Performance and serum parameters of calves (*Bos taurus*) subject to milk restriction associated with supplementation with 2-hydroxy-4-methylthiobutanoic acid

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Abstract: Our aim, with this study was to evaluate the consumption, performance, quantitative
characteristics of carcasses, biochemical profile, plasma levels of ghrelin and leptin, expression of
the receptor for ghrelin (GHS-R1a) in the hypothalamus and duodenum, and the number of goblet
cells in the duodenum of calves subjected to milk volume restriction and supplemented with 2-
hydroxy-4-(methylthio)butanoate acid (HMTBa). We used 21 Holstein-mixed breed calves, aged
between 3 and 15 days with an average weight of 36.8 kg, and housed in pens with troughs for hay,
concentrate, and water. The study included two consecutive experimental periods (P1 and P2) of 21
days each, with seven days of adaptation to the diet and facilities. The calves were distributed in a
completely randomized design in three treatments with seven repetitions. 1 - Control: 6 L of
milk/day during P1 and 6 L of milk/day during P2; 2 – RES (milk restriction): 3 L of milk/day during P1
and 6 L of milk/day during P2; 3 - RES + HMTBa: 3 L of milk/day during P1 and 6 L of milk/day during
P2 + 3.3 g of HMTBa/day in both periods. HMTBa was supplied in milk, and the amount of
concentrated ration and hay provided and leftovers were recorded daily to estimate dry matter
(DM) and crude protein (CP) consumption. Mean daily weight gain (DWG), final weight (FW), and
feed conversion (FC) were obtained at the beginning and at the end of each 21-day period. Plasma
concentrations of ghrelin and leptin, triglycerides, total protein, urea, lactate, creatinine, alkaline
phosphatase, and cholesterol were measured for P1 and P2 at the end of each 21-day period. At the
end of P2 animals were slaughtered; sections of the duodenum were collected to evaluate the
expression of GHS-R1a and quantity of goblet cells; hypothalamus was used to evaluate the
expression of GHS-R1a; rumen was used to evaluate the thickness of epithelium, keratin, density,
and height and width of ruminal papillae. In P1, total DM consumption, FW, DWG, glucose and
triglycerides were lower in the RES and RES+HMTBa groups (P < 0.001). In P2, there was an
improvement in the FC of the RES+HMTBa group (compared to Control and RES) and a lower urea
concentration in the RES group (compared to Control and RES+HMTBa) (P < 0.001). No differences
were observed among groups regarding hormonal concentrations, histological parameters, and GHS-
R1a expression in the duodenum and hypothalamus. Therefore, milk restriction combined with
HMTBa supplementation promoted greater compensatory gain by a mechanism independent of changes in GHS-R1a expression and hormone levels of ghrelin and leptin.

Keywords: performance, compensatory gain, intestine, rumen
| Abbreviation | Definition |
|--------------|------------|
| CP           | crude protein |
| DM           | dry matter |
| HMTBa        | 2-hydroxy-4-(methylthio) butanoate |
| P1           | first period |
| P2           | second period |
| RES          | milk restriction |
| DWG          | daily weight gain |
| FW           | final weight |
| FC           | feed conversion |
| CNS          | central nervous system |
| GHS          | growth hormone secretagogue |
| NDF          | neutral detergent fiber |
| ADF          | acid detergent fiber |
| AOAC         | Association of Official Analytical Chemists |
| EDTA         | ethylenediaminetetraacetic acid |
| ELISA        | enzyme linked immuno sorbent assay |
| HE           | hematoxylin-eosin |
| Acronym | Description                        |
|---------|------------------------------------|
| PAS     | periodic acid Schiff               |
| BSA     | bovine serum albumin               |
| PBS     | phosphate-buffered saline          |
| HRP     | horseradish peroxidase             |
| SDS     | sodium dodecyl sulphate            |
Introduction

To improve calves’ weight gain, several approaches are used such as food supplementation and restriction of milk supply during calf development. Methionine supplementation is an alternative that may have additive effects on protein metabolism, resulting in better growth rates (Silva et al., 2018). However, since the DL-methionine is degraded in rumen, the alternative is to supplement with HMTBa, which is converted to methionine in various tissues (Dibner and Knight, 1984). Moreover, HMTBa has an advantage of being more resistant to ruminal degradation compared to DL-methionine (Lapierre et al., 2011). In dairy cattle, HMTBa is used to increase production and levels of protein and fat in milk (Baldin et al., 2018). Indeed, dietary supplementation of essential amino acids aims to complete the dietary amino acid profile and to compensate potential amino acid imbalances (Rychen et al., 2018). Similarly, milk restriction during calf development is an approach that has proven effective to stimulate compensatory weight gain (Alves, 2003; Alves Costa et al., 2019). The compensatory weight gain (WG) results in economic benefits, since the milk saved during the calves feeding period, can be used for other economic destinations.

One hypothesis for the compensatory gain mechanism in animals, as also in humans, is the hormonal action, like those involved in the hunger mechanism. In this scenario, gastric release of ghrelin is involved with both food intake (Wertz-Luz et al., 2006) and secretion of growth hormone (GH) (Thidar Myint et al., 2006). Ghrelin, in cattle, is a peptide composed of 27 amino acid residues. Its biological activity occurs through acylation of the third amino acid residue (serine) by addition of an octanoic site (Kojima and Kangawa, 2005). The ghrelin receptor (GHS-R) is a G-protein-coupled receptor and presents two isoforms: GHS-R1a (functional isoform for ghrelin) and GHS-R1b. Studies demonstrated the peripheral expression of GHS-R1a in several organs such as stomach and intestine, (Date et al., 2000; Guan et al., 1997). In the central nervous system, GHS-R1a expression was found in the hypothalamus and anterior pituitary, corroborating its role in GH
release. In addition, GHS-R1a was found in the hypothalamus’ arched nucleus, thus elucidating its importance in controlling appetite (Muccioli, et al., 1998; Katayama, et al., 2000). Although Alves Costa et al. (2019) reported no statistical differences for GHS-R1a expression in hypothalamic tissues of calves submitted to milk restriction, the expression of the receptor in hypothalamus is of interest due to the fact that GHS-R1a expression is differentially regulated in different regions of the brain during aging (Sun et al., 2007). Thus the data obtained could help future researches as a parameter. Other tissues have GHS-R1a receptors, such as pancreas, kidneys, and circulatory system. However, this study did not focus on those sites because they, apparently, have no influence on the digestive tract development and on animal performance.

