Changes in hematological, biochemical, and blood gases parameters in response to progressive inclusion of nitrate in the diet of Holstein calves

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Abstract

Background and Aim: Nitrate (NO₃⁻) reduces enteric methane emissions and could be a source of non-protein nitrogen in ruminant feeds. Nonetheless, it has a potential toxic effect that could compromise animal health and production. The purpose of this study was to determine the effects of progressive inclusion of NO₃⁻ in the diet on the hematological, biochemical, and blood gases parameters, in turn, the effects on feed intake and live weight gain (LWG) in Holstein calves.

Materials and Methods: Eighteen Holstein heifers and steers (nine animals/treatment) were maintained in individual pens for 45 days. Animals were randomly allocated to either a control or nitrate diet (ND) (containing 15 g of NO₃/kg of dry matter [DM]). The biochemical parameters and blood gases were analyzed only in the NO₃ group on days: -1, 1, 7, 13, 19, and 25 corresponding to 0, 20, 40, 60, 80, and 100% of the total inclusion of NO₃ in the diet, respectively. In addition, DM intake (DMI) and LWG were evaluated among dietary treatments.

Results: Feeding the ND did not influence DMI or LWG (p>0.05). Methemoglobin (MetHb) and deoxyhemoglobin increased according to the NO₃ concentrations in the diet (p<0.05), while an opposite effect was observed for oxyhemoglobin and carboxyhemoglobin (p<0.05). Hematocrit levels decreased (p<0.05), while albumin, alanine aminotransferase, and gamma-glutamyl transpeptidase concentrations were not modified (p>0.05). However, glucose, urea, aspartate aminotransferase (AST), and retinol concentrations increased (p<0.05) according to the NO₃ concentrations in the diet.

Conclusion: This study confirmed that the progressive inclusion of 123 g of NO₃/animal/day in the diet could be safe without affecting DMI and LWG of Holstein calves. In turn, a dose-response effect of the MetHb, glucose, urea, AST, and retinol was observed, but these values did not exceed reference values. These results highlighted the importance of using a scheme of progressive inclusion of NO₃ in the diet of calves to reduce the risks of NO₃ toxicity.

Keywords: dry matter intake, liver function, methemoglobin, nitrate toxicity.

Introduction

The use of nitrate (NO₃⁻) in ruminants’ diet decreases enteric methane (CH₄) emissions, which tested in in vitro and in vivo studies, showing effective and persistent results as an option in methane mitigation [1]. This reduction relies on that NO₃⁻ consumes more electrons at the expense of CH₄ production, by reducing it to nitrite (NO₂⁻), and also to ruminal ammonia (NH₃) [2]. In this sense, the presence of NO₃⁻ in the rumen drives a shift in the use of hydrogen (H₂) toward NH₃ production instead of CH₄ production [2]. However, when the input of NO₃⁻ exceeds the ruminal microbiota ability for NO₂⁻ reduction, this mechanism is altered, causing the NO₃⁻ to accumulate in the rumen and pass into the bloodstream, resulting in increased methemoglobinemia in ruminants [3]. Signs of NO₃⁻ toxicity may appear when more than 20% of the hemoglobin is converted to methemoglobin (MetHb) [4]. Symptoms depend on the degree of exposure to NO₃⁻, such as decreased feed intake resulting in reduced live weight gain (LWG), susceptibility to infections, reproductive inefficiency, brown mucous membrane discoloration, respiratory distress, coma, cyanosis, and even death [5].

A key condition for the use of NO₃⁻ or other additives as anti-methanogenic agents is that they do not develop harmful effects on animal health and performance [6]. Several strategies have been developed...
to reduce the risks of toxicity by NO\textsuperscript{3} inclusion in the diet, such as NO\textsuperscript{3} encapsulation [7] and forages sprayed with NO\textsuperscript{3} [8]. In addition, the gradual adaptation to NO\textsuperscript{3} in the diet could be an alternative to minimize negative effects. The previous study has shown that progressive inclusion of NO\textsuperscript{3} in the diet did not compromise animal performance, produced no toxic effects, and had no cumulative effects on the animal products [9]. However, these previous studies were focused only on assessing the effects on the abatement capacity of enteric CH\textsubscript{4} emissions, and monitoring of blood MetHb as the unique indicator of NO\textsuperscript{3} toxicity [10,11]. In this sense, many studies have often ignored hematological, biochemical, and blood gases changes during the adaptation period to dietary NO\textsuperscript{3}; although the importance of the inclusion of these compounds on animal health.

