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# Patterns of milk productivity associated with biochemical parameters and expressions of the HSPs and Toll Like genes in dairy buffaloes

*Padrões de produtividade do leite associados a parâmetros bioquímicos e expressões dos genes HSPs e Toll Like em búfalas leiteiras*

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## Abstract

The aim of this study was to verify milk productivity associated with biochemical parameters and expression of stress marker genes (HSP70, HSP90α, HSP90β) and immunity (TLR-2 and TLR-4) in dairy buffaloes. For this purpose, 12 buffaloes with an average production of 5.93 liters/day (highest production group, n=6) and 3.46 liters/day (lowest production group, n=6) were evaluated. As a result, the groups did not differ (P>0.05) regarding the biochemical analysis of proteins (4.16% versus 4.61%, p=0.13), lipids (5.80% versus 4.87%, p=0.69), lactose (4.74% versus 4.43%, p=0.83) and cortisol quantification (0.67 versus 0.79, p=0.12). The somatic cell count (106.4 versus 139, p=0.36) was within the normal range, indicating the absence of an inflammatory process in the breasts. Relative quantification was higher in the lower yield group compared to the higher yield group (P<0.05) in all genes studied: HSP70 (0.96 versus 0.58, p=0.02), HSP90α (0.98 versus 0.03, p=0.003), HSP90β (0.95 versus 0.48, p=0.03), TLR-2 (1.02 versus 0.16, p=0.008) and TLR-4 (1.26 versus 0.07, p=0.007). Furthermore, Pearson's linear correlation test indicated a negative association between production and expression of HSP90α (r=-0.89, p=0.0001), HSP90β (r=-0.66, p=0.018), TLR-2 (r=-0.72, p=0.001) and TLR-4 (r=-0.67, p=0.01). Despite the difference in production between the groups, milk quality did not differ, nor did cortisol levels, suggesting the absence of physiological stress. However, according to the negative association identified, the higher the daily milk production, the lower the expression of stress and immunity marker genes, suggesting that stress at the cellular level impairs milk production in buffaloes.

## Resumo

Objetivou-se verificar a associação entre a produção e qualidade do leiteprodutividade leiteira em búfalas com os níveis de cortisol parâmetros bioquímicos e a expressão de genes marcadores de estresse (HSP70, HSP90α, HSP90β) e imunidade (TLR-2 e TLR-4). Para isso, foram avaliadas 12 búfalas, divididas em dois grupos com médias de produção diária de 5,93 litros/dia (grupo de maior produção, n=6) e 3,46 litros/dia (grupo de menor produção, n=6). Os grupos não apresentaram diferenças (P>0,05) na análise bioquímica de proteínas (4,16% versus 4,61%, p=0,13), lipídios (5,80% versus 4,87%, p=0,69), lactose (4,74% versus 4,43%, p=0,83) e na quantificação de cortisol (0,67 versus 0,79, p=0,12). A contagem de células somáticas (106,4 versus 139, p=0,36) estava dentro da faixa normal, indicando ausência de processo inflamatório nas glândulas mamárias. A quantificação relativa foi mais alta no grupo de menor produção em comparação ao grupo de maior produção (P<0,05) em todos os genes estudados: HSP70 (0,96 versus 0,58, p=0,02), HSP90α (0,98 versus 0,03, p=0,003), HSP90β (0,95 versus 0,48, p=0,03), TLR-2 (1,02 versus 0,16, p=0,008) e TLR-4 (1,26 versus 0,07, p=0,007). Além disso, o teste linear de Pearson indicou uma associação negativa entre a produção e a expressão de HSP90? (r=-0,89, p=0,0001), HSP90? (r=-0,66, p=0,018), TLR-2 (r=-0,72, p=0,001) e TLR-4 (r=-0,67, p=0,01). Apesar da diferença na produção entre os grupos, a qualidade do leite não diferiu, assim como os níveis de cortisol, sugerindo ausência de estresse fisiológico. No entanto, de acordo com a associação negativa identificada, quanto maior a produção diária de leite, menor a

expressão dos genes marcadores de estresse e imunidade, sugerindo que o estresse a nível celular compromete a produção de leite em búfalas.
**Pavaras-chave:** composição do leite, estresse térmico, TLR-2, TLR-4.