Leptin, is another hormone involved in energy consumption and metabolism regulation, whose actions are antagonistic to ghrelin. Leptin is a 16-kD peptide (Zhang et al., 1994), whose receptors are expressed in the CNS, activating the center of satiety in hypothalamus (Dias-Salman et al., 2007). According to Hayashi et al. (2020) leptin, secreted by the abomasum, acts on the hypothalamus and causes short-term appetite inhibition. Also the presence of leptin in the gastric juice increases the peptide transporter 1 (PepT-1) and inhibits glucose transportation by SGLT-1 in the small intestine (Ducroc et al., 2005). Specifically in suckling calves, it is suggested that leptin produced in the abomasum acts via the endocrine and exocrine systems (Hayashi et al., 2020). Altogether, it is evident that the regulation of ghrelin, leptin and GH levels is relevant with regard to animal production, because of its impact on weight gain and body size growth. Therefore, it is important to investigate how HMTBa supplementation and milk restriction influences hormonal profile, especially ghrelin and leptin.

In addition to hormonal profile, integrity of intestinal mucosa is an important factor for verifying livestock performance. Goblet cells of the mucosa produce mucus whose functions include lubrication, digestion, absorption, hosting of intestinal microflora, and protection from toxins and pathogens (Nag and Prasad, 2016).
Moreover, small intestine mucosa has villi and microvilli, which are specialized structures to ameliorate nutrient absorption (Bühler et al., 1998). At the base of the villi are the crypts, which are responsible for the constant cell renewal in the villus. According to Naburrs (1995), it is desirable that the mucosa has high villi and shallow crypts. Interestingly, the food type can influence intestinal morphology. Other important absorption site is the rumen. Though, at birth, calves do not have the physiological and anatomical characteristics that characterize them as ruminants and milk is their main nutrient source (Jasper and Weary, 2002). The transition to the condition of functional ruminant occurs around eight weeks age and depends on consumption of solid food, which leads to short chain fatty acids (SCFA), specially butyric and propionic (Baldwin et al., 2004). The mean length of the calves’ ruminal papillae is about 1.0 mm however; it can reach 5 to 7.0 mm at eighth week (Beharka et al., 1998). The papillae increases absorption surface and are responsible for absorption of most of the SCFA (Harrison et al., 1960). Thus, alternatives to fasten papillae development would improve calves’ growth. Therefore, the supplement with HMTb and milk restriction can influence the intestinal and ruminal mucosa, improving the nutrient absorption. However, little is known about the mechanisms of action behind both approaches. In this way, this study aimed to evaluate the effect of milk restriction, with or without supplementation with 2-hydroxy-4-methylthiobutanoic acid (HMTBa) on food intake, plasma levels of total ghrelin, leptin, GHS-R1a expression in the hypothalamus and duodenum, and also histology of rumen and duodenum of calves.

**Material and Methods**

All procedures and protocols used in this experiment were approved by the Animal Ethics and Experimentation Committee of the Federal University of Goias, under protocol number 028/15.
Animals and Feeding

The study was conducted in the Experimental Shed at the School of Veterinary Medicine and Animal Science of the Federal University of Goias (UFG), Campus Samambaia, between February and March 2015. We used 21 mixed Holstein X Jersey calves, aged between 3 and 15 days and an average weight of 36.8 kg, identified and housed in individual bays, provided with milk and water buckets and troughs for hay and concentrate (Table 1). Hay and concentrate were provided ad libitum for all treatments. The animals were donated by dairy farmers from different properties in the region within 50 km of Goiânia. The animals had an adaptation period of 7 days to both the diet and the facilities. Animals were distributed in a completely randomized design with three treatments and seven repetitions. Treatments consisted of 1- Control: 6 L of milk/day in the first period (P1) and 6 L of milk/day in the second period (P2); 2- RES (milk restriction): 3 L in P1 and 6 L of milk/day in P2; 3- RES + HMTBa: 3 L in P1 and 6 L of milk/day in P2 with 3.3 g of HMTBa/day (Mintrex™Zn feed supplement with 83% HMTBa and 2.4% Zinc; Novus International Inc., St. Charles, MO). Milk was provided over 42 days in buckets and divided into the first and second period of 21 days each. The daily amount of milk and HMTBa supplement treatment were divided into two portions at 8 AM and 4 PM. In the first period, the control group received two meals of 3 L of milk, while the RES and RES+HMTBa groups received two meals of 1.5 L each. In the second period, the animals of all groups received two meals of 3 L each. The nutritional composition of milk supplied to calves as a percentage of dry matter (% DM) was as follows: 13.24 dry matter; 3.67 crude protein; 4.69 lactose and 4.31 fat. Mintrex™Zn (Novus International Inc., St. Charles, MO) is a compound that has in its chemical structure calcium ionically bound to two molecules of HMTBa calcium bis(2-hydroxy-4-(methylthio)) butyrate and Zn chelated to two molecules of 2-hydroxy-4-(methylthio) butanoic acid (chelated Zn). Mintrex™Zn is a powder with density of 0.65–0.75 g/cm³ and is supplied in milk.
Animal consumption and performance

The estimated consumption of dry matter (DM) and crude protein (CP) of each animal was obtained by daily measurements of the amount provided of concentrated feed and tifton hay, and their leftovers (considering 10% of leftovers). Dry matter, CP, ether extract (EE), neutral detergent fiber (NDF), and acid detergent fiber (ADF) (AOAC, 1990) were determined in samples from the concentrate and hay supplied. To obtain the performance variables such as DWG and FC, DM kg/weight gain kg, animal weights were obtained at the beginning and end of each 21-day period.

