., 2017), mare's milk is considered as a health-promoting animal product and thus, it has frequently become a desirable ingredient for cosmetic and pharmaceutical industries.

α-Lactalbumin (α-LA) is a small protein (molecular mass ~14 kDa), which is unique for mammals' milk and is usually one of the most abundant milk whey proteins among all mammalian species. For example, in cow's milk α-LA accounts for over 50% of all whey proteins, whereas within the same fraction of mare's milk its percentage is around 30%. The structure of α-LA is similar to that of the c-type lysozyme and therefore, this protein is classified to the lysozyme super-family. Similarly to human and cattle lactalbumin, the equine α-LA precursor consists of 142 amino acids (AA), whereas the mature protein contains 123 AA. To date, three protein variants (A, B and C) of the equine α-LA have been described. These genetic forms differ slightly in their AA composition (Uniacke-Lowe et al., 2010). Based on the GenBank database record (NC_009149.3), the gene encoding equine α-LA (*LALBA*) is located on chromosome 6. Its structure comprises 4 exons and the total physical length of the gene is 2431 bp (including the 5'- and 3'-untranslated regions).

Similarly to other whey proteins (e.g., lysozyme and lactoferrin), α-LA may be classified as multifunctional. It plays an important role in the transport of vitamins, metabolites and microelements. Like several other milk proteins (e.g., αs1-, αs2- and β-casein) α-LA exhibits an ability of Ca^{2+} ions binding. Moreover, it is also involved in the regulation of the final step of lactose synthesis (glucose and galactose linkage) (Pieszka et al., 2016; Redington et al., 2016). From the nutritional point of view α-LA seems to be an important milk protein, especially within the context of the newborn protection against infectious agents, since peptide products of its digestion show antibacterial and immunostimulatory properties (Lönnerdal and Lien, 2003). On the other hand, the α-LA (similarly to β-lactoglobulin and caseins) is considered as a potential allergenic factor; however, the reported percentage of patients suffering from hypersensitivity to milk α-LA varies strongly between different experiments (Fiocchi et al., 2010).

According to the review by Uniacke-Lowe et al. (2010), the level of α-LA in mare's milk is about 2.4 g/kg, which is comparable to the values recorded for human breast milk (~2.5 g/kg) and about two-fold higher than that observed for cow's milk (~1.2 g/kg). However, it should be underlined that noticeable differences in equine milk α-LA occur between various investigations. For example, in a study by Summer et al. (2005) the mean concentration of α-LA was 1.6 g/l, while in the experiment by Markiewicz-Kęszycka et al. (2013) this protein was more abundant (2.2 g/l). Moreover, a significant variability in α-LA content was observed between various mares analysed in the same study. As it was shown in the above-mentioned paper by Markiewicz-Kęszycka et al. (2013) based on the Polish Coldblood Horse breed, the difference in α-LA abundance between milk samples collected from various animals may exceed 2 g/l. To date, no scientific data regarding the potential role of the genetic background in mare's milk α-LA content variability has been reported.

Taking into consideration the numerous examples of associations described for the α-LA genetic variants and some milk production traits of ruminant species e.g., milk yield, fat and protein contents or lactose concentration (Voelker et al., 1997; Dettori et al., 2015), the *LALBA* gene can be considered as an obvious candidate for mare's milk composition. Although many previous experiments were based only on polymorphisms located within the coding sequences of milk protein genes, currently substantial attention is focused on the genetic variants present in the regulatory elements of these genes. This is related with the potential impact of such variants on both gene expression and milk composition traits (Cosenza et al., 2016; Noce et al., 2016). Therefore, the main aim of this study was to verify whether the 5'-flanking variants of the equine *LALBA* gene may affect its expression (measured at mRNA and milk protein levels). Moreover, this study was attempted to find associations between the discovered polymorphisms and basic mare's milk composition traits (protein, fat and lactose contents). Finally, we assessed the influence of horse breed and lactation stage on the *LALBA* gene mRNA level and α-LA milk abundance.