## 1 | Introduction

Buffaloes are the second largest source of milk production in the world, behind only cows, and contribute 11% of the world's total milk production (Zhang et al., 2022) and, in the current scenario of climate change, rising temperatures and humidity have been a constant challenge to milk productivity in buffaloes (Hassan et al., 2019), and other ruminants in tropical and subtropical countries (Mishra et al., 2011). Although buffaloes are highly adapted to their environments, they also suffer from the effects of direct solar radiation exposure (Kapila et al., 2016).

The problem lies in prolonged exposure to high temperatures, which can induce heat stress and impair milk productivity and quality (Kapila et al., 2016; Collier et al., 2019; Daltro et al., 2020), altering parameters of biochemical (Arbello et al., 2021) and cellular composition (Pegolo et al., 2021). However, there are few studies on the relationship between heat stress and milk quality in Brazil, especially in the Amazon region (Vinicio et al., 2021), considering its conducive conditions of tropical climate, high humidity, temperature, and heavy rainfall (Santos et al., 2024), ideal for the development of thermal stress in animals.

Heat stress activates various physiological responses in animals such as increased sweating, secretion of the hormone cortisol, among others (Becker et al., 2020), as well as cellular responses such as increased expression of heat shock proteins (HSPs) in buffalo tissues (Lan et al., 2022) and other animal species (Astakhova et al., 2015; Hall et al., 2016; Kapila et al., 2016). HSPs aid in the folding of newly synthesized proteins and are involved in signal transduction pathways that control cellular homeostasis, proliferation, differentiation, and cell death (Hu et al., 2022).

Another cellular response to stress is the expression of Toll-like receptors (TLR-2 and TLR-4), which are endogenous ligands of HSPs and together act to combat the deleterious effects of heat stress on the cell by activating anti-inflammatory mechanisms related to the animal's immune system (Avishek et al., 2015; Khandia et al., 2017). Given the above, the aim of this study is to assess the association of milk productivity and quality in buffaloes (Bubalus bubalis) with physiological and molecular indicators of stress

## 2 | Materials and Method

### 2.1 | Ethics and specimen collection

This research was approved by the Ethics Committee on the Use of Animals of the Federal Rural University of the Amazon (CEUA/UFRA), with process N°. 2820150222/2022. A total of 12 animals belonging to the same group of contemporaries, aged between three and six years, were collected from a dairy farm in the municipality of Bujarú, Belém (Brazil) (1°37'40''S, 48°13'21''W).

The buffaloes were in the lactation period and were divided into two groups according to the average daily milk production: HMP Group buffaloes with highest milk production of 5.93 liters (n=6); LMP Group buffaloes with lowest milk production of 3.46 liters (n=6). The average daily milk production was calculated based on the values collected between the months of August and October.

### 2.2 | Milk quality and CCS analysis

Individual milk samples were collected once from each animal in 40mL vials containing bronopol and left for 10 minutes at room temperature until bronopol was completely dissolved and then the samples were stored in a styrofoam box containing ice sheets, keeping the temperature at 6°C.

At the Food Engineering Laboratory of the Federal University of Pará, somatic cell count (SCC) and chemical composition analyses were conducted on the same day as the collection. SCC analysis was performed using flow cytometry method on Dairy Spec FT and Somacount FC (Bentley Instruments, Inc. Minnesota, USA), following the recommended standard norms (ISO 13366-2 | IDF 148-2). Meanwhile, centesimal composition was analyzed using mid-infrared absorption spectrometry method using Dairy Spec FT and Somacount FC equipment (Bentley Instruments, Inc. Minnesota, USA), following the recommended standard norms (ISO 9622 | IDF 141).

