Milk production and hematological and antioxidant profiles of dairy cows supplemented with oregano and green tea extracts as feed additives

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ABSTRACT - We aimed to evaluate the effects of the addition of oregano (Origanum vulgare) or green tea (Camellia sinensis L.) extracts (separately and associated) on feed intake, milk production, and hematological and antioxidant profiles of dairy cows. For that purpose, 16 Holstein and 16 Holstein-Gyr cows with 526.3±10.2 kg and within the first third of lactation were distributed according to a complete block design with measurements repeated in time. Treatments were control (CON), addition of 0.056% of oregano extract (OR), addition of 0.028% of green tea extract (GT), addition of a mixture of OR and GT extract (0.056% each) in the diet (MIX). Hematological and antioxidant profiles were monitored. Data were subjected to ANOVA, with block, treatment, days, and their interactions considered as fixed effects and animal and the residue as random effects. In Holstein cows, GT increased feed intake and milk yield compared with CON; in Holstein-Gyr crossbred cows, OR showed increased intake and GT increased milk yield compared with CON. Compared with CON, GT and OR decreased eosinophils concentration; OR showed the highest neutrophils concentration and neutrophils to leukocyte ratio. Compared with CON, OR presented increased catalase (CAT) activity, while GT increased the reduced glutathione concentration. The MIX treatment reduced CAT activity compared with OR, presented the lowest concentration of oxidized dichlorofluorescein in the erythrocytes (DCFER) and plasma (DCFPLA), and increased eosinophils concentration compared with GT and OR. Extracts differently affected feed intake and milk yield depending on genetic group. Feeding green tea and oregano extracts separately or associated distinctly affects the antioxidant indicators of lactating dairy cows.

Keywords: Holstein, Holstein-Gyr crossbred, plant extracts, redox state

1. Introduction

The first third of lactation is a critical period for dairy cows, because although they usually are able to meet nutrient requirements after the peak of lactation, they still produce large amounts of...
milk and should initiate reproductive activity by this time. Moreover, Holstein cows may be challenged by heat stress (Stumpf et al., 2021), increasing oxidative stress (Abreu et al., 2020; Gross and Bruckmaier, 2019).

The increase in demand for animal-based products without residual substances that can be harmful to human health and to the environment have been stimulating the use of plant extracts in animal nutrition (Saeed et al., 2017). In this sense, secondary compounds produced by plants, such as essential oils and polyphenols, have been studied due to their therapeutic functions for both humans and animals (Aristatile et al., 2015). Oregano extract contains the essential oils carvacrol and thymol, which present antimicrobial (Cho et al., 2020), antimetanogenic (by directly inhibiting ruminal methanogens (Patra and Yu, 2012); Kolling et al., 2018) and antioxidant properties (Paraskevakis et al., 2015); green tea extract, in its turn, is rich in catechins, L-theanine, and caffeine and presents antioxidant and anti-inflammatory properties (Oh et al., 2017).

In ruminants, the majority of in vitro and in vivo trials emphasizes the potential of essential oils and polyphenols in modifying rumen fermentation, decreasing methane emissions (Kolling et al., 2018). Based on previous reports, the addition of oregano and green tea extracts is expected to improve the redox state (Paraskevakis et al., 2015) without negatively affecting behavior, feed intake, and milk production (Kolling et al., 2018; Stivanin et al., 2019; Vizzotto et al., 2021).

The effects of oregano and green tea extracts on ruminants’ immune and antioxidant systems still need to be better clarified. Paraskevakis et al. (2015) added dried oregano leaves (1 mL of essential oil) to dairy goats’ diet and observed an improvement in enzymatic and non-enzymatic antioxidant defenses in blood and milk. Vizzotto et al. (2021) observed distinct effects on redox status biomarkers of dairy cows receiving oregano or green tea extracts during the transition period. To the best of our knowledge, no other study investigated the existence of additive effects of green tea and oregano extracts on hematological and antioxidant profiles of dairy cows.