The mitigation potential of NO\textsuperscript{3} can not only be beneficial in intensive milk and meat production systems but can also be especially interesting in pasture-based livestock systems that use low protein forages to maintain animal production (mainly during the dry season), because the ruminal microbiota of the host animal can benefit from NO\textsuperscript{3} as a non-protein nitrogen source and use it for microbial protein synthesis. Therefore, the use of NO\textsuperscript{3} would not only reduce the environmental impact but also improve animal performance, such as was evidenced in the study by Wang et al. [12].

Our study highlights the importance of the adaptation period and animal response to NO\textsuperscript{3} in the diet. We hypothesize that the progressive inclusion of NO\textsuperscript{3} in the diet allows an effective adaptation of the NO\textsuperscript{3} reducing ruminal microbiota, which causes a dose-response effect on hematological, biochemical, and blood gases parameters without reaching toxicity levels for the animal, and without causing changes in animal performance. Thus, the aim of this study was to evaluate the effects of progressive inclusion of NO\textsuperscript{3} in the diet on the hematological, biochemical, and blood gases parameters, in turn, the effects on feed intake and LWG in Holstein calves.

Materials and Methods

Ethical approval

This study was performed in accordance with international recommendations specified in the guidelines for the use and care of animals. All the animal procedures used in this study were approved by the Committee for Use and Care of Experimental Animals (Protocol CICUAE/124-2017; Approval date September 12, 2017) of the National Institute of Agricultural Technology (INTA) of Argentina.

Study location, period, experimental design and animal procedures

The experiment was conducted at the Experimental Dairy Centre of the Balcarce Agricultural Experimental Station of INTA, Argentina (37°45’37”S; 58°17’55”W), during the period from October 20 to December 4, 2017. Eighteen calves (seven heifers and 11 steers) of 8.1±0.5 months of age (mean-standard deviation) and with 214±13.5 kg live weight were used. The calves were considered clinically healthy based on physical examination and blood sample results (biochemical and hematological parameters). During the study period, daily physical examination of the animals was performed, and potential lack of appetite, mucosal color or other abnormal signs were recorded. As a precautionary protocol, against intoxication of NO\textsuperscript{3}, a solution of methylene blue was prepared for emergency use, at a dose of 15 mg/kg of body weight (intravenous administration).

The animals were randomly allocated to either a control diet (CD; including five steers and four heifers) or a nitrate diet (ND; including six steers and three heifers). The CD group received a total mix ration (% of dry matter [DM]) of corn ground, soybean meal, premix, and urea (79.6%), and grass hay (20.4%). In turn, the ND group received CD (98.5%) plus 1.5% of NO\textsuperscript{3} (as calcium NO\textsuperscript{3}, YaraLiva Calcinit®, Yara Argentina S.A.) (Table-1). The intermediate level of NO\textsuperscript{3} inclusion in the diet was selected for this study because it was previously used to mitigate enteric CH\textsubscript{4} emissions in Holstein cattle without compromising animal health [10,13].

To reduce the risks of toxicity, the amount of NO\textsuperscript{3} was gradually increased (Table-2). The animals were fed ad libitum twice a day (08:00 AM and 4:00 PM) in individual pens (36 m\textsuperscript{2}) provided with individual feeders and shared drinking troughs. The trial included 30 days of adaptation period to the diet and handling, followed by a 15 days measurement period (from day 31 to day 45).

