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Hemato-biochemical profile of meat cattle submitted to different types of pre-loading handling and transport times

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ABSTRACT

Pre-loading handling and conditions of transport are related to welfare, disease risk and product quality of production animals. These steps continue to be one of the major animal management problems in Brazil. This study evaluated the effects of different types of pre-loading handling and road transport times on the haematological and biochemical traits of cattle. Eighteen male cattle were submitted to three travel times (24, 48 and 72 h) in a truck soon after load using different types of pre-loading handling: traditional (rough handling), training (gentle handling) and use of flags to movement cattle. Haematological traits, blood biochemical measures as well as blood and faecal cortisol were analysed in order to assess animal welfare and physiological status. The traditional management showed to be more stressful, also had animals with a greater number of neutrophils and lower numbers of lymphocytes than handling with flags, showing that animals submitted to more stressful situations can have compromised immune system. Serum aspartate aminotransferase concentrations were within the reference levels and when taken together with increased creatine kinase patterns observed indicate muscle damage in traditional management. Decrease in glucose concentrations over time from traditional management to flag management was observed, while fructosamine was increased in traditional management with 72 h of travel. When taken together, all reported factors, immune, enzymatic, energetic and hormonal, indicate that the quality of pre-loading handling and time of transport were determinant for animal welfare, its homeostatic balance and sanitary conditions.

1. Introduction

Pre-loading handling, transport and the time that the cattle travel to their final destination or slaughterhouse, represent critical stages in the production chain. These are some of the main causes of stress with negative repercussions on animal welfare, including the health of the cattle [1]. The option of gentle rather than aggressive management has a direct effect on homeostasis, leading to adaptive responses mediated by physiological processes aimed at avoiding the onset of stressful processes [2]. These responses act as indicators of animal welfare and are targeted for stress analysis [3].

In countries where beef cattle is raised extensively, the animal has the liberty to make choices, but also do not have much contact with human being. This can become a problem during pre-loading handling of these individuals, who can become reactive, stressed and dangerous, injuring themselves and others and bringing hazard not only to the

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Abbreviations: ALB, albumin; AST, aspartate aminotransferase; CK, creatine kinase; Cort-FZ, faecal cortisol; Cort-SG, blood cortisol; ERT, erythrocyte; ALP, alkaline phosphatase; FLAG, flag handling; FrAm, fructosamine; GLI, glucose; Hb, haemoglobin; LEU, leukocytes; LINF, lymphocytes; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; NEU, neutrophils; PCV, packed cell volume; PLAQ, platelets; TPP, total plasma protein; TRAD, traditional management (rough handling); TRAIN, training management (gentle handling)

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handlers, but also for the whole group [4]. These animals are more susceptible to stressful situations, and it can be reflected in higher cortisol levels, decrease in feed intake, body weight gains, immune function and in agitation during simple routine husbandry procedures or even during transport [5]. Animals undergoing stressful conditions have their hypothalamic-pituitaryadrenal axis stimulated, culminating with the release of catecholamines, glucocorticoids and other hormones that may alter the blood biochemical and cellular composition, energy metabolism, and immune responsiveness [6] and therefore have a strong effect on meat characteristics [7].

Pre-loading handling, transportation and unloading are necessary and crucial steps in the production chain of cattle destined for slaughter and, therefore, the best conditions to reduce stress and promote the maintenance of good welfare of the animals must be observed, contributing to the quality of the final product. This study aimed to investigate the effects of different types of pre-loading handling and transport times on cellular and biochemical traits in the blood of beef cattle.

2. Material and methods

All animal use in this project have been approved by the appropriate ethics committee and have therefore been performed in accordance with ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. The present study was approved by the Ethics Committee on Animal Use of the University of Brasília (CEUA-UnB), case n° 152862/2014.

The experiment was carried out in Planaltina-DF, Brazil (15.67° S, 47.58° W and 1240 m altitude) on Federal District roads, during the dry season, from May to July, with temperatures between 13 and 28 °C.