Blood collection, biochemical, and hormonal analyses

Blood collection was performed via a venipuncture of jugular vein in vacuum tubes (Vacutainer®) containing sodium fluoride with EDTA and siliconized without anticoagulants (20 mL, for serum biochemical concentrations) and with anticoagulants (20 mL, for plasma concentrations of ghrelin and leptin hormones) at 6 AM and at the end of P1 and P2. Soon after collection, blood samples were immediately placed on ice (4°C), followed by centrifugation (2,000 g, 15 min, 4°C). Then transferred by pipette to 2 mL plastic tubes and stored at -20°C for biochemical analyzes and -80°C for hormonal analyzes with subsequent serum and plasma samples frozen (−80 °C). Serum concentrations of glucose, triglycerides, total protein, urea, lactate, creatinine, alkaline phosphatase, and cholesterol were carried out using Labtest Diagnostica SA® kits as per manufacturer’s directions, and analyzed in an automatic biochemical apparatus LABMAX PLENNO, Labtest Diagnostica SA®. The total serum ghrelin and plasma leptin were obtained using the Enzyne Linked Immune Sorbant Assay (ELISA) sandwich technique (Ozturk et al., 2013). In a 96-well polystyrene plate, 100 μL of rat anti-ghrelin antibody (AAU93610 RayBiotech, Norcross, GA, 100μg/mL) were added per well, and the sealed plate incubated overnight at 4°C. Rat ghrelin has been used and validated to detect bovine ghrelin in another study (Miura et al., 2014). The plate
was washed five times using washing buffer (50 mM Tris-HCl containing Tween-20) and blocked with 1% bovine serum albumin (BSA) for 1 h. Subsequently, 20 μL of serum samples and standards were added to the wells and the plate was incubated overnight at 4°C, followed by five washes with washing buffer. Sequentially, 100 μL of detection antibody (0.25 μg/mL; Peprotech®) was added to all wells. The plate was then covered and incubated for 4 h at room temperature in a shaker at a moderate speed. After incubation, the plate was washed five times and 100 μL of enzyme solution (streptavidin-poly HRP80 conjugated peroxidase in phosphate-buffered saline-PBS) was added and incubated for 1 h at room temperature. Finally, the plate was washed five times, and 100 μl/well of substrate solution (3,3′,5,5′-tetramethylbenzidine in PBS with H2O2) was added. After 30 min of development, 100 μL of stop solution (0.3 M HCl) were rapidly added and the plate was read in a Genios plate reader (Phoenix Research Products, Candler, NC), Multi Skan Go® microplate reader (Thermo Scientific®, Software 2.4) with an excitation wavelength of 535 nm and an emission filter of 590 nm. For the leptin analyses the anti-human leptin antibody (500-P86 Peprotech®, Rocky Hill) was used and we followed the same methodology used for ghrelin analyses. Note that leptin human homology with bovine leptin was validated in another study (Miura et al., 2014).

**Tissue collection**

At the end of P2 animals were transported to a slaughterhouse with a State Inspection Service. After 12 h of fasting, they were slaughtered by cerebral concussion, followed by jugular and carotid venesection, according to the Normative Instruction N°3 of 01/13/2000 (Brazil, 2000). Animals that entered the slaughter line were divided into two half-carcasses that underwent washing, identification, weight measurement, and subsequent cooling in a cold room at 1°C, for 24 h. After this period, carcasses were weighed again to obtain the cold carcass weight. Hot carcass yields were obtained by the relationship between hot, cold carcass weight and live body weight.
multiplied by 100. The left half-carcass was then separated into the following cuts: front portion, needle tip, and special hind portion. Pieces of the dorsal sac of the calves’ rumen were collected for quantifying histological variables of the rumen (papillary density and thickness of epithelium and keratin). These pieces were placed in PBS for papillae count and in fixative solution (10% formaldehyde) for histological analysis. Quantification of ruminal papillae was performed in pieces of 1 cm² observed using binocular magnifying glasses. Papillae counting per cm² was carried out by three observers to obtain the average papillae density (papillae’s/cm²). Samples collected from the intestine were submerged in 10% formaldehyde solution to count goblet cells using PAS staining. This also allowed for confirming ghrelin receptor (GHS–R1a) expression throughout the intestinal mucosa by immunohistochemistry. Samples from intestine (medial portion of the duodenum) and hypothalamus were collected, immediately frozen in liquid nitrogen and stored at −80°C, prior to determining GHS–R1a expression using Western blot technique.

**Histological analysis**

**Rumen and Duodenum**

After 24 h of fixation, the tissues from rumen and duodenum were washed in running water and maintained in 70% alcohol until histological processing for inclusion in paraffin. Moreover, 3-mm-thick tissue fragments were dehydrated in increasing concentrations of alcohol (70%–100%), cleared in xylol, and immediately included in paraffin at 58–60°C. Paraffin blocks were cut by a rotating microtome to obtain 5-µm-thick histological cuts, which were mounted on silanized slides. After dewaxing and hydration, rumen cuts were stained using the Hematoxylin-Eosin (HE) technique (hematoxylin stained for 4 minutes and followed by eosin staining for 1
minute, followed by a dehydration step in increasing series of alcohol and mounted on synthetic resin) to obtain morphometric measurements. Moreover, imaging was performed under the Leica DM 4000B microscope with a 40X objective for morphometric measurements: height and width of papillae and keratin and epithelium thickness. To quantify goblet cells of the duodenal mucosa, cuts were dewaxed and hydrated for further staining using the Periodic Acid Schiff (PAS) technique (slides where deparaffinized and hydrated to water, oxidized in periodic acid solution, placed in Schiff reagent, and washed in lukewarm tap water, where the magenta color was highlighted in the positive PAS material) (Prophet et al., 1992). Image capture was performed under the Leica DM 4000B microscope with a 20X objective. For analysis, six fields were randomly selected in two cuts of each animal. The quantification of PAS positive areas, number of PAS positive cells, and the relationship of the area with the number of cells were performed with the help of Image-Pro Plus®.

