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The effects of diet supplementation with yeast on the expression of selected immune system genes in the milk somatic cells of dairy goats*

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The aim of the study was to evaluate the impact of diet supplementation with active yeast culture (*Saccharomyces cerevisiae*) on the expression of selected immune system genes in goat milk somatic cells (MSC) and on milk production traits. Eighteen dairy goats were divided by breed and parity into two groups: control (n = 9) and supplemented (n = 9). Each group consisted of 5 Polish White Improved (PWI) and 4 Polish Fawn Improved (PFI) goats. Four goats (2 PWI and 2 PFI) were in their second lactation and five (3 WPI and 2 PFI) in more than second lactation. Beginning from a week before expected kidding, supplemented does were fed 10g yeast/day/goat until 100 day post-partum, after which the dose was increased to 20g/day/goat. On day 7, 30, 80, 120 and 240 of lactation milk samples were collected and used to SC isolation, microbiological analysis and to determine the physico-chemical composition of milk. Total RNA was isolated from SC and expressions of β1-defensin, β2-defensin, bactenecin 7.5, bactenecin 5, hepcidin and lyzozyme genes were measured with qRT-PCR using cyclophilin A (*PPM*) as a reference gene.
Supplementation with yeast did not affect milk yield and composition (P>0.05). Genes encoding β2-defensin, bactenecin 7.5 and hepcidin were influenced by yeast diet supplementation resulting in their increased expression in MSC (P≤0.05) regardless of the amount of supplement. The environmental bacteria did not affect the level of gene transcripts. However, the presence of β2-defensin, bactenecin

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5 and 7.5, hepcidin and lysozyme transcripts in milk cells derived from pathogen-free udders proved their constitutive expression. The expression of β1-defensin gene was not found indicating that it is not constitutively expressed in milk cells and does not participate in the defense of the udder against environmental bacteria. These findings support that β2-defensin, bactenecin 5, bactenecin 7.5 and hepcidin may play a role in maintaining the health of the goat mammary gland.

KEYWORDS: gene expression / goat / milk SCC / yeast supplementation

Probiotics are a group of viable microorganisms with a beneficial effect on the prevention and treatment of specific pathological conditions in humans and animals. The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) define probiotics as live microorganisms that in sufficient quantity have a beneficial effect on the health of the host. Yeasts are often used as probiotics in human and animal nutrition. Yeast-derived products generally contain *Saccharomyces cerevisiae* (the most common species), in a concentration higher than 10^9 colonies per gram or *Saccharomyces boulardii*, an especially important probiotic administered to prevent antibiotic-associated diarrhea and recurrent intestinal infections with *Clostridium difficile* [Czerucka *et al.* 2007].

Yeast cultures are also used as probiotics in ruminants' diets to create a rumen environment more conductive to the growth and activity of anaerobic cellulolytic bacteria. Although they compete with autochtonous ruminal species for energy, the addition of yeast to the diet facilitates the stabilization of the rumen pH by stimulating lactate-utilizing bacteria [Kowalik *et al.* 2012]. The stable environment of the rumen is one of the most important factors both in optimum milk yield and good animal health [Doležal *et al.* 2011]. Mannan oligosaccharides (MOS) and β-glucan in the yeast cell walls have a positive effect on the health status of the host. MOS is a competitive ligand for the binding site for gram-negative bacteria. It causes the immediate removal of pathogens from the digestive tract and prevents its colonization. Franklin *et al.* [2005] supplemented dry cows with MOS and observed an enhancement of humoral immune response to rotavirus and a tendency for enhanced transfer of rotavirus antibodies to calves. β-Glucan seems to have an immunomodulatory effect when supplemented in pig and avian diets [Nocek *et al.* 2011] and in the diets of other animals and humans [Soltanian *et al.* 2009]. In the studies on dairy cows it was found that the somatic cell count in milk was lower after supplementation the diet with yeast culture and enzymatically hydrolyzed yeasts. Moreover, new clinical cases of mastitis were numerically lower in cows fed a diet with this additive [Nocek *et al.* 2011]. However, Waller *et al.* [2003], who infused β-1,3-glucan at drying off into udders of cows infected with *Staphylococcus aureus*, showed no therapeutic effect on quarters afflicted with the chronic subclinical mastitis.