Eighteen male Nellore cattle, not castrated, aged between one and one and a half years were submitted to different handling and transportation times. The animals presented an average weight at the beginning of the experiment of 229.22 ± 5.32 kg. Three different preloading treatments were applied to the animals: 1) rough handling, called traditional management (TRAD), which consisted of the use of the methods commonly practised on beef cattle farms: rod with metallic stinger, whistles and shouts. 2) The training (gentle handling) management (TRAIN) consisted of the movement and loading of the cattle only with quiet voice commands and movement of the farmhand with respect to the animal's safety zone and stimulation to the movement of the animal group leader (this method was developed and executed in all of its extension by our scientific group members). This was obtained after training all animals with different commands four hours a day for a week. 3) Flag handling (FLAG) management consisted of the movement and loading of animals using a flag attached to a staff that was agitated to the extent necessary to control and move the animals. Between the application of one type of pre-loading handling and the next, the animals remained resting on grass pasture for a week. Prior to the beginning of the studies, the animals remained in the handling corral for 72 h. Tifton hay *ad libitum* and concentrated ration twice a day were offered. Prior to transportation, all animals were deprived of water and food for one to three hours before being loaded.

Cattle were submitted to one type of pre-loading management and, subsequently, travelled 24, 48 or 72 h in a latin square scheme. All animals were submitted to all treatments. Animals were rested in the corral with *ad libitum* water, hay and mineral salt, for the same duration as the previous transport. The journeys travelled were of approximately 200 km/day. Immediately prior to loading and subsequent to unloading, blood and faeces samples were taken from all individuals. Samples were obtained from puncture of the ventral coccygeal vein using vacuum tubes (Vacutainer®) with EDTA and without anticoagulant. After collection, the tubes were packed in a thermal box for further analysis. Packed Cell Volume (PCV) – obtained by the use of a microcentrifuge and erythrocyte count (ERT), leukocytes count (LEU), platelets count (PLAQ) and haemoglobin concentration (Hb) were performed in automatic counter (ABCVet-ABX®, Montpellier, France). Mean corpuscular volume (MCV), mean corpuscular haemoglobin (HCM), and mean corpuscular haemoglobin concentration (MCHC) were determined by calculation. The differential leukocyte count was performed manually by a trained technician using an optical microscope and the total plasma protein (TPP) was measured using a refractometer.

For biochemical analyses, the serum was obtained from EDTA-free blood centrifuged at 2000 rpm for 5 min. Aspartate aminotransferase (AST), albumin (ALB), alkaline phosphatase (ALP), glucose (GLI), and creatine kinase (CK) were quantified using specific kits (LABTEST®, Lagoa Santa, MG, Brazil) by spectrophotometry in a semi-automatic biochemical analyser (Cobas C111, Roche®). Faeces were submitted to a cortisol extraction protocol as recommended by Palme and Möstl [8]. Extracted faeces and serum samples were then analysed for ELISA using commercial kits (Biochem®) and spectrophotometry (Biotek EL800 – Winooski, VT, USA).

Statistical analyses evaluated the effects of fixed factors (handling type, travel time, time of samples collection, loading and unloading) on variable factors (haematological, biochemical and cortisol parameters). The SAS program (v9.3, Cary, North Carolina, USA) was used for analysis of variance (PROC MIXED) with subsequent comparison of means by the Tukey test ($P < 0.05$) and for the data with non-parametric distribution PROC GLIMMIX used with subsequent application of the Dunn test. In addition, principal component analysis (PROC PRINCOMP) in order to investigate possibly correlation between traits measured.

3. Results

Haematological parameters measured are presented on Table 1. Higher levels of stress (TRAD and 72 h of transportation) presented higher ($P < 0.001$) PCV values (Table 1). There was a tendency for lower values of PCV with pre-loading handling with flags. The MCHC ($P < 0.05$) and Hb ($P < 0.001$) showed similar behaviour to PCV, with higher values for traditional handling at all transport times and with the trend of values decreasing for the management with flags, with higher Hb at the end of the 72 h of transport. There was a difference between load and unload (Fig. 1) for PCV ($P = 0.03$), erithrocytes ($P = 0.01$), MCHC (P = 0.02) and fructosamine ($P < 0.0001$). TRAD treatment showed higher number of NEU (4859.6) when compared ($P < 0.001$) with TRAIN (3807.3) and FLAG (2713.5). Lower lymphocytes (LINF) ($P < 0.001$) were seen in for TRAD (7580.9) when compared with FLAG (9008.7).

Biochemical parameters of cattle submitted to different types of preloading handling and transport time are on Table 2. When assessing biochemical measures, such as AST ($P < 0.05$) and CK, interactions were observed between the types of pre-loading handling and travel time (Table 2).