## 2.3 | Cortisol measurement

Blood samples (5mL) were collected from the caudal vein in vacuum tubes with heparin. The blood samples of the animals were collected in the afternoon and stored in a styrofoam containing ice sheets and sent to the laboratory for cortisol analysis.

For the cortisol assay, Animal Cortisol Radioimmunoassay Kits (Beijing North Institute of Biological Technology, Beijing, China) were used to determine cortisol levels according to the manufacturer's instructions. Radioactivity was determined using a gamma counter (Cobra 5005; Perkin Elmer Life and Analytical Sciences, Beijing, China). Cortisol levels for individual assays were calculated by interpolation from the standard curves using the RiaSmart software (PerkinElmer Life and Analytical Sciences).

## 2.4 | RNA extraction

Milk fat separation was performed from 600mL of milk in 1.5mL eppendorf tubes and centrifuged at 12000rpm at 4°C for eight minutes. In this step, there are two phases in the tube, at the top is the fat, which has been carefully removed with a spatula. The remaining milk was transferred to new 1.5mL Eppendorf tubes, then 600µL of Trizol was added and stored at -80°C for one hour for the next step. Total RNA was isolated from milk cells using the Trizol method, as described by Chomczynski and Sacchi (1987). RNA purity was performed using Spectrophotometer (Biodrop µLite, Biodrop, Cambridge, England) and calculated based on the absorption ratio, samples with a ratio of 1.8 to 2.0 were considered pure and suitable for RNA.

## 2.5 | Reverse Transcriptase Real-Time Polymerase Chain Reaction (RTq-PCR)

Total RNA (1µl) from milk cells was reverse transcribed into complementary DNA (cDNA) using the Revert cDNA synthesis kit (Thermo Scientific, USA) according to the manufacturer's protocol. Specific primers for GAPDH, TRL-2, TRL-4, β-ACTIN and heat shock proteins (HSP70, HSP90α, and HSP90β) were used, as shown in Table 1.

**Table 1**: Primer sequences used according to GenBank ID for gene expression analysis

| Genes | Forward and Reverse primers (5'-3') | Amplicon | GenBank |
|---|---|---|---|
| *HSP70* | F: AACATGAAGAGGCCGTGGAGG<br>R: GTTACACACCTGCTCCAGCTCC | 171 bp | JN604432.1 |
| *HSP90-α* | F: GGCGCTGATATCTCCATGAT<br>R: ACTTCTAGAGGCCTGGCACA | 74 bp | AB072368.1 |
| *HSP90-β* | F: ACAGCCGTTCTCTTGAGTCAC<br>R: TGGGCAATTTCTGCCTGGAA | 176 bp | NM_001034034.1 |
| *GAPDH* | F: AGGTCATCCCTGAGCTCAACGG<br>R: TCGCAGGAGACAACCTGGTCCTCA | 199 bp | NM_001034034.2 |
| *β-ACTIN* | F :GACATCCGCAAGGACCTCTA<br>R: GGGCAGTGATCTCTTTCTGC | 227 bp | NM_001290932. |
| *TLR-2* | F: CAGTTTAACCCAGTGCCTTC<br>R: CTCCAAAGTCTTCAGCTGCT | 241 bp | AF368419.1,<br>NM_001290891.1 |
| *TLR-4* | F:TGCCTCCACTACAGGGACTTT<br>R: TGGGACACCACGACAATAAC | 101 bp | AY634630.1,<br>NM_001290903.1 |

Bp: base pairs

The RTq-PCR reaction was performed on Applied Biosystems® 7500RT-PCR using For and Rev primers (0.5µl each) of the target genes, cDNA template (1µl), water grade PCR without nuclease

(8µl) and mixed with SYBR Green (10µl). The RT-PCR protocol used was: initial heating at 43°C for 30 min and 95°C for 5 min, and then the content was amplified for 40 cycles at the appropriate hybridization temperature. The hybridization temperature for all genes studied was 60°C.