For many years, numerous companies have been engaged in the production of food additives for animals, and promoting yeast as an agent optimizing the microflora in the contents of gastrointestinal tract. This has a great impact on animal health as it prevents diarrhea caused by *Escherichia coli* and reduces the urea level in milk. It has been shown that when the diet of dairy cows is supplemented with yeast, milk yield

is elevated and its protein and fat contents increase [Desnoyers *et al.* 2009, Nocek *et al.* 2011].

The innate immunity represented by antimicrobial peptides and proteins constitutes the first line of defense against pathogens, *i.e*. as the immediate response to invading pathogens. One of these immunological proteins is lysozyme, present also in milk. Lysozyme exhibits antifungal and antibacterial activity. This immunological protein, found in some animal tissues and secretions (tears, urine, nasal secretions, and cervical mucus) as well as in serum and milk, exhibits activity against human immunodeficiency *virus* (HIV) [Lee and Yang 2002]. Hepcidin, the peptide produced in the mammalian liver [Fujita *et al.* 2007], kidney [Kulaksiz *et al.* 2005] and heart [Merle *et al.* 2007] such as in human airway epithelial cells [Frazier *et al.* 2011] and alveolar macrophages [Nguyen *et al.* 2006, Sow *et al.* 2007] as a response to inflammation and iron overload, has been shown to possess also direct antimicrobial activity. Sow *et al.* [2007] found that hepcidin inhibited *Mycobacterium tuberculosis* growth *in vitro* and caused structural damage to the mycobacteria. Defensins and cathelicidins are two large families of antimicrobial peptides (AMPs) that are also involved in the innate immune response. They are stored in neutrophils and macrophages, while in epithelial tissues they are released into the intercellular spaces from the epithelial cells. The defensins family, which is the only class of antimicrobial peptides conserved between vertebrates, invertebrates and plants demonstrate, similarly to cathelicidins, antimicrobial activity against bacteria, enveloped viruses, fungi and some kinds of neoplastic cells [Ganz 2003]. The role of defensins [reviewed by Bagnicka *et al.* 2010 and Jarczak *et al.* 2013] and cathelicidins [reviewed by Kościuczuk *et al.* 2012] concerns the microbicidal and antiviral activity, an induction of cytokines, the regulation of C1 (the first component of human serine protease inhibitor), the corticostatic effect, activating mast cells, and the immunoenhancing effect. In this study, two representatives from each family of AMPs were selected (β1- and β2 defensins and cathelicidins – bactenecin 5 and 7.5) and the expressions of their genes (mRNA levels) were measured in milk somatic cells (MSC). The role of these genes in protecting the mammary gland against mastitis was proved in a previous reports [reviewed by Bagnicka *et al.* 2010].

It was shown that MSC derived from pathogen-free goat milk contain about 35% leucocytes, with a high content of neutrophils and monocytes, and a much lower content of eosinophils and lymphocytes (15%, 10%, 3% and 2% of total MSC, respectively). The rest of the SC (65%) was a fraction of exfoliated epithelial cells. The presence of bacterial pathogens causes an increase in the percentage of leucocytes, particularly of neutrophils and eozynophiles, in the total number of MSC [Bagnicka *et al.* 2011]. The cells isolated from the goat milk are therefore considered an excellent experimental model to study the expression of the genes involved in the animals' defense system.

The aim of this study was to evaluate the effect of diet supplementation with active yeast culture (*Saccharomyces cerevisiae*) on milk production traits and the expression of immune system genes in the milk somatic cells of dairy goats.

