The effect of oral or respiratory exposure to limonene on goat kid performance and meat quality

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Short title: limonene effect on goat meat quality

Abstract

Pasture contains a lot of terpenes, able to pass throughout oral assumption and inhalation into meat. The aim of this paper was to verify if limonene accumulates in meat after oral or respiratory exposure and to assess its effects on animal oxidative status and meat quality. Thirty-six goat kids were involved and assigned for 1 week to three treatment groups: control group (CG); an oral group (OG), where limonene was administered directly into the mouth (drenched); and a respiratory group (RG), in which limonene was administered via inhalation. The meat limonene concentration for the OG was the highest ($P < 0.01$), although RG showed the highest rate of transfer ($P < 0.001$). Limonene gives the possibility to delay lipid oxidative processes, reducing discoloration and rancidity in meat. However, the respiration route seems to be able to guarantee a greater limonene transfer into meat compared to the oral one.

Keywords: terpenes, oxidative profile, terpenes' transfer to meat

1. Introduction

The management of pasture for the production of ruminant meat is an important factor that affects consumer purchasing behaviour and end-product value. Extensive pasture systems support human health, sustainable environments, and a high level of animal welfare, when compared to intensive production systems (Karoui & De Baerdemaeker, 2007). Plants, fresh forages, and vegetable by-products are rich in bioactive compounds, such as terpenes (also known as terpenoids or isoprenoids) (Mewalal et al., 2017). They are secondary metabolites naturally produced by plants and are involved in defence mechanisms against parasites, insects, and herbivores of the animal kingdom (Gershenzon & Dudareva, 2007). Permanent, diversified grasslands have a rich and unique terpene fingerprint (Serrano, Cornu, Kondjoyan, Agabriel, & Micol, 2011), and diet is considered to be the main transfer route of terpenes into animal tissues (Poulopoulou, Evangelos, Styliani, Theofilakto, & Ioannis, 2020).

Limonene, is a monocyclic monoterpene and one of the most abundant terpenes found in nature (Arrieta, López, Ferrándiz, & Peltzer, 2013). For this reason, it was selected for use in this study. Specifically, limonene is a proven biomarker that enables both the respiratory and the oral routes of exposure to be investigated. It is noted that the oral/dietary route is likely to be the main pathway for accumulation in the tissue, with the contributions from the inhalation of terpenes likely to be comparatively lesser.

Terpenes, when included in animal feed, have antioxidant activities whereby they can scavenge free radicals, delay oxidative phenomena, and in doing so, modify the sensory and organoleptic profile of meat and animal products (Brewer, 2011; Manuelian et al., 2020). Based on these actions, there is an imperative to determine the effect of terpenes on meat quality and shelf-life. In fact, recent studies show that natural substances with declared antioxidant activity could be used to reduce oxidative stress in fattening animals (Kafantaris et al., 2017; Maggiolino et al., 2021a; Salzano et al., 2021), improve product stability during aging (Maggiolino et al., 2020b; Maggiolino et al., 2021b; Quiñones et al., 2019; Sgarro et al., 2022), and reduce greenhouse emissions without compromising

58 animal productivity (Maggiolino et al., 2019a). These outcomes may result from the impact of feed
59 antioxidant activities on the rumen and gut microbiota *viz.* growth rate and population (Ferrara et al.,
60 2021; Tagliapietra, Cattani, Hansen, Bittante, & Schiavon, 2013); as well as the oxidative stability
61 and sensory quality of meat and other animal products, that occurs with the accumulation of these
62 compounds in the tissues (Brewer, 2011; Natrella, Gambacorta, De Palo, Maggiolino, & Faccia,
63 2020). Meat is a perishable product that begins to deteriorate from the initial conversion of the muscle
64 to meat (Cunha et al., 2018). The obvious characteristics of perished (spoilt) meat are discoloration,
65 texture changes, development of off-flavors and malodors, loss of nutritive value, and production of
66 undesirable secondary compounds(Domínguez et al., 2020; Domínguez et al., 2019; Gómez &
67 Lorenzo, 2012). Methods to extend the shelf-life of meat and delay its spoilage would therefore are
68 advantageous to capturing market share and assuring consumer satisfaction. Consequently, the
69 untested capacity for limonene to change the physicochemical and sensory properties of meat merits
70 investigation. For this study we utilised goat kids because they are usually reared on pasture and,
71 therefore, together with sheep, they represent the animals that could benefit most from the effects of
72 terpenes on meat.
73 Therefore, this study aimed to i) verify if limonene accumulates in meat after oral or respiratory
74 exposure; ii) assess limonene's effects on animal oxidative status; and iii) to evaluate the effects of
75 limonene on meat physical and sensory properties.

76 **2. Materials and Methods**

77 *2.1. Animal management and experimental design*

78 All animals and procedures used this study were approved by the Ethical Committee for
79 Animal Welfare of Animals employed in scientific research of the Department of Veterinary
80 Medicine of the University of Bari (Approval n. 08/2020).

81 Experimental Saanen goat kids (n = 36) were randomly allocated to one of three
82 chronologically separate replicates (each replicate included 12 kids) (Table 1). Kids within each

replicate were then randomly allocated to one of three treatment groups (each treatment group included 4 kids) (Fig. 1). Individual treatment groups were held in a sealed chamber, positioned apart from each other so to avoid the potential cross-contamination of volatile compounds. The three treatment groups included a control group (CG), with no exposure to limonene; an oral group (OG), where limonene was administered directly into the mouth (drenched); and a respiratory group (RG), in which limonene was administered via inhalation. For each replicate, kids were held for a 2 days adaptation period prior to the commencement of the 7 d treatment period. Throughout the study, kids were fed, *ad libitum*, a basal diet of oat hay (18.5% of crude protein on a dry matter basis, DM) and a commercial pelleted concentrated mixture (20.3% crude protein DM, 10.8% crude fiber DM, 2.7% crude fat DM, and 7.2% ash DM). The gross composition of feed samples were determined in accordance to standard procedures for DM (AOAC, 2005) (method 930.15), ash (AOAC, 2005) (method 942.05), the Soxhlet extraction method for fat content (AOAC, 2005) (Method 991.36), and the Kjeldahl N × 6.25 method for crude protein (AOAC, 2005) (Method 968.06).

2.2. Limonene administration protocols

For both treatments (RG and OG), the limonene was obtained by the cold and vacuum distillation of grapefruit peels (Exentiae srl, Catania, Italy), which resulted in the product having an oil content of > 98.9% (determined by SPME-GC/MS). For the OG treatment, 0.5 mL of the limonene-containing essential oil was administered *in bolus* with a syringe, directly in the mouth of each animal (drenched). The administration was performed twice daily (each 12 h) to result in a total of 14 administrations (7 mL) during the treatment period (Fig. 1). For the RG treatment, a diluted mixture of essential oil was prepared (10% concentration) and kept in a sealed container until use, to minimize the risks of evaporation and contamination. Then, 300 mL of the diluted mixture was placed in an electric diffuser, which was turned on 6 h before the start of the treatment period, to ensure the presence in the air of a saturated and constant quantity of limonene and its clear smell. The RG

treatment was constantly exposed to the limonene-saturated atmosphere, for a total of 164.5 h which included the 30 min daily exception, when the chamber was refreshed and measurements recorded.