Material and methods

Screening for polymorphism and animal genotyping

Screening for polymorphism in the 5'-flanking region of the equine *LALBA* gene was performed on a multi-breed panel of 96 DNA samples representing

12 horse breeds – Polish Primitive Horse (PPH, n = 8); Polish Coldblood Horse (PCH, n = 8); Polish Warmblood Horse (PWH, n = 8); Silesian (SIL, n = 8); Hucul (HUC, n = 8); Fiording (FIOR, n = 8); Haflinger (HAFL, n = 8); Shetland Pony (SHET, n = 8); Welsh Pony (WELS, n = 8); Arabian (ARAB, n = 8); Thoroughbred (THOR, n = 8) and Percheron (PER, n = 8). The material was derived from the Horse Genetic Markers Laboratory collection (Poznań University of Life Sciences, Poznań, Poland). Based on the equine *LALBA* gene nucleotide sequence (GenBank NC_009149.2 – recently updated to NC_009149.3 version) the two PCR primer pairs were designed using the Primer3 tool (Koressaar and Remm, 2007). Oligonucleotides were synthesized by Sigma-Aldrich (St. Louis, MO, USA). In total, the analysis harboured the 1115 bp of the *LALBA* gene 5'-flanking region. PCR amplification was carried out in a Bio-Rad T100 thermocycler (Bio-Rad, Hercules, CA, USA) using the following conditions: initial denaturation (95 °C, 5 min); 35 cycles of denaturation (95 °C, 1 min), primer annealing (58°C, 1 min) and elongation (72 °C, 1 min); and final synthesis (72 °C, 10 min). Afterwards, the samples were cooled and stored at 4 °C until further analyses. Amplification of both fragments was conducted in the total volume of 10 µl using 1 U of Perpetual Taq DNA Polymerase (EURx, Gdańsk, Poland). The primer sequences and other amplification details are shown in Table 1. The PCR product specificity was tested by electrophoresis (120 V, 45 min) in 1.5% agarose gel stained with ethidium bromide. Afterwards, PCR products were cleaned from unused primers and nucleotides using Thermosensitive Alkaline Phosphatase and Exonuclease I digestion (Thermo Fisher Scientific, Waltham, MA, USA) under the following incubation conditions: 37 °C, 30 min; 80 °C, 15 min.

The sequencing reaction, based on the BigDye® Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA), was carried out in the above-mentioned thermocycler applying the following conditions: initial denaturation (95 °C, 5 min); 25 cycles of denaturation (95 °C, 30 s), primer annealing (50 °C, 10 s) and DNA synthesis (60 °C, 4 min). Afterwards, samples were filtered through a 96-well plate with Sephadex (Sigma-Aldrich, St. Louis, MO, USA) by centrifugation (3180 *g*, 3 min) followed by an electrophoretic separation using an ABI Prism 3130 Genetic Analyzer instrument (Applied Biosystems, Foster City, CA, USA). Obtained electropherograms were analysed in the Lasergene SeqMan Pro (version 12.2.0) software (DNASTAR, Madison, WI, USA).

The PCR and sequencing procedures described above were also used to genotype discovered polymorphisms in a group of over 70 mares from which milk samples were collected in order to analyse gene

Table 1. PCR amplification details

Primer (probe) sequence	PCR product size, bp	Primer annealing temp., °C	No. of cycles	Polymerase
PCR (screening for polymorphism/genotyping)				
LALBAIF: AACTCCTCCTGGGCTTTGTT LALBAIR: GGGTGGCAGAGAACAGGAT	582	60	35	Perpetual Taq (EURx, Gdańsk, Poland)
LALBAIIF: TTTGCTATCAGGACCTTCTG LALBAIIR: ATGGCCCCAGGATCAGAG	601	60	35	
Real-Time PCR (gene expression studies)				
LALBARTF: ATCTGTGGCATCTCCTGTAACAAGTT LALBARTR: GCTTATGAGCCAACCAGTAGTCAA LALBARTprobe: TACTGATGACGTGATGTGTGCCAAGAAGA	112	60	45	LightCycler® 480 Probes Master (Roche, Mannheim, Germany)
ACTBRTF: TCCTTCCTGGGGCATGGAATC ACTBRTR: TCCTGTCGGCGATGCCT ACTBRTprobe: CCGTAAGGACCTGTACGCCAACACAGT	146	60	45	
GAPDHRTF: GAGGACCAGGTTGTCTCCTGC GAPDHRTR: ATGAGCTTGACAAAGTGGTCGTT GAPDHRTprobe: ACCCACTCTTCCACCTTCGATGCT	101	60	45	
KRT8RTF: ACCCAGGAGAAGGAGCAGAT KRT8RTR: GCTCCACTTGGTCTCCAGAA KRT8RTprobe: GCATCTGGAACAGCAGAACA	108	60	45	
TOP2BF: GCCAGCTGACAATAAACAGAGG TOP2BR: TGCCTTTCCCATTATTCCAA TOP2Bprobe: TTGATCCTGAATCTAACATTATAAGCA	101	60	45	

expression and milk composition traits. This sample set is described in detail below.