Based on the abovementioned studies, we hypothesized that the use of oregano and green tea extracts may reduce oxidative stress in lactating cows at the beginning of lactation, without negative effects on feed intake, milk yield, and hematological indicators. Moreover, based on previous results showing distinct effects of these plants extracts on antioxidant defenses, we also hypothesized that these extracts, when associated, may present additive effects that are not presented by the same extracts when consumed separately. The objective of this study was to evaluate the effects of the addition of oregano or green tea extracts (separately and associated) on feed intake, milk production, and hematological and antioxidant profiles of lactating dairy cows.

2. Material and Methods

Research was conducted according to the institutional committee on animal use (case n. 18.510). The experiment was conducted in Coronel Pacheco, Minas Gerais, Brazil (Latitude: 21°38’9” S, Longitude: 43°19’9” W), from January to February 2015 (hot season in the southern hemisphere) and lasted 58 days (first 14 days as the adaptation period and the subsequent 44 days as the experimental period). Details on this subject are better described by Kolling et al. (2018).

The data of air temperature (°C), relative air humidity (%), wind speed (km/h), and precipitation (mm/day) during the study were collected at the meteorological station A577 of INMET in Coronel Pacheco, Minas Gerais, Brazil. The mean values (means±SD) of air temperature, relative air humidity, and wind speed were 26.2±4.8 °C, 72.9±15.7%, and 0.6±0.8 km/h, respectively, and cumulative rainfall over the entire experimental period was 211.6 mm.

In a free-stall barn, 32 lactating dairy cows (primiparous and multiparous) in the first third of lactation were blocked by genetic group (16 Holstein cows and 16 crossbred Holstein-Gyr), and then allocated into one of four treatments, each one with four Holstein and four crossbred...
Holstein-Gyr cows; cows were stratified by body weight (BW), days in milk (DIM), parity, and previous milk production.

Treatments were control (CON) – without plant extracts in the diet; oregano extract (OR) – addition of 10 g per cow or 0.056% of commercial oregano added to the diet (dry matter basis); green tea (GT) – addition of 5 g per cow or 0.028% of green tea extract added to the diet; and mixture (MIX) – addition of 10 g per cow or 0.056 g of oregano extract plus 5 g per cow or 0.028% of green tea extract added to the diet. The extracts and doses used in the present study are consistent with the levels recommended by feed additive suppliers; they were also the same used in a previous study, which was conducted with dairy cows in the transition period (Stivanin et al., 2019; Vizzotto et al., 2021).

At the beginning of the experiment, CON cows averaged (± SD): 529.8±51.2 kg of BW, 60.7±22.0 DIM, 28.2±7.5 kg of milk yield, 5.3±2.6 years, and 3.1±1.7 lactations; GT cows averaged 519.7±51.8 kg of BW, 59.3±21.4 DIM, 21.2±3.7 kg of milk yield, 5.7±2.6 years, and 3.6±2.3 lactations; OR cows averaged 526.2±74.6 kg of BW, 55.5±21.3 DIM, 28.2±3.5 kg of milk yield, 5.4±2.2 years, and 3.1±1.5 lactations; MIX cows averaged 514.4±71.5 kg of BW, 53.4±25.0 DIM, 25.0±4.4 kg of milk yield, 5.3±2.2 years, and 3.1±1.9 lactations.

Oregano extract (Orego Stim®, Meriden Animal Health, commercialized by Advet Animal Nutrition, São José dos Campos, SP) contained 80-82% carvacrol, 2.5-3.0% thymol, 3.5-9.0% p-cymene, and 2.0-5.0% Y-terpinene. Green tea extract (commercialized by Seiva Brazilis, São Paulo, SP) had an approximate concentration of 56% (±2.5%) of polyphenols. Extract composition was based on general manufacturer’s information. Quercetins and cafein were determined by high-performance liquid chromatography (HPLC Shimadzu Prominence 20AT module (Kyoto, Japan), while carvacrol and thymol were determined by gas chromatography coupled to ion trap mass spectrometry (Fiori et al., 2013). To supply plant extracts to the cows, they were mixed with 1 kg (as fed) of concentrate, which was top-dressed onto the total mixed ration (TMR). During the 14 days of adaptation period, all cows fed the same diet, without plant extracts.