### 2.6 | Statistical analysis

Data on production, biochemical composition, SCC and cortisol were analyzed using simple descriptive statistics and compared using Student's t-test, with a significance level of $P<0.05$. The relative quantification of all genes was performed using the 2-ΔΔCt method (Arocho et al., 2006), where ΔΔCt = (Ct of Medium Target – Ct of Medium endogenous) – CT of Medium Calibrator. The analyses of production, biochemical composition, SSC, cortisol and relative expression were correlated using Pearson's linear correlation test in the BioEstast 5.0 software (Ayeres et al., 2005).

## 3 | Results

### 3.1 | Milk production and quality parameters and cortisol dosage

When evaluating the groups of animals considered to have higher (HMP) and lower (LMP) productivity, a difference was observed in production measured in liters of milk per day ($p=0.00003$). However, the HMP and LMP groups did not differ in terms of nutritional parameters of milk, such as lipids, proteins, and lactose ($P>0.05$). Similarly, there was no difference in the quality parameter SCC ($P>0.05$) and in blood cortisol levels ($P>0.05$), as illustrated in Table 2.

**Table 2.** Milk production and quality parameters and cortisol dosage of buffaloes from the Highest (HMP) and Lowest Productivity (LMP) groups

| Parameters | HMP (Mean±DP) | LMP (Mean±DP) | P-values |
|---|---|---|---|
| Milk Production (Liters/day) | 5.93±0.1 | 3.46±0.4 | 0.00003 |
| Protein (%) | 4.16±0.07 | 4.61±0.5 | 0.13 |
| Lipids (%) | 5.80±0.4 | 4.87±0.1 | 0.69 |
| Lactose (%) | 4.74±0.05 | 4.43±0.4 | 0.83 |
| Milk somatic cell count/mL$10^3$ | 106.4±0.07 | 139±0.1 | 0.36 |
| Cortisol concentration (µg/dL) | 0.67±0.01 | 0.79±0.2 | 0.12 |

*Values expressed as mean ± standard deviation (DP) and P value (probability).

### 3.2 | Relative gene expression

The relative gene expressions were higher in the LMP group compared to the HMP group, such as the HSP70 gene (0.96±0.36 versus 0.58±0.08, $p=0.02$), HSP90α (0.98±0.54 versus 0.03±0.01, $p=0.003$), HSP90β (0.95±0.42 versus 0.48±0.13, $p=0.03$), TLR-2 (1.02±0.59 versus 0.16±0.06, $p=0.008$), and TLR-4 (1.26±0.80 versus 0.07±0.04, $p=0.007$), respectively, as shown in Figure 1.

### 3.3 | Relationship of yield and quality parameters with gene expression

A negative correlation was observed between milk production and the relative expression of the genes HSp90α, HSP90β, TLR-2, and TLR-4 ($p<0.05$), as illustrated in Table 3.

## 4 | Discussion

Discovering the impacts of heat stress on animals and milk production is the goal of several researchers, and it is a subject that has been gaining great proportion. In this context, this study advances the understanding of the relationship between heat shock proteins (HSPs) and buffalo milk production, in which it is demonstrated that the group of animals with the lowest average milk production had higher expression of heat shock proteins, which indicates that these animals were under stress conditions. Therefore, in order to minimize damage, cells activate

gene synthesis mechanisms in an attempt to maintain animal homeostasis (Mayer and Bukau, 2005).