AST was shown for all cases within the reference values without showing a fixed pattern throughout the experiment. Particular cases such as unloading at 24 h of transport, after traditional handling (88.9 U/L) had the highest value and loading for 72 h of transport, in the flag handling (65.9 U/L) were the extreme and most highly different values. CK presented a different behaviour to AST, with higher concentrations when the animals were more stressed, such as when the animals were submitted to traditional handling at unloading after 48 h of transport (707.9 U/L) or when the animals remained longer (72 h) in the truck (TRAD – 671.6; TRAIN – 451.8 and FLAG – 705.8 U/L) (Table 2), with a tendency towards a decrease in concentrations, the highest very with traditional handling, intermediate values in training handling and lower values in flag handling (Fig. 2). All values found were higher than the reference values, which indicates that the movement of cattle in the corral or during transportation was reason enough to change the blood values of CK.

The amount of GLI present in the blood had a progressive decrease

Table 1
Haematological measures of bovine submitted to different types of pre-loading handling and transport time.

Handling	Transport (h)						Reference Values[*]
	24		48		72		
	L	UL	L	UL	L	UL	
PCV (%)							[†]24–46
TRAD	38.94^{a}	36.67^{abcd}	36.44^{abcd}	36.83^{abcd}	36.00^{abcd}	38.56^{ab}	
TRAIN	34.06^{abcd}	35.44^{abcd}	39.06^{a}	37.24^{abc}	34.94^{abcd}	39.17^{a}	
FLAG	33.61^{abcd}	35.00^{abcd}	32.94^{bcd}	36.17^{abcd}	31.18^{d}	32.33^{cd}	
CHCM (%)							[†]30–36
TRAD	31.35^{ab}	31.34^{ab}	29.62^{bc}	29.79^{bc}	30.45^{bc}	30.85^{abc}	
TRAIN	32.85^{a}	31.36^{ab}	30.36^{bc}	31.32^{ab}	30.61^{bc}	29.43^{cb}	
FLAG	30.79^{abc}	29.21^{c}	31.45^{ab}	30.73^{abc}	30.11^{bc}	29.64^{bc}	
Hb (g/100 mL)							[†]8–15
TRAD	10.42^{abc}	10.49^{abc}	9.81^{abc}	9.80^{abc}	9.81^{abc}	10.64^{abc}	
TRAIN	10.08^{abc}	10.34^{abc}	10.81^{ab}	10.34^{abc}	9.97^{abc}	10.90^{a}	
FLAG	9.67^{abc}	9.56^{abc}	9.54^{abc}	9.96^{abc}	8.98^{c}	9.22^{cb}	
ERT ($\times 10^6/\mu L$)							[†]5–10
TRAD	9.03^{abc}	9.23^{abc}	8.84^{abc}	9.14^{abc}	8.86^{abc}	9.23^{abc}	
TRAIN	8.42^{bc}	8.94^{abc}	9.48^{ab}	8.98^{abc}	8.86^{abc}	9.86^{a}	
FLAG	8.42^{bc}	8.77^{abc}	8.51^{abc}	8.92^{abc}	7.97^{c}	8.28^{bc}	
PLAQ ($\times 10^3/mm^3$)							[†]100–800
TRAD	705.8^{a}	697.1^{a}	534.8^{ab}	521.8^{ab}	349.4^{b}	607.2^{ab}	
TRAIN	491.9^{ab}	519.6^{ab}	501.1^{ab}	427.4^{b}	416.2^{b}	460.4^{ab}	
FLAG	380.0^{b}	397.6^{b}	375.2^{b}	445.0^{ab}	411.5^{b}	413.0^{b}	
NEU ($/\mu L$)							600–4.000
TRAD	5234.4^{ab}	6404.2^{a}	5525.5^{ab}	3553.2^{abc}	4255.9^{abc}	4184.7^{abc}	
TRAIN	3054.0^{abc}	5502.4^{ab}	3987.1^{abc}	4084.7^{abc}	3208.2^{abc}	3007.4^{abc}	
FLAG	2814.8^{abc}	3302.4^{abc}	3460.0^{abc}	2440.2^{abc}	1946.6^{c}	2317.0^{bc}	
LEU ($\times 10^3/\mu L$)							4–12
TRAD	13.60^{ab}	15.53^{a}	13.49^{ab}	12.13^{b}	12.87^{ab}	12.78^{ab}	
TRAIN	10.87^{b}	11.13^{b}	12.81^{ab}	13.24^{ab}	12.17^{ab}	11.86^{b}	
FLAG	12.86^{ab}	12.80^{ab}	12.75^{ab}	12.74^{ab}	11.72^{b}	12.46^{ab}	

L-Loading; UL-Unloading; TRAD-traditional handling; TRAIN-training handling; FLAG-flag handling; PCV-packed cell volume; MCHC-mean corpuscular haemoglobin concentration; Hb-haemoglobin; ERT-erythrocyte count; VCM-mean corpuscular volume; PLAQ-platelets; NEU-neutrophils; LEU-leukocytes; Means by haematological parameter followed by different letters (a–d) are significantly different using Tukey test ($P < 0.05$).