Screening for potential transcription factor binding sites spanning the discovered single nucleotide polymorphism (SNP) locations was conducted using the MatInspector software (Cartharius et al., 2005) based upon the default settings.

Gene expression and milk composition analyses

Mare milk samples were collected from 74 individuals representing three horse breeds (PPH, n = 20; PWH, n = 27; PCH, n = 27). Mares originated from four Polish national studs located in western and north Poland in Wielkopolskie (Kobylniki, Sieraków, Racot) and Kujawsko-Pomorskie (Nowe Jankowice) voivodeships. The horses were kept under similar environmental conditions. Mares were milked manually in the morning (7:00–9:00) three times during lactation (at weeks 5, 10 and 15 *postpartum*). Thus, the total number of 222 milk samples (100 ml each) was obtained. During the milking procedure mares and foals remained in visual contact. Immediately after milking, samples were partly (15 ml) frozen in liquid nitrogen (for gene expression studies). The remaining part was stored at −20 °C (for milk composition analyses). During milk sampling, mares remained under veterinary control and did not show any disease symptoms. This study was approved by the National Commission for Ethics of Animal Experimentation, Local Ethics Committee for Animal Research (Poznań, Poland; permission number: 39/2012).

Total RNA was extracted from the milk somatic cells using the TriPure Isolation Reagent (Roche, Mannheim, Germany) according to the procedure described previously (Cieslak et al., 2016). After cDNA synthesis the relative transcripts level (Real-Time PCR) analysis was carried out for *LALBA* and four reference genes (*ACTB, GAPDH, TOP2B* and *KRT8*). The procedure of reference gene set selection was described in detail previously (Cieslak et al., 2015). The Real-Time PCR amplification (based on TaqMan® probes designed and synthesized by TIB Molbiol, Berlin, Germany) was carried out in duplicates. Obtained results were normalized to the geometric mean of relative mRNA abundances for the above listed reference genes according to the method recommended by Vandesompele et al. (2002).

The basic composition of mare's milk (protein, fat and lactose contents) was determined using automated infrared analysis in a Milkoscan FT2 instrument (Foss Electric, Hillerød, Denmark). The level of α-LA protein in 222 investigated milk samples was assessed with the application of high-performance liquid chromatography (HPLC) according to the methodology described before (Puppel et al., 2016). Briefly, tubes containing 10 ml of milk were centrifuged (4000 *g*, 15 min). After the fat layer removal, the remaining part of each sample was heated to 40 °C. In order to precipitate the casein fraction the 10% solution of acetic acid was added and then the tubes were centrifuged (3000 *g*, 5 min). The supernatant was filtered through a nylon filter and used in further steps of the analysis. The α-LA milk concentration was measured in triplicates using an Agilent 1100 Series reverse phase high-performance liquid chromatograph (Agilent Technologies, Santa Clara, CA, USA). The identification of α-LA peaks was based upon the comparison with the bovine milk α-LA standard (Sigma-Aldrich, St. Louis, MO, USA).

Statistical analyses

After determination of *LALBA* gene genotype and allele frequencies distribution obtained results were checked for compliance with Hardy-Weinberg Equilibrium (HWE).The potential impact of the *LALBA* gene polymorphisms on its relative transcript level (measured in milk somatic cells), milk α-LA protein content and basic milk composition traits (protein, fat and lactose concentrations) was checked using a mixed model. The model also contained the fixed effect of breed and/or sampling time (weeks 5, 10 or 15 *postpartum*) as a repeated-measure factor. Firstly the significance of each factor included in the statistical model was tested using non-parametric Kruskall-Wallis and Friedman tests (due to the fact that the analysed trait didn't have normal distribution). The REML (restricted maximum likelihood) method was applied in order to estimate the unknown variance components. Hypotheses were tested with the application of the F test and the multiple comparison procedure based on least significant differences (LSDs) followed by the Tukey-Kramer adjustment. Genotype groups containing fewer than 5 horses (15 measurements) were excluded from the association study.

