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Virendra Kumar Singh
Assistant Professor, Department of Veterinary Physiology and Biochemistry, College of Veterinary Science and Animal Husbandry, Navsari Agricultural University, Navsari, Gujarat, India

Sandhya S Chaudhary
Professor and Head, Department of Veterinary Physiology and Biochemistry, College of Veterinary Science and Animal Husbandry, Navsari Agricultural University, Navsari, Gujarat, India

Tanvi D Manat
Assistant Professor, Department of Veterinary Physiology and Biochemistry, College of Veterinary Science and Animal Husbandry, Navsari Agricultural University, Navsari, Gujarat, India

Corresponding Author:
Virendra Kumar Singh
Assistant Professor, Department of Veterinary Physiology and Biochemistry, College of Veterinary Science and Animal Husbandry, Navsari Agricultural University, Navsari, Gujarat, India

Comparative analysis of quantitative gene expression of prolactin, leptin and glutathione peroxidase in buffalo calves supplemented with different yeast derivatives

Virendra Kumar Singh, Sandhya S Chaudhary and Tanvi D Manat

Abstract
The present study was conducted on 9 apparently healthy Surti young buffalo female calves aged (6-12 months) divided into 3 groups of 3 each categorized as group I (control), group II (supplemented with rumen specific yeast *Saccharomyces cerevisiae* CNCM I-1077 @ $4x10^9$ cfu/animal/day and group III (supplemented with selenoyeast-inactivated yeast *Saccharomyces cerevisiae* containing selenomethionine ensuring dietary inclusion of selenium @ 0.3 ppm). Whole blood was collected at day 0 of study (beginning), day 45 and day 90 of study (end). Total RNA was isolated, cDNA was synthesized and quantitative gene expression of prolactin, leptin, and GPx1 was studied using real time PCR. Downregulation of prolactin was observed in all groups at all stages wherein between groups, down regulation during 0-45 days was maximum in group II and minimum in group III.; during 45-90 days maximum in group III and minimum in group II and during 0-90 days between groups the down regulation was maximum in group II and III followed by control. Within groups down regulation increased in group I and III and decreased in II from 0-45 days to 45-90 days of study. Between the groups leptin was upregulated during 45-90 days and 0-90 days of study. Maximum upregulation was in group II and minimum in control. During 0-45 days the upregulation was almost similar in all the groups. Within groups the upregulation increased in group II and decreased in group III and group I from 0-45 days to 45-90 days of study. GPx1 was upregulated in all groups and at all stages but between groups was maximum in group III. Thus it was concluded that in female Surti buffalo calves supplementation of rumen specific yeast had more positive influence on prolactin and leptin gene expression whereas selenoyeast supplementation positively impacted glutathione peroxidase expression.

Keywords: Gene expression, prolactin, leptin, glutathione peroxidase, yeast derivatives female Surti buffalo calves

Introduction
Livestock comprises an important industry that contributes to the agriculture based economy of country like India. Dairy activities are of prime importance in it. Milk production from buffalo (*Bubalus bubalis*) forms a sizeable portion in the national milk production. The foundation of good lactation performance is laid during early life in buffalo female calves. It is ideal to have faster growth rate during young age without accumulating any stress and attain early puberty. Thus nutrition plays an essential role in this and has been reported by Birthal and Jha (2005) [1] that issues related to feeding cripple the realisation of potential of dairy animal. It also has been supported by the study of Garg *et al.* (2012) [2]. During young age especially before six months the rumen development is on the verge of completion. During this time an impetus in the form of probiotic supplementation can increase dry matter intake and may optimise the nutritional status of young female buffalo calves directly as well as indirectly. Leptin from adipocytes being an antiobesity hormone is of prime importance for studies related to gain in body weight as in young calves. Prolactin secreted from pituitary as well as extra pituitary sources mediates stress to some extent apart from its major effect on growth especially involving mammogenesis. The level of these hormones may indirectly reflect the nutritional state as an additional aspect apart from regulation in physiological processes. Glutathione peroxidase, a selenium dependent antioxidant enzyme (GPx) is considered the major peroxide-removing enzymes located in the cytosol. Since the hormonal level in blood is affected by their respective gene expression, it is desirable to know the quantification of gene expression.

Feeding of probiotic is quite common but rumen specific yeast and selenoyeast feeding have gained importance recently. Thus the present study was planned to study the nutrigenomic effects of feeding probiotics (rumen specific yeast and selenoyeast) in Surti buffalo female calves on genes of prolactin, leptin and Glutathione peroxidase.

Materials and Methods

Location of study

The study was conducted in the Department of Veterinary Physiology and Biochemistry, NAU, Navsari, Gujarat following ethical guidelines and was approved by IAEC vide NAU/NVC/IAEC/6/2015 01/08/2015.

Experimental design

9 apparently healthy Surti young buffalo female calves aged (6-12 months) were randomly selected and divided into 3 groups of 3 each categorized as group I (control), group II (supplemented with rumen specific yeast *Saccharomyces cerevisiae* CNCM I-1077 @ $4x10^9$ cfu/animal/day and group III (supplemented with selenoyeast-inactivated yeast *Saccharomyces cerevisiae* containing selenomethionine ensuring dietary inclusion of selenium @ 0.3 ppm). Calves were maintained at Livestock Research Station of university and standard conditions of feeding and management were adopted. The duration of study was 90 days during which supplementation of both yeast derivatives was done in group II and III calves.