[*] Benchmark values according to Kaneko et al. [9].

[†] Benchmark values according to Jones and Allison [10].

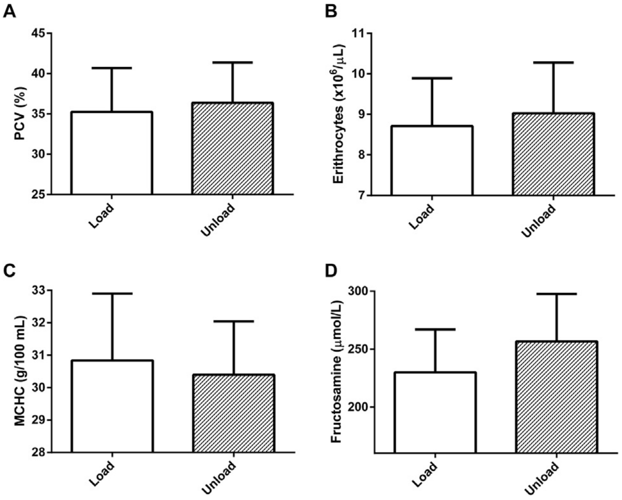

Fig. 1. Comparison between loading (Load) and unloading (Unload) for PCV (A), Erythrocytes (B), MCHC (C) and Fructosamine (D). PCV – Packet cell volume; MCHC – Mean corpuscular haemoglobin concentration.

when compared with the most stressful (TRAD) handling in the period of 24 h of transportation, through training, until reaching the considered less stressful (with flags) in the period of 72 h of transport (Table 3). Also, in all periods of travel with TRAD (rough handling) and TRAIN (gentle handling) the GLI remained above the reference levels. The evaluation of fructosamine (FrAm) ($P < 0.001$) showed just one change, an increase above the reference limits (287.7 µmol/L), with traditional handling and 72 h of travel, however, not corresponding to high levels of albumin at the same time. There was an interaction between management and travel time in the analysed cortisol measurements, both serum ($P < 0.001$) and faecal ($P < 0.05$), with pre-loading handling with flag associated with 48 and 72 h of transport causing greater increase of faecal and serum cortisol (Table 3).

4. Discussion

When the cattle were submitted to higher levels of stress (TRAD), the PCV was higher, seen when we observe the effect of traditional management (rough handling) and the longer stay in the truck (72 h of transportation). The observed increase in the mean values of PCV and Hb may be clue of dehydration of the animals, being more evident in the animals with traditional handling treatment compared with the animals where the flags were used. This is indicative of the importance of pre-loading handling independent of transport time, demonstrating that previous stressful handling may adversely affect animal physiological condition and attempts to maintain homeostasis during transport. When PCV and Hb values were compared during loading and unloading, higher values were seen at the arrival of the animals, probably because of water imbalance resulting from a pre-loading stress allied to the travel times. Similar results of changes were found by Bernardini

Table 2
Biochemical parameters of cattle submitted to different types of pre-loading handling and transport time.

Handling	Transport (h)						Reference Values[*]
	24		48		72		
	L	UL	L	UL	L	UL	
AST (U/L)							78–132
TRAD	76.8^{a,b}	88.9^{a}	79.81^{a,b,c}	72.6^{a,b}	81.0^{a,b}	80.5^{a,b}	
TRAIN	71.5^{a,b}	85.8^{a,b}	88.3^{a}	81.4^{a,b}	81.5^{a,b}	75.4^{a,b}	
FLAG	74.9^{a,b}	74.6^{a,b}	68.4^{a,b}	70.8^{a,b}	65.9^{b}	73.7^{a,b}	
CK (U/L)							< 94
TRAD	427.2^{a,b,c}	707.9^{a}	551.8^{a,b,c}	336.8^{a,b,c}	238.5^{a,b,c}	671.6^{a,b,c}	
TRAIN	434.9^{a,b,c}	257.7^{a,b,c}	384.7^{a,b,c}	311.9^{a,b,c}	189.6^{a,b,c}	451.8^{a,b,c}	
FLAG	285.0^{a,b,c}	132.2^{b,c}	164.94^{a,b,c}	108.2^{c}	132.1^{b,c}	705.8^{a,b}	
ALP (U/L)							0–196
TRAD	81.2^{ef}	73.9^{f}	95.2^{def}	78.6^{ef}	106.8^{bcdef}	77.2^{f}	
TRAIN	146.9^{ab}	110.9^{bcdef}	89.2^{ef}	94.7^{def}	78.3^{ef}	101.4^{cdef}	
FLAG	150.7^{a}	140.8^{abc}	118.3^{abcde}	133.7^{abcd}	157.9^{a}	105.8^{bcdef}	