The TMR was formulated to meet the nutritional requirements for 30 kg of milk with 3.8% of fat (NRC, 2001). Forage to concentrate ratio was 60:40, being the forage composed by corn silage (94%) and Tifton hay (6%). Water was provided in automatic drinkers and animals had continuous access to them. Diet was individually supplied once a day in automatic feeding troughs, at 08:30 h, allowing 5 to 10% of refusals. In each trough, 1 kg of concentrate containing or not the plant extracts was top-dressed on the total mixed diet at 08:30 h. The TMR given to the cows were identical, except for the added plant extracts. The concentrate was formulated with ground corn (43.6 kg/100 kg), soybean meal with 46% of crude protein (47.9 kg/100 kg), mineral salt Guabiphos Lactage Gold® (4.3 kg/100 kg), sodium bicarbonate (1.4 kg/100 kg), limestone (1.1 kg/100 kg), magnesium oxide (0.7 kg/100 kg), ammonium sulfate (0.6 kg/100 kg), and urea (0.4 kg/100 kg) (Table 1).

Daily dry matter intake was evaluated as the difference between the amount of food supplied and the amount of orts left in the trough, using electronic Calan gates (American Calan Inc.; Northwood, NH, USA). Milk production (kg) was daily recorded at the morning and evening milkings using DeLaval milk meter MM25 (DeLaval, Campinas, SP, Brazil).

Body weight and body condition score (BCS) evaluations were performed once a week, after morning milking. An electronic scale was used for weighing, and BCS was evaluated on a scale of 1 to 5, according to Wildman et al. (1982), always by the same researcher.

At days 14, 29, 44, and 58 of the experiment, before the morning feeding, blood samples were taken from the coccygeal vein of each animal in 5-mL Vacutainer tubes (Becton-Dickinson, Rutherford, NJ) containing EDTA anticoagulant. Samples were immediately placed on ice and transported to the laboratory to be processed within 60 min after collection. Leukocyte, eosinophils, segmented neutrophils, lymphocytes, and monocytes counts were analyzed. Analyses were performed according to Lopes et al. (2007) using the automated method (Urit 3000 plus, Medtek, São Paulo, SP).
At days 14, 35, and 56 of the experiment, before the morning feeding, blood samples were taken from the coccygeal vein in 5-mL Vacutainer tubes (Becton-Dickinson, Rutherford, NJ) containing heparin. Immediately after blood collection, plasma and erythrocytes were separated by centrifugation at $1,000 \times g$ during 10 min at $4^\circ$C. Plasma fraction was aliquoted and stored at $-80^\circ$C for further biochemical assays. Platelet and leucocytes fractions were removed and discarded from the intermediate fraction, and the remaining fraction contained the erythrocytes, which were diluted at 1:10 (v/v) with 0.9% commercial saline (ADV FARMA, Juiz de Fora, MG) and centrifuged at $1,000 \times g$ during 10 min at $4^\circ$C, three times; at each step, the supernatant was removed and discarded. At the end of the last centrifugation, the erythrocytes were resuspended in saline solution at a final dilution of 1:10, and then stored at $-80^\circ$C until the biochemical assays described below were performed.

Protein concentration was measured according to Lowry et al. (1951), and data are expressed as mg protein/mL. Reactive oxygen and nitrogen species were measured in erythrocytes (DCFER) and plasma (DCFPLA) using 2',7'-dichlorofluorescein diacetate (DCFH2-DA) according to LeBel et al. (1992). The following parameters were determined in the erythrocytes: activities of enzyme superoxide dismutase (SOD) (Misra and Fridovich, 1972), catalase (CAT) (Aebi, 1984), and glutathione peroxidase (GPx) (Wendel, 1981). Concentration of reduced glutathione (GSH) in erythrocytes was measured according to Browne and Armstrong (1998); and carbonylated protein content (CARBO) was measured in plasma, according to Reznick and Packer (1994) and adapted by Stone et al. (2016) for reading in 96-well microplates. All the experiment results, in absorbance (all methods, except GSH) or fluorescence (for GSH), were measured in a microplate Spectrophotometer Spectramax M5.