Material and methods

The experiment was conducted during lactation period of dairy goats (January to September 2011). The goats were obtained from the herd maintained at the Institute of Genetics and Animal Breeding of the Polish Academy of Sciences, Jastrzębiec, Poland.

Animals and diets

The study was conducted on 18 Polish White Improved (PWI) and Polish Fawn Improved (PFI) dairy goats in their second to fifth lactation. The mean milk yield during the 280-day lactation was about 800 kg, with 3.35% fat and 3.20% protein. There were no differences in milk yield or composition between the two breeds. All animals were free of the caprine arthritis and encephalitis (CAE) virus. The goats were kept in loose housing, machine milked twice a day and fed according to the INRA system. The basic diet consisted of corn silage, wilted grass silage and concentrates, supplemented with a mineral and vitamin mixture. Water was available *ad libitum*.

Eighteen dairy goats were equally divided into two analogous groups according to their breed and parity. All animals had similar milk yield and somatic cell count (SCC). Each group consisted of 5 PWI and 4 PFI goats. Four of them (2-PWI and 2-PFI) were in their second lactation and five (3-WPI and 2-PFI) in more than second lactation (Tab. 1).

Table 1. Number of animals according to group, breed and parity

Parity	Group	Breed		Total
		PWI	PFI	
2	control	2	2	4
	experimental	2	2	4
>2	control	3	2	5
	experimental	3	2	5
	total	10	8	18

PWI – Polish White Improved.
PFI – Polish Fawn Improved.

The control group was fed a basic diet and the experimental group was fed a diet supplemented with active yeast *Saccharomyces cerevisiae*. The experiment lasted from one week before kidding until day 240 of lactation. Beginning with week before expected kidding supplemented does were fed 10g/day/goat until day100 post-partum, after which the dose was increased to 20g/day/goat roughly calculated based on the dose recommended for dairy cows[1]. The milk samples were taken on day 7, 30, 80,

[1]The recommended daily dose for a dairy cow is between 100 and 200 g of yeast throughout lactation and beginning at least three weeks before parturition. Because the live weight of an adult goat is about one tenth of that of an adult cow, the daily dose for a goat should also be lower. However, as the metabolic rate of goats is higher than that of cows (about 30%), their daily dose should probably be respectively higher. The daily dose for a goat should therefore not be less than 10 g or higher than 26 g of yeast.

120 and 240 of lactation. Altogether 90 samples were collected during experiment (5 per goat).

In order to check the microbiological status of the mammary gland, foremilk samples were collected just before the morning milking and examined for the presence of bacteria. To identify the pathogens Columbia agar supplemented with 5% sheep blood and MacConkey agar (BioMérieux, France) were used. The plates were incubated at 37°C for 24-36 hours. Vitek2 equipment was used to identify the bacteria strains (BioMérieux, France).

Total protein, fat and lactose contents were estimated using MilkoScanFT2 (FOSS, Denmark). The total somatic cell count (SCC) was estimated using IBC_M automated fluorescent microscopic somatic-cell counter (BENTLEY INSTRUMENTS, USA).

Isolation of somatic cells from milk

One liter of milk was taken from each goat and used to isolate somatic cells. Milk was centrifuged at 1500g for 30 min to separate fat and milk cell fractions. Somatic cell pellet was washed with phosphate buffered saline (PBS) and centrifuged at 1100 g for 15 min. The procedure was repeated twice. Purified pellet was dissolved in 1 ml of Trizol reagent and stored at -80°C.