**Immunohistochemistry**

Immunohistochemistry for marking of GHS-R1a was performed on duodenum pieces. Cuts on silanized slides were dewaxed and hydrated. After antigenic recovery with a sodium citrate buffer (pH 6.0), slides were incubated with 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity. The primary antibody (1:500, Antibody/GHS-R1, BIOSS BS-11529R, USA) was incubated overnight at 8°C. As a secondary antibody, the Max PolimerDetectionSystem (Novo Link Kit, Novocastra®, UK) was used. Specimens were then stained against hematoxylin, dehydrated in ethanol, cleared with xylol, and the slides were covered with Entellan and coverslip. Rat pituitary, known to express ghrelin, was used as a positive control, and a specimen that received a buffered solution of PBS rather than the primary antibody was used as a negative control. A positive result was indicated by the brown coloration at the binding site of the antibody throughout the length of the captured image, which represented the intestinal mucosa of the
calves. The areas, in pixels, of colored tissue section (brown color) in six fields were randomly selected in two cuts from each animal and quantified with the help of Image-Pro Plus.

**Western Blot**

To confirm the effect of diet on GHS-R1a protein synthesis in the hypothalamus and duodenum, portions of these tissues were analyzed using the Western Blot technique, as per the methodology proposed by Macedo et al. (2016). Specific extraction buffers were used to homogenize portions of duodenum [PBS (phosphate saline buffer) 1X, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate)] and hypothalamus (Hepes 50mm, MgCl$_2$ 1mm, EDTA 10mm, 0.5% Triton X-100). After obtaining the protein extract, samples were quantified and 40 µg of proteins per sample were fractionated by polyacrylamide gel electrophoresis. Next, the obtained bands were transferred to nitrocellulose membranes for incubation with primary and secondary antibodies. Membranes were incubated overnight at 4°C with the primary Ghrelin receptor antibody (1:1000, BIOSS Antibodies, USA) and with secondary anti-rabbit IgG HRP peroxidase conjugated antibodies, (1:4000, GE Healthcare, UK). Immunoreactivity was detected by chemiluminescence using enhanced luminol-based chemiluminescent (ECL) as a substrate. The protein bands were quantified by densitometry analysis using ImageJ (National Institute of Mental Health, USA) and normalized using GAPDH as an internal control (1:1000, Santa Cruz Biotechnology, USA).
Statistics

Data on consumption, performance, carcass, biochemical profile, rumen, and intestinal mucosa development were submitted to variance analysis, followed by Duncan’s test with 5% significance using PROC GLM from the SAS (Statistical Analysis System) program. For analyzing food consumption variables, initial body weights were used as a covariate to obtain the final corrected means. Data on ghrelin and leptin hormones, and relative GHS-R1a expression of protein in duodenum and hypothalamus were submitted to one-way ANOVA, with post-test Tukey using GraphPad Prism 6.1. The Spearman correlation coefficient, which measures the degree of association of variables with 5% significance, was used for correlation analysis, utilizing SAS.

Results

Milk restricted calves, whether supplemented with HMTBa or not, did not influence the dry matter consumption (DMC) of solid foods expressed kg.day\(^{-1}\) in both periods (Table 2). In P1, consumptions of total dry matter (concentrate + bulk feed + milk) and total crude protein of animals in groups RES and RES + HMTBa were lower compared to animals in the control. In P1, regardless of HMTBa addition, animals under milk restriction had lower average daily weight gain (DWG) and final weight (FW; Table 3). In P2, the evaluated groups presented statistical difference in FC (P < 0.001), with the RES+HMTBa group presenting the best FC compared to the other groups. Hot carcass weights (HCW) and cold carcass weights (CCW) and their yields (Table 4) were not influenced by milk restriction and/or supplementation with HMTBa. The primary cuts (front portion, special hind portion, and needle tip) and their yields were similar (P > 0.05) for the three groups. In P1 concentrations of glucose (P = 0.0365) and triglycerides (P = 0.0006) were lower in the animals of RES and RES + HMTBa groups (Table 5). In P2, urea concentration was lower for the RES group (P = 0.047). In both periods (P1 and P2) the concentrations of cholesterol, total protein,
alkaline phosphatase, creatinine, and lactate did not differ (P > 0.05) among groups. There was no difference for the hormonal concentrations of total ghrelin and leptin (Figure 1) between the evaluated groups. Moreover, there was no correlation between the serum levels of ghrelin and leptin and the variables of production and biochemical profile (Table 6) between the groups. There were no differences (P > 0.05) regarding the thicknesses of the epithelium, keratin, nor regarding the density of papillae of the dorsal portion of the rumen among the evaluated groups (Table 7). The average density of papillae did not show differences among the evaluated groups. Milk restriction did not influence the height (P = 0.214) and width (P = 0.409) of ruminal papillae (Figure 2). No significant differences (P > 0.05) for area and numbers of PAS positive cells in the duodenum were seen among the groups (Table 8 and Figure 3). In the duodenum, mucosal cells were reported to be distributed in the epithelium of crypts and villi. Immunopositive areas for GHS-R1a in villi and crypts (Figure 4) of the duodenum mucosa were observed in the three groups without significant differences. No significant changes in the GHS-R1a expression in the duodenum and hypothalamus were observed (Figure 5).

Discussion

We aimed to evaluate the effect of milk restriction, with or without supplementation with 2-hydroxy-4-methylthiobutanoic acid (HMTBa) on food intake as well as plasma levels of total ghrelin, leptin, and GHS-R1a expression in the hypothalamus of calves.