All statistical procedures were conducted using SAS® (Statistical Analysis System, version 9.4). The power analysis of the sample size was calculated using the POWER procedure. Data were tested for
normal distribution using PROC UNIVARIATE (Shapiro-Wilk test) and for homogeneity of variances using PROC GLM (Levene’s test and Welch option). Data were analyzed considering the completely randomized block design with four replicates per block. Data collected over time were subjected to repeated measurements ANOVA, with treatment and block as main effects, using PROC MIXED. Block (n = 2 genetic groups), treatment (n = 4), days of evaluations, and their interactions (treatment by day of evaluations, treatment by block, block by day, and the triple interaction) were considered as fixed effects, while animal and the residue were considered as the random effects. Parity, DIM, BW, and milk yield measured before treatments were included in the model as covariates. Covariance structures were tested using the Bayesian information criterion. The hematological variables lymphocytes and eosinophils failed normality test and were logarithmically transformed before being statistically analyzed. The GSH was analyzed by non-parametric statistics using the test of Wilcoxon as it did not show normal distribution even after being transformed.

3. Results

The sample sufficiency was confirmed by a power analysis with the result of 0.9 to 0.99 for all hematological and antioxidant variables. The interactions block by day, treatment by day of evaluation, and the triple interaction were not significant for the variables evaluated (P ≥ 0.05).

There was treatment by block (genetic group) interaction for feed intake (P<0.01) and milk yield (P<0.01). The GT Holstein cows showed increased feed intake and milk yield compared with CON (P<0.05), while OR Holstein-Gyr crossbred cows presented increased feed intake, and GT increased milk yield compared with CON (P<0.05). Compared with CON, cows in MIX had similar (P>0.05) feed intake. Moreover, compared with CON, Holstein cows in MIX had lower milk yield, while Holstein-Gyr crossbred cows had higher milk yield (P<0.05; Table 2).

Compared with CON, GT and OR cows showed decreased eosinophils concentration (P<0.05), and OR cows showed the highest neutrophils concentration and neutrophils to leukocyte ratio (P<0.05; Table 3).

Compared with CON, OR increased CAT activity (P<0.05), while GT increased GSH concentration (P<0.05). Cows in MIX showed reduced CAT activity compared with OR cows (P<0.05), and the lowest concentration of oxidized dichlorofluorescein in the erythrocytes (DCFER) and in plasma (DCFPLA) (P<0.05) and increased eosinophils concentration when compared with GT and OR (P<0.05) cows. Cows in MIX and CON had similar values (P>0.05) of CAT and GSH (Table 3).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment (T)</th>
<th>SEM</th>
<th>P-values for effects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Block (B)</td>
<td>CON</td>
<td>GT</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td></td>
<td>514</td>
<td>533</td>
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<tr>
<td>Body condition score (1 to 5)</td>
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<td>3.2</td>
<td>3.2</td>
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<tr>
<td>Dry matter intake (kg)</td>
<td>Hol</td>
<td>16.8b</td>
<td>18.3a</td>
</tr>
<tr>
<td></td>
<td>HG</td>
<td>17.7b</td>
<td>18.0b</td>
</tr>
<tr>
<td>Milk yield (kg/d)</td>
<td>Hol</td>
<td>25.3b</td>
<td>27.5a</td>
</tr>
<tr>
<td></td>
<td>HG</td>
<td>26.2c</td>
<td>27.4b</td>
</tr>
</tbody>
</table>

1 CON - control; GT - 0.028% diet (dry matter basis) of green tea extract; OR - 0.056% diet (dry matter basis) of oregano extract; MIX - 0.056% diet (dry matter basis) of OR + 0.028% diet (dry matter basis) of GT.