Table-1: Dietary ingredients (% of DM) and nutritional composition of experimental diets (% of DM).

| Variable          | CD    | ND    |
|-------------------|-------|-------|
| Ingredients       |       |       |
| Grass hay         | 20.4  | 20.4  |
| Ground corn       | 69.4  | 68.3  |
| Soybean expeller  | 8.0   | 8.4   |
| Urea              | 0.8   | 0.2   |
| Calcium nitrate*  | 0.0   | 1.5   |
| Premix*           | 1.1   | 1.1   |
| Total             | 100   | 100   |
| Composition       |       |       |
| Dry matter (% of FM) | 90.4  | 90.5  |
| Organic matter (% of DM) | 94.5  | 94.5  |
| Crude protein (% of DM) | 12.4  | 12.2  |
| Neutral detergent fiber (% of DM) | 25.9  | 25.8  |
| Starch (% of DM)  | 48.3  | 47.6  |
| GE (MJ/kg of DM)  | 21.3  | 21.3  |

DM=Dry matter, FM=Fresh matter, GE=Gross energy, CD=Control diet, ND=Nitrate diet. *Calcium ammonium nitrate, 5Ca (NO\textsubscript{3})\textsubscript{2}•NH\textsubscript{4}NO\textsubscript{3}•10H\textsubscript{2}O; 75% NO\textsubscript{3} on dry basis; estimated composition 11.3 g NO\textsubscript{3}/kg DM for nitrate treatment. *Composition of Premix (per kg of premix): Calcium 23%, Sodium 8%, Phosphorus 1%, Magnesium 3.1%, Vitamin A 150000 UI, Vitamin D3 15000 UI, Vitamin E 150 UI, Iron 960 ppm, Magnesium 900 ppm, Zinc 900 ppm, Copper 150 ppm, Iodine 24 ppm, Cobalt 15 ppm, Selenium 6 ppm.
Blood sampling and analysis

Blood samples were taken only from the ND group. For blood gas (MetHb, oxyhemoglobin [O₂Hb], carboxyhemoglobin [COHb], and deoxyhemoglobin [HHb]), hematocrit, and glucose monitoring, the sampling was performed 3 h post-feeding on days-1 (control day), 1, 7, 13, 19, and 25, by jugular vein puncture using Vacuette® tubes with lithium heparin (Greiner Bio-One GmbH – Germany), and placed on ice directly after sampling. The analytes were determined using the Cobas-b221 blood gas system (Roche Diagnostics, USA).

In addition, the serum concentration of urea, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT), and retinol was monitored, to examine liver function. Blood sampling on days-1 (control day), 7, and 25 were transferred into tubes with clot activator and gel separator (Greiner Bio-One GmbH - Germany). After clotting, serum was separated by low-speed centrifugation (3500×g) for 15 min at 4°C and stored at −20°C until analysis. The concentrations of urea and albumin were determined by the enzymatic method UV-glutamate dehydrogenase and colorimetrically with bromine cresol-sulfophalein, respectively [14], while AST and ALT and GGT were determined using an automatic biochemical analyzer [15,16]. Retinol was determined as an indicator of Vitamin A by high-performance liquid chromatography.

Evaluation of DM intake (DMI), LWG, and diets analysis

DMI was calculated as the difference between the daily offered and residual feed. Only measures of DMI from day 31 to day 45 (post-adaptation period) were considered for the analysis of the data. The results were expressed in kilograms of DMI/day. The LWG was determined as the difference between the final and initial weight during 45 days of evaluation and was expressed in kilograms of LWG/day.

The ingredients of the diets were dried in a forced-air oven at 55°C and milled to pass a 1-mm screen. DM analysis by oven drying (105°C) and ash by incineration at 550°C for 4 h were determined, according to AOAC [17]. Total nitrogen content was determined by combustion type auto-analyzer (Leco FP-2000, Leco Corp., St. Joseph, MI). In addition, we assessed neutral detergent fiber in a fiber analyzer ANKOM® 220 (ANKOM Technology, Macedon NY-USA) [18], and starch was analyzed by an enzymatic method [19].