L-Loading; UL-Unloading; TRAD-traditional handling; TRAIN-training handling; FLAG-flag handling; ALP-alkaline phosphatase; AST-aspartate aminotransferase; CK-creatine kinase; Means by biochemical parameter followed by different letters (a–f) are significantly different using Tukey test ($P < 0.05$).

[*] According to Kaneko et al. [9].

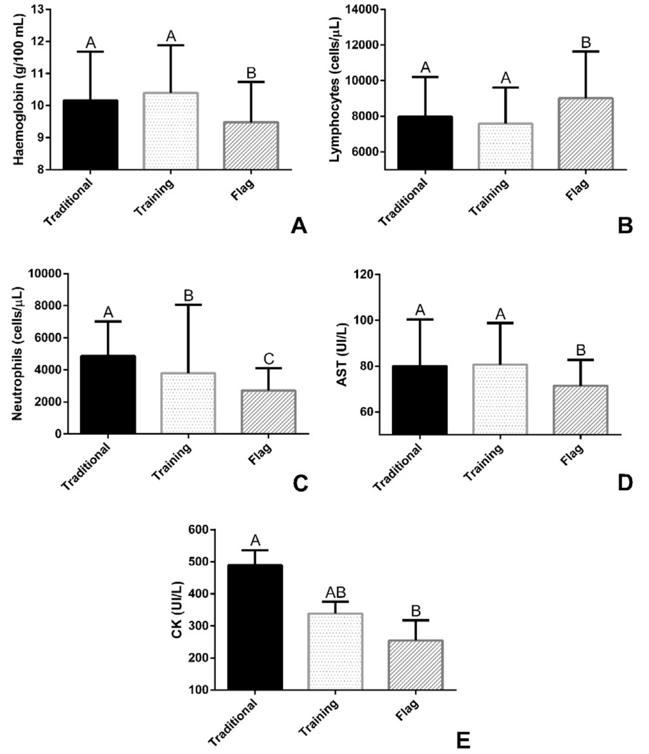

Fig. 2. Haematological traits from cattle loaded using traditional, training or flag methods. CK – creatine kinase; AST – aspartate aminotransferase.

et al. [11] after a 19-h travel period, in which transportation led to increased blood measures related to dehydration.

Dehydration in animals transported in the final stage of production is a factor of extreme relevance, having a direct influence on the weight of these individuals and in the final quality of the carcass at slaughter. Studies indicate that the loss of water during transportation cannot be recovered during the rest period commonly allowed before slaughter, as this recovery can take from 10 to 36 days, when the total loss is above 6% of the live weight [12]. In fact, when the animals, submitted to a long journey, are exposed to suffering because they stay for long periods without food and water, and are confined to small spaces in the truck, this can cause exhaustion [13], besides being a moment of intense psychological stress permeated by fear that animals may feel during handling procedures for loading and transportation [14]. Thus, official regulations that oblige the industry to create stopping stations on routes of more than 24 h are necessary and these should allow the animals to rest, be fed and have water available *ad libitum*.

Values for ERT and for PLAQ, neutrophils (NEU) and LEU followed the same trend as for PCV, MCHC and Hb with increase in the amount of ERT, PLAQ, NEU and LEU in the traditional management, perhaps, due to the amount of stress generated and the greater duration of transport, with a tendency to decrease haemoconcentration with the less stressful training and flagging treatments. Earley et al. [15], in their work describe the effects of transport on characterizing haematological parameters due to dehydration, and found that both the haemoglobin concentration and erythrocyte count were within the reference limits, coinciding with similar data found in this study, having increased in some cases, but keeping within the reference values.