All statistical analyses were carried out using the SAS 9.3 package (SAS Institute Inc., Cary, CA, USA).

Results

Equine *LALBA* gene 5'-flanking variants

Sequencing analysis revealed the presence of 4 previously unknown SNPs in the *LALBA* gene 5'-flanking region. Distribution of polymorphisms was uneven across the analysed horse breeds, for

Table 2. Interbreed distribution of the equine LALBA 5'-flanking variants

SNP/genotype		Genotype and allele frequencies											
		PPH (n = 28)	PWH (n = 34)	PCH (n = 32)	FIOR (n = 8)	HUC (n = 8)	HAFL (n = 8)	ARAB (n = 8)	PER (n = 8)	SIL (n = 8)	SHET (n = 8)	WELS (n = 8)	THOR (n = 8)
c.−165G>C	GG	1.00	0.94	0.81	0.75	0.63	0.88	0.88	0.75	1.00	1.00	1.00	0.88
	CG	0.00	0.06	0.19	0.25	0.25	0.12	0.12	0.25	0.00	0.00	0.00	0.12
	CC	0.00	0.00	0.00	0.00	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	MAF	0.00	0.03	0.10	0.13	0.25	0.06	0.06	0.13	0.00	0.00	0.00	0.06
c.−222A>G	AA	0.86	0.74	0.94	0.88	1.00	0.88	1.00	1.00	0.75	1.00	1.00	1.00
	AG	0.14	0.26	0.06	0.12	0.00	0.12	0.00	0.00	0.25	0.00	0.00	0.00
	GG	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	MAF	0.07	0.13	0.03	0.06	0.00	0.06	0.00	0.00	0.13	0.00	0.00	0.00
c.−357C>A	CC	0.93	1.00	1.00	1.00	1.00	1.00	1.00	0.88	1.00	1.00	1.00	1.00
	CA	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.12	0.00	0.00	0.00	0.00
	AA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	MAF	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00
c.−928C>T	CC	0.42	0.38	0.00	0.88	0.63	0.38	0.50	0.88	1.00	0.88	0.50	0.50
	CT	0.29	0.50	0.06	0.12	0.37	0.38	0.25	0.12	0.00	0.12	0.25	0.38
	TT	0.29	0.12	0.94	0.00	0.00	0.24	0.25	0.00	0.00	0.00	0.25	0.12
	MAF	0.44*	0.37	0.03	0.06	0.19	0.43	0.38	0.06	0.00	0.06	0.38	0.31

PPH – Polish Primitive Horse, PWH – Polish Warmblood Horse, PCH – Polish Coldblood Horse, FIOR – Fiording, HUC – Hucul, HAFL – Haflinger, ARAB – Arabian, PER – Percheron, SIL – Silesian, SHET – Shetland Pony, WELS – Welsh Pony, THOR – Thoroughbred; MAF – Minor Allele Frequency; *– significant deviation from Hardy Weinberg Equilibrum ($P < 0.05$)

example: c.−928C>T substitution was found in almost all breeds studied (except for the SIL), whereas the c.−357C>A SNP segregated exclusively in the PPH and PER breeds (Table 2). The lowest number of polymorphic sites (1) was described for the SIL, SHET and WELS breeds, while the highest number of SNPs (3) was found in FIOR, PPH, PWH, PCH, HAFL and PER. Among the breeds, for which gene expression studies and milk composition traits measurements were conducted (PPH, PCH and PWH), the highest level of variability was recorded for c.−222A>G (the presence of all 3 genotypes was observed in each of above mentioned breeds). Interesting differences in allele distribution were found for c.−928C>T polymorphism, as in the case of PPH and PWH the predominant allele was C (frequencies of 0.57 and 0.63, respectively), whereas within PCH this allele turned out to be minor (0.03). In the case of PPH the c.−928C>T SNP genotype distribution showed a significant ($P < 0.05$) deviation from the HWE.