**Table-2:** Scheme of progressive adaptation to a diet with NO\textsubscript{3} inclusion.

| Phase | 1 | 2 | 3 | 4 | 5 | 6 |
|-------|---|---|---|---|---|---|
| Day   |   |   | 13-18 | 19-24 | 25-30 | 31-45 |
| Calcium nitrate* (%) | 20 | 40 | 60 | 80 | 100 | 100 |
| g/animal/day | 24.6 | 49.2 | 73.8 | 98.4 | 123 | 123 |

*Percentage of NO\textsubscript{3} inclusion in each phase was according to the total intake on day 25 (15 g of NO\textsubscript{3}/kg of DM)

Statistical analysis

The results of DMI and LWG were analyzed with PROC MIXED SAS software version 13.1 (SAS Institute Inc., Cary NC, USA 2013) [20] with treatment as fixed effect and animals as random effect according to the model:

\[ Y_{ij} = \mu + \text{Treat}_i + \text{Anim}_j(\text{Treat}) + e_{ij}, \]

where: \( Y_{ij} \)=response variable; \( \mu \)=general mean of the experiment; \( \text{Treat}_i \)=Treatment, CD versus ND \((i=2)\); \( \text{Anim}_j(\text{Treat}) \)=animals within the treatment \((j=18)\); \( e_{ij} \)=experimental error.

Urea, albumin, retinol, AST, ALT, and GGT data were analyzed with the time factor as a repeated measure using PROC GLM of the SAS version 13.1 (SAS Institute Inc., Cary NC, USA 2013) [20], according to the following model:

\[ Y_{ij} = \mu + \text{Anim}_j + \text{Time}_k + e_{ij}, \]

where: \( Y_{ij} \)=response variable; \( \mu \)=general mean of the experiment; \( \text{Anim}_j \)=animals \((i=9)\); \( \text{Time}_k \)=time factor: sampling day \((k=6 \text{ or } 3)\); \( e_{ij} \)=experimental error, in turn, followed by Dunnett’s multiple comparison tests.

The data that did not meet the assumption of normality and homogeneity of variance, such as Methb, O₂Hb, COHb, and HHb, glucose and hematocrit were analyzed using the Friedman test and a comparison between median was performed using Wilcoxon signed-rank test in R software version 3.6.1 [21]. Differences among mean and median were considered significant when \( p<0.05 \). In addition, Spearman’s correlation analysis was used to evaluate the association between variables using the `corrplot` function in R.

Results

Effect on DMI and LWG

DMI and LWG did not differ among dietary treatments in Holstein calves \((p=0.117 \text{ and } p=0.439, \text{ respectively; Table-3})\). Likewise, the initial and final weight of the calves did not differ significantly among dietary treatments \((p=0.960 \text{ and } p=0.832, \text{ respectively})\).

Effect on hematological, biochemical, and blood gases parameters

An incremental effect was observed for Methb (Figure-1), where levels increased numerically until day 19, though not significantly compared to day-1. In contrast, on day 25, there was a significant increase compared to day-1 \((p<0.001)\). Moreover, an opposing effect was observed for the O₂Hb level, because it decreased according to NO\textsubscript{3} concentrations in the diet, although there was a significant decrease only on day 25 compared to day-1 \((p=0.001; \text{Table-4})\). In turn, the COHb values decreased significantly on days 1, 13, 19, and 25 with respect to day-1 \((p=0.003)\), but...
statistically significant differences were not found between day 7 and day-1. Conversely, HHb increased significantly from day 1 to day 13 compared to day-1, and then there was a slight decrease toward day 25, but remained higher than day-1 (p=0.005).

The hematocrit was reduced according to NO$_3^-$ concentrations in the diet (p=0.001). This reduction was not associated with the hemolysis of blood samples because they were verified during laboratory analyses. In turn, glucose concentrations increased with NO$_3^-$ inclusion (p=0.001), being most evident on days 13, 19, and 25, which corresponded to 60, 80, and 100% of NO$_3^-$ inclusion (Figure-2).