2.3. Determination of limonene concentration in the air of the respiratory treatment chamber

Atmospheric limonene levels inside the RG treatment chamber were measured at different time points, across the treatment period (at 24, 72 and 120 h), and the results used to calculate the respiratory route's transfer efficiency. Specifically, atmosphere limonene levels were measured and monitored by applying the NIOSH 1552 method (Ashley & O'Connor, 2017). The chemical analysis was carried out by GC/FID, preceded by a desorption phase of the solid sorbent tube with Carbon Disulfide (CS2) solvent. The active sampling of the atmosphere was performed using a pump unit equipped with flexible tubing (ACTI-VOC low-flow pump, Markes International Ltd, UK), set at a flow rate of 0.01 L/min, and carried out on solid sorbent tubes (SKC Ltd., UK - sorbent coating Anasorb CSC/Coconut Charcoal, glass tube, 7 cm long, 6 mm o.d., 4 mm i.d., flame-sealed ends, 2 sections coconut shell charcoal - sorbent 100 mg/50 mg) that were suitable for CS2 solvent desorption. After exposure to the atmosphere of the RG treatment chamber and active sampling for 60 min, individual glass sample vials were sealed, stored under refrigeration (2 °C), and transported to the chemical laboratory for analysis.

Before the quantification of limonene using the GC/FID technique, each sampling tube was eluted by chemical desorption with 1 mL of CS2 - chromatographic grade and kept 30 min with occasional agitation. The analysis was carried out using a chemical desorber coupled with a GC equipped with an FID detector (Agilent GC System 7820A, Agilent Technologies Inc., USA). The analytical operating conditions were injection volume, 1 µL; injection temperature, 250 °C; detector temperature, 300 °C; detector column, 35 °C to 135 °C at 10 °C/min; carrier gas, He-30 mL/min; column, Stabilwax - 30 m, 0.53-mm ID, 3 µm film. To quantify the samples, the calibration curves were prepared by injecting the standard solutions of Limonene in CS2, as described in the NIOSH 1552 method (Ashley & O'Connor, 2017). The concentration of limonene was derived the ratio of the

mass of limonene (µg), corrected for Desorption Efficiency (DE), and the atmosphere volume sampled, V (L). The mass of limonene was assessed by the equation between the mass of analyte found in the sample front (Wf) and back (Wb) sorbent sections, and in the average media blank front (Bf) and back (Bb) sorbent sections (Wf+Wb-Bf-Bb).

2.4. Estimation of the level of orally and respiratory limonene administered

The amount of limonene administered to the OG treated animals was calculated to be 6,923 mg per kid, across the experimental period. This calculation multiples the total number of limonene administrations (14) by the limonene concentration of 989 mg per each ml administered of the oral treatment (1 mL of an essential oil with minimum limonene purity level of 98.9%).

The amount of limonene administered to the RG treated animals was estimated by first considering the limonene concentration measured, during the experimental period, in the atmosphere of the RG treatment chamber. By the analytical method described in the previous paragraph, the value of this concentration was found to be 0.112 mg/m^3 (= 0.112 ng/mL) on average (± 0.006), which is well above the lowest odour threshold values reported in the literature for limonene (Fuller, Steltenkamp, & Tisserand, 1964). In addition, it was considered that, for a goat kid, the tidal volume is 15 mL/kg of live weight (Boesch et al., 2009), which implies that the mean volume of air that moved in or out of the lungs of each of our experimental kids (weighing 26.4 kg on average) with each breath was about 366 mL. As the normal respiration rate for a goat kid at rest is 40 breaths per minute (Khalek & Khalifa, 2004), one can approximate that the amount of limonene plausibly inhaled by each goat kid in one minute of treatment was equal to 1639.7 ng/min [= 0.112 (ng) * 366 * 40]. Moreover, assuming that in goat kids, like in humans (Falk-Filipsson, Löf, Hagberg, Hjelm, & Wang, 1993), the relative pulmonary uptake of limonene after short-term inhalation is about 70% of the amount supplied, the amount of limonene actually retained in the lungs of each goat kid in one minute of treatment was estimated to be 1147.78 ng. Accordingly, considering the time exposure of the

156 experimental trial (164.5 h), 11.36 mg was the amount of limonene inhaled and retained by each goat
157 kid.

158 *2.5. Blood sampling and analysis*

159 Initial blood samples were collected from each kid (Day 0) and repeat samples collected on
160 the morning of Days 2, 4 and 7, of the experimental period. Blood was collected from the jugular
161 vein into sterile Vacutainer ® tubes (9 mL, lithium-heparin, Becton Dickinson and Co., Oxford, UK).
162 Sample tubes were immediately centrifuged (on site) and the plasma decanted and stored at -20 °C.
163 Plasma TBARS concentrations were determined fluorometrically according to Gondim,
164 Zoppi, dos Reis Silveira, Pereira-da-Silva, and Vaz de Macedo (2009). Plasma reactive carbonyl
165 derivative (RCD) levels were determined according to Faure and Lafond (1995), using the carbonyl
166 reagent 2,4-Dinitrophenylhydrazine (DNPH). Plasma hydroperoxide concentrations were determined
167 using the method of Maggiolino et al. (2021a). Non esterified fatty acid (NEFA) analysis was
168 performed using a Randox kit (Randox Laboratories, UK) as described by De Palo et al. (2018a).
169 *2.6.Meat sampling and analysis*

170 At the end of the treatment period, the kids were transported to a commercial abattoir, located
171 approximately 25 km or < 45 min away, where they were slaughtered at a European Community
172 approved abattoir, in compliance with European Community laws on Animal Welfare in Transport
173 (1/2005EC) and the European Community regulation on Animal Welfare for the Slaughter of
174 Commercial Animals (1099/2009EC).

175 Within 24 h post-slaughter, the left m. *longissimus thoracis et lumborum* (LTL) was removed
176 from each carcass. Initial samples of 100 g were removed, wrapped in aluminium foil, vacuum
177 packaged, and then held frozen (-20 °C) until the analysis for intramuscular fat and limonene
178 concentrations. The remaining LTL were divided in three equal portions, that were individually
179 vacuum packaged, and randomly allocated one of three ageing times (1, 3 or 7 days) at 2-4 °C.

180 *2.6.1. Determination of intramuscular fat and meat limonene concentration*

181 Muscle limonene concentration was determined as per the method of Serrano et al. (2007).
182 This utilised a Triplus RSH autosampler (ThermoFisher Scientific, Rodano, Italy) for extraction at
183 37 °C for 15 min using a divinylbenzene/carboxen/polydimethylsiloxane 50/30 μm SPME fiber
184 assembly (Supelco, USA) after 10 min of pre-equilibration phase. The fiber was desorbed at 220 °C
185 for 2 min in the injection port of the GC, operating in the splitless mode. The GC-MS apparatus used
186 was a Trace1300 chromatograph equipped with an ISQ Series 3.2 SP1 mass spectrometer
187 (ThermoFisher Scientific, Rodano, Italy). The compounds were separated on a VFWAX MS thermo
188 capillary column (60 m x 0.25 mm i.d., 0.25 μm film thickness), under the following conditions:
189 injection port temperature, 220 °C; oven temperatures, 40 °C for 0.1 min then 4 °C min^{-1} to 140 °C,
190 10 °C min^{-1} to 220 °C and a final isothermal for 7.5 min. Data were acquired using a Single Ion
191 Monitoring mode (SIM), the recorded ions were m/z = 68, 93, 136; using 136 as qualifier and all
192 three as quantifier. A linear calibration curve (r^2 = 0.9993) was obtained using limonene standard
193 solutions (Exentiae s.r.l., Messina, Italy) at concentrations ranging from $5.61 \cdot 10^3$ to $5.61 \cdot 10^{-8}$ μg L^{-1}
194 to quantify the molecule. The formula used for quantification was:

195 $$x = \frac{y + 0.1652}{0.4701}$$

196 Where *x* is limonene concentration; *y* is the limonene area of the chromatogram peak; 0.1652 is the
197 *Y*-intercept; and 0.4701 is the gradient of the line.