Bioinformatic analysis applying the MatInspector software showed that 3 of 4 discovered polymorphisms (c.−357C>A, c.−222A>G and c.−165G>C) can potentially alter the consensus sequences for transcription factors (TFs) (Table 3). The fourth polymorphism (c.−928C>T) did not influence any TF binding site. Although we were unable to confirm this *in silico* prediction by molecular analyses, it may partly justify the further association study of the discovered SNPs and the *LALBA* gene expression levels.

Table 3. *In silico* prediction of transcription factor binding sites depending on alleles in 3 discovered *LALBA* single nucleotides peptides (SNPs) (according to MatInspector software)

SNP/allele Transcription factors binding sites					
c.−357C>A		c.−222A>G		c.−165G>C	
C	A	A	G	G	C
–	OVOL1 HNF1 MYBL1	FHXB SOX5	RU49 NRSF02	PEA3	POU3F3 TEF_HLF CEBPB CEBPE_ATF4 OCT1

Impact of breed and lactation stage on *LALBA* gene expression and milk composition

Analysis of the *LALBA* gene expression showed the highest transcript level and milk α-LA protein concentration at the week 5 of lactation (Figure 1). In the case of α-LA protein a significant gradual decrease in its milk content was noticed between weeks 5 and 15 *postpartum* ($P < 0.05$). The results of relative *LALBA* transcript abundance analysis do not correlate fully with the above-mentioned protein concentration profile, since the lowest mRNA level was recorded for the week 10 of lactation (with a statistically significant difference recorded between weeks 5 and 10, $P < 0.05$). Afterwards, the *LALBA* transcript level increased slightly at the last considered time-point (week 15 *postpartum*).

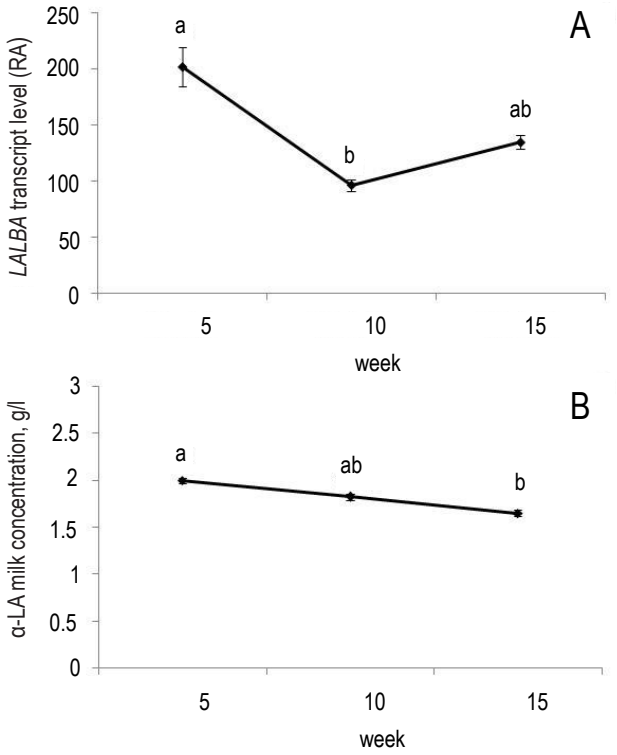

Figure 1. Mean *LALBA* relative transcript levels (A) and milk α-lactalbumin (α-LA) concentrations (B) at three different time-points of lactation (weeks 5, 10 and 15 postpartum)

Presented values are means and their standard errors (SEM); RA – relative abundance, [ab] – values with different superscripts are significantly different at $P < 0.05$

The interbreed comparison revealed identical trends for both α-LA expression levels (mRNA and protein). The lowest expression was observed for PCH, whereas the highest – for the PWH breed, respectively. However, recorded interbreed differences were much pronounced for milk α-LA concentration ($P < 0.01$) when compared to those noticed for relative transcript abundance ($P < 0.05$).