2 Triple interaction was not significant for all variables.
4. Discussion

The present study highlights the effects of oregano and green tea extracts supplied separately or associated on oxidative stress when supplemented in diets of different genetic groups. The main contribution of this study is to evidence that the effects of green tea and oregano extracts on productive indicators (e.g., feed intake and milk yield) were dependent on genetic group and form of supply (separated or associated). Moreover, green tea and oregano consumed separately stimulated some of the main biomarkers with antioxidant activity such as CAT activity and GSH concentration, while when they were supplied in association, they decrease oxidative stress by lowering reactive species in the erythrocytes and in plasma.

Feed intake of Holstein and Holstein-Gyr crossbred cows were distinctly affected by plant extracts. Holstein cows in GT presented the highest feed intake, while Holstein-Gyr crossbred cows in OR consumed more feed. As basal TMR were the same for the four groups, feed intake might have been affected by volatile substances such as carvacrol present in oregano extract or catechins and tannins in green tea. However, most of the published studies do not report significant differences in feed intake in Holstein cows fed diets supplemented with oregano extract and its essential oil carvacrol (Benchaar, 2020). Conversely, when these extracts were given at the beginning of lactation, oregano extract tended to increase feed intake and reduced time spent eating the concentrate compared with control (Stivanin et al., 2019).

The increase in milk production in Holstein cows in GT and OR, as well as the higher milk yield observed in Holstein-Gyr crossbred cows in GT might be due to higher feed intake observed in cows fed green tea, as well as to the higher digestible dry matter intake and lower methane emissions in these groups as reported by Kolling et al. (2018).

It is worth noticing that before the beginning of the trial, Holstein and Holstein-Gyr crossbred cows presented similar averages for milk yield, BW, and parity. However, Holstein cows might have been more challenged by the environment (heat stress) than Holstein-Gyr crossbreds as the trial took place during the hot season (Stumpf et al., 2021), thus, partially explaining the interaction between genetic variation and environmental conditions.

Table 3 - Means and standard error of the means (SEM) of hematological and antioxidant profiles of lactating cows fed CON diet or diet supplied with OR extract, GT extract, or their association

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment (T)</th>
<th>SEM</th>
<th>P-values for effects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
<td>GT</td>
<td>OR</td>
</tr>
<tr>
<td>Leukocytes (µL)</td>
<td>12941</td>
<td>11778</td>
<td>12322</td>
</tr>
<tr>
<td>Eosinophils (µL)</td>
<td>614a</td>
<td>314b</td>
<td>278b</td>
</tr>
<tr>
<td>Lymphocytes (µL)</td>
<td>7599</td>
<td>7575</td>
<td>6738</td>
</tr>
<tr>
<td>Monocytes (µL)</td>
<td>420</td>
<td>370</td>
<td>293</td>
</tr>
<tr>
<td>Neutrophils (µL)</td>
<td>4099ab</td>
<td>3378b</td>
<td>4964a</td>
</tr>
<tr>
<td>Neutrophils/Lymphocytes</td>
<td>0.32b</td>
<td>0.31b</td>
<td>0.41a</td>
</tr>
<tr>
<td>SOD (U/mg)</td>
<td>13.0</td>
<td>14.59</td>
<td>18.28</td>
</tr>
<tr>
<td>CAT (U/mg)</td>
<td>1.1b</td>
<td>1.1b</td>
<td>1.4a</td>
</tr>
<tr>
<td>GPx (U/mg)</td>
<td>2.8</td>
<td>2.4</td>
<td>3.3</td>
</tr>
<tr>
<td>GSH (nmol/mg)</td>
<td>0.9b</td>
<td>1.3a</td>
<td>0.7b</td>
</tr>
<tr>
<td>DCFER (nmol/mg)</td>
<td>8975a</td>
<td>8928a</td>
<td>9629a</td>
</tr>
<tr>
<td>DCFPLA (nmol/mg)</td>
<td>2430a</td>
<td>2145ab</td>
<td>2267ab</td>
</tr>
<tr>
<td>CARBO (nmol/mg)</td>
<td>2.8</td>
<td>3.2</td>
<td>3.3</td>
</tr>
</tbody>
</table>

DCFER - oxidation of dichlorofluorescein in the erythrocytes; SOD - superoxide dismutase; CAT - catalase; GPx - glutathione peroxidase; GSH - reduced glutathione; DCFPLA - oxidation of dichlorofluorescein in plasma; CARBO - carbonyl; B - block (genetic groups); D - day; T×B - interaction treatment by block; T×D - treatment by day interaction; B×D - block by day interaction.