Primer design

Genes encoding the antimicrobial peptides of the defensin (*GBD1*, *GBD2*) and cathelicidin (*BAC5*, *BAC7.5*) families, hepcidin (*HAMP*) and lysozyme (*LZ*) were chosen to check the influence of yeast supplementation on the dairy goat immune system (Tab. 2). Primers were determined by matching the coding regions of the target

Table 2. Sequence of primers used in Real-Time PCR for amplification of target and housekeeping genes

Gene symbol	Primer sequence	Forward (F) Reverse (R)	Product size (bp)	GenBank accession No.
	Target genes			
Bac5	CAGTCACCTTGGATCCATCCA	F	192	Y18873.1
	CAGGAAATGGTCCTATGGGT	R		
Bac7.5	GATCCATCCAATGACCAGTTT	F	91	AJ243125.1
	TTGGCCTTGGCAAACGT	R		
GBD1	ACTCAAGGAATAAGAAGTCG	F	110	Y17679.1
	CATTTTACTGGGGGCCCGAA	R		
GBD2	CTCAAGGAATAATAAATCA	F	110	AJ009877.1
	CATTTTACTGGGGGCCCGTG	R		
LZ	TGAAGGCTCTCATTATTCTG	F	446	GQ889414.1
	TTACACTCCACAACCCTG	R		
HAMP	ACCTGCCTTCTGCTCCTTGT	F	103	GQ901053.1
	CTCCAGCTGTGTGCTGAGTTT	R		
	Housekeeping gene			
PPM	GGATTTATGTGCCAGGGTGGTGA	F	120	AY 247029.1
	CAAGATGCCAGGACCTGTATG	R		

mRNAs to within two neighboring exons so as to prevent interference from genomic DNA amplification. The Primer BLAST(http://www.ncbi.nlm.nih.gov/tools/primer-blast/) programme was used to design the primer sequences. These had to meet the following conditions: size 18-25 base pairs (bp), annealing temperature 56-64°C, GC percentage 50-60%, and a PCR product length 100-450 bp. All primers were designed on the basis of *Capra hircus* sequences available in GenBank, except for those for the *HAMP* gene, which were designed on the basis of *Ovis aries* sequences. The cyclophilin A (*PPM*) gene was used as the reference one [Finot *et al.* 2011]. The sequence of primers for *GBD1* and *GBD2* were taken from Zhao *et al.* [1999].

RNA isolation and cDNA synthesis

The high-quality total RNA was isolated from milk somatic cells using a Pure Link RNA Mini Kit (AMBION, USA) according to the manufacturer's instructions. The cell pellet was homogenized in 1 ml of Trizol reagent. After 5 min incubation at room temperature 200 µl of chloroform was added to each sample. The mixture was centrifuged at 12,000 g for 15 min. at 4°C and the upper, transparent phase containing RNA was recovered. A DNAse digestion step was carried out to remove genomic DNA using a PureLinkDNase Set (Invitrogen). RNA was eluted from spin columns with 40 µl of RNase-free water.

A qualitative and quantitative assessment of the isolated RNA was conducted using a NanoDrop (NANO DROP, USA) spectrophotometer. Only samples with more than 50 ng RNA and absorbance ratios $A_{260/280}$ and $A_{260/230}$ of around 2.0 were used for further analyses. To confirm the RNA quality, a quick electrophoretic analysis was conducted using a BioAnalyzer (AGILENT, USA). The RIN (RNA Integrity Number) of all samples occurred higher than 7.5. RNA was reverse transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kit (ROCHE, Switzerland). RNA in the amount of 0.5 µg was firstly denatured at 65°C for 10 min in the presence of 50 µM oligo(dT). After that, reverse-transcription was conducted in a total volume of 20 µl of mixture composed of 13 µl of RNA, 4 µl of reverse transcriptase buffer, 2 µl of 10 mM deoxynucleotides, 0.5 µl of protector RNase Inhibitor (40U/µl), and 0.5 µl of reverse transcriptase (20U/µl). The mixture was incubated in 50˚C for 60 min, then at 85°C for 5 min, and finally stored at -20°C.

Real-Time PCR

Real-Time PCR using a Light Cycler System (ROCHE, Switzerland) was used to measure levels of transcripts of reference and target genes. The reaction mixture consisted of 7.5 µl of 200 nM primers, 10 µl of SYBR Green Master Mix (ROCHE, Switzerland), 3 µl of pure water, and 5 µl of cDNA. Reactions were performed in triplicate, with a non-template control, on 96-well optical reaction plates. The Real-Time PCR programme consisted of initial denaturation, amplification, and a melting curve step (Tab. 3).