On the other hand, the changes of AST activity (Figure-3) and retinol concentrations (Figure-4) on day 7 (corresponding to 40% of total NO$_3^-$) in comparison to day-1 were not different. However, on day 25, there was a significant increase with respect to day-1 (p=0.004 and p=0.025, respectively). Similarly, there was a significant increase in urea concentrations from day 7 to day 25 compared to day-1 (p=0.001). However, the levels of NO$_3^-$ inclusion in the diet did not modify albumin concentrations, and ALT and GGT activity (p=0.387, p=0.673, and p=0.779, respectively) in Holstein calves (Table-5).

Table-3: Dry matter intake and live weight gain in Holstein calves fed with a control diet (n=9) and nitrate diet (n=9).

| Parameters                  | Diets        | SEM | p-value |
|-----------------------------|--------------|-----|---------|
| Dry matter intake (kg/day)  | CD 8.8 ± 0.24| 0.24| 0.117  |
|                             | ND 8.1 ± 0.24|     |         |
| Initial weight (kg)         | 214 ± 14 4.76|     | 0.960  |
| Final weight (kg)           | 268 ± 5.81 |     | 0.832  |
| Live weight gain (kg/day)   | 1.2 ± 0.10  |     | 0.439  |

SEM=Standard error of the mean, CD=Control diet, ND=Nitrate diet

Table-4: Effect of a progressive inclusion of NO$_3^-$ in the diet on blood gases (%) and hematocrit (%) levels in Holstein calves (n=9).

| Parameters | Monitoring days (medians±IQR)* | p-value | Reference values |
|------------|---------------------------------|---------|------------------|
| O$_2$Hb    | Day-1 96 ± 1.5                 |        | N/A              |
|            | Day 1 94 ± 2.0                 |        |                  |
|            | Day 7 95 ± 2.9                 |        |                  |
|            | Day 13 92 ± 3.7                |        |                  |
|            | Day 19 93 ± 3.4                |        |                  |
|            | Day 25 90 ± 3.3                | 0.001   |                  |
|            | p-value                        |         |                  |
| COHb       | Day-1 1.5 ± 2.4                |        | N/A              |
|            | Day 1 0.4 ± 0.3                |        |                  |
|            | Day 7 0.7 ± 0.3                |        |                  |
|            | Day 13 0.4 ± 0.2               |        |                  |
|            | Day 19 0.3 ± 0.3               |        |                  |
|            | Day 25 0.4 ± 1.1               | 0.003   |                  |
| HHb        | Day-1 0.8 ± 0.1                |        | N/A              |
|            | Day 1 2.7 ± 1.1                |        |                  |
|            | Day 7 2.1 ± 1.7                |        |                  |
|            | Day 13 5.5 ± 4.0               |        |                  |
|            | Day 19 3.5 ± 2.5               |        |                  |
|            | Day 25 1.7 ± 3.9               | 0.005   |                  |
| Hematocrit | Day-1 32 ± 1.7                 |        | 30-36% Kahn and Line [38] |
|            | Day 1 30 ± 0.8                 |        |                  |
|            | Day 7 29 ± 1.8                 |        |                  |
|            | Day 13 29 ± 0.8                |        |                  |
|            | Day 19 29 ± 1.3                |        |                  |
|            | Day 25 29 ± 0.5                | 0.001   |                  |

O$_2$Hb=Oxyhemoglobin, COHb=Carboxyhemoglobin, HHb=Deoxyhemoglobin. IQR=Interquartile range. N/A=Not applicable. *Day-1=Without NO$_3^-$ in the diet (control day); Day 1=With 20% of total NO$_3^-$ in the diet; Day 7=With 40% of total NO$_3^-$ in the diet; Day 13=With 60% of total NO$_3^-$ in the diet; Day 19=With 80% of total NO$_3^-$ in the diet; Day 25=With 100% NO$_3^-$ in the diet. a,b=Medians within a row with different superscripts differ (p<0.05) from Day-1 (Wilcoxon signed-rank test).