A similar analysis conducted for the basic milk components confirmed the significant effect of lactation stage on protein, fat and lactose concentrations (Table 4). In the case of protein and fat, a constant decrease in their milk contents was noticed within the investigated time-points (weeks 5–15 of lactation). An opposite trend was recorded for milk lactose abundance, with the lowest values recorded at the week 5 and the highest at the week 15 *postpartum*, respectively. The interbreed comparison showed significantly elevated protein ($P < 0.01$) contents and decreased fat concentrations ($P < 0.05$) in milk samples collected from PWH mares. No significant differences between investigated horse breeds were observed for milk lactose concentration ($P > 0.05$).

Table 4. Basic milk components in tested samples

Milk component, g/l	Week *postpartum*			Breed		
	5	10	15	PCH	PPH	PWH
Protein	18.9^{a} (± 0.3)	16.9^{b} (± 0.2)	16.0^{c} (± 0.2)	16.5^{A} (± 0.3)	17.1^{A} (± 0.3)	18.0^{B} (± 0.2)
Fat	17.4^{a} (± 0.8)	15.0^{b} (± 0.6)	14.6^{b} (± 0.5)	15.9^{ab} (± 0.6)	17.8^{b} (± 0.8)	13.9^{a} (± 0.5)
Lactose	63.0^{a} (± 0.3)	64.8^{b} (± 0.2)	66.1^{c} (± 0.2)	64.7 (± 0.2)	64.5 (± 0.3)	64.7 (± 0.3)

Presented values are means and their standard errors (SEM). PCH – Polish Coldblood Horse, PPH – Polish Primitive Horse, PWH – Polish Warmblood Horse; [abc, ABC] – means with different superscripts within the row (for lactation stage and horse breed separately) are significantly different at $P < 0.05$ and $P < 0.01$, respectively

Association analyses

Although the detected polymorphic sites are located in the 5'-regulatory region of the *LALBA* gene and three of them potentially affect the consensus sequences for transcription factors, we found no significant relationships between genotypes and gene expression measured at mRNA and milk protein levels ($P > 0.05$). However, interesting associations were noticed for the c.−928C>T variant genotypes

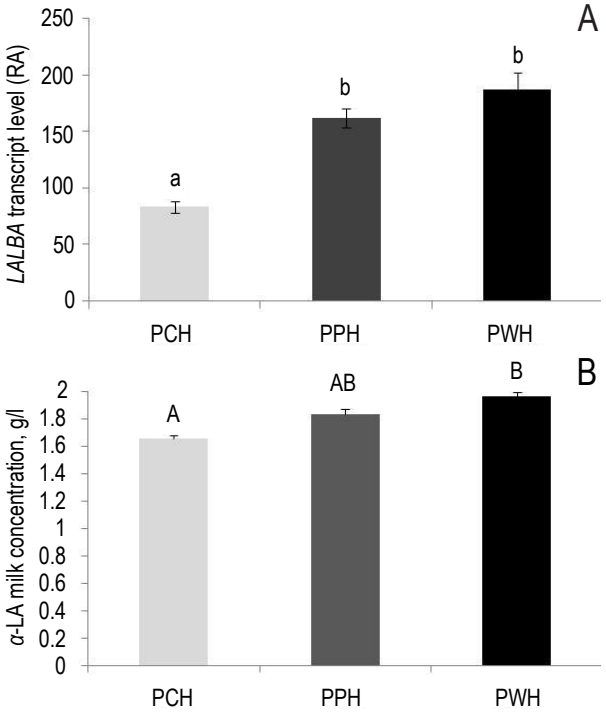

Figure 2. The interbreed comparison of mean LALBA relative transcript levels (A) and milk α-LA concentrations (B)

PCH – Polish Coldblood Horse, PPH – Polish Primitive Horse, PWH – Polish Warmblood Horse; presented values are means and their standard errors (SEM); RA – relative abundance; [ab, AB] – values with different superscripts are significantly different at $P < 0.05$, $P < 0.01$, respectively

Table 5. Association of found *LALBA* gene 5'-flanking variants with gene expression and milk composition