Approximately 5 ml of whole blood from each animal was collected from jugular vein into vacutainers i.e. K_3EDTA. Whole blood was used for total RNA isolation for quantitative gene expression studies of prolactin, leptin, GPX_1 against housekeeping GAPDH.

Isolation of total RNA and synthesis of cDNA

Total RNA was isolated from whole blood using Trizol reagent TRI Reagent BD (Sigma-Aldrich, USA) as per recommended protocol taking maximum care to avoid RNAase contamination during procedure. Total RNA isolated in the form of pellet was dissolved in 30 µl nuclease free water. Quantification of RNA and determination of its purity was done by (Nanodrop spectrophotometric (Thermo Scientific ND 2000C) and samples having 260/280 ratio ≥1.7 were selected for reverse transcription and quantitative expression by real time PCR. Assessment of integrity of RNA was done by agarose gel electrophoresis. The first strand cDNA was synthesized from the isolated total RNA. RT-PCR was done using Quanti Tect Reverse Transcription Kit (Qiagen, India) following manufacturers instruction.

Quantitative gene expression and its analysis

The qRT PCR for Prolactin, Leptin and GPx1 gene was performed against housekeeping gene GAPDH which also acted as endogenous control using Applied Biosystems 7500 software v 2.0.5. For real time PCR, Quanti Fast SYBR Green PCR Kit (qiagen) was used.

The bovine specific primers were commercially synthesized (Eurofins Genomics, India). Published sequences for prolactin and leptin gene and designed sequences for GPx1 and GAPDH gene were used for primer synthesis. The specificity of primers was checked by NCBI blast program (http://www.ncbi.nlm.nih.gov/BLAST/). Following primers were used for qPCR:

Table 1: Primer pair sequences, amplicon length and accession number/reference of genes under study

Target Gene	Sequence of nucleotide (5'-3')	Fragment size (bp)	EMBL/Reference
Prolactin Forward	CGAGTCCTTATGAGCTTGATTCTT	156	Mitra (1994) [3] NM_001290885.1
Prolactin Reverse	GCCTTCCAGAAGTCGTTTGTTTTC		
Leptin Forward	GGCTCCACCCTCTCCTGAGT	123	Dhanoa *et al*. (2016) [4] NM_173928
Leptin Reverse	CCCGGAGGTTCTCCAGGTCA		
GPx1 Forward	ACGAGGAGATCCTGAATTGC	91	JQ031269.1
GPx1 Reverse	CCATTCACCTCGCACTTTTC		
GAPDH Forward	TCATTGACCTTCACTACATGGTC	109	HQ434960.1
GAPDH Reverse	GCCTTTCCATTGATGACGAG		

Relative quantification of a target gene to a reference gene was done according to Livak and Schmittgen (2001) [5] and Pfaffl (2001) [6]. Housekeeping gene i.e. GAPDH was used as reference gene for normalization of target gene for relative quantification. The ΔCq values were calculated as ΔCq = Cq target gene transcript–Cq reference gene transcript

Calculations for relative quantification (RQ) was done by using following formula

Fold increase/decrease in target = $2^{-\Delta\Delta Cq}$ (Livak and Schmittgen, 2001) [5]

Statistical analysis

The collected data were compiled, tabulated and analyzed by using SAS 9.3 software. Statistical analysis was carried using repeated measure two-way ANOVA with PROC GLM procedure.

Results

The fold increase or decrease in gene transcripts of prolactin, leptin, and GPx1 in Surti buffalo calves were calculated and are presented in table 2.

Table 2: Fold increase/decrease of gene transcripts in Surti buffalo calves

Prolactin			
	0-45 day	45-90 day	0-90 day
Group I	0.91±0.04	0.88±0.09	0.80±0.07
Group II	0.87±0.07	0.96±0.09	0.82±0.04
Group III	0.95±0.02	0.87±0.06	0.82±0.08
Leptin			
	0-45 day	45-90 day	0-90 day
Group I	1.19±0.08	1.08±0.02	1.28±0.08
Group II	1.18±0.08	1.25±0.14	1.44±0.08

Group III	1.18±0.16	1.11±0.03	1.31±0.15
GPx1			
	0-45 day	45-90 day	0-90 day
Group I	1.38±0.17	1.08±0.04	1.50±0.21
Group II	1.39±0.20	1.18±0.14	1.68±0.41
Group III	1.59±0.15	1.25±0.16	2.03±0.45

Group I (Control-no dietary supplement); Group II (diet supplemented with rumen specific yeast *Saccharomyces cerevisiae* CNCM I-1077@4x10^9 cfu/animal/day and Group III (diet supplemented with selenoyeast-inactivated yeast *Saccharomyces cerevisiae* containing selenomethionine ensuring dietary inclusion of selenium @ 0.3 ppm) (n=3)
Mean bearing different superscripts in upper case letters differ significantly between groups and in lower case letters differ significantly within groups; Mean bearing superscripts with '*' differ significantly at $P \leq 0.01$ and without '*' at $P \leq 0.05$.