**Figure-1**: Box and whisker plots showing levels of methemoglobin (% of total hemoglobin) in blood of nine Holstein calves measured on day-1, 1, 7, 13, 19, and 25 with 0, 20, 40, 60, 80, and 100% of total NO$_3^-$ in the diet, respectively. The median is indicated by the middle line, the mean is indicated by the symbol (○), and the 75th and 25th percentiles by the upper and lower edges of the boxes. The whiskers show the 95% confidence interval. Comparison of medians, box and whisker with different letters above ("a" or "b") differs (p<0.05) from day-1 (range test signed by Wilcoxon).
Correlation analyses were performed with the hematological, biochemical, and blood gases variables corresponding to day 25 (Figure-5). DMI was positively associated with the level of MetHb ($r=0.34$), $O_2$Hb ($r=0.32$), AST ($r=0.38$), ALT ($r=0.44$), and albumin ($r=0.38$). In contrast, it was negatively associated and in less degree with the concentration of glucose ($r=-0.12$), HHb ($r=-0.30$), hematocrit ($r=-0.15$), urea ($r=-0.10$), and retinol ($r=-0.13$). LWG was positively associated with glucose concentration ($r=0.41$), $O_2$Hb ($r=0.6$), and COHb ($r=0.57$).
while, negatively with the concentration of MetHb (r=-0.2), HHb (r=-0.27), Urea (r=-0.13), AST (r=-0.23), and GGT (r=-0.5). MetHb was negatively associated with glucose concentration (r=-0.73), and less so with O₂Hb (r=-0.15), COHb (r=-0.13), HHb (r=-0.49), and AST (r=-0.25), but positively with retinol (r=0.39). Urea concentration was positively associated with O₂Hb (r=0.6) and ALT (r=0.38), and negatively with HHb (r=-0.31) and albumin (r=-0.39). AST activity was positively correlated with ALT activity (r=0.7) and albumin concentration (r=0.68), and negatively with retinol (r=-0.41). GGT activity was negatively associated with ALT (r=-0.53) and COHb (r=-0.54), and positively with retinol (r=0.45).

Discussion

The physiological response to NO₃⁻ in animals was variable because the level of NO₃⁻ toxicity depends on several factors: Dietary NO₃⁻ dose levels, the rate of NO₃⁻ intake, an incomplete reduction of NO₃⁻ and NO₂⁻ to NH₃ in the rumen, and a low rate of rumen content passage, which results in higher retention of NO₃⁻ or NO₂⁻ in the rumen [22]. Thus, in this study, it was possible to control the majority of these risk factors by the progressive inclusion of NO₃⁻ in the diet because it allowed the DMI and LWG not to differ between dietary treatments, despite observing a 7% numerical reduction in the DMI in the DN, which could be attributed to the organoleptic characteristics of NO₃⁻[5]. Similar results were found in the previous studies [23,24].

A linear relationship between levels of blood MetHb and dietary NO₃⁻ was observed in a meta-analysis study [1]. In addition, Newbold et al. [25] showed that CH₄ emissions decreased linearly with increasing dietary NO₃⁻ level, but the risk of poisoning also increased with daily doses >2.4 g of NO₃⁻/kg of DM. However, when NO₃⁻ was used in intermediate doses (13-21 g of NO₃⁻/kg of DM/day) and adequate dietary

| Parameters | Monitoring days (means)* | SEM | p-value | Reference values |
|------------|--------------------------|-----|---------|------------------|
| Urea (mg/dL) | 14ᵃ | 23ᵇ | 21ᵇ | 0.93 | 0.001 | 10-25 Kahn and Line [38] |
| Albumin (g/L) | 38 | 39 | 37 | 0.09 | 0.387 | 25-38 Kahn and Line [38] |
| ALT (UI) | 16 | 16 | 17 | 0.97 | 0.673 | 11-40 Kaneko et al [37] |
| GGT (UI) | 22 | 21 | 22 | 0.83 | 0.779 | 6.1-17.4 Kaneko et al [37] |

ALT=Alanine aminotransferase, GGT=Gamma-glutamyl transpeptidase. SEM=Standard error of the mean.