Single nucleotide polymorphism (SNP)*	Genotype	α-Lactalbumin						Basic milk components, g/l									
		mRNA (RA)			milk protein concentration, g/l			protein			fat			lactose		
		PCH	PWH	PPH	PCH	PWH	PPH	PCH	PWH	PPH	PCH	PWH	PPH	PCH	PWH	PPH
c.–165G>C	GG	87.6 ± 11.0	–	–	1.7 ± 0.1	–	–	16.7 ± 0.3	–	–	16.5 ± 1.3	–	–	64.6 ± 0.2	–	–
	GC	66.5 ± 20.4	–	–	1.7 ± 0.1	–	–	16.0 ± 0.5	–	–	15.8 ± 0.7	–	–	64.8 ± 0.4	–	–
c.–222A>G	AA	–	172.1 ± 32.4	–	–	2.0 ± 0.1	–	–	18.0 ± 0.2	–	–	14.2 ± 0.7	–	–	64.7 ± 0.3	–
	GA	–	223.6 ± 53.8	–	–	2.0 ± 0.1	–	–	17.9 ± 0.3	–	–	13.2 ± 0.9	–	–	64.9 ± 0.5	–
c.–928C>T	CC	–	182.9 ± 43.4	162.3 ± 23.6	–	2.0 ± 0.1	1.8 ± 0.1	–	17.5 ± 0.2	18.5^{a} ± 0.3	–	15.1 ± 0.8	19.8^{a} ± 0.9	–	64.0 ± 0.4	64.4 ± 0.4
	CT	–	186.3 ± 39.0	160.6 ± 33.0	–	1.9 ± 0.1	1.8 ± 0.1	–	18.3 ± 0.2	16.9^{ab}± 0.4	–	13.1 ± 0.7	15.0^{b} ± 1.2	–	65.3 ± 0.4	64.8 ± 0.5
	TT	–	–	168.1 ± 57.1	–	–	1.8 ± 0.2	–	–	15.5^{b} ± 0.7	–	–	15.4^{ab}± 2.2	–	–	64.0 ± 0.8

Presented values are means and their standard errors (SEM). PCH – Polish Coldblood Horse, PPH – Polish Primitive Horse, PWH – Polish Warmblood Horse; *The c.–357C>A SNP was not analysed due to very low frequency (only 2 heterozygous individuals were found within PPH breed); RA – relative abundance; '–' not analysed due to insufficient genotype frequency; [ab] – values marked with different superscripts within the column (for each SNP separately) are significantly different at $P < 0.05$

and milk composition traits (protein and fat contents) in the PPH breed. It turned out that CC genotype carriers have significantly ($P < 0.05$) elevated milk protein contents (18.5 ± 0.3 g/l) when compared with TT homozygous animals (15.5 ± 0.7 g/l). In the case of milk fat content the most pronounced difference ($P < 0.05$) was recorded between CC and CT genotype carriers (19.8 ± 0.9 vs. 15.4 ± 2.2 g/l).

The other investigated 5'-flanking variants either showed no association with milk composition traits or the distribution of particular genotypes was insufficient to perform statistical analyses (Table 5).

Discussion

Equine milk as valuable animal product. Although cattle remain the major source of milk consumed by people all over the world, the chemical composition of ruminants' milk is significantly different from that of human breast milk, therefore an increased interest in the potential utility of milk of other (non-ruminant) species is currently observed (Uniacke-Lowe et al., 2010). Additionally, cow's milk is considered as one of the most important sources of food allergens due to their significant amounts present in both casein and whey protein fractions (including α-LA) (Caira et al., 2012). In contrast, tolerability of equids' (horse and donkey) milk in children suffering from cow's milk protein hypersensitivity is very high (82–100%) (Salimei and Fantuz, 2012). Apart from children with allergies, mare's milk is recommended e.g., for people suffering from metabolic disorders, skin problems, increased cholesterol levels, hepatitis and gastric ulcers (Rad et al., 2013; Pieszka et al., 2016).

Variability of milk α-LA content and *LALBA* gene expression level. While it is well known that α-LA is one of the most abundant whey proteins present in milk of various mammalian species, its concentration can vary strongly between particular animals. A previously published study by Markiewicz-Kęszycka et al. (2013) indicated that in the case of mare's milk this variability can be substantial (1.46–3.49 g/l). The difference between minimum and maximum α-LA concentrations observed in the present study was even greater (0.63–2.94 g/l); however, it should be underlined that we used samples collected from 3 horse breeds at 3 different time-points of lactation, whereas the cited study was based on milk samples derived from PCH mares in the late lactation stage only. As it was confirmed by the statistical analyses, both factors (breed and sampling time) have a significant impact

on mare's milk α-LA level and *LALBA* gene relative transcript abundance (Figures 1 and 2). Recorded significant decrease of the *LALBA* gene mRNA level between the weeks 5 and 10 of lactation is concordant with the results of similar experiment performed on bovine milk-purified mammary epithelial cells (Sigl et al., 2012). The highest expression of *LALBA* gene (noticed for both – mRNA and protein stages) during early lactation may reflect the increased need for anti-inflammatory proteins at the beginning of newborn foal life. Similar trend was observed before for lysozyme and lactoferrin bioactive proteins (Cieslak et al., 2017).