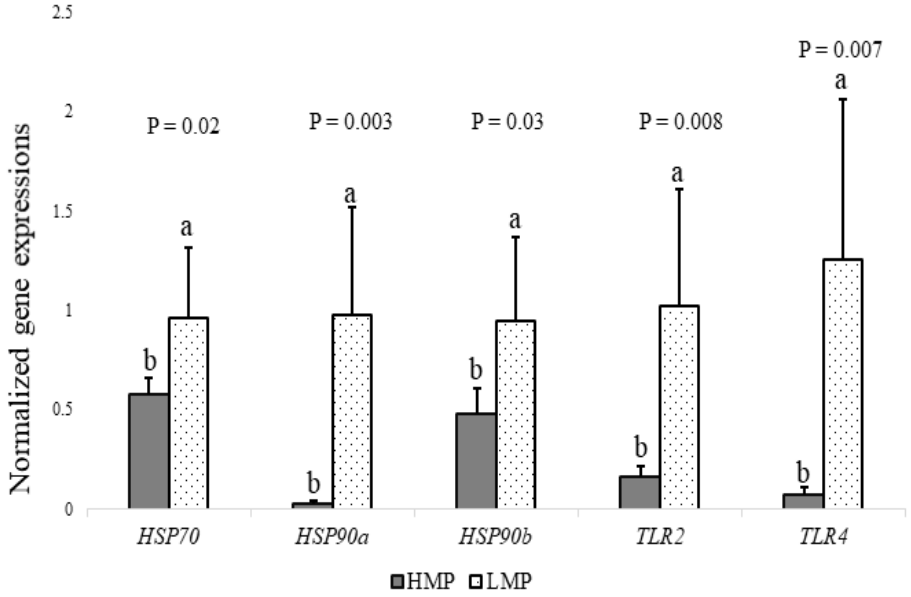


**Figure 1.** Relative gene expressions between the HMP and LMP groups. Lowercase letters indicate significant differences between groups ($p<0.05$).

**Table 3**. Pearson's correlation coefficients between the parameters of milk production, fat, protein, lactose and gene expression

| Correlation | r (Pearson) | P-values |
|---|---|---|
| Production vs. *HSP70* | -0.47 | 0.1157 |
| Production vs. *HSP90α* | -0.89 | 0.0001** |
| Production vs. *HSP90β* | -0.66 | 0.018** |
| Production vs. CCS | -0.21 | 0.49 |
| Production vs Cortisol | -0.41 | 0.17 |
| *HSP70* vs. Lipid | -0.28 | 0.36 |
| *HSP70* vs. Protein | -0.26 | 0.41 |
| *HSP70* vs. Lactose | 0.03 | 0.90 |
| *HSP70* vs. CCS | 0.09 | 0.75 |
| *HSP70* vs. Cortisol | -0.12 | 0.69 |
| Cortisol vs. CCS | 0.09 | 0.76 |
| *TLR-2* vs. Production | -0.72 | 0.001** |
| *TLR-4* vs. Production | -0.67 | 0.01** |
| *TLR-2 vs.* Lipid | -0.03 | 0.91 |
| *TLR-2* vs. Protein | 0.37 | 0.22 |
| *TLR-2* vs. Lactose | -0.47 | 0.11 |
| *TLR-4 vs.* Lipid | -0.24 | 0.43 |
| *TLR-4* vs. Protein | 0.29 | 0.35 |
| *TLR-4* vs. Lactose | -0.23 | 0.45 |

* SCC stands for Somatic Cell Count. ** Indicates significance ($p<0.05$).

Regarding milk production, both groups of buffaloes produced about 3.46 to 5.93 liters of milk/day. This difference in production between the groups (HMP and LMP) may be motivated by

environmental or genetic factors (Andrade et al., 2007). Regarding environmental aspects, several authors have reported that milk production is influenced by factors such as feeding, management and location (Castellano et al., 2019; Cavalcante et al., 2019; Safari et al., 2019). In a study with cattle, it was shown that the season with higher temperatures negatively influenced milk production and quality (Alhussien and Dang, 2018). It has also been reported that dairy cows that fed on rations containing mycotoxins decreased milk production and fat, protein and lactose contents (Wu et al., 2022). Similarly, in buffaloes, high temperature and humidity also caused heat stress and decreased productivity (Choudhary and Sirohi, 2019).

Therefore, the literature makes it clear that, like cattle, buffaloes are also susceptible to heat stress, which can even affect their immunity and consequently milk production and quality. In this way, animal production can be optimized by improving the quality of feed, as well as management conditions and the environment (Eldawy et al., 2021).