1CON - control; GT - 0.028% diet (dry matter basis) of green tea extract; OR - 0.056% diet (dry matter basis) of oregano extract; MIX - 0.056% diet (dry matter basis) of OR + 0.028% diet (dry matter basis) of GT.

2Triple interaction was no significant for all variables.
group and treatment. Borges et al. (2015) highlighted significant differences in the metabolism and milk production between Holstein and Holstein-Gyr cows.

The greater neutrophils concentration and neutrophils to lymphocytes ratio observed in OR were probably linked to the higher somatic cell count (SCC) of cows in the OR group ($P<0.05$) compared with cows in CON (1,406,000±199,000 and 596,000±189,000 cells/mL of milk, respectively), as reported by Kolling et al. (2018), and these results should be considered with caution. Normal neutrophils to lymphocytes ratio in adult bovine is 1:2 or 0.5 (Roland et al., 2014). Also, the lower eosinophils in OR and GT might be related to SCC, as Holtenius et al. (2004) found lower eosinophils in herds with high compared with herds with low mastitis incidence.

Lactating cows’ metabolism produces large amounts of free radicals that might cause cell damage with loss of biological function. Oxygen metabolism generates reactive oxygen species that can be determined with the oxidation of the dichlorofluorescein (Lebel et al., 1992). The lower values of oxidized dichlorofluorescein in the erythrocytes and in plasma of MIX cows show the effectiveness of oregano and green tea extracts in decreasing the levels of oxidants, probably due to the stimulus to the antioxidant capacity. Vizzotto et al. (2021) reported lower concentration for oxidized dichlorofluorescein in the erythrocytes and in plasma of dairy cows supplemented with similar dose of oregano extract. However, in the mentioned study, the authors did not supply oregano associated with green tea extract.

Endogenous antioxidant system, especially the enzymes SOD, CAT, and GPx as well as the non-enzymatic antioxidant compound GSH (Aristatile et al., 2015) play an important role in protecting cells against oxidative damage. Catechins and carvacrol stimulated the endogenous antioxidant system and reduced the effects of free radical production on cells and tissues in calves (de Paris et al., 2020) and in Jersey cows during the transition period (Vizzotto et al., 2021). These effects were partially confirmed in the present study, as greater activity of CAT and higher concentration of GSH were observed in OR and GT groups, respectively. Against our expectation, oregano and green tea extracts supplied together did not enhance enzyme activity of the antioxidant system or concentration of antioxidant compounds, except that they decreased the concentration of oxidized compounds (DCF) in plasma and erythrocytes. To our knowledge, there are no previous studies about the effects of oregano and green tea extracts fed associated on redox status. On the other hand, it is noticeable the increasing number of studies about supplying blends of essential oils to ruminants and measuring their effects on performance, rumen microbes as well as immune and health indicators, reporting synergism, additivity, and antagonism between essential oils (El-Azrak et al., 2021).

This study evidenced the occurrence of antagonistic effects of plant extracts associated (MIX) on some enzyme activity and antioxidant compounds (CAT and GSH), probably due to competition for receptor sites (Friedman, 2014) or counteracted effects (Benchaar and Greathead, 2011), as well as some additive effects of plant extracts on oxidized compounds (diminishing DCF in plasma and erythrocytes).

5. Conclusions

Oregano and green tea extracts when given separately or associated to lactating dairy cows present distinct effects on feed intake, milk yield, and on antioxidant systems. Plant extracts distinctly affect the performance depending on genetic group. Plant extracts do not expressively affect hematological variables. Plant extracts given separately improve antioxidant enzymes and GSH, but when given associated, decrease reactive oxygen and nitrogen species.

Conflict of Interest

The authors declare no conflict of interest.
Author Contributions


Acknowledgments

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