The minimum milk supply recommended for calves is 6 L per day (Azevedo et al, 2016; Leão et al., 2018), distributed in two meals. This methodology was adopted in the present study for the control group. Based on previous studies, milk volume was restricted to observe the occurrence of compensatory gain. Compensatory gain is represented by a rapid increase in animal performance after a period of low performance. This compensatory gain effect is signaled when, in
the second experimental period, animals that have undergone food restriction accelerate daily weight gain without increasing the consumption of dry matter (Table 3). We observed that glucose and triglycerides were significantly lower in the first experimental period in food restricted animals (Table 5). This is another parameter confirming the effect of food restriction as a suitable methodology for inducing compensatory gain. The DMC of solid foods was similar among the evaluated groups, not being influenced by milk restriction or HMTBa supplementation. This consumption may have been reflected in ruminal development as the consumption of solid foods interferes with development of ruminal papillae (Khan et al., 2011). This fact justifies the similar values observed among the groups in regard to measured rumen variables. The mean values found were as follows: epithelium thickness (26.62 µm), keratin (13.34 µm), papillary density (192.33 papillae cm⁻²), height (2.30 mm) and width of the ruminal papillae (0.82 mm). These values are in accordance with results obtained by other researchers (Maciel et al., 2016). Total DMC (tDMC), kg d⁻¹, was lower in calves submitted to milk restriction: 0.607 kg versus 0.966 kg. This is because of milk restriction, which is consistent with previous results with calves receiving smaller amounts of milk/or milk substitutes (Ozkaya and Toker, 2012; Silva et al., 2015; Schaff et al., 2016). The total protein consumption tCPC, kg d⁻¹, is a reflection of tDMC and so, it was smaller in animals under milk restriction in P1. Evaluation of calves’ performance is relevant, as according to Restle et al. (2005), heavier males at weaning have a reduction in the slaughter age and heavier females have a reduction in the age at puberty. The age reduction at puberty indicates a reduction in the age of first parturition, thus indicating an anticipation of the productive life of the animal. In the first period, the lowest tDMC of animals subjected to milk restriction (RES and RES + HMTBa) is reflected in the daily mean weight gain (DMG) (0.400 and 0.500 kg) when compared to the unrestricted group (0.820 kg). Previous research has shown that 4 L of milk/day provide nutrients only for maintenance and weight gain of 200–300 g/day under thermal conditions (15–25 °C; Drackley, 2008). In this study, 450 g/day gain was observed in calves receiving 3 L of milk daily. This difference of 250 and 150 g/day higher than the aforementioned study is possibly attributed to
consumption of concentrate and bulk foods. This demonstrates the importance of providing a solid diet for calves during this initial phase. According to Ozkaya and Toker (2012), the lower weight gain obtained by calves receiving lower amounts of milk resulted from a reduction in digestion capacity and absorption of nutrients, which are reflected in animal growth. Thus, the supply of concentrated and bulk foods possibly influenced digestion capacity and absorption of nutrients. In the first period, supplementation with HMTBa did not show efficacy on performance, since there was no difference in weight gain between RES and RES + HMTBa groups. This indicates that methionine was not a limiting nutrient in our research. In fact, Molano et al. (2020) evaluated several methionine sources, HMTBa included, and found no difference from the control group in the weight gain from birth to weaning. No improvement in calves’ performance were reported by Silva et al. (2018) when supplementing calves with methionine (5.3 g), lysine (17 g), glutamate (0.67 g) and glutamine (0.67) in calf milk substitutes. In the second period, animals that were submitted to milk restriction in the first period but were supplemented with HMTBa obtained better FC, indicating greater efficiency of transforming nutrients into weight gain suggesting the occurrence of compensatory gain. The HMTBa is absorbed from the digestive tract and partially converts to methionine in the liver (Baldin et al., 2018). Data observed here may indicate a better hepatic activity caused by this mechanism, favoring expression of mRNA for synthesizing muscle mass. These metabolic activities may express a mechanism related to the compensatory gain effect. The compensatory gain was evident when, in the second experimental period, the animals that were submitted to food restriction, accelerated the daily weight gain without increasing the dry matter consumption (Table 3).

Quantitative characteristics of bovine carcasses are primarily affected by the slaughter weight of animals (Vaz et al., 2008). The mean values of hot (36.47) cold (34.31) carcass weights and hot (55.32) and cold carcass yield (52.82) are in accordance with values reported by another study when evaluating calf carcass in this phase (Maciel et al., 2016). These results indicate similar
development among calves, despite milk restriction and contradicts the idea that compensatory gain would only be the result of greater growth in the viscera.

As per the biochemical profile of calves, the restriction in milk volume, regardless of the supply of HMTBa, altered the concentrations of glucose and triglycerides, being lower in these restricted animals compared to the animals in the control group, which has also been observed in another study (Khan et al., 2011). Calves submitted to milk restriction showed lower glucose and triglycerides concentrations compared to animals fed ad libitum (Schaff et al., 2016). Mean value of triglycerides of 16.5 mg/dL for animals subjected to milk restriction are within the normal range (16.3 – 34.8 mg/dL; Pogliani and Birgel Junior, 2007). Lower glucose and triglycerides concentrations were observed in milk restricted calves (Alves Costa et al., 2019). As per these researchers, low triglycerides concentrations resulted from the use of triglycerides as an energy source (Alves Costa et al., 2019). Mean glucose concentrations of 86.67 mg/dL observed in animals subjected to milk restriction are within the reference range for newborn calves (62.6 - 88.3 mg/dL; Pogliani and Birgel Junior, 2007). Serum urea concentrations of milk restricted calves supplemented HMTBa were similar to the control group, with mean values of 20.36 mg/dL, which are within the normal range of 20–30 mg/dL(Kaneco et al., 2008). This indicates similarity in protein metabolism between control group and milk restricted animals with and without HMTBa supplementation. Alkaline phosphatase, an important indicator of bone tissue formation or body construction (Hill et al., 2007), presented similar concentrations between groups, indicating milk restriction with or without HMTBa supplementation did not interfere in bone growth. Moreover, it was observed that serum alkaline phosphatase concentrations were not influenced by amino acid supplementation to milk replacer (Silva et al., 2018). The remaining serum parameters were within the normal range for calves 1 to 8 weeks of age (Klinkon and Ježek, 2012), demonstrating that animals were in nutritional balance.
The duodenum is the site of various signs that regulate hunger, food intake, and action-mediated satiety of ghrelin and its GHS-R1a receptor (Hayashida et al., 2001; Lely, 2004; Alam et al., 2012). In this study, some atrophy in the duodenum was expected due to the results presented by Steinhoff-Wagner et al. (2015) however, this was not observed. The increase in milk supply quickly restores the morphology and function of the intestine (repair of intestinal atrophy and normalization of intestinal permeability) (Steinhoff-Wagner et al., 2015).