Prolactin expression was not affected much however down regulation to some extent was observed in all the groups and at all stages. Between groups, down regulation during 0-45 days was maximum in group II (yeast supplemented) (0.87±0.07) and minimum in group III (selenoyeast supplemented) (0.95±0.02). For the duration of 45-90 days, maximum in group III (0.87±0.06) and minimum in group II (0.96±0.09) and during 0-90 days between groups the down regulation was maximum (0.82±0.04) in group II and III followed by control (group I) (0.80±0.07). Within group the extent of down regulation increased in group I and III and decreased in group II from 0-45 days to 45-90 days of study.

Between the groups the expression of leptin was upregulated during 45-90 days and 0-90 days of study. Maximum upregulation was in group II (1.25±0.14) and minimum in control (group I) (1.08±0.02). During 0-45 days the upregulation was almost similar in all the groups. Within groups the upregulation increased in group II and decreased in group III and group I from 0-45 days to 45-90 days of study.

The expression of GPx1 was up regulated in all the groups and at all the stages but between groups was maximum in group III i.e.1.45±0.09 for 0-45 days, 1.17±0.07 for 45-90 days and 1.74±0.20 for 0-90 days.

Discussion

The expression of prolactin was down regulated at all stages in all groups. Between groups down regulation in group II during 0-45 day (yeast supplemented) was more than in group I (control). Within group II the prolactin expression increased from 0-45 day to 45-90 day. Both pituitary (Peers *et al*., 1990) [7] and extra-pituitary sites (Soares *et al*., 2007, Ben-Jonathan *et al.*, 1996) [8, 9] have known to express prolactin gene. However in the present study the immune cells especially the lymphocytes from whole blood were more appropriate for prolactin gene expression. Prolactin acts on several tissues to regulate energy metabolism, brain function, reproduction, osmoregulation, immune response, growth, and angiogenesis (Carré and Binart, 2014) [10]. However mammary gland growth is not very significant so it was not reviewed. In the absence of any disease the variation cannot be attributed to immune responses but as the growth in terms of body weight of group II animals were better, the marginal increase in prolactin expression in group II can be viewed as correlated to increased body weight gain. Even though the study on quantitative expression of prolactin gene concerning body growth especially in bovines is lacking but evidence from study of Freemark *et al.* (2001) [11] may to some extent support the present results wherein they found that absence of prolactin receptors in knockout mice was accompanied by a small (5-12%), but progressive, reduction in body weight after 16 weeks of age. In the same study it was noticed that Females were affected to a greater degree than males.

Group III (selenoyeast supplemented) showed minimal downregulation of prolactin gene between groups during 0-45 days. Selenium supplementation has been shown to positively impact growth (Castellan *et al.*, 1999; Ganie *et al.*, 2010) [12, 13] and may have led to associated upregulation in prolactin gene expression which also is an important mediator for growth (Freemark *et al.*, 2001) [11].

Leptin expression was upregulated at all stages in all groups. Group II had higher upregulation of leptin gene between groups during 0-90 days than group I. The appropriate reason for this could be increased dry matter intake owing to yeast supplementation in group II which also had led to increased body weight gain which may have led to higher leptin expression. This effect can be visualized well by the fact that leptin gene and letpin hormone concentration is synonymous to anti-obesity (Friedman and Halaas., 1998) [14]. Leptin's role on milk yield, body growth and other parameters has been analysed in cattle (Liefers *et al.*, 2002; Buchanan *et al.*, 2002; Liefers 2004; Choudhary *et al.*, 2005) [15, 16, 17, 18].

Group III had high leptin upregulation than group I for the duration of 0-90 day. However the differences between groups were non-significant but also very minimal.

Expression of GPx1 gene was upregulated at all stages and was highest in group III followed by group II and group I. GPx and SOD act as antioxidants which prevent from oxidative damage, however in the absence of any visible stress the increase in levels may be associated with improved feeding. This may rationalize that yeast supplementation would increase dry matter intake thus leading to optimum nutritional status and subsequently higher antioxidant GPx1 expression. Bernabucci *et al.* (2005) [19] have concluded from their study that higher body condition possesses higher GPX levels and in the present study also the group II calves after yeast supplementation had higher body weights. Bermingham *et al.* (2014) [20] reviewed extensively role of selenium in various forms affecting the GPx levels in various species and concluded that GPx can act indirectly as selenium levels. So, a direct effect of selenium supplementation that too in a higher bioavailable form *i.e.* selenoyeast containing predominantly selenomethionine would increase the level of GPx expression. Yuan *et al.* (2012) [21] also showed that selenium enriched food when supplemented increases expression of glutathione peroxidase gene. In the present study the increase was not significant probably because of lesser number of samples.

Conclusion

Thus it was concluded that in female Surti buffalo calves supplementation of rumen specific yeast had more positive influence on prolactin and leptin gene expression whereas selenoyeast supplementation positively impacted glutathione peroxidase expression.

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