198 Limonene concentrations were calculated using the formula LMC = IMFL/100 × IMF, where
199 LMC is the limonene meat concentration; IMLC is the intramuscular fat limonene concentration; and
200 IMF is the intramuscular fat content expressed as %. The IMF and meat limonene concentrations for
201 samples from the OG and RG treatments were calculated minus the limonene concentration registered
202 in the IMF (0.79 ± 0.02 mg/kg) and meat (0.11 mg/100g of meat) of CG.

203 *2.6.2 Chemical composition*

For samples from each ageing period, the epimysium was first removed, the denuded sample triturated in a domestic blender, and chemical composition (moisture, protein content, intramuscular fat and ash) determined as described by (Maggiolino et al., 2019b).

2.6.3. pH determination

The pH of the aged meat samples was determined using a digital portable meat pH-meter (Hanna Instruments, Eibar, Spain) with a glass electrode attachment, calibrated using pH 4 and 7 standard solutions (Crison, Lainate, Italy) with adjustments made muscle temperature – as per De Palo, Maggiolino, Tateo, and Centoducati (2014).

2.6.4. Colorimetric analysis

For color determination muscle sample surfaces were fresh cut and exposed to air for ~ 20 min. CIE colorimetric coordinates (L*, a*, and b*) were determined as described by Gálvez et al. (2020), using a Minolta CR-300 colorimeter (Minolta Camera Co., Osaka, Japan), set to Illuminant D-65, standard observer 10°, and with an aperture of 8 mm diameter. Triplicate measures were made at different sites on each sample. This was repeated three times, whereby the colorimeter was rotated 90° between repeats, to result in a total of 9 measurements per sample (De Palo et al., 2017).

2.6.5. Water holding capacity (WHC), cooking loss, thawing losses and acid hematin

Water holding capacity was determined as described by De Palo, Tateo, Maggiolino, and Centoducati (2014). This involved the centrifugation, of 0.3 g samples at 30 000 × *g*, for 1 h, and the calculation of centrifugation loss, being the difference in weight before and after centrifugation.

Cooking loss was determined using cubic meat samples (cross-sectional area: 1.5 cm^2) that were weighed and then cooked in plastic bags in a water bath set to 80 °C, until they achieved an internal temperature of 75 °C, measured by a copper constantin fine-wire thermocouple fixed in the geometrical center of the sample (Model 5SC-TT-T-30–36; Omega Engineering Inc., Stamford, CT, USA) (De Palo et al. (2018b). Cooked samples were cooled, dried, and reweighed for the calculation of cooking loss as a percentage. Post-thawing losses were calculated as the percentage change in sample weight before freezing and after thawing.

A sample of 5 g was used to determine the acid hematin concentration according the method of Hornsey (1956). All procedures were conducted in darkness conditions. Briefly, after trimming off the fat tissue, it was minced and mixed with 20 mL of acetone and 1 ml of water. After, 2 ml of HCl at 7% were added and samples remained at darkness conditions for 1h. After, samples were centrifuged at 4000 rpm for 10 minutes. A solution of 80% acetone and 20% water was used as blank. The surnatant obtained and the blank were red at absorbance of 640 nm and values multiplied by the factor 680 to give the concentration of total pigments in the meat as ppm of haematin.

2.6.6. Thiobarbituric Acid Reactive Substances (TBARS), Hydroperoxides, Protein Carbonyls Analyses and NEFA

The TBARS were determined as per Tateo et al. (2020). Briefly, 5 g samples were first homogenized with 15 mL deionized distilled water and then a 1 mL aliquot of homogenate was transferred to a glass tube with 0.05 mL of butylated hydroxytoluene (7.2% in ethanol) and 1.95 mL of thiobarbituric acid (TBA)/trichloroacetic acid (TCA)/HCl (0.375% TBA, 15% TCA, and 0.25 N HCl). After incubation (90 °C for 15 min) and refrigeration, samples were centrifuged (2000 × *g* × 15 min) and the supernatant absorbance at 531 nm was corrected against a blank containing 2 mL of TBA/TCA/HCl solution in 1 mL of distilled water. Sample TBARS concentrations were calculated against a standard curve constructed with 1,1,3,3-tetramethoxypropane, and reported as mg of malondialdehyde (MDA) per kg of meat.

Hydroperoxide quantification was performed according to Maggiolino et al. (2020a). Briefly, 2 mL of homogenate (prepared for TBARS determination) were combined with 4 mL of CH_3OH and 2 mL of $CHCl_3$. Once vortexed, samples were combined with 2 mL of $CHCl_3$ and 1.6 mL of 0.9% NaCl, agitated and then centrifuged. The lipid extract (2 mL) was sampled from the lower chloroform phase and then processed with 1 mL of $CH_3COOH/CHCl_3$ and 50 µL of KI (1.2 g/L in 1 mL distilled water). Samples were stored for 5 min in a dark room, vortexed with 3 mL of 0.5% of CH_3COOCd, and then centrifuged at 4500 × g for 10 min at 40 °C. Absorbance was measured at 353 nm and the results corrected against a sample blank.

Protein carbonyls determination was performed as per Maggiolino et al. (2020a). Briefly, 2 g of meat were homogenized in 20 mL of 0.15 M KCl for 2 min. Two 50 µL aliquots were then combined with 1 mL 10% TCA and centrifuged (1200 × *g* for 3 min at 4 °C). The samples were incubated for 1 h at room temperature (15 to 30 °C) before the addition of 1 mL of 10% TCA. The samples were vortexed for 30 s and centrifuged 3 times at 1200 × *g* for 3 min at 4 °C, so that the supernatant could be removed. The pellet was washed with 1 mL of ethanol:ethyl acetate (1:1), shaken, centrifuged 3 times at 1200 × *g* for 3 min at 4 °C, and the supernatant again removed. The pellet was dissolved in 1 mL of 20 mM sodium phosphate 6 M guanidine hydrochloride buffer and the sample, shaken, and then centrifuged at 1200 × *g* for 3 min at 4 °C. Carbonyl concentration was calculated on the DNPH treated sample at 360 nm with a Beckman Coulter DU800 (Beckman Instruments Inc., Brea, CA, USA) and expressed as nmol carbonyl per mg protein.