In addition to generating knowledge about duodenal ghrelin receptor expression, it is important to investigate the mucous layer. This layer coats the inner surface of the duodenum and is designed for maximum absorption because it is covered with villi protruding into the lumen resulting in increased surface area. It is known that maintaining the integrity of the duodenum’s mucosa is essential for calf development because digestive processes and absorption of nutrients is linked to productive performance of animals. Of the duodenum’s mucosa, the crypt layer is an area of continuous cell renewal and proliferation. Cells that move from crypts to villi transform into enterocytes, goblet cells, Paneth cells, or enteroendocrine cells (Collins and Badireddy, 2018) as ghrelin-producing cells. With regard to goblet cells, they protect the intestinal epithelium from the action of digestive enzymes and abrasive effects of digestion (Robertis and Hib, 2001). These cells, located in the intestinal crypts along the mucous surface, synthesize and secrete mucus, which contains “mucin,” i.e., a substance composed of neutral and acidic mucopolysaccharides. Neutral mucin has a positive reaction by the periodic acid staining method of Schiff (PAS). In this study, the goblet PAS positive cells were identified in the duodenum in similar numbers in the mucosa of calves among the evaluated groups. This result indicates that milk restriction and HMTBa supplementation did not alter neutral mucin production. The treatments tested in this study did not alter the expression of GHS-R1a in the hypothalamus. Other researchers reported no statistical differences for GHS-R1a expression in hypothalamic tissues of calves submitted to milk restriction (Alves Costa et al., 2019). Leptin concentrations were similar among the calves, with mean leptin values in calves were 0.22 ng/ml in P1 and 0.16 ng/ml in P2. This indicates that food restriction was
not sufficient to alter concentration of this hormone. Leptin concentrations in plasma remain ~2.3 ng/mL through 1 year of age, indicating that leptin is regulated by postnatal nutrition (Block et al., 2003).

Milk restriction did not change plasma ghrelin concentrations with mean values of 17.17 ng/mL for P1 and 11.51 ng/mL for P2. Increased plasma ghrelin concentration in cattle have been observed with food deprivation (Wertz-Lutz et al., 2008). The blood collection methodology did not favor the observation of the ghrelin release pattern with greater accuracy. Other factors reported to alter ghrelin concentrations include body size, body composition, feeding frequency, diet composition, or feed quantity offered (Wertz-Lutz et al., 2006). Sugino et al. (2002) reported low plasma ghrelin concentrations in cattle with ad libitum feed and higher concentrations when intake was fractional. In the current study, in addition to milk restriction, milk was supplied across two meals daily, which could have favored increases of ghrelin concentrations. The free access to concentrate and hay probably influenced secretion of ghrelin, causing the observed variation in the results. Another important point that may have influenced ghrelin concentrations is the presence of solid foods in the gastrointestinal tract as the production of this hormone is inversely proportional to the amount of feed consumed (Heredia et al., 2015). Spearman correlations between serum ghrelin and leptin levels, and the productive variables and the biochemical profile did not link these parameters to serum hormone levels under the conditions of the present study.
Conclusion

This study demonstrated that milk restriction along with HMTBa supplementation induced compensatory gain although the mechanism by which this occurred was not elucidated by the study.

Acknowledgments

The authors express their gratitude to Marcus Ferreira Junior for his assistance regarding imaging, and the Foundation for Research Support (FUNAPE) - UFG for the financial support.
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**Figure 1:** Plasma concentrations of total ghrelin (A) and leptin (B) in calves without milk restriction (Control), with milk restriction (RES) and with milk restriction and HMTBa supplementation (RES + HMTBa). Mean ± SEM, N = 21.

**Figure 2:** Photomicrography of ruminal papillae (point of the arrows) of calves without milk restriction (Control), with milk restriction (RES) and with milk restriction and HMTBa supplementation (RES + HMTBa). Scale bar = 50 µm.

**Figure 3:** Photomicrography of Periodic Acid Schiff - PAS positive cells in the duodenum of calves without milk restriction (Control), with milk restriction (RES) and with milk restriction and HMTBa supplementation (RES + HMTBa). Scale bar = 100 µm.

**Figure 4:** Photomicrography and quantification of immunoreactive area for GHS-R1a (arrows) in the duodenum (simple columnar epithelium) of calves without milk restriction (Control), with milk restriction (RES) and with milk restriction and HMTBa supplementation (RES + HMTBa). Mean ±SEM. Scale Bar = 100 µm.

**Figure 5:** Expression of ghrelin receptors (GHS-R1a) in the duodenum (A) and in the hypothalamus (B) of calves without milk restriction (Control), with milk restriction (RES) and with milk restriction and HMTBa supplementation (RES + HMTBa). Mean ±SEM.
Table 1: Chemical composition, g kg\(^{-1}\) MS, of concentrate and hay used in the experiment.

| Nutrient                   | Concentrate\(^1\) | Tifton Hay |
|----------------------------|-------------------|------------|
| Dry matter                 | 857.0             | 898.0      |
| Crude protein              | 186.0             | 92.0       |
| Neutral detergent fiber    | 107.3             | 777.4      |
| Acid detergent fiber       | 23.7              | 400.4      |
| Ether Extract              | 31.6              | 13.3       |

\(^1\)Proportion of ingredients in concentrate: Ground corn, cornmeal (70.0%); Soybean meal (29.0%); Mineral premix without additive (1.0%)
Table 2: Consumption of dry matter and crude protein of calves subject to milk restriction with or without supplementation of HMTBa.