PLAQ at the beginning of the experiment increased with traditional pre-loading handling. In general, the increase in PLAQ in cattle is associated with situations of exercise, stress or inflammatory conditions, and is part of the physiological response after the release of adrenaline [10], which is produced by rough (traditional) handling in the physical, behavioural and psychological condition of the animals.

The evaluation of white blood cells has been used in the detection of inflammation, toxins presence and emotional changes [16]. The data showed intense leucocytosis, characterized by neutrophilia caused possibly by the stress presented at the moment of the pre-loading handling and the unloading with traditional handling with 24 h of transport. High values were also observed after 72 h of transportation at the loading and unloading with traditional management, above the reference values, data that coincides with findings observed by Earley et al. [15] in cattle subjected to 18 h of road transport, even though cattle are described as having lower immune responses to stress [17]. The high average values of neutrophils found at particular moments indicate which type of management and road transport are determining factors stressing the animals.

Excessive release of neutrophils occurs through the action of endogenous or exogenous glucocorticoids, causing mobilization of the marginal neutrophils of the microvasculature, as well as the induction of increased reserve release of these cells from the bone marrow [17]. When the cattle were transported for 72 h and considering that the neutrophil half-life is short, around 9 h [17], the lower values observed in 72-h journeys may be caused by the short time the animals had to recover from previous transport periods. These were not enough to induce a new episode of neutrophilia, concomitant with a possible

Table 3
Blood values of fructosamine, glucose, serum cortisol and faecal cortisol of cattle submitted to different types of pre-loading handling and transport time.

Handling	Transport (h)						Reference Values[*]
	24		48		72		
	L	UL	L	UL	L	UL	
Alb							27–28
TRAD	25.9^{bc}	26.6^{bc}	25.4^{c}	27.3^{bc}	30.4^{ab}	28.4^{abc}	
TRAIN	26.7^{bc}	27.5^{bc}	26.9^{bc}	32.9^{a}	28.2^{abc}	29.9^{abc}	
FLAG	28.5^{abc}	30.6^{ab}	28.8^{abc}	29.8^{abc}	29.0^{abc}	26.0^{bc}	
FrAM (μmol/L)							213.4–265
TRAD	$204.4^{c,d}$	$230.6^{b,c,d}$	198.94^{d}	$247.7^{a,b}$	$245.1^{a,b,c}$	287.7^{a}	
TRAIN	$239.9^{b,c,d}$	$231.9^{b,c,d}$	$258.40^{a,b}$	$264.1^{a,b}$	$244.2^{a,b,c}$	$270.5^{a,b}$	
FLAG	$230.9^{a,b}$	$252.0^{a,b}$	$235.6^{b,c,d}$	$255.85^{a,b}$	$238.4^{b,c,d}$	$255.9^{a,b}$	
Glucose (mg/dL)							45–75
TRAD	105.1^{a}	105.9^{a}	83.8^{b}	81.0^{b}	$75.1^{b,c}$	86.6^{b}	
TRAIN	86.6^{b}	84.7^{b}	83.3^{b}	$74.4^{b,c}$	$75.0^{b,c}$	$74.4^{b,c}$	
FLAG	$60.3^{c,d}$	$70.3^{b,c,d}$	$69.6^{b,c,d}$	55.9^{d}	55.7^{d}	$61.4^{c,d}$	
Cort-SG (μg/dL)							–
TRAD	$4.02^{e,f,g}$	$4.43^{b,c,d,e,f}$	$4.43^{b,c,d,e,f}$	$4.44^{b,c,d,e,f}$	$4.38^{c,d,e,f,g}$	$4.00^{f,g}$	
TRAIN	3.88^{g}	$4.82^{a,b,c}$	$4.31^{d,e,f,g}$	$4.23^{d,e,f,g}$	$4.50^{b,c,d,e}$	$4.42^{b,c,d,e,f}$	
FLAG	$4.24^{d,e,f,g}$	$4.27^{d,e,f,g}$	$4.59^{a,b,c,d}$	5.09^{a}	5.08^{a}	4.87^{ab}	
Cort-FZ (μg/dL)							–
TRAD	3.57^{i}	2.78^{j}	2.69^{j}	$4.36^{d,e}$	3.83^{h}	$3.89^{g,h}$	
TRAIN	$3.67^{h,i}$	$4.08^{g,f}$	$4.14^{e,f}$	$4.07^{g,f}$	$4.36^{d,e}$	4.78^{c}	
FLAG	$4.18^{e,f}$	4.74^{c}	4.46^{d}	5.60^{a}	5.34^{b}	4.85^{c}	

L-Loading; UL-Unloading; TRAD-traditional handling; TRAIN-training handling; FLAG-flag handling; Alb-Albumin; FrAM-Fructosamine; Cort-SG-Serum cortisol; Cort-FZ-Faecal cortisol; Means by biochemical parameter followed by different letters (a–h) are significantly different using Tukey test ($P < 0.05$).