2.6.7. Superoxide Dismutase, Catalase and Glutathione Peroxidase Activity Evaluation

Duplicate 400 mg muscle samples were homogenized with 4 mL saline, centrifuged at 7000 × *g* for 20 min at 4°C, and the supernatant collected for analysis. Superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and glutathione peroxidase (GPx, EC1.11.1.9.) activity determined as per Maggiolino et al. (2020a). SOD activity was quantified by its ability to inhibit the autoxidation of epinephrine, with 1 U of SOD defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50%. CAT activity was measured following the decrease in absorbance of H_2O_2 at 240 nm (e = 40 M-1 cm^{-1}), with 1 U of CAT defined as the amount of enzyme that is required to degrade 1 µmol of H_2O_2 in 1 min. The GPx activity was measured according to the rate of GSH oxidation by tert-butyl hydroperoxide, catalyzed by GPx.

2.7 Sensory analysis

A duo-trio ortho-olfactive assessment (O'Mahony, 1990) of samples was performed using 24 trained assessors to perform qualitative discrimination testing. The offered samples compared the OG and RG samples to CG samples. Each sample was identified by a four-digit code. The assessors were

asked to record whether there was any difference perceptible between the CG sample and the other samples (OG and RG). The assessors who detected a difference were then asked to smell the sample pairs and describe the reason for the perceived difference.

2.8 Statistical analysis.

According to the study design, the smallest experimental unit was the chamber in which the animals were housed.

The meat chemical, rheological, colorimetric, and oxidative parameters were subjected to analysis of variance (ANOVA) according to General Linear Model (GLM) procedure as reported by the following model:

$$Y_{ijk} = \mu + \alpha_i + T_j + A_k + (T \times A)_{jk} + \varepsilon_{ijk},$$

where Y_{ijk} represents the meat dependent variables (chemical composition, rheological parameters, oxidative profile and antioxidant ensymes), μ is the overall mean; α_i is the i^{th} block random effect ($i = 1,\ldots3$), T_j is the effect of the j^{th} administration treatment ($j = 1,\ldots3$), A_k is the k^{th} aging time effect ($k = 1,\ldots3$), $(T \times A)_{jk}$ is the binary interaction effect of jk^{th} ($1,\ldots9$) treatment × aging time, and ε_{ijk} is the error term. A Tukey test was applied to evaluate the differences among means when the effect of time or the binary interaction of treatment × time was significant.

Liver enzyme activities and limonene concentration data were subjected to analysis by ANOVA according to the GLM procedure as reported the following model:

$$Y_{ij} = \mu + \alpha_i + T_j + \varepsilon_{ij},$$

where Y_{ij} represents the liver enzyme activities (variables), μ is the overall mean; α_i is the i^{th} block random effect ($i = 1,\ldots3$), and ε_{ij} is the error term. Limonene concentration data, without control group, were subjected to this same model where T_j was the effect of the j^{th} administration treatment ($j = 1, 2$).

Plasma enzymes profile data were subjected to analysis by ANOVA according to GLM procedure as reported by the following model:

306 $$Y_{ijk} = \mu + \alpha_i + T_j + A_k + (T \times A)_{jk} + \varepsilon_{ijk},$$

307 where Y_{ijk} represents the plasma enzymes, μ is the overall mean; α_i is the i^{th} block random
308 effect ($i = 1,\ldots3$), T_j is the effect of the j^{th} oral administration treatment ($j = 1,\ldots3$), A_k is the k^{th} time
309 effect ($k = 1,\ldots4$), $(T \times A)_{jk}$ is the binary interaction effect of jk^{th} ($1,\ldots12$) treatment × time, and ε_{ijk}
310 is the error term. A Tukey test was applied to evaluate the differences among means when the effect
311 of time or the binary interaction of treatment × time was significant.

312 Significance was set at $P < 0.05$ and the results were reported as the mean and standard error
313 of the mean. All of the analysis were performed using SAS software (SAS, 2011).

314 Data from the duo-trio ortho-olfactive test was analysed as per the Guidelines for Sensory
315 Analysis (Appendix A), according to the Fisher test (Carpenter, Lyon, & Hasdell, 2000).

316 **3. Results**

317 The meat limonene concentration for the OG treated animals was higher than was observed
318 for the other groups ($P < 0.01$) (Fig. 2a). The rate of limonene transfer was higher for the RG
319 treatment, compared to the OG treatment ($P < 0.001$) (Fig. 2b).

320 There were no significant first or second order effects of group and aging time on the tested
321 chemical parameters of the meat ($P > 0.05$) (Table 2).

322 Meat pH was observed to decrease, in all the treatment groups, from the initial levels at Day
323 0 ($P < 0.001$) (Table 3). L* increased ($P = 0.002$) across the experimental period, irrespective of
324 treatment groups. The a* was unchanged by treatment group, experimental period, or their two-way
325 interaction ($P > 0.05$). Sample b* values decreased across the experimental period ($P = 0.007$), and
326 after 7 days the OG treatment b* values were higher than observed for the other groups ($P = 0.002$).
327 The WHC of all samples was lowest at Day 4 of aging ($P = 0.002$). There were no significant
328 treatment group, experimental period or interaction effects on sample thaw losses, concentration, or
329 cooking loss.

330 Fig. 3 and 4 show the oxidative profile and antioxidant enzymes results. The TBARS
331 concentration increased during aging in all experimental groups ($P < 0.01$), with lower values after 4
332 and 7 days in OG meat compared to other groups ($P < 0.01$). The SOD activity decreased during
333 aging in all experimental groups ($P < 0.01$) showing ever higher values in OG meat ($P < 0.01$). Also
334 Cat and GSPx activity decreased in all groups, but after 7 days ($P < 0.01$), the CAT values registered
335 at 0 and 7 aging days in the OG group were higher compared to the other groups ($P < 0.01$).

336 Plasma TBARS concentrations were higher for the OG treatment than was observed for the
337 RG treatment ($P = 0.005$) and concentrations were shown, on average, to decline as the experimental
338 period increased ($P = 0.040$) (Table 4). No difference in oxidation metabolites was observed due to
339 the method of limonene administration or aging time ($P > 0.05$).

340 Liver enzymes activity is reported in Table 5. The OG animals showed the highest SOD, CAT
341 and GSPx activity ($P < 0.01$). Moreover, the RG animals showed higher CAT ($P < 0.01$) and SOD
342 ($P < 0.05$) activity than the CG ones.

343 Fig. 5 shows that plasma NEFA concentrations increased in OG animals after 2 days,
344 achieving higher values than was observed for the other treatment groups, before then decreasing
345 with additional exposure to limonene ($P < 0.05$).

346 The sensory analysis results performed by the duo-trio test are reported in Table 6. On the
347 basis of this analysis, 20.7 of 24 panellists were found to correctly identify OG and RG from CG
348 samples. After Day 4, 20.3 panellists identified the OG samples ($P < 0.01$) and 19.3 the RG samples
349 ($P < 0.05$). Moreover, at Day 7, 19 panellists recognised CG and RG samples ($P < 0.05$).

350 **4. Discussion**

351 It is particularly interesting to observe that although a greater concentration of limonene was found
352 in the meat of OG treated animals, a greater ratio of limonene administered/concentration was found
353 in meat from RG treated animals. The OG goat kids received a total amount of 6,923 mg of limonene,

but by contrast the RG animals ingested an estimated 11.56 mg of limonene. So, the OG animals received ~ 600 × the limonene received by RG animals. These results suggest that the higher amount of limonene ingested by the OG kids contributed to their meat having a higher limonene concentration. However, if we consider the ratio of limonene administered to that found in the IMF and meat, it seems that the respiratory route of exposure facilitated greater limonene accumulation in the IMF, and consequently in the meat – indeed, more limonene was found in the RG tissue samples than for the OG. It must be remembered that limonene is one of the most represented monoterpenes in pasture and hay, and its presence was also found in CG meat. However, several authors have suggested that, in general, terpene accumulation in tissues is very weak, but that differences in results obtained can be ascribed to multiple metabolism patterns depending on their chemical structure (monoterpenes are more extensively eliminated than sesquiterpenes) (Serrano et al., 2007), to the adipose tissue considered, the species, breed and precocity of the animals, as well as to animal management and experimental design (Prache, Cornu, Berdagué, & Priolo, 2005). All these factors and their interaction may have resulted in the multiple and conflicting findings that are reported in the literature.

Terpenes from forages eaten by ruminants have been found in both milk (Poulopoulou, Zoidis, Massouras, & Hadjigeorgiou, 2012) and meat (Larick et al., 1987), with their transfer into milk occurring rapidly after ingestion (Vialloninsta et al., 2000). These substances are lipophilic (Lu, Chiang, Huang, & Li, 2014), so they have been found in different fatty tissue deposits such as perirenal, subcaudal, and intramuscular (Priolo et al., 2004; Sebastian, Viallon-Fernandez, Berge, & Berdague, 2003; Young, Stagsted, Jensen, Karlsson, & Henckel, 2003). The results of the current study confirm these deposits, with detection of limonene in the IMF. It must be remembered that this experiment simulates a short-term treatment period (only 7 days) of terpene administration by two different ways, and that we considered only meat, consequently, intramuscular fat limonene accumulation. In fact, according to past research, a preferential accumulation of terpenes was observed in perirenal and intraperitoneal fat, when compared to intramuscular and subcutaneous fat

deposits (Serrano et al., 2007). Overall, these findings prove that oral and respiratory routes of exposure both ensure a rapid transfer (measurable in days) of the monoterpene limonene into goat kid meat. In addition, it was found that the respiratory route of exposure is more efficient, resulting in limonene accumulation, in the meat, more so than the equivalent amount of limonene administered orally. At the detected concentrations, limonene administration and or the method of administration did not affect the chemical composition, of the meat. Similarly, the administration of other substances with an antioxidant potential, that are likewise derived from aromatic plant by-products and/or essential oil, have been shown to have no effect on the chemical composition of meat from kids (Cimmino et al., 2018; Salzano et al., 2021; Smeti et al., 2021) or lambs (Maggiolino et al., 2021a; Yagoubi et al., 2018a; Yagoubi et al., 2018b).

Meat acidity and pH is affected by multiple factors, both pre- and *post-mortem*, that are able to influence glycogen synthesis and glycogenolytic pathways in the muscle (De Palo et al., 2016; Maggiolino et al., 2021a; Pastsart, De Boever, Claeys, & De Smet, 2013) and in doing so, impact on meat quality (Yakan et al., 2016). Limonene administration or method of administration did not affect meat pH after slaughtering. The pH values observed were similar to those reported by investigations of small ruminant meat (Quiñones et al., 2019; Smeti et al., 2021; Yakan et al., 2016). There was a drop in pH values during aging in all experimental groups, with no difference in the decline trend. It is well known that ultimate pH values and its decline can affect some rheological parameters such as WHC (De Palo, Maggiolino, Centoducati, & Tateo, 2013; Maggiolino et al., 2021b; Revilla, Plaza, & Palacios, 2021), so the nil effect of the limonene administration on the rate of pH decline is reflected in the nil effect on WHC. A faster pH decline is associated to lower WHC values (Frylinck, Strydom, Webb, & du Toit, 2013). However, WHC decreased only in the control group during aging, although no differences among experimental groups were observed. Despite the absence of variations in pH, the WHC can be influenced by the action of the antioxidant itself (Karami, Alimon, Sazili, Goh, & Ivan, 2011; Yakan et al., 2016). In fact, these substances tend to maintain a greater WHC, which could also result in a greater juiciness of the meat.

406 Color represents the sensorial property that consumers most associate with a meat product's
407 freshness (Lobo et al., 2020), and therefore, it affects consumer's perception and willingness to
408 purchase (Maggiolino et al., 2021a; Maggiolino et al., 2020b). In all treatment groups, lightness
409 increased with aging period, showing no apparent differences between limonene exposure routes or
410 control goat kids. The increase in L* values over time is linked to the cell membrane denaturation in
411 muscle myofibrils that allows the passage of water from the intracellular space to the extracellular
412 one (Mortensen, Andersen, Engelsen, & Bertram, 2006; Tateo, De Palo, Maggiolino, & Centoducati,
413 2013). Differently, redness and yellowness decreased in all treatment groups during aging, although
414 yellowness decreased slowly in meat of OG animals which had higher concentration of limonene in
415 the meat. It is noted that a* and b* are linked to sensory degradation and consequently, to the visual
416 acceptability of meat to the consumers (Insausti, Beriain, Lizaso, Carr, & Purroy, 2008; Luciano et
417 al., 2009). Myoglobin concentration and its redox status represent the main factors that affect color
418 variation during aging (Mancini & Hunt, 2005) and b*, particularly, is linked to meat oxidative
419 stability, both to myoglobin stability and lipid oxidation processes (Qin et al., 2020). The higher b*
420 values observed in aged OG meat suggests that the higher limonene meat content, derived from oral
421 administration, could suppress meat oxidation processes. In fact, as well as TBARS, b* increased
422 during aging in all treatment groups, but only the meat from the OG group showed lower values at
423 Day 7. These results are consistent with an effect of limonene on oxidative stability of the meat,
424 particularly in OG kids. Several studies that used natural dietary antioxidants (i.e. essential oils) to
425 enhance meat oxidation stability by decreasing lipid peroxidation and improving antioxidative status
426 in different species have reported similar results (Holman et al., 2019; Maggiolino et al., 2020b;
427 Maggiolino et al., 2021b; Smeti et al., 2021). Collectively, these findings confirm that these dietary
428 antioxidants and their metabolites can be applied to inhibit lipid peroxidation and prolong the shelf-
429 life of red meat (Amensour, Sendra, Pérez-Alvarez, Abrini, & Fernández-López, 2015; Rant et al.,
430 2019). Meat enzymes residual activity represents the *in vivo* cell defence system against oxidative
431 damage (Descalzo et al., 2005) and, after slaughtering, there is a loss of physiological activity due

both to intracellular protease denaturation (proteolysis) and hydrolysis processes (Imazaki, Douny, Elansary, Scippo, & Clinquart, 2018; Jabalbarezi Hukerdi, Fathi Nasri, Rashidi, Ganjkhanlou, & Emami, 2019) and to their redistribution between cellular compartments after cell wall rupture (Daun, Johansson, Önning, & Åkesson, 2001). All treatment groups showed the same decreasing trend with aging, although OG meat was characterized by higher values of SOD and GSPx. It is postulated that the oral exposure route increased the concentration of limonene in the meat and thus, it resulted in comparatively enhanced enzymic activity in muscle cells. This is supported by the liver tissue results, whereby SOD, GSPx and CAT activity were observed to be higher in samples from OG kids. Further, this outcome aligns to past research that found the antioxidant status of ruminant meat can be positively influenced by dietary consumption of substances with antioxidant activity, and that increase the enzyme activity in different tissues, such as plasma and meat (Jabalbarezi Hukerdi et al., 2019; Zhao et al., 2018).

This study demonstrated that plasma NEFA levels increased for the first 2 d of the experimental period, and thereafter decreased. Although these values fell in the physiological range for the species (Celi, Di Trana, & Claps, 2008; Davis, Sahlu, Puchala, & Tesfai, 1998), the statistical differences of NEFA values are attributed to the limited variability of these data. Plasma NEFA concentration increases when dietary requirements are not met (Schlumbohm & Harmeyer, 2008) and an animal's response to this event is usually very rapid (Laporte-Broux et al., 2011). Consequently, the limonene dosages used in this study may have initially induced a partial inhibition of the rumen bacterial activity. The hypothesis is that, just after a first inhibition of ruminal activity, a process of adaptation of the rumen microbiota may be established. So, the quantity of limonene administered could have negatively affected digestion processes through its intrinsic potential inhibition of bacterial activity, with a temporary rise of NEFA, although it was a slight rise and remained low and in the physiological range for goat kids.

The sensory evaluation using the duo-trio test showed that both RG and OG samples were different compared to the CG samples, in all aging times considered, although the number of right answers

458 decreased during aging. It is important to note that the limonene concentration in the meat was over
459 the lowest odour threshold values reported in the literature for limonene (5.8×10^{-6} mg) (Fuller et al.,
460 1964) in all of the treatment groups. So, the observed differences could be due to the limonene
461 concentration. It should be pointed out that this decreasing trend in right answers was evident in both
462 RG and OG samples, although significance decreased first in the RG samples (after 4 days) and then
463 in OG samples (after 7 days). Limonene is a volatile substance and its lower concentration in RG
464 samples together with its volatile characteristics and human perception capacity probably lead to a
465 lower capacity to recognize samples of treated animals from those untreated (CG) after some aging
466 days.

467 **Conclusion**

468 Essential information emerges from these results concerning terpenes transfer and their effect
469 on meat in goat kids, which should be taken into account before considering studies on a larger scale.
470 Considering limonene antioxidant activities, its effect in the preservation of goat kids' meat is
471 important, giving the possibility to delay lipid oxidative processes and consequently reducing
472 discoloration processes, production of oxidation metabolites and rancidity in meat. On this point,
473 terpenes consumed by animals, particularly at pasture, could improve animals' oxidative status (both
474 at plasma and liver level). So, pasture and associated consumption of natural terpenes can result in a
475 more attractive meat from both quality and animal health point of views. Moreover, a short-term
476 treatment (at relatively high doses) of essential oil can induce terpene enrichment in intramuscular
477 fat, and so in meat of goat kids. Although at lower doses, the respiration route seems to be able to
478 guarantee a greater limonene transfer into meat compared to the oral one, and this highlights the
479 possibility that limonene (and probably other terpenes) which they inhale, when breathing, partitions
480 and accumulates more readily in the meat than had the same concentrations been consumed.

481

482 **References**

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721 **TABLES**

722

723 **Table 1.** The mean (± standard deviation) body weight and age of each treatment group (*n* = 12 per
724 treatment group). Abbreviations include control group (CG), respiratory group (RG), and oral group
725 (OG).

726

727

	Body weight (kg)	Age (days)
CG	24.3±0.8	94.2±1.7
RG	24.9±0.3	94.5±1.3
OG	24.5±0.7	94.0±1.6
CG	24.8±1.2	95.0±1.4
RG	24.6±0.7	94.5±1.0
OG	24.7±0.8	94.3±1.7
CG	23.8±0.4	95.5±1.0
RG	24.3±1.0	95.0±1.4
OG	23.9±0.8	94.3±1.7

728

729 **Table 2.** Effect of limonene exposure routes on the chemical composition of goat kid meat, when
730 aged for up to 7 d *post-mortem*. Means, standard error or means (SEM), and level of significance
731 (*P*-value) are shown. Other abbreviations included control group (CG), respiratory group (RG), and
732 oral group (OG).

Group	Day 0	Day 4	Day 7	SEM	*P*-value		
		Moisture (g/100g)			**Group**	**Aging**	**Group × Aging**
CG	74.1	72.8	72.4				
OG	73.6	72.6	73.2	0.54	0.804	0.098	0.692
RG	73.2	72.9	72.5				
		Proteins (g/100g)					
CG	20.6	21.7	22.2				
OG	21.5	22.2	20.2	0.75	0.740	0.904	0.155
RG	22.0	21.1	22.3				
		Intramuscular fat (g/100g)					
CG	1.6	1. 9	1.6				
OG	1.7	1.6	1.9	0.23	0.993	0.555	0.148
RG	1.8	1. 7	1.6				
		Ash (g/100g)					
CG	1.2	1.2	1.2				
OG	1.2	1.3	1.2	0.07	0.263	0.563	0.318
RG	1.3	1.2	1.4				

733

Table 3. Effect of limonene exposure routes on the CIE color coordinates and rheological parameters of goat kid meat, when aged for up to 7 d *post-mortem*. Means, standard error or means (SEM), and level of significance (*P*-value) are shown. Other abbreviations included control group (CG), respiratory group (RG), and oral group (OG).[1]

Group	Day 0	Day 4	Day 7	SEM	*P*-value		
					Group	Aging	Group × Aging
		pH					
CG	6.01^{A}	5.53^{B}	5.47^{B}				
OG	6.19^{A}	5.61^{B}	5.44^{B}	0.67	0.619	<.001	0.055
RG	6.12^{A}	5.78^{B}	5.46^{B}				
		Lightness (L*)					
CG	43.66^{A}	47.38^{A}	54.48^{B}				
OG	43.30^{A}	44.69^{A}	54.32^{B}	1.30	0.137	0.002	0.754
RG	42.37^{A}	43.22^{A}	53.20^{B}				
		Redness (a*)					
CG	13.45^{a}	14.04^{a}	11.19^{b}				
OG	13.42^{a}	14.68^{a}	11.15^{b}	0.71	0.542	0.559	0.712
RG	14.61^{a}	13.95^{a}	11.03^{b}				
		Yellowness (b*)					
CG	1.55^{a}	1.60^{a}	0.67^{bX}				
OG	1.56^{a}	1.46^{a}	1.13^{bY}	0.64	0.033	0.007	0.002
RG	1.42^{a}	1.54^{b}	0.70^{bX}				
		Thawing losses (%)					
CG	7.33	7.77	10.20				
OG	7.37	11.37	5.47	1.27	0.939	0.170	0.054
RG	7.17	8.83	8.67				
		Water holding capacity (%)					
CG	83.48^{a}	77.33^{b}	78.97^{ab}				
OG	80.52	78.73	81.73	1.23	0.110	0.002	0.206
RG	79.73	75.53	79.40				
		Hematin (mg/kg)					
CG	147.38	139.06	160.68				
OG	157.29	168.39	199.19	20.05	0.152	0.825	0.459
RG	189.38	189.63	162.07				
		Cooking loss (%)					
CG	37.97	35.83	37.27				
OG	38.27	37.67	37.17	0.78	0.457	0.050	0.666
RG	38.07	35.70	37.13				

[1] Means with different superscripts, within rows (^{a,b}), were significantly different ($P < 0.05$). Means with different superscripts (^{X,Y}), within columns, were significantly different ($P < 0.05$).

741 **Table 4** Effect of limonene exposure routes on the plasma oxidative parameters of goat kids, when
742 exposed for up to 7 d. Means, standard error or means (SEM), and level of significance (P-value)
743 are shown. Other abbreviations included control group (CG), respiratory group (RG), and oral
744 group (OG).[1]

Group	Day 0	Day 2	Day 4	Day 7	SEM	*P*-value		
						Group	Time	Group × Time
TBARS (nmol/ml plasma)								
CG	1.62	1.48	1.57	1.47				
OG	1.53	1.28	1.32	1.31	0.09	0.005	0.040	0.123
RG	1.42	1.54	1.15	1.20				
Hydroperoxides (µmol/ml plasma)								
CG	5.92	6.89	6.12	5.94				
OG	5.48	7.20	5.78	6.51	0.38	0.476	0.010	0.550
RG	6.17	6.73	6.54	6.59				
Protein Carbonyls (µmol/ml plasma)								
CG	113.67	104.32	101.20	104.82				
OG	103.43	100.22	101.46	98.58	3.62	0.161	0.436	0.569
RG	102.10	103.07	103.36	104.37				

745

746 **Table 5.** Effect of limonene exposure routes on the activity of superoxide dismutase, catalase, and
747 glutathione peroxidase in the liver tissue. Means, standard error or means (SEM), and level of
748 significance (*P*-value) are shown. Other abbreviations included control group (CG), respiratory
749 group (RG), and oral group (OG).[1]

Parameters	CG	OG	RG	SEM	*P*-value
Superoxide dismutase (U/mg protein)	160.6^{Aa}	171.5^{B}	164.5^{Ab}	1.10	0.001
Catalase (U/mg protein)	62.5^{a}	97.5^{c}	72.0^{b}	0.98	< 0.001
Glutathione peroxidase (µmol NADPH ox/mg protein)	0.22^{a}	0.28^{b}	0.23^{a}	0.004	< 0.001

750 [1] Means with different superscripts, within rows (^{a,b,c}), were significantly different ($P < 0.05$).

751 **Table 6.** Orthonasal olfaction test at vacuum bag opening according to duo-trio method considering
752 the CG as reference experimental group. Other abbreviations included control group (CG),
753 respiratory group (RG), and oral group (OG).[1]

	***n* correct answers**		
Comparison	**T1**	**T4**	**T7**
CG vs RG	20.7**	19.3 *	19.0 *
CG vs OG	20.7**	20.3**	19.0 *

754 [1]Level of significance is denoted as ** = $P < 0.01$; and * = $P < 0.05$.

755

FIGURES

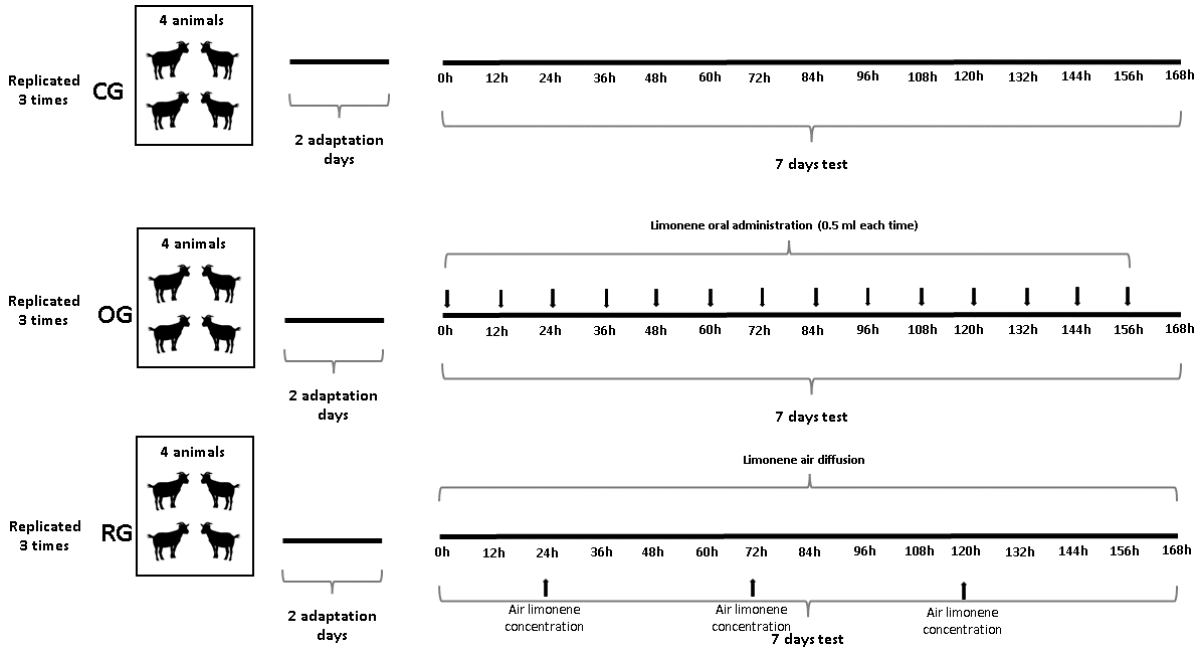


Figure 1. A schematic diagram of the experimental design. Abbreviations include control group (CG), respiratory group (RG), and oral group (OG).

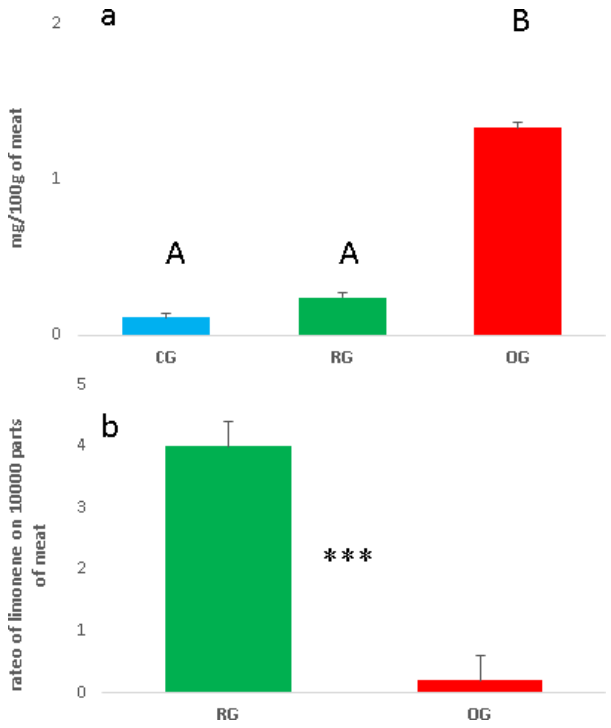


Fig. 2. Effect of limonene exposure routes on goat kid meat a) limonene concentrations; and b) the transfer efficiency ratio. Means and standard error of means (error bars) are plotted. Columns with different superscripts (^{A,B}) were significantly different ($P < 0.05$), or alternatively denoted by *** = $P < 0.001$. Abbreviations included control group (CG), respiratory group (RG), and oral group (OG).

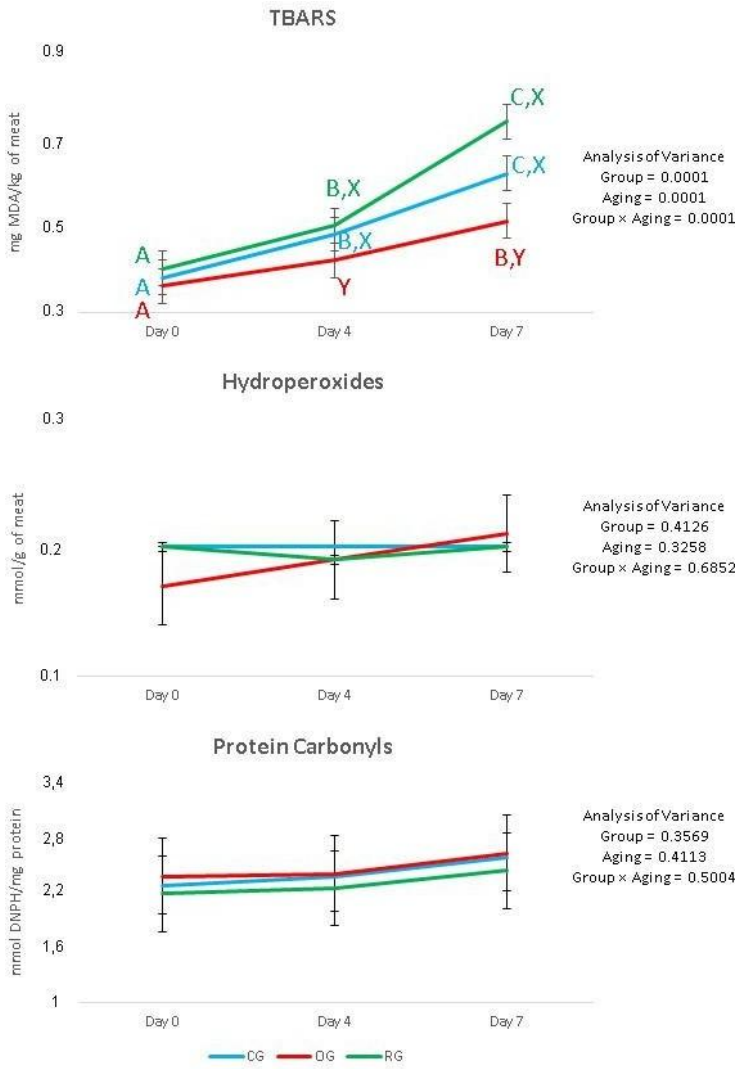
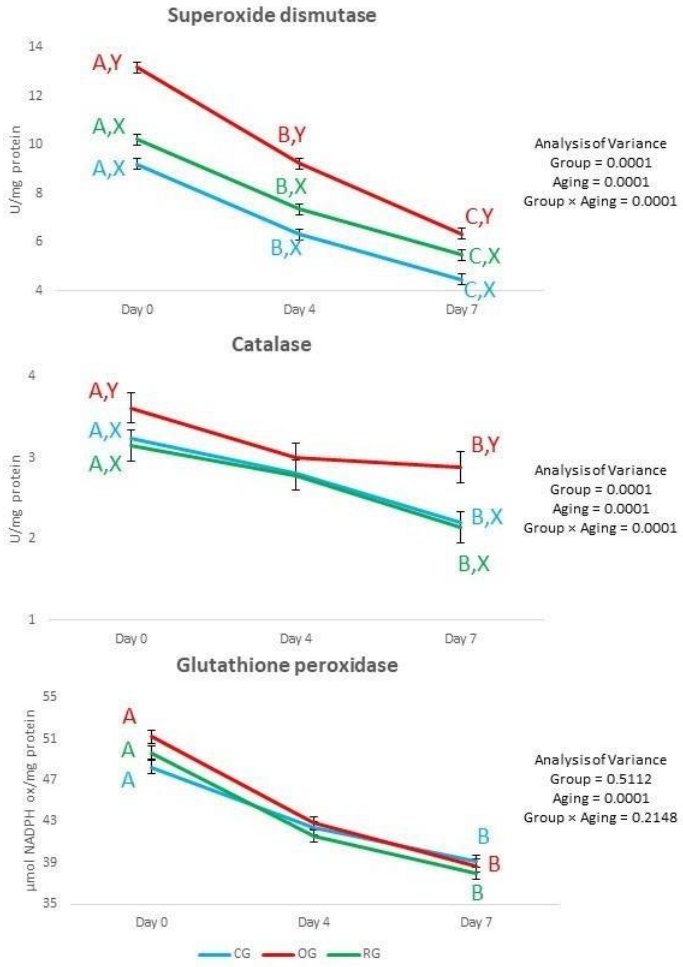


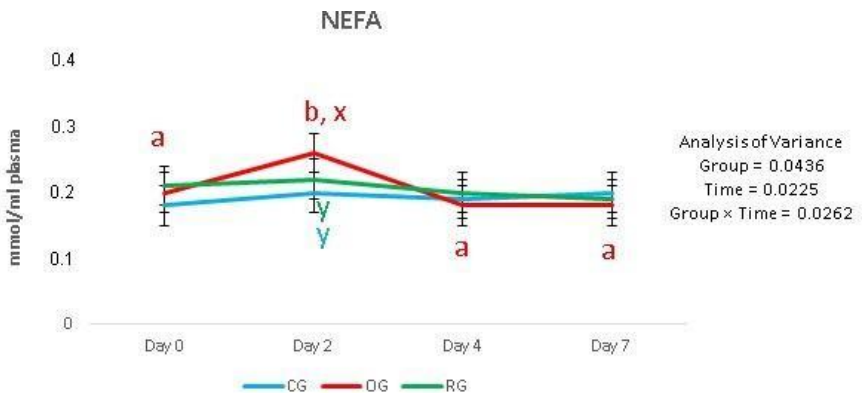
Fig. 3. Effect of limonene exposure routes on goat kid meat oxidative status a) thiobarbituric reactive substances (TBARS); b) hydroperoxides; and c) protein carbonyls. Means and standard error of means (error bars) are plotted. Data points with different superscripts (^{A,B}) were significantly different ($P < 0.05$). Abbreviations included control group (CG), respiratory group (RG), and oral group (OG).



777

778 **Fig. 4.** Effect of limonene exposure routes on goat kid meat antioxidant enzyme activity a)
779 superoxide dismutase; b) catalase; and c) glutathione peroxidase. Means and standard error of
780 means (error bars) are plotted. Data points with different superscripts (^{A,B}) were significantly
781 different ($P < 0.05$). Abbreviations included control group (CG), respiratory group (RG), and oral
782 group (OG).

783



784

785 **Fig. 5.** Effect of limonene exposure routes on goat kid plasma non esterified fatty acid (NEFA)
786 concentrations, measured across a total of 7 d. Means and standard error of means (error bars) are
787 plotted. Data points with different superscripts (^{A,B}) were significantly different ($P < 0.05$).
788 Abbreviations included control group (CG), respiratory group (RG), and oral group (OG).

789