*Day-1=Without NO₃⁻ in the diet; Day 7=With 40% of total NO₃⁻ in the diet; day 25=With 100% NO₃⁻ in the diet.ᵃᵇMeans within a row with different superscripts differ (p<0.05) from Day-1 (Dunnett’s test).
adaptation studies showed a 14-25% decrease in enteric CH4 emissions without affecting animal performance and animal health [10,11,26,27]. In this study, blood MetHb did not exceed the upper tolerance limits for cattle (<10% of total hemoglobin) when fed daily with 15 g of NO3/kg of DM, but we noted that the individual response was variable (CV=38.4%). Moreover, the percentage of MetHb was positively associated with DMI, and negatively with LWG, HHb, O2Hb, and COHb. Furthermore, although NO3 did not affect LWG, the numerical difference (~100 g) could be explained partially by the negative association between these variables. In the previous studies in beef and dairy cattle under a system of progressive adaptation to dietary NO3 reported levels <6% of blood MetHb [11,24]. Similar results were found using encapsulated NO3[7].

The increase in serum urea concentrations according to the increase in dietary NO3 was expected, since NO3 in the rumen is reduced to NH3 by NO3-reducing microorganisms. Therefore, these increases can be attributed to a higher concentration of rumen NH3, as it is absorbed and converted to urea in the liver, then excreted through the urine [28]. Furthermore, the increased concentration of glucose is probably related to high urea concentration. This mechanism in ruminants is well documented and has been attributed either to a lower release of pancreatic insulin [29] or to increased glucose production in the liver [30]. However, some authors hypothesized the beneficial effects of using NO3 and NO2 as precursors of nitric oxide, at blood and tissue level, on glucose uptake and increased insulin sensitivity in humans and rats [31,32], but not confirmed yet in ruminants [33].

Overall, we can affirm that the progressive inclusion of 123 g of NO3/animal/day, caused an animal metabolic adjustment, due to a reduction of available oxygen caused by the increase of blood MetHb induced a higher glucose concentration and a reduction of hematocrit because aerobic metabolism at cellular and tissue level was affected by the reduction of oxygen availability [34]. However, animals with higher MetHb levels did not always induce higher glucose concentration, showing a negative correlation between both variables. The reason for these findings remains unclear.

Recently, González Delgado et al. [34] studied the acute effects of NO3 poisoning in Wistar rats. The
authors observed an increase in glucose, cholesterol, triglycerides, LDH, AST, and ALT that associated with changes in liver metabolism caused by liver damage. Moreover, other study reported that levels of LDH, AST, and ALT were increased under the chronic condition of NO₃⁻ exposure in pregnant cows [35]. However, in this study, no significant increase in liver enzymes was observed after NO₃⁻ inclusion. These different results are probably due to NO₃⁻ exposure time, physiological status of the animal, animal species, and dose levels of NO₃⁻, time of adaptation to NO₃⁻ among other factors.

We can confirm the hypothesis that the progressive inclusion of NO₃⁻ allows an effective adaptation of the NO₃⁻-reducing ruminal microbiota, without reaching toxicity levels for the animal, nor causing changes in animal performance. There were no changes in DMI, LWG, nor ALT and GGT activity, or albumin concentration by NO₃⁻ inclusion in the diet, except for MetHb, urea, glucose, AST, and retinol concentrations that were significantly increased. However, these increases did not exceed the reference values of clinically healthy cattle [36-38].

Conclusion

This study confirmed that the progressive inclusion of 123 g of NO₃⁻/animal/day in the diet could provide safe supplementation for Holstein calves without affecting DMI and LWG. In turn, a dose-response effect of the MetHb, glucose, urea, AST, and retinol was observed, but these values did not exceed reference values. These results highlighted the importance of using a scheme of progressive inclusion of NO₃⁻ in the diet of calves to reduce the risks of NO₃⁻ toxicity.

Authors’ Contributions

AO designed and performed the experiments, analyzed, and wrote the manuscript. GM, GD, and FS contributed in handling the animals, sample collection, and analyzed the data. MDT, JG, CF, and SLC contributed reagents/materials and analyzed the data. AC designed the experiment and analyzed the data. MEC conceived and designed the experiments, analyzed the data. MDT, JG, CF, and SLC contributed in handling the animals, sample collection, and analyzed the data.

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Competing Interests

The authors declare that they have no competing interests.

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