[*] According to Kaneko et al. [9].

depletion of bone marrow reserves as the production time of the neutrophils is 4 to 6 days in cattle [17].

Excessive release of neutrophils at time of stress and their subsequent depletion may weaken the individual's immune defence system [18], leaving them susceptible to infections and inflammatory processes. However, studies differ concerning the changes that occur, and lymphopenia with neutropenia has been reported [19], as well as neutrophilia accompanied by lymphopenia [2], caused by the excessive release of adrenaline [10] as well as by emotional changes or excess muscular effort [17] at the time of loading and during transportation.

Transport as a stressful condition can lead to many physiological changes in cattle with drastic homeostatic alterations, increasing the number of white cells and haemoglobin and decreasing the number of lymphocytes and their functions in cattle of different ages at unloading [20]. On the other hand, neutrophils can be found in excess in the inflammatory processes during tissue damage concomitantly with leucocytosis due to a decrease in the number of lymphocytes and also by neutrophilia indicating an interruption of the neutrophilic balance in response to stress in cattle [21]. In general, transport alters the cellular and humoral immune response because it is a highly stressful situation for cattle [22]. In this experiment, the traditional handling being more stressful was also determinant in the higher number of NEU when compared with the result for trained animals and those where the flag handling was used, and on the contrary, lower LINF were seen in more stressful situations such as traditional handling when compared with flag handling, that is, more stressed animals were more susceptible to illness due to the process of cellular immunosuppression.

On the other hand, cortisol reduces mobilization of circulating leukocytes by inhibition of production, and binding of coupling molecules to cellular receptors decreases the phagocytic and bactericidal activity of neutrophils, although increasing the fraction of these circulating cells by stimulating their release from the bone marrow. Cortisol decreases the number of circulating lymphocytes, particularly T-helper cells involved in response to foreign substances, and also decreases their function, so all cell-mediated immunity is depressed. The mechanism of depression of this response is complex but includes a reduction in the production of intercellular mediators that activate the immune system and block the progression in the cell cycle of the cells involved [22]. Increases in the total number of white cells such as neutrophils and basophils, cell volume, haemoglobin and decreased lymphocytes, eosinophils and monocytes are commonly observed in cattle of different ages after transport. The increase in neutrophils has been observed in tissue damage processes [22] as possibly due to pre-loading stress in the more aggressive type of handling and for longer periods of transport. A marked neutrophilic leucocytosis may occur simultaneously with a decrease in the number of lymphocytes. This may indicate disruption of neutrophilic balance in response to stress in cattle [22]. The relation between the increase of neutrophils and the tissue damage can be evidenced by the high concentration of creatine kinase due to the damage of the muscular tissue occurred by the pre-loading stress and, in other cases, by the duration of transport, as observed below.

Variations in blood biochemical parameters may indicate that animals are undergoing stressful conditions and this situation may become pathological. Traditional pre-loading handling and submission to travel is an example of this, which can lead individuals to extreme situations with loss of physiological balance and, consequently, changes in their welfare.

In a study carried out with Holstein-Friesian cows with 19-h road transport from Poland to Italy, the highest values of aspartate aminotransferase were found at the end of the journey [11]. Uetake et al. [23], in an experiment with crossbred cows, found similar results proving that intense exercise can increase AST. In this experiment, AST remained within the reference standards, however, the highest concentrations of the enzyme were found after the animals underwent stressful situations, either traditional handling or transport (72 h) (Table 2). In most cases, ALP was shown to decrease after transport and the higher CK levels observed in traditional handling may indicate the occurrence of muscle damage, which has already been described in previous transport studies and can therefore be a good indicator of the degree of welfare during the whole management procedure and subsequent transportation [3].