Although many studies on milk production have been developed, a few has focused on the effect of heat stress on buffalo milk production, especially in the Amazon region, where the tropical environment is conducive to triggering thermal discomfort in buffaloes, because the high environmental temperature causes a decrease in production. On the other hand, many studies on bovine milk production have been developed (Pereira et al., 2020).

Part of this scarcity may be related to the lack of regularization of milk quality standards in buffaloes in Brazil and other tropical countries. However, a study conducted with dairy buffaloes in the city of Belém, state of Pará, Brazil, analyzed the physical and chemical parameters of milk using the titration method, pH, acidity, soluble solids and proximate compositional (Rosa et al., 2015). This work opened the door for other studies to be developed, since the lipid (5.05 to 8.48%), protein (3.51 to 4.48%) and lactose (4.02 to 5.63%) contents served as a basis for the compositional analysis of buffalo milk (Rosa et al., 2015; Nasr, 2016; Cavalcante et al., 2019). Therefore, the findings of this study regarding the chemical composition of milk from both groups of higher and lower production showed similar percentages of protein, lipid and lactose ($P>0.05$), and were within the parameters described in the literature.

Although the groups exhibited different averages of daily milk production (5.93 versus 3.46), there was no difference in milk chemistry composition in both groups. Probably, because the animals are under the same environmental conditions such as ambient temperature, handling, nutrition and biological conditions such as age and stage of lactation are similar.

As previously reported, genetic factors such as polymorphic alterations may be directly related to milk production (Lima et al., 2006). In a study with dairy cows, it was observed that animals that presented cytosine (C-) deletion in the promoter of the HSP70.1 gene had lower total milk production compared to homozygous animals (CC) ($P<0.05$) (Deb et al., 2013).

Therefore, genetic variation within dairy animals at specific points for thermotolerance and milk production may allow genetic alterations to improve thermotolerance and productivity, increasing animal resilience and welfare. In addition, animal genetic selection for thermotolerance provides cumulative and permanent solutions in milk production at a relatively low cost (Carabaño, 2016; Ansari et al., 2019; Hariyono and Prihandini, 2022).

Another factor that can impair milk production is the quality of the animals' breasts (Costa et al., 2020). Therefore, milk somatic cell counts were performed to quantitatively verify the degree of infection of the mammary gland. The data acquired from both groups were within the normal range, indicating the good condition of the mammary glands and the absence of infection and mastitis in the animals (139.0 to 106.4 x $10^3$ CCS/mL). This is considered a well-established parameter that indicates udder health and milk quality (Pegolo et al., 2021). Several studies that have carried out this methodology have indicated that the amount of 200,000 SCC/mL is the maximum limit that indicates the absence of inflammatory processes due to mastitis in buffaloes, and values above this limit indicate an inflammatory response to subclinical mastitis (Tanamati et al., 2019).

In addition, another physiological parameter that can indicate animal welfare is the serum level of cortisol in the blood (Mishra et al., 2011). Cortisol is a steroid hormone synthesized from cholesterol, and secreted by the cortex of the adrenal glands; its plasma level is elevated in response to stress due to intense heat or cold, infection, diseases, among others (Yadav et al., 2013). In this context, the cortisol levels of the buffaloes' blood were measured. Nonetheless, there was no significant difference

between the two groups and did not show serum cortisol concentrations compatible with physiological stress, since cortisol ranged from 0.67 to 0.79µg/dl, being below 2µg/dl considered a reference value according to studies that measured cortisol concentration by means of radioimmunoassay and ELISA (Thinh et al., 2011; Chen et al., 2018). Again, these values may be because the animals shared the same environment, handling, and feeding.