Table 3. Real-Time PCR programme for reference and target genes

Gene name	Real-Time PCR program		
	preincubation	primer annealing	melting curve
PPM	95°C for 5 min – 1 cycle	58°C for 15 sec – 45 cycles	95°C for 5 sec 65°C for 1 min – 1 cycle
BAC5		58°C for 15 sec – 45 cycles	
BAC7.5		58°C for 15 sec – 45 cycles	
GBD1		58°C for 15 sec – 45 cycles	
HAMP		60°C for 15 sec – 45 cycles	
LZ		60°C for 15 sec – 45 cycles	

Efficiency of Real-Time PCR and slope values were determined for each primer. Amplification efficiency was calculated from the slope of the standard curve using the formula $E=10^{(-1/slope)}$. The standard curve was generated using a series of 10-fold dilutions. A melting curve was produced to confirm the presence of a single gene-specific peak and the absence of primer dimers. All data, including the raw cycle threshold (Ct), which was obtained using Light Cycler 480 Software, were used for the comparative Ct method [Pfaffl 2001].

Statistical

Relative gene expression level was measured in relation to the reference gene and was shown as the mean relative mRNA content and standard error (SE). All traits were tested for normality of distribution and only somatic cell count was transformed to natural logarithm values. Analysis of variance was conducted with the Student's t-test (SAS/STAT) to check the influence of yeast supplementation on the relative expression of target genes and milk production traits. Other effects, such as goat breed, parity, stage of lactation, and presence of environmental bacteria in milk, were also taken into consideration in the statistical model:

$$y_{ijklmn} = \alpha + YS_i + BR_j + P_k + SL_l + BA_m + e_{ijklmn}$$

where:

y_{ijklmn} – trait value;

α – overall mean;

YS_i – fixed effect of i-th yeast supplementation (i = 1,2);

BR_j – fixed effect of j-th goat breed (j = 1,2);

P_k – fixed effect of k-th parity (k = 1,2);

SL_l – fixed effect of l-th stage of lactation (l = 1,…,5);

BA_m – fixed effect of m-th presence/lack of environmental bacteria (m = 1,2);

e_{ijklmn} – random error.

Results and discussion

An impact of the diet supplementation with yeast upon the presence of bacteria in milk and upon the milk production traits

The microbiological examination of milk samples showed that almost 67% of them did not contain any bacterial pathogens. The remaining contained bacteria such as *Staphylococcus simulans* (the one most commonly found in goat milk), *Staphylococcus caprae, Staphylococcus chromogenes, Staphylococcus warnieri, Staphylococcus epidermidis, Staphylococcus xylosus* and *Lactococcus garviae* (Fig. 1). Bacterial strains isolated from milk were likely environmental in origin as they are not commonly associated with *mastitis*. These results confirmed those of our earlier study on this herd [Bagnicka *et al.* 2011]. Yeast supplementation had no effect on the absence or presence of environmental bacteria in goat milk.