| Variable       | Control | RES  | RES + HMTBa | SEM  | P       |
|----------------|---------|------|-------------|------|---------|
| DMC\(^2\), kg d\(^{-1}\)P1 | 0.171   | 0.223| 0.171       | 0.284| 0.891   |
| tDMC\(^3\), kg d\(^{-1}\)P1 | 0.956\(^a\) | 0.637\(^b\) | 0.596\(^b\) | 0.038| <0.001  |
| CPC\(^4\), kg d\(^{-1}\)P1 | 0.031   | 0.040| 0.030       | 0.006| 0.5272  |
| tCPC\(^5\), kg d\(^{-1}\)P1 | 0.057\(^a\) | 0.055\(^b\) | 0.045\(^b\) | 0.006| <0.001  |
| DMC, kg d\(^{-1}\)P2   | 0.497   | 0.488| 0.364       | 0.111| 0.6414  |
| tDMC, kg d\(^{-1}\)P2   | 1.318   | 1.308| 1.184       | 0.111| 0.6414  |
| CPC, kg d\(^{-1}\)P2   | 0.089   | 0.087| 0.064       | 0.646| 0.5272  |
| tCPC, kg d\(^{-1}\)P2   | 0.119   | 0.117| 0.094       | 0.006| 0.6465  |

HMTBa = 2-hydroxy-4-(methylthio)butanoate acid; Control = 6 L of milk/day during P1 and 6 L of milk/day during P2; RES (milk restriction) = 3 L of milk/day during P1 and 6 L of milk/day during P2; RES + HMTBa: 3 L of milk/day during P1 and 6 L of milk/day during P2 + 3.3 g of HMTBa/day in both periods; \(^1\)Standard error of the mean; means followed with the same letter within a row do not differ from each other by Duncan test (P > 0.05); \(^2\)Dry matter consumption (Concentrate + Hay); \(^3\)Total dry matter consumption (Concentrate + hay + milk); \(^4\)Crude protein consumption (Concentrate + Hay); \(^5\)Total crude protein consumption (Concentrate + hay+ milk).
Table 3: Performance variables, quantitative characteristics, primary carcass cuts of calves subject to milk restriction with or without supplementation of HMTBa.

| Variable                | Control  | *RES  | RES + HMTBa | SEM^1 | P     |
|-------------------------|----------|-------|-------------|-------|-------|
| tDMC^2, Kg d⁻¹P1       | 0.956^a  | 0.637^b | 0.596^b     | 0.038 | <0.001|
| IW^3, Kg P1            | 37.70    | 34.20 | 38.50       |       |       |
| FW^4, Kg P1            | 53.64^a  | 46.78^b | 45.53^b     | 2.108 | 0.005 |
| DAWG^5, Kg d⁻¹P1       | 0.82^a   | 0.50^b  | 0.40^b      | 0.100 | 0.002 |
| TFC^6, kg MS/ Kg P1    | 1.36     | 1.59   | 1.32        | 0.286 | 0.825 |
| tDMC^2, Kg d⁻¹P2       | 1.318    | 1.308  | 1.184       | 0.111 | 0.6414|
| IW^3, Kg P2            | 53.64    | 46.78  | 45.53       |       |       |
| WF^4, Kg P2            | 68.76    | 64.55  | 64.42       | 3.058 | 0.340 |
| TFC^6, kg MS/ Kg P2    | 1.74^b   | 1.65^b | 1.28^a      | 0.062 | 0.001 |
|                |       |       |       |       |       |
|----------------|-------|-------|-------|-------|-------|
| DAWG, Kg d⁻¹ P2 | 0.755 | 0.802 | 0.925 | 0.061 | 0.113 |
| TWG            | 31.828| 27.428| 26.782| 3.091 | 0.679 |
| TADG           | 0.758 | 0.653 | 0.637 | 0.451 | 0.679 |
| FC kg MS/ kg   | 3.089 | 2.807 | 2.927 | 0.141 | 0.512 |

*RES = milk restriction; ¹Standard error of the mean; Means followed with the same letter within a row do not differ from each other by Duncan test (P>0.05); ²Total Dry Matter Consumption; ³Starting Weight; ⁴Final Weight; ⁵Daily Average Weight Gain; ⁶Total Food Conversion; ⁷Total weight gain; ⁸Total average daily gain; ⁹Food conversion of total experimental period (42 days).
Table 4: Quantitative characteristics, primary carcass cuts of calves subject to milk restriction with or without supplementation of HMTBa.

| Variable                  | Control. | *RES  | RES + HMTBa | SEM\(^1\) | P     |
|---------------------------|----------|-------|-------------|------------|-------|
| HCW\(^2\), Kg             | 39.00    | 33.42 | 37.00       | 1.875      | 0.137 |
| HCY\(^3\), %              | 55.45    | 54.56 | 55.95       | 1.047      | 0.434 |
| CCW\(^4\), Kg             | 37.25    | 32.02 | 33.48       | 1.415      | 0.393 |
| CCY\(^5\), %              | 54.37    | 52.18 | 51.92       | 2.260      | 0.482 |
| Front portion, Kg         | 15.20    | 12.82 | 14.05       | 1.111      | 0.807 |
| Special hind portion, Kg  | 19.31    | 16.75 | 17.01       | 0.842      | 0.155 |
| Needle tip, Kg            | 2.74     | 2.41  | 2.68        | 3.684      | 0.346 |
| Front portion\(^6\), %    | 40.06    | 40.06 | 41.78       | 1.974      | 0.806 |
| Special hind portion\(^6\), % | 51.93 | 52.33 | 50.43       | 2.037      | 0.712 |
| Needle tip\(^6\), %       | 7.52     | 7.603 | 7.77        | 0.410      | 0.909 |

*RES = milk restriction; \(^1\)Standard error of the mean; Means followed with the same letter within a row do not differ from each other by Duncan test (P>0.05); \(^2\)Hot Carcass Weight; \(^3\)Hot Carcass Yield in kg 100 kg\(^1\)LP; \(^4\)Cold Carcass Weight; \(^5\)Cold Carcass Yield in kg 100 kg\(^2\)PV; \(^6\)kg 100 kg\(^3\)cold carcass.
Table 5: Biochemical profile of mixed-breed calves submitted to milk restriction with or without supplementation with HMTBa in different periods.