Taking into consideration the high ($h^2 > 0.5$) heritability of α-LA content in cows'milk described in the scientific literature (Schopen et al., 2009), we hypothesized that the variability observed for mare's milk may be strongly related to genetic factors (e.g., polymorphisms located in regulatory regions of the *LALBA* gene). The thesis on the importance of the genetic component is also supported by the recorded interbreed differences in milk α-LA concentrations and *LALBA* gene relative transcript abundance, as the three investigated horse breeds (PWH, PPH and PCH) represent phylogenetically distinct horse groups, thus obviously their genetic backgrounds differ significantly. Analogous between-breed differences in milk α-LA abundance were observed previously for various cattle breeds (Bleck et al., 2009).

Similarly to our previous studies on other equine milk proteins (Cieslak et al., 2015, 2017) in the present experiment we have noticed that results of *LALBA* gene expression measured at transcript level do not fully correlate with the observed α-LA milk concentration (Figure 1). This is in agreement with the current knowledge regarding complexity of mechanisms involved in regulation of gene expression, which are responsible for the lack of simple, linear relationship between mRNA abundance and given protein synthesis rate (Maier et al., 2009). Thus, we can conclude that each experiment should be possibly carried out for both expression stages (transcript and protein). Otherwise, obtained results should be interpreted very carefully.

Association of 5'-flanking variants with gene expression and milk composition. Since recently published studies based on ruminants (Cosenza et al., 2016; Noce et al., 2016) have confirmed the potential impact of polymorphisms located within the regulatory regions of milk protein genes on their expression and variability in milk composition traits (e.g., protein, fat and lactose contents), we decided to focus on the 5'-flanking region of the equine *LALBA* gene. Although we found 4 novel SNPs in the investigated sequence (unevenly distributed across analysed horse breeds), none of them turned out to be associated with the equine *LALBA* gene expression (measured at mRNA and milk protein levels). The lack of association may be partly related with the relatively small sample size and low frequencies of the investigated SNPs (which in several cases made the statistical analysis impossible or only two genotype groups could be compared). Although due to the crucial role of α-LA in lactogenesis (Hayssen and Blackburn, 1985) we expected a potential relationship between detected polymorphisms and milk lactose concentration variability, the only significant association was recorded for fat and protein contents in the PPH breed (c.−928C>T polymorphism). Also for this particular variant, the significant deviation from HWE was noticed in the PPH breed (which may be e.g., a subtle sign of past selection for unknown milk-related trait). Therefore analyses of this promising SNP should be continued. On the other hand, results of previously published studies regarding the impact of *LALBA* gene polymorphisms on the variability in ruminants' milk traits are often not consistent. For example, in a study by Dettori et al. (2015) on the Sarda goat breed a significant association was described between several 5'-flanking variants and milk production traits (milk yield, lactose content, curd forming time). In contrast, in a study by Zhou and Dong (2013) the SNP found in exon 4 of the *LALBA* gene revealed no association with milk composition traits in Chinese Holstein cows.

Conclusions

Despite the fact that our results do not confirm any direct influence of the equine *LALBA* gene 5'-flanking variants on its expression (measured at mRNA and protein levels), these investigations should be continued. Since many previous studies, including recent experiment regarding the equine α-s2 casein gene (Cieslak et al., 2016), have proven that also polymorphisms located within the coding sequence may affect milk protein genes expression, it seems necessary to extend the present investigation to include other regions of the *LALBA* gene. Moreover, preliminary results on the association between one of the detected polymorphisms (c.−928C>T) and some of analyzed milk traits variability (protein and fat concentrations) indicate that the gene encoding equine α-LA should remain a major candidate gene for mare's milk composition.

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