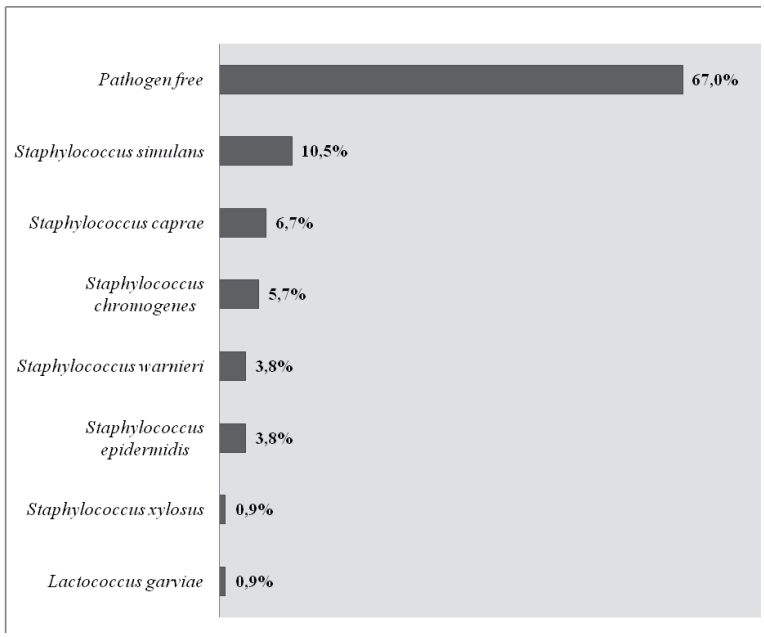

Fig. 1. Frequency and strains of bacteria spread in goat milk in studied groups.

Moreover, the yeast supplementation did not affect neither yield nor composition of the milk, even after doubling the dose (Tab. 4). This contrasts with the results on dairy cows, where it has been shown that when their diet is supplemented with yeast, milk yield is increases and its protein and fat contents are higher [Cooke *et al.* 2007, Desnoyers *et al.* 2009, Lehloenya *et al.* 2008, Nocek *et al.* 2011]. However, as expected, the stage of lactation influenced the milk production traits.

Table 4. Least squares means (LSM) and standard errors (SE) for milk production traits according to control and experimental groups and stage of lactation

Trait	Time of sample taking	Group		SE
		control	experimental	
		LSM	LSM	
Milk yield in morning milking (kg)	1	1.23	1.18^{A}	0.09
	2	1.05	0.83^{B}	
	3	1.05	0.98^{B}	
	4	1.18	1.06	
	5	1.20	1.08	
lnSCC	1	4.45^{A}	4.49^{A}	0.44
	2	4.73^{A}	5.44	
	3	6.64	5.48	
	4	6.43^{B}	6.06^{B}	
	5	5.79^{B}	5.25	
Fat (%)	1	4.68^{A}	4.80^{A}	0.20
	2	4.86^{A}	4.81^{A}	
	3	4.15^{B}	$4. 28^{A}$	
	4	3.93^{B}	4.37^{A}	
	5	2.83^{C}	2.67^{B}	
Protein (%)	1	3.39^{A}	3.14^{A}	0.09
	2	2.83^{B}	2.72^{B}	
	3	2.95^{B}	2.66^{B}	
	4	2.77^{B}	2.80^{B}	
	5	3.15^{A}	3.04^{A}	
Lactose (%)	1	4.88^{A}	4.91^{A}	0.05
	2	4.77^{A}	4.74^{A}	
	3	4.76^{A}	4.75^{A}	
	4	4.61^{A}	4.58^{B}	
	5	4.40^{B}	4.48^{B}	

^{ABC}Different letters within columns and within traits indicate differences at $P \leq 0.01$.

Impact of yeast supplementation on the expression of immune system-related genes

In this study the presence of environmental bacteria, the breed of goat, and the parity and stage of lactation had no effect on the expression levels of the studied genes involved in goat immune system. This indicates the constitutive expression of *GBD2*, *BAC5*, *BAC7.5, LZ* and *HAMP* genes in goat milk somatic cells, and possibly both in leukocytes and the mammary gland epithelium.

In the present study lack of expression of the *GBD1* gene in goat milk somatic cells was found both derived from milk not containing bacteria and that from containing environmental bacteria. Previously, we also did not detect the expression of the *GBD1* gene in milk somatic cells while the expression of the *GBD2* gene in cells derived from the milk of goats with both low and high SCC estimates was found, although the presence of bacteria was not examined. In that study the expression of the *GBD2* gene did, however, parallel increase with the somatic cell number during lactation [Bagnicka *et al.* 2006]. Probably, in that study goat milk contained major pathogen bacteria, even milk with low SCC. In study conducted between 2005-2008 we found

that the bacterial pathogens were presented in about 20% of milk samples containing low SCC (below 1×10^6/mL) [Bagnicka *et al.* 2011]. After yeast supplementation regardless of the amount added, one cathelicidin gene (*BAC7.5*) and one defensin genes (*GBD2*) and hepcidin showed an increased expression (Fig. 2).