| Variable                 | Control | *RES  | RES + HMTBa | SEM  | P      |
|--------------------------|---------|-------|-------------|------|--------|
| Glucose P1, mg/mL        | 102.14a | 84.93b | 88.42b      | 4.589| 0.036  |
| Glucose P2, mg/mL        | 126.00  | 124.52| 127.16      | 5.132| 0.932  |
| Cholesterol P1, mg/dL    | 115.34  | 128.09| 102.84      | 9.456| 0.170  |
| Cholesterol P2, mg/dL    | 104.16  | 117.86| 100.15      | 9.235| 0.310  |
| Triglyceride P1, mg/dL   | 30.64a  | 17.5  | 15.50b      | 2.544| <0.001 |
| Triglycerides P2, mg/dL  | 28.11   | 26.04 | 28.42       | 2.431| 0.455  |
| Total Protein P1, g/dL   | 6.13    | 6.21  | 6.62        | 0.335| 0.728  |
| Total Protein P2, g/dL   | 6.31    | 6.15  | 6.56        | 0.327| 0.745  |
| Alkaline phosphatase P1, mg/dL | 279.49 | 256.9 | 287.71      | 42.208| 0.644 |
| Alkaline phosphatase P2, mg/dL | 376.50 | 396.25| 439.17      | 45.889| 0.135 |
| Creatinine P1, mg/dL     | 0.79    | 0.88  | 0.83        | 0.055| 0.546  |
| Creatinine P2, mg/dL     | 0.79    | 0.70  | 0.71        | 0.056| 0.266  |
|                         | Urea P1, mg/dL | Urea P2, mg/dL | Lactate P1, mg/dL | Lactate P2, mg/dL |
|-------------------------|----------------|----------------|-------------------|-------------------|
|                         | 25.29          | 21.95<sup>a</sup> | 22.35             | 18.41             |
|                         | 19.86          | 17.25<sup>b</sup> | 26.37             | 15.46             |
|                         | 21.39          | 18.78<sup>a</sup> | 21.66             | 18.33             |
|                         | 2.236          | 1.243           | 2.460             | 1.265             |
|                         | 0.097          | 0.047           | 0.413             | 0.106             |

*RES = milk restriction; P1 = first period; P2 = second period; <sup>a</sup> Means followed with the same letter within a row do not differ from each other by Duncan test (P>0.05).
Table 6. Spearman correlations between serum ghrelin and leptin levels, and productive variables and biochemical profile.

| Variable                          | Ghrelin   |       | Leptin   |       |
|-----------------------------------|-----------|-------|----------|-------|
|                                   | ρ         | P     | ρ        | P     |
| tDMC, Kg·day P1                   | 0.115     | 0.618 | -0.002   | 0.999 |
| DAWG, Kg·day P1                   | 0.044     | 0.846 | 0.156    | 0.499 |
| TFC kg MS/ kg GP P1               | -0.068    | 0.766 | -0.055   | 0.810 |
| Glucose P1                        | 0.227     | 0.321 | 0.327    | 0.147 |
| Total protein P1                  | 0.263     | 0.248 | 0.054    | 0.814 |
| Cholesterol P1                    | 0.161     | 0.485 | 0.250    | 0.273 |
| Triglycerides P1                  | 0.371     | 0.097 | 0.244    | 0.284 |
| tDMC, Kg·day P2                   | 0.245     | 0.283 | 0.109    | 0.637 |
| DWG, Kg·day P2                    | 0.381     | 0.087 | 0.330    | 0.143 |
| TFC kg MS/ Kg GP P2               | -0.238    | 0.296 | -0.150   | 0.617 |
| Glucose P2                        | -0.092    | 0.690 | 0.087    | 0.706 |
| Total protein P2                  | 0.045     | 0.844 | 0.024    | 0.915 |
| Cholesterol P2                    | -0.073    | 0.750 | 0.037    | 0.871 |
| Triglycerides P2                  | -0.221    | 0.334 | 0.026    | 0.908 |

P1 = first period; P2 = second period; ¹Total Dry Matter Consumption; ²Daily Average Weight Gain; ³Total Food Conversion; ⁴Correlation Coefficient
Table 7: Variables of rumen of calves subject to milk restriction with or without supplementation with HMTBa.

| Variable                  | Control (n=7) | *RES (n=7) | RES + HMTBa (n=7) | SEM\(^1\) | P   |
|---------------------------|---------------|------------|-------------------|-----------|-----|
| Epithelium, µm            | 24.37         | 27.87      | 27.62             | 11.42     | 0.110|
| Keratin, µm               | 12.81         | 14.00      | 13.21             | 0.932     | 0.480|
| Epit+Keratin, µm          | 36.87         | 40.73      | 39.90             | 1.363     | 0.132|
| Density, papillae/cm\(^2\)| 196.7         | 184.33     | 197.00            | 19.87     | 0.913|
| HRP\(^2\), mm             | 2.041         | 2.367      | 2.497             | 0.351     | 0.214|
| WRP\(^3\), mm             | 0.827         | 0.859      | 0.798             | 0.094     | 0.409|

*RES = milk restriction; means followed with the same letter within a row do not differ from each other by Duncan test (P>0.05). 1Standard error of the mean; 2Height of rumen papilla; 3Width of rumen papilla.
Table 8: Area, cell numbers and area PAS positive cell ratio in duodenum of calves subject to milk restriction with or without supplementation with HMTBa.

| Variable                          | Control (n = 7) | RES* (n = 7) | RES + HMTBa (n = 7) | SEM¹ | P     |
|----------------------------------|----------------|--------------|---------------------|------|-------|
| PAS positive area²               | 12.44          | 34.97        | 37.51               | 9,399| 0.273 |
| No. PAS positive cells           | 27.43          | 34.16        | 37.64               | 3,429| 0.200 |
| Area/ PAS positive cells (mm²)   | 0.45           | 1.02         | 0.99                | 0.244| 0.250 |

*RES = milk restriction; ¹Standard error of the mean; ²mm²; means followed with the same letter within a row do not differ from each other by Duncan test (P>0.05).
Figure 4

[Image: Showing immunoreactive area comparison between Control, Res, and Res + HMTBa groups with bar graphs and accompanying tissue samples.]