However, several studies have found the relationship between heat stress, elevated cortisol levels and the effects on milk production. In a study with dairy cows subjected to a high-temperature environment, the animals exhibited levels of 2.9µg/dl of cortisol in the milk, compared to control cows which was 1.5µg/dl, concluding that heat stress results in higher cortisol concentrations (Chen et al., 2018). Similarly, studies with calves subjected to heat stress showed higher cortisol concentrations compared to non-exposed calves (Kovács et al., 2019). On the other hand, although studies show that heat stress increases serum cortisol levels, it is still not enough to say that this is the only factor that may be related. Other stressors can cause them to end up having higher concentrations of this hormone in their blood (Saqib et al., 2022).

In this study, the expression levels of genes related to thermotolerance (HSPs) and immunity (TLR-2 e TLR-4) were also evaluated in order to understand the mechanisms of response to heat stress and its impact on milk production and quality. As a result, we observed that the group with the lowest mean production (LMP) had the highest level of expression of all the genes studied ($P<0.05$), which are directly and indirectly related to stress responses at the cellular level.

In addition, a negative correlation, i.s., inversely proportional, was observed between the productivity parameter and the expression of HSP90α (-0.89; p=0.0001) and HSP90β (-0.66; p=0.01). These results directly demonstrate the relationship between heat stress and milk production, i.e., when animals have high levels of genes related to heat stress, production tends to be affected.

Similarly, other studies have also demonstrated the correlation between genes and milk production (Pawar et al., 2014), in their study with buffalo (Murrah) showed that milk production decreased with increasing temperature, concomitant with the increase in HSP70 expression, so they deduced a negative correlation between milk production and HSP70 (Wang et al., 2020), in order to explore the levels of circRNA expression of cows under heat stress, concluded that heat stress increased the concentration of HSP70, reduced not only the production but also the quality of the milk (Habimana et al., 2023), highlighted a large number of genomic regions and candidate genes responsible for climate adaptation, which may improve milk production and genetic adaptation in tropical countries.

In addition, heat stress also affects the expression of other genes, such as those of the immune response, activating immunity by expressing Toll-Like receptors (TLRs). Previous studies have shown that pigs exposed to consecutive periods of heat stress had a significant increase in the expression of TLR2 and TLR4 compared to non-exposed pigs ($P<0.05$), as well as plasma levels of inflammatory cytokines (Ju et al., 2014). This is due to heat stress triggering a series of cellular responses initiated by HSP proteins which activate TLR receptors, and together possibly act to combat the deleterious effects of heat stress at the cellular level (Avishek et al., 2015).

Therefore, according to our results, stress at the cellular level was detrimental to milk production in the lower production group (LMP) of buffalo. However, this impairment occurred only in the quantity of milk produced and not in the quality of the milk, since none of the composition parameters such as lipids, proteins and lactose were related to gene expressions.

On the other hand, due to the lack of studies related to HSPs and TLRs genes in somatic cells of milk in buffaloes, and also the lack of other parameters such as simultaneous measurement of THI and physiological indices (e.g., body temperature) in this study, it would be very useful for the understanding that only the production is affected and the composition is not, as well as explaining whether the cell was under heat stress or another type of stress, since HSPs are not just limited to heat stress

## 5 | Conclusion

Milk production can be influenced by cellular stress. From this point of view, the cells would be overloaded with energy expenditure to elicit the stress response and, therefore, ensure their homeostasis, on the other hand, compromising their capacity to produce milk.

The expression levels of heat stress indicator genes (HSPs) and immunity (Toll like receptors) were higher in the lower production group, influencing the productivity of these animals. However, none of the parameters analyzed influenced the milk quality of both groups.

Regarding HSP genes, we can conclude that they can be used as a marker for stress. In addition to environmental, physiological and feeding factors, another hypothesis about differentiation in yield may be related to polymorphisms in genes related to heat stress and milk quality. However, more studies are needed to understand the basic mechanisms induced by heat stress related to buffalo milk production and quality.

## 6 | Conflict of Interest Statement

The authors confirm that there are no conflicts of interest regarding the publication and/or funding of this manuscript.

## 6 | Ethics Committee

This research was approved by the Ethics Committee on the Use of Animals of the Federal Rural University of the Amazon (CEUA/UFRA), with process Nº. 2820150222/2022.

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