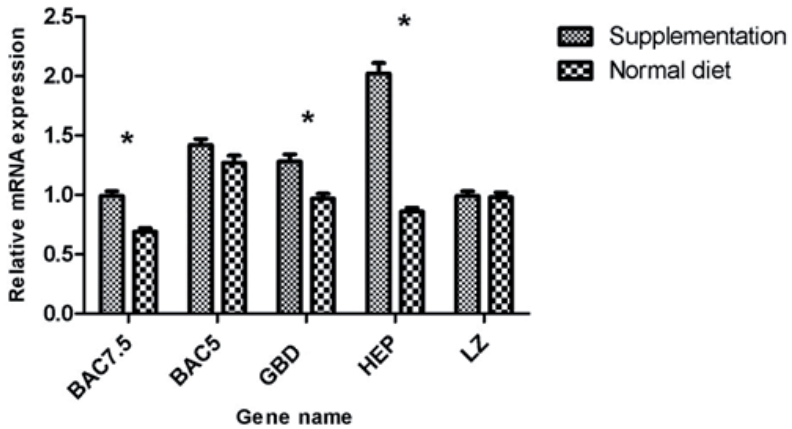

Fig. 2. Relative expression of selected genes in the milk somatic cells.
*Different at $P \leq 0.05$.

According to our knowledge, hepcidin transcripts were found in goat milk somatic cells for the first time. In this study equal amounts of hepcidin mRNA were detected in cells derived from healthy mammary glands and from milk containing environmental bacteria. Furthermore, the hepcidin gene (*HAMP*) showed an increased expression after yeast supplementation. This is an evidence that yeast diet supplementation has a positive effect on the health status of the mammary gland.

Lysozyme, present in milk exhibits antifungal and antibacterial activity. This immunological protein, found in some animal tissues and secretions as well as in blood serum and milk, exhibits activity against HIV [Lee and Yang 2002]. In present study, there were no differences in mRNA transcripts of *LZ* gene between control and experimental groups that may indicate a lack of effect of yeast supplementation on its expression in milk somatic cells, but its constitutive expression could confirm its participation in maintaining of health status of mammary gland.

Only limited information on the expressions of the genes involved in the immune response of the goat mammary gland has so far been available. Moreover, there is no information on the effect of yeast diet supplementation on gene expression. There is therefore no possibility of comparing the obtained results with other research. There is, however, some information on the positive effect of diet supplementation with different additives (e.g. protein, oils, and selenium) on the expressions of genes involved in the immune system and on goat mammary gland metabolism [Nnadi *et al.* 2007, Bernard *et al.* 2009, Ollier *et al.* 2009, Ren *et al.* 2011]. These promising results justify further studies to prove our results.

Concluding, the results presented here demonstrate the importance of nutrigenomic studies in conjunction with gene expression analysis and show that there is a need to examine the effect of feeding on gene expression more closely. The increased expression of genes encoding the antimicrobial peptides β2-defensin, bactenecin7.5 and hepcidin, influenced by active yeast diet supplementation, may justify a role for this food additive in maintaining the health status of the goat mammary gland. The presence of β2-defensin, bactenecin5 and 7.5, hepcidin, and lysozyme transcripts in milk cells derived from pathogen-free mammary glands proved their constitutive expression. The presence of environmental bacteria in milk did not affect the expression of the studied genes. No β1-defensin gene transcript was detected in our study, indicating that it is not constitutively expressed in milk cells and does not participate in the defense of the udder against environmental bacteria.

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