Regarding travel times, there were no differences in the concentrations of CK found for the different times of transport, which may be indicative of muscle damage in all cases. Van de Water et al. [13] found an increase in the CK enzyme after transport with an average duration of two and a half hours, and Bernardini et al. [11] in a 19 h transport study, indicating that even short travel times can cause tissue injuries and decrease in the welfare conditions of individuals due to transport fatigue. Soares [24] and Gruber et al. [25] found that the highest levels of CK were obtained from animals that suffered muscular damage during the pre-slaughter and transportation procedures. AST is a mitochondrial and cytosolic enzyme, therefore, in order to find higher concentrations in the blood, the muscular damage should be much more severe when compared to the release of CK, a small cytosolic enzyme that passes easily through the cell membrane with the lesion in the muscular fibres, with maximum values between 24 and 36 h after the lesion [26].

The association between stress and glycaemic changes has been proven [1], since the stressful situation requires greater energy input [27]. It was clear in the present study that there is a glucose requirement during the loading and transportation periods, and the first moment of travel, 24 h of transportation with traditional pre-loading handling, was the moment of greatest stress of the animals, with highest mean glycaemic value. Less stressful pre-loading allows the maintenance of energy reserves with baseline blood glucose levels. It was also observed that the increasing transport time was definitive for the decrease of blood glucose concentrations, showing that the animals undergoing longer transport periods were very close to exhaustion. However, similarly, some studies show changes in GLI values after several periods of transport [1,3,11,27,28], as well as the periods of rest [29]. It seems that, in the present study, the handling with flag associated with all travel times, was the least stressful considering the glycaemic index, with lower concentration of blood glucose. These variations are of extreme importance to the meat industry, since glycogen, a precursor of glucose, is essential at the time of muscle transformation into meat in the post-mortem process. The requirement of muscle glucose during transport with the use of this glycogen can directly affect the quality of the meat, which is directly dependent on the transport time of the animals and the handling that preceded it [25,30].

FrAm, as a stable ketamine derived from a non-enzymatic sugar reaction with amine groups of proteins, especially ALB, is directly related to serum glucose and serum albumin concentration. Thus, FrAm levels increase in cases of hyperglycaemia or hyperalbuminemia, and may also reduce in the inverse conditions [9,31]. In this study, when higher values of FrAm were found, they correspond to hyperglycaemia and not hyperalbuminemia.

Interestingly, FLAG treatment associated to 48 and 72 h of transport caused greater increase of faecal and serum cortisol. This is completely unexpected as we considered FLAG as the pre-loading handling less stressful and then, cortisol concentrations should be lower. In most determinations, the values of faecal or serum cortisol were increased after transport when compared with values at the time of loading. In the same way, for almost all moments, the trend of the values found in the faecal cortisol (Cort-FZ) was to accompany the values found in the blood cortisol (Cort-SG).

After the release of cortisol into the bloodstream and after producing its effects in different organs of the animal, it is transformed into metabolites that will be excreted either in the faeces or urine. According to Palme et al. [32] and Möstl and Palme [33], the maximum concentration of cortisol metabolites found in excreta varies according to the time of intestinal passage, according to each animal species and depending on the physiology of each individual, with peaks in ruminants about 12 h after the event causing excessive release of cortisol.

The higher values of cortisol on unloading demonstrate that travel is stressful with events highly impacting on homeostasis and animal welfare, data that coincide with those already presented by other authors [2,3]. Schwartzkopf-Genswein et al. [34] who found that animals not conditioned to handling and transport time had higher concentrations of cortisol compared with previously conditioned animals.

The highest values of Cort-FZ or Cort-SG blood or faecal cortisol were present with the lowest concentrations of GLI and high values of FrAM. This may be related to the longer transportation time and not to the type of pre-loading handling, when flags and 48 or 72 h of transport were used.

5. Conclusions

Homeostatic changes due to pre-loading handling and transport times were observed, with effects on immune defence factors, leaving animals more susceptible to stressful challenges. Enzymatic alterations indicative of possible muscular damage were seen due to the stress prior to loading and the time in which the animals were transported. The sum of these factors (immune, enzymatic and energetic) indicate that the quality of pre-loading handling and the time of transport are determinant for the welfare of the animals. This, together with the energy imbalance, endangers the quality of the final product at the slaughterhouse. Thus, gently pre-loading procedures as FLAG treatment are recommended aiming to reduce stress and improve animal welfare.

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Competing interest

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The manuscript has been read and approved by all authors and there are no other persons who satisfied the criteria for authorship but are not listed. Moreover, there are no impediments to publication, including intellectual property and ethical aspects involving experimental animals.

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