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# Thymus and meat physicochemical measurements to discriminate calves treated with anabolic and therapeutic doses of dexamethasone

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ABSTRACT

To preserve the Europe consumers' health, the use of glucocorticoids as growth promoters is prohibited in cattle fattening. In 2008, the Italian Ministry of Health associated to the official control a national monitoring plan based on the histological thymus analysis to identify animals illegally treated with corticosteroids. However, since corticosteroids are authorized and widely used for therapeutic purposes, it is necessary to verify whether the thymus histological test and some physicochemical traits in meat are able to discriminate doped calves from dexamethasone therapeutic treated ones. The aims of this study were (i) to establish whether the therapeutic and illicit corticosteroid treatments of calves could be differentiated through histological evaluation of thymus and by physicochemical meat traits; (ii) to identify a restricted number of physicochemical traits that could differentiate dexamethasone treated from untreated calves. Three groups of 15 calves each were included in this study: group dexamethasone therapeutic treatment treated with dexamethasone 21-phosphate disodium salt at a therapeutic dose (2 mg/kg of live weight for three consecutive days); group dexamethasone anabolic treatment orally treated with dexamethasone 21-phosphate disodium salt according to a presumed anabolic protocol (0.4 mg/day per animal for 20 days); group placebo control treated with a placebo served as control. Results demonstrated that groups could be easily discriminated by thymus microscopy as well as by two meat markers, namely, cooking loss and shear firmness or Warner-Bratzler shear force. The combination of thymus microscopic features and meat physicochemical traits could be used as a practical, economic and accurate screening strategy to discriminate between meat from illegally and therapeutically treated calves. This new reliable and simple tool could contribute to identify animals treated with dexamethasone in those countries where glucocorticoids are illegally used as growth promoters. More in general, this system could be included in the framework of official controls, and applied to verify suppliers' reliability by the meat industry.


## Implications

European countries banned the use of glucocorticoids alone or in association with anabolic steroids as growth promoters, in calves and beef production since 1981 to protect the health and safety of consumers. The study investigated whether histological thymus analysis and physicochemical traits of meat could differentiate therapeutic and illicit corticosteroid treatments of calves. Results evidence that thymus markers and meat physicochemical traits could be used both in the frame of monitoring plan and meat industry for identifying meat from illegally treated animals.

## Introduction

In the livestock industry, growth promoters are used in some parts of the world to enhance the performance of beef cattle. For the protection of consumers health in 1981, the European Union (**EU**) adopted the first restrictions on the use of hormones as growth promoters in beef production (Directive 81/602/EEC) and, later, in 1989, fully implemented ban on imports of animal's meats treated with enhancing growth promoters from non-European countries (Directive 88/299/EEC). Among the drugs illegally used,

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corticosteroid, especially dexamethasone (**DEX**), administered alone at low concentration or in association with steroids or beta agonists in cocktails, is able to improve the productive performances of animals and to enhance carcass and meat quality traits (Gottardo et al., 2008; Girolami et al., 2010). However, the use of DEX in the EU is approved in livestock only for specific therapeutic indications, provided that the use should be expressly recorded, traced and therefore its residual concentration in muscle should not exceed regulated maximal residue limits (0.75 μg/kg) (Commission Regulation (EU) No. 37/2010).

The evidence of DEX illicit use is highlighted in the last Efsa report (EFSA 2020) where 19 non-compliant bovine samples were recorded for B2f (bovine: 0.15%). To date, gold standard methods to identify corticosteroid residues in edible tissues are gas chromatography-mass spectrometry or liquid chromatography-tandem mass spectrometry. Unfortunately, these methods may give unsatisfactory results due to rapid drug excretion and to the low dosage used in illicit practice (Pinel et al., 2010). To solve this problem, as EU legislation states that the efficient control of residues is an essential component of the maintenance of a high level of consumer protection, new screening methods are needed to detect illicit treatments of farm animals.

According to the National Residue Control Plan, in order to detect the abuse of drugs such as 17β-oestradiol or corticosteroids, only analytical screening and confirmatory methods should be applied. For several years, the scientific community has been proposing approaches based on the detection of biological effects rather than molecule effects of abused drugs (Pezzolato et al., 2013). In order to avoid penalties, illegal protocols changed gradually by reducing the dosage of illegal drug administration. As a consequence, the detection of residues in biological samples is gradually becoming a difficult task. For this reason, it is important to develop new strategies to control the issue. For example, new methods based on the study of the effects rather than the detection of drugs residues in animals have to be tested in order to implement the currently in use official strategies to detect illegal treatment in cattle. So, the changes of meat qualitative parameters could be an interesting topic to correlate with the thymus changes as atrophy and substitution with adipose tissue in the same organ to detect the combination potential indirect markers for the illegal abuse of growth promoters.

Therefore, the aims of this study are (i) to establish whether the therapeutic and illicit corticosteroid treatments of calves could be differentiated through histological evaluation of thymus and by physicochemical traits of meat; (ii) to identify a limited number of physicochemical traits that could differentiate from calves treated and untreated DEX. This new reliable and simple tool could contribute to identify animals treated with dexamethasone in those countries where glucocorticoids are illegally used as growth promoters. More in general, this system could be included in the framework of official controls, and applied to verify suppliers' reliability by the meat industry

## Material and methods

### *Animals and treatments*

The experiment was carried out in accordance with the European Council Directive Directive 2010/63/EU 86/609 (D.Lgs 26/2014) and was authorized by the Italian Ministry of Health and the Ethics Committee of the University of Turin. At the end of the sampling procedure, the carcasses of the treated animals were destroyed according to the law in force (Directive 2003/74/EC). The study was a randomized controlled blind clinical trial.

Overall, 45 Friesians male calves 2 months old were recruited, randomly divided into three groups of 15 animals and raised in multiple pens under the same controlled conditions. Each pen had its own crib, multiple drinking troughs, and a dedicated automated milk feeder system. Animals were fed with milk powder and enriched dried maize silage from 50 days of age to slaughter (185 days as average). Clinical controls were carried out daily by a veterinarian and treatments for occurring infections were performed without using hormonally active substances.

During the last month before slaughtering, the animals were orally treated with: dexamethasone 21-phosphate disodium salt at a dose of 0.4 mg/day per animal for 20 days dexamethasone anabolic treatment group (**DX** group), according to a presumed anabolic protocol of treatment; dexamethasone 21-phosphate disodium salt at a therapeutic dose of 2 mg/kg of live weight for 3 consecutive days dexamethasone therapeutic treatment group (**DT** group), to simulate a dose shock; placebo control group (**CO** group). All animals were slaughtered in a commercial EU-licensed abattoir in the same week. Animals of DX group were slaughtered about 10 days after the last drug administration, animal of DT group after 28 days after the last drug administration (based on withdrawal period of drug) and control animals were slaughtered in the same days (Commission Regulation (EU) No. 37/2010).

### *Thymus collection and histological analyses*

At the slaughterhouse, the thoracic thymus of 15 DX, 12 DT, and 15 CO calves was collected and fixed in 10% neutral buffered formaldehyde. The thymus samples were processed for routinely histological analyses: embedded in paraffin box, sectioned in 3–5 μm slices and stained with haematoxylin and eosin. Morphology of the thymus parenchyma was blinded analysed at low magnification (1× and 4×) by three pathologists to attribute a grading that evaluates the adipose tissue infiltration: grade 1 – minimal or mild invasion of adipose tissue localized within the thymus septa; grade 2 – moderate invasion of adipose tissue in septa with minimal invasion of cortex part of the thymus; grade 3 – severe invasion of adipose tissue in the cortex of the thymus with invasion of the medullar part (Bozzetta et al., 2011; Vascellari et al., 2012). The cortical/medulla ratio was also calculated. To obtain this index, the slides were examined at low magnification (4×) using a digital micro-imaging device (Leica DMD108 Digital micro-imaging device for clinical diagnostics labs); for each slide, five lobules were randomly selected and measured against a graduated line, starting and ending at level of the interlobular connective tissue; a second line was drawn just in correspondence of the first to measure medullar diameter. Cortex thickness was obtained by subtracting the medullar diameter from the corresponding diameter of the entire lobule (Richelmi et al., 2017).

In addition, 3 μm sections from each tissue were stained using anti-cleaved-Lamin A. Antigen retrieval was performed using buffer solution pH6 at 97 °C for 30 min. The slides were incubated with a Rabbit Polyclonal Cleaved-Lamin A antibody (Cleaved-Lamin A, Small Sub unit, Cell Signaling Technology) applied at 1:100 dilution for 60 min at room temperature. The EnVision System Kit (Dako) for polyclonal and monoclonal antibodies was used as detection system. Each run included a positive control, consisting of a lymphnode from an adult animal, and a negative control, obtained by omitting the primary antibody during the staining steps. Digital images of each slide were obtained with a Nikon DS-Fi1 colour digital camera (Nikon Instruments) and recorded to perform a semi-quantitative analysis of positive cells. The positive cells, identified as a dark brown deposit in the nuclei of cortical thymocytes, were counted in 5 randomly selected fields (HPFs; 400×; 2.37 $mm^2$) using Image-Pro-Plus software.

*Meat sampling and physicochemical analyses*

27 calves (9 DX, 9 DT, and 9 CO) out of 42 were randomly selected to perform the physicochemical analyses. At the end of the slaughter line, meat colour measurements were carried out with a Minolta CR-331C Chroma Meter set with D65 illuminant and the 2° standard observer. Measurements were taken at 1 hour *postmortem* on the external right side of *Rectus abdominis* (**REA**) muscle. Results were expressed as lightness ($\boldsymbol{L^*_{REA}}$), redness ($\boldsymbol{a^*_{REA}}$), yellowness ($\boldsymbol{b^*_{REA}}$) values in the CIELAB colour space model (AMSA, 2012). Greater $L^*$ values correspond to lighter meat, while lower $L^*$ values correspond to darker meat. The parameters Chroma ($\boldsymbol{C^*}$), related to the intensity of colour, and hue angle ($\boldsymbol{h^*}$), related to the change of colour from red to yellow, were calculated as follows: $C^*_{REA} = (a^{*2} + b^{*2})^{0.5}$; $h^*_{REA} = \tan^{-1} b^*/a^*$. The greater values of Chroma and hue angle indicate a more vivid colour and a less red colour, respectively.

From the right side of each carcass, samples of *Longissimus thoracis et lumborum* (**LTL**) muscle between the 8th thoracic and the 1st lumbar vertebra were obtained. The day after slaughter, each sample was divided into the subsamples required to perform the following physicochemical analyses: colour, water holding capacity, tenderness, ultimate pH, haem iron content and proximate composition.

The colour of LTL muscle was measured using a Minolta CR-331C Chroma Meter after 1 h of blooming on the freshly cut surface of a 3.5 cm thick steak. The instrument was set as previously described, $L^*_{LTL}$, $a^*_{LTL}$ and $b^*_{LTL}$ values were recorded, and $C^*_{LTL}$ and $h^*_{LTL}$ were calculated.

In addition, the reflectance values from 400 to 700 nm were acquired by a Minolta CM600d Spectrophotometer set with specular component excluded, D65 illuminant and 10° standard observer. Reflectance values at wavelengths not given by the instrument (474, 525 and 572 nm) were calculated using linear interpolation. The reflectance values were converted to $K/S$ ratios (absorption and scattering coefficient ratios) using the following equation: $K/S = (1 - R)^2 \div 2R$, where $R$ represents the % reflectance expressed as decimal. The ratio of $K/S_{474} \div K/S_{525}$, $K/S_{572} \div K/S_{525}$ and $K/S_{610} \div K/S_{525}$ was used to estimate Deoxymyoglobin (**DMb**), Metmyoglobin (**MMb**) and Oxymyoglobin (**OMb**), respectively (AMSA, 2012). We used only the ratios so that they can be obtained from each sample reflectance spectra without further modification. Lower $K/S$ ratios indicate more myoglobin in the given state.

Water holding capacity was measured by drip loss (**DL**) and cooking loss (**CL**) according to the methods described by Honikel (1998). For DL determination, meat samples stored at +4 °C for 24 h in a plastic box provided with a double bottom were weighed before and after storage. DL was defined as % losses during storage, calculated as 100 × (1 − weight after storage/weight before storage).

To determine CL, meat samples were weighed, vacuum-sealed in a polyethylene bag and cooked in a water bath, set at 75 °C until reaching an internal core temperature of 70 °C monitored with a thermocouple. After cooking, the samples were cooled under cold water, while still in bags. Then the samples were removed from the bags, blotted and reweight. CL was defined as % losses during cooking, calculated as 100 × (1 − weight after cooking/weight before cooking).

The instrumental tenderness was measured according to the AMSA guidelines (AMSA, 2015). From the steaks used for CL determination, at least six 1 $cm^2$ cross-section cores were removed parallel to the muscle fibre orientation. The cores were sheared perpendicular to the muscle fibre orientation using an Instron (Model 5543, Instron Corp., USA) with a Warner–Bratzler shear device and crosshead speed set at 200 mm/min. The Warner-Bratzler shear force (**WB**; kgf), maximum shear force recorded during analysis, was determined. Lower shear force values indicate a more tender meat. From the Warner-Bratzler force–deformation curves, the following textural parameters were calculated: shear firmness (SF; kgf/s), as the slope of the line drawn from the origin of the curve to the maximum peak force; and total energy (TE; kgfxmm), the total work necessary for the total sample cut (area under the force-deformation curve). Higher shear firmness values correspond to lower elasticity and indicate a tough meat (Brady and Hunecke, 1985), whereas total energy describe the total energy consumed to chew the meat until it could be swallowed.

The ultimate pH ($pH_u$) was measured 24 h *postmortem* using a Crison portable pH-meter (Crison Instruments, S.A., Alella, Spain) fitted with a spear-type electrode and an automatic temperature compensation probe. The haem iron content (Fe; μg/g) was estimated measuring the absorbance (at 410 nm) of aqueous extracts according to Hudzik (1990). The proximate composition was determined following AOAC (2006) official methods: moisture content (Water; method 950.46) by drying samples at 125 °C for 5 h; CP content by Kjeldahl method (CP; method 928.08) using a Büchi Distillation Unit K-355 (Flawil, Switzerland); crude fat content (Ether Extract; method 991.36) by petroleum ether extraction using a Soxhlet apparatus (Büchi Extraction System B-811; Flawil, Switzerland).

*Statistical analysis*

Statistical analysis of the histological findings and physicochemical traits was performed with IBM SPSS Statistics, version 25.0. (Armonk, NY: IBM Corp) and XLSTAT 2019.2.2 (AddinSoft, Paris, France). The histological thymus data were tested for normality using Shapiro Wilk test. Since the data were not normally distributed, differences among groups were tested using Wilcoxon rank-sum test for multiple comparisons with Bonferroni's correction method to determine which means are significantly different ($P < 0.05$).

The physicochemical data were tested for outliers, normality and homogeneity, and then subjected to a one-way analysis of variance using the GLM procedure in IBM SPSS according to the following model: $y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$ where: $y_{ij}$ is the physicochemical trait analysed; $\mu$ is the overall mean; $\alpha_i$ is the fixed effect of the treatment; $\varepsilon_{ij}$ is the random error.

The least-square means of fixed effect were compared using Tukey's test ($P < 0.05$).

A stepwise linear discriminant analysis (**LDA**) using all physicochemical traits was applied in order to obtain a restricted number of variables that best highlighted differences among groups. An a priori equal probability for a sample to be in one group independently of the group size was considered and the criterion for the selection of variables was Wilk's lambda (F-probability to enter and out value of 0.05 and 0.10, respectively). Wilk's lambda is a measure of a variable's potential and smaller values indicate the variable is better at discriminating between groups. A cross-validate test was used to validate the accuracy of the LDA classification.

Finally, the retained variables in the LDA model were used to build a decision tree. In this approach, the exhaustive chi-squared automatic interaction detection procedure was utilized. The data set was split between a learning set and an evaluation set using a stratified random sampling method, with 2/3 and 1/3 of initial observations. The first set helped us to design the decision tree, and the second set was used to assess the performance of the model. The decision tree analysis (**DTA**) splits data into binary branches according to the values of variables and continues splitting branches in an iterative process that leads to the target value. Each split depends on the value of only one variable (Combes et al.

2007). The position of the variables is determined hierarchically by locating the most important variable at the root of the tree.

## Results

### *Histological analyses of thymus*

All results of the histological analyses are reported in Table 1. The scores 1 and 2 of atrophy of thymus were present in CO and DT groups, whereas the score 3 was attributed only to the thymus of the DX animals. The amount of atrophy of the thymus tissue was significantly associated only with the DX treatment. The parameters of cortical/medulla ratio resulted significantly different between DX group and CO and DT groups; the results put in evidence that the cortical/medulla ratio was lower in the treated animals compared to the control ones. The analysis of immunohistochemical results showed a significant difference in positive cell number between CO and DX and DT. In particular, apoptosis decreased in DX and DT groups (Fig. 1).

### *Physicochemical analyses of meat*

The least-square means showing the effects of DEX treatment on meat physical traits are presented in Table 2. The results revealed that there were no significant differences for the colour of the REA muscle among treatments, whereas, for LTL muscle, significant differences ($P < 0.05$) were found for $C^*_{LTL}$, showing higher values in DX than in DT calves. Concerning the water holding capacity, significant differences ($P < 0.01$) were observed for CL, higher in CO and DT calves, while no differences were found for DL.

Texture parameters analysed with the Warner-Bratzler test did not differ among treatment groups, except for shear firmness which values were higher in DT group ($P = 0.02$). The effects of DEX treatment on $pH_u$, proximate composition and haem iron are presented in Table 3. The results showed that the treatment affected significantly ($P < 0.05$) only CP, which was higher in CO and lower in DX calves.

### *Multivariate analyses of physicochemical traits of meat*

The stepwise procedure of LDA produced two discriminant functions and selected 11 out of 23 variables, as the best discriminant traits between the three groups (Wilks's lambda = 0.010, $P < 0.01$), the trace of Pillai ($P < 0.0001$), and the trace of Hotelling-Lawley ($P < 0.0001$). The standardized coefficients for the two LDA factors are reported in Table 4. The first LDA factor (F1) accounted for 77.32% of total variability between groups (eigenvalue equal to 16.76; canonical $R^2 = 0.971$) and was mainly determined, in decreasing order of their contribution to the first LDA factor by CL, SF, P, $L^*_{REA}$, OMb, $b^*_{REA}$, DMb, W, Fe, WB and $pH_u$.

The second LDA factor (**F2**) accounted for 22.68% of total variability (eigenvalue equal to 4.92; canonical $R^2 = 0.912$). The discriminant functions had a good classification with 100% of original cases correctly classified. The ability of the discrimination model was tested by a leave-on-out cross-validation method. The results indicate that 96.30% of animals in cross-validation were correctly classified. In fact, all DT and DX animals were correctly classified, while 1 animal out 9 of CO was included in DT group.

The LDA distribution of samples on plane given by the two discriminant functions is reported in Fig. 2. The first function was able to discriminate DX from DT and CO. In fact, the centroid of DX group was located in the left side of the plot while the centroids of CO and DT groups were located in the right side. The second function was able to discriminate DT from DX and CO. In fact, DT group was located in the right bottom quadrant, while DX and CO groups were located in the right and left top quadrant,

**Table 1**
Thymus score distribution in different groups of calves (CO control; DT, corticosteroid at therapeutic dose; DX, corticosteroid) and descriptive statistics for cortex-medulla ratio and apoptosis of thymus samples, by treatment group.

| | Group | *N* | Median | Min | Max |
|---|---|---|---|---|---|
| Grading thymus atrophy | CO | 15 | 1[a] | 1 | 2 |
| | DT | 12 | 2[a] | 1 | 2 |
| | DX | 15 | 2[b] | 1 | 3 |
| Cortex-medulla ratio | CO | 15 | 1.64[a] | 1.07 | 2.56 |
| | DT | 12 | 1.415[a] | 0.95 | 2.26 |
| | DX | 15 | 0.91[b] | 0.52 | 2.04 |
| Apoptosis | CO | 15 | 107.4[a] | 65.0 | 171.2 |
| | DT | 12 | 65.9[c] | 24.0 | 141.0 |
| | DX | 15 | 36.2[b] | 19.2 | 59.2 |

[a,b,c] Different superscripts indicate a statistical difference, $P < 0.05$.

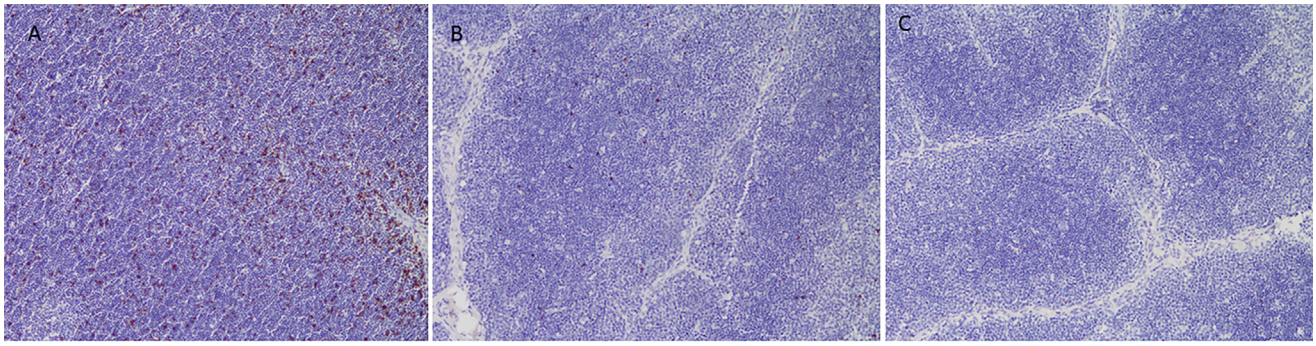


**Fig. 1.** Immunohistochemical anti-cleaved-Lamin A: the immunohistochemistry showed positive cells, identified as a dark-brown deposit in the nuclei of thymocytes. Positive cells were significantly increased in control group (A) with respect to calves treated with therapeutic doses of dexamethasone (B) and calves treated with anabolic doses of dexamethasone (C) (10×).

**Table 2**
Meat physical traits of the different groups of calves (CO control; DT, corticosteroid at therapeutic dose; DX, corticosteroid).

| | *N* | CO | DT | DX | Mean | SEM | *P* |
|---|---|---|---|---|---|---|---|
| Colour | | | | | | | |
| $L^*_{REA}$ | 27 | 43.32 | 45.59 | 42.64 | 43.85 | 0.587 | 0.121 |
| $a^*_{REA}$ | 27 | 19.99 | 19.77 | 20.87 | 20.21 | 0.305 | 0.310 |
| $b^*_{REA}$ | 27 | 2.62 | 2.19 | 2.05 | 2.29 | 0.177 | 0.406 |
| $C^*_{REA}$ | 27 | 20.19 | 19.90 | 21 | 20.36 | 0.294 | 0.302 |
| $h^*_{REA}$ | 27 | 7.65 | 6.38 | 5.64 | 6.56 | 0.553 | 0.343 |
| $L^*_{LTL}$ | 27 | 51.74 | 52.66 | 50.42 | 51.61 | 0.672 | 0.405 |
| $a^*_{LTL}$ | 27 | 20.65 | 19.31 | 21.49 | 20.48 | 0.370 | 0.071 |
| $b^*_{LTL}$ | 27 | 6.82 | 5.97 | 6.69 | 6.494 | 0.212 | 0.230 |
| $C^*_{LTL}$ | 27 | $21.79^{ab}$ | $20.22^{a}$ | $22.54^{b}$ | 21.52 | 0.361 | 0.044 |
| $h^*_{LTL}$ | 27 | 18.48 | 17.29 | 17.20 | 17.66 | 0.603 | 0.629 |
| DMb | 27 | 0.85 | 0.86 | 0.83 | 0.85 | 0.011 | 0.570 |
| OMb | 27 | 0.42 | 0.43 | 0.39 | 0.42 | 0.009 | 0.159 |
| MMb | 27 | 1.44 | 1.44 | 1.45 | 1.44 | 0.003 | 0.709 |
| Water Holding Capacity | | | | | | | |
| DL (%) | 27 | 1.76 | 1.65 | 2.07 | 1.82 | 0.135 | 0.431 |
| CL (%) | 27 | $17.19^{B}$ | $17.94^{B}$ | $13.64^{A}$ | 16.26 | 0.344 | 0.000 |
| Texture | | | | | | | |
| WB (kg) | 27 | 4.89 | 5.01 | 4.64 | 4.85 | 0.339 | 0.927 |
| SF (kg/s) | 27 | $1.44^{a}$ | $2.77^{b}$ | $1.38^{a}$ | 1.86 | 0.159 | 0.020 |
| TE (kgxmm) | 27 | 17.67 | 15.98 | 16.57 | 16.74 | 1.081 | 0.812 |

Abbreviations: $L^*_{REA}$ = Lightness *Rectus abdominis* muscle; $a^*_{REA}$ = redness *Rectus abdominis* muscle; $b^*_{REA}$ = Yellowness *Rectus abdominis* muscle; $C^*_{REA}$ = Chroma *Rectus abdominis* muscle; $h^*_{REA}$ = *Hue Rectus abdominis* muscle; $L^*_{LTL}$ = Lightness *Longissimus thoracis et lumborum* muscle; $a^*_{LTL}$ = redness *Longissimus thoracis et lumborum* Muscle; $b^*_{LTL}$ = Yellowness *Longissimus thoracis et lumborum* muscle; $C^*_{LTL}$ = Chroma *Longissimus thoracis et lumborum* muscle; $h^*_{LTL}$ = Hue *Longissimus thoracis et lumborum* muscle; DMb = Deoxymyoglobin; OMb = Oxymyoglobin; MMb = Metamyoglobin; DL = drip loss; CL = cooking loss; WB = Warner-Bratzler shear force; SF = shear firmness; TE = total energy.
Least-square means in the same row with different superscripts are significantly different; $^{a,b}$, $P < 0.05$; $^{A,B}$, $P < 0.01$.

**Table 3**
Chemical meat traits of the different groups of calves (CO, control; DT, corticosteroid at therapeutic dose; DX, corticosteroid).

| | *N* | CO | DT | DX | Mean | SEM | *P* |
|---|---|---|---|---|---|---|---|
| pHu | 27 | 5.61 | 5.61 | 5.61 | 5.61 | 0.006 | 0.926 |
| Fe (μg/g) | 27 | 4.35 | 4.14 | 4.50 | 4.33 | 0.190 | 0.738 |
| Proximate composition | | | | | | | |
| Water (%) | 27 | 77.03 | 77.10 | 77.25 | 77.13 | 0.109 | 0.709 |
| CP (%) | 27 | $21.53^{b}$ | $21.22^{ab}$ | $20.76^{a}$ | 21.17 | 0.110 | 0.028 |
| Ether Extract (%) | 27 | 0.54 | 0.44 | 0.53 | 0.51 | 0.041 | 0.580 |

Abbreviations: pHu = ultimate pH; Fe = haem iron.
Least-square means in the same row with different superscripts are significantly different; $^{a,b}$, $P \leq 0.05$.

**Table 4**
Standardized canonical discriminant function coefficients and the explained percentage of variance of linear discriminant analysis (LDA) in decreasing order of their contribution to the first LDA factor. Variables selected by the stepwise procedure of the linear discriminant analysis to discriminate the three groups of calves (CO, control; DT, corticosteroid at therapeutic dose; DX, corticosteroid).

| Rank order | Variable | F1 | F2 |
|---|---|---|---|
| 1 | CL | 2.088 | −0.029 |
| 2 | SF | −0.053 | −2.433 |
| 3 | CP | 2.595 | 0.754 |
| 4 | $L^*_{REA}$ | −2.001 | −0.832 |
| 5 | OMb | 1.420 | 0.640 |
| 6 | $b^*_{REA}$ | 1.573 | 0.684 |
| 7 | DMb | 2.595 | 0.429 |
| 8 | W | 1.644 | 0.369 |
| 9 | Fe | 2.542 | 0.785 |
| 10 | WB | 0.561 | 2.162 |
| 11 | pHu | 1.474 | −0.187 |
| | Explained % of variance | 77.32 | 22.68 |

Abbreviations: CL = cooking loss; SF = shear firmness; $L^*_{REA}$ = Lightness *Rectus abdominis* muscle; OMb = Oxymyoglobin; $b^*_{REA}$ = Yellowness *Rectus abdominis* muscle; DMb = Deoxymyoglobin; W = water; Fe = haem iron; WB = Warner-Bratzler shear force; pHu = ultimate pH.

respectively. Finally, DT and DX groups showed a more homogeneous distribution, while CO group was more spread.

The final decision tree (Fig. 3) had three remaining leaves. The CL was selected as the first splitting variable to partially discriminate DX group from CO and DT groups. The second splitting variable was WB. It allowed to separate definitively DX from the other two groups. In both learning and evaluation sets, all the CO calves were misclassified in DT group while all DX calves were correctly classified. The misclassification rate of DT calves was 33% for evaluation set because 1 DT animal was misclassified in DX group. The resultant model yielded an overall predictive accuracy of 67% and 56% for learning and evaluation sets, respectively.

## Discussion

The veal industry is an important sector of animal husbandry, closely related to dairy production since its basic resource are pure-bred young male dairy calves which are fed predominantly liquid, milk-replacer diet and some fibrous feed from the beginning to the end of the fattening period (Council Directive 2008/119/CE).

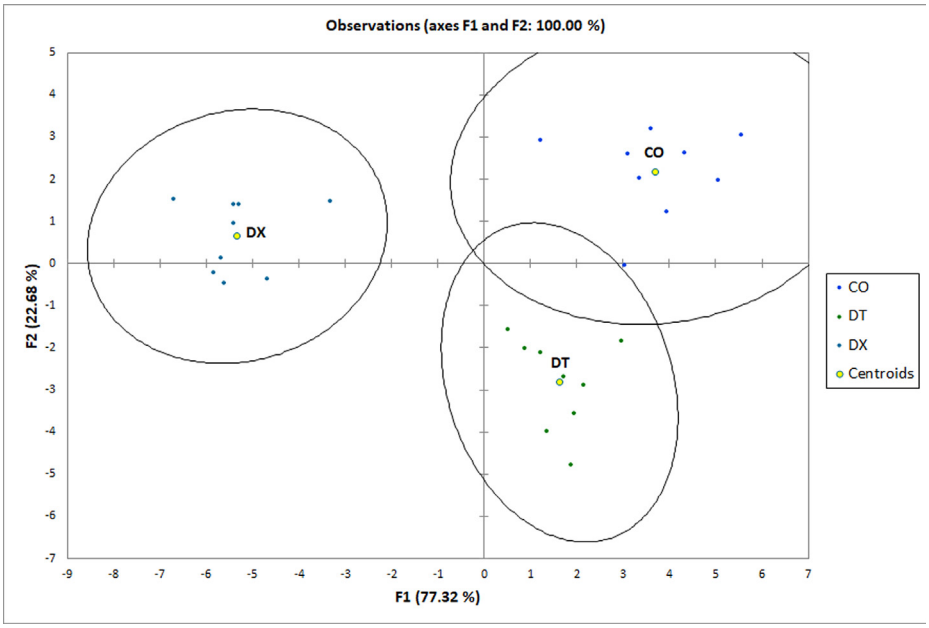


**Fig. 2.** Linear discriminant analysis (represented by canonical discriminant functions) of veal samples from the three groups of calves (CO, control; DT, corticosteroid at therapeutic dose; DX, corticosteroid).

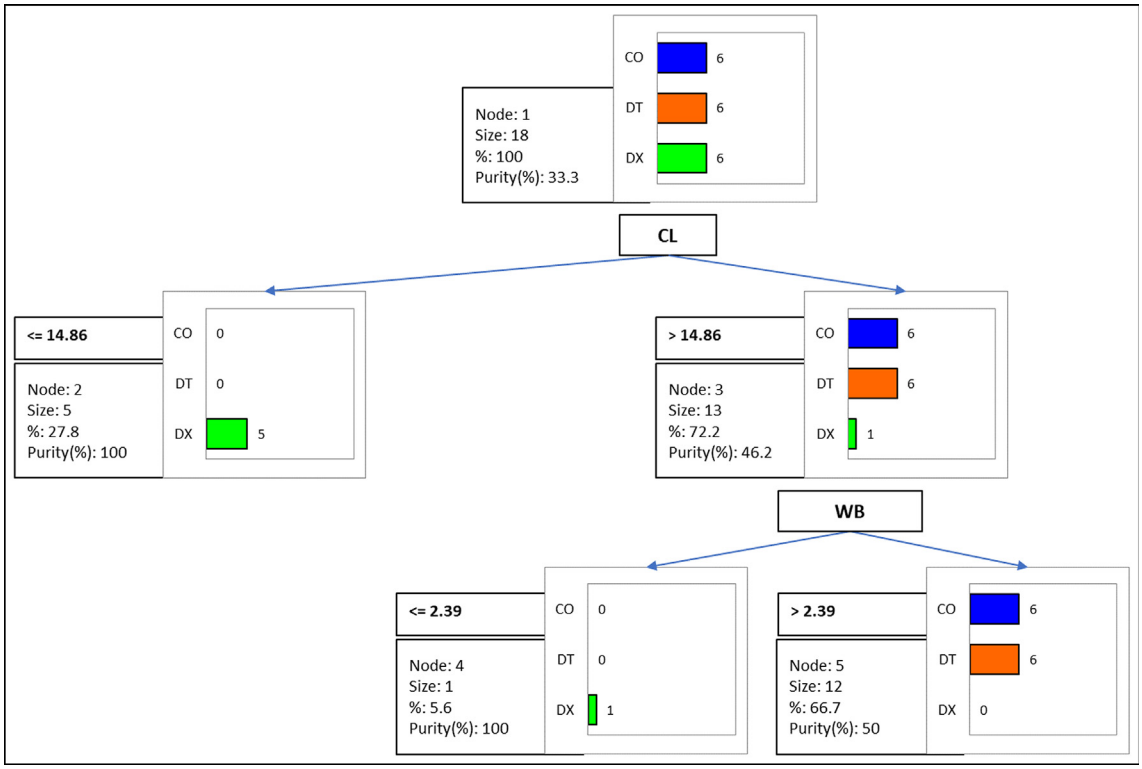


**Fig. 3.** Decision tree obtained by chi-squared automatic interaction detection procedure built using the variables selected by linear discriminant analysis and performed on learning set. Variables selected by decision tree analysis (CL = cooking loss; WB = Warner-Bratzler shear force) to discriminate the three groups of calves (CO, control; DT, corticosteroid at therapeutic dose; DX, corticosteroid).

Thanks to this peculiar feeding strategy and the young age of the animals, the resulting meat is characterized by pale pink colour, high tenderness, and mild flavour, which make it greatly appreciated by the Italian consumers (Dalle Zotte et al, 2017). Nevertheless, the veal market has been declining since the 2000s; the main reason for the consumers' reluctance to buy veal is that veal is perceived as less healthy than other meat for the potential presence of hormone and/or other illicit substances (Chever et al, 2014).

To overcome these problems, the beef industry needs to cooperate consistently to supply high-quality products, healthful but also and above all safe. To preserve consumers in the EU, the use of

glucocorticoids in farm animals is strictly regulated by Reg. 37/2010. Effective surveillance and monitoring of the ban require not only the availability of validated analytical methods for screening and confirmation able to detect residues, but also indirect methods to enhance the control efficiency.

Literature reported that low doses of exogenous glucocorticoids induced thymus atrophy (Cannizzo et al., 2008; Bozzetta et al., 2011). The evaluation of thymus atrophy based on adipose tissue infiltration in our study confirms these data, instead animals treated with therapeutic DEX dose (DT) showed a slight to moderate fat deposition along the interstitial without significant difference with control. Also, the cortex-medulla ratio showed a significant reduction in the group DX as reported in literature (Richelmi et al. 2017; Zanardello et al., 2018).

The fat infiltration score and the morphometric analysis were co-existing findings in dexamethasone treated calves; our experiment confirms that this ratio could be associated with low-dose DEX illicit treatment and did not relate with therapeutic treatments. These results indicate that histopathology is a valid screening tool for the abuse of corticosteroids in veal production and highlight that therapeutic treatment does not alter the accuracy of this approach.

At the same time, fat infiltration and morphometry results have been correlated with apoptosis of thymocytes, but the mechanism of corticosteroid-induced apoptosis is only partially understood (Distelhorst, 2002). Our results revealed a significant reduction of specific processes of cell death in both DX and DT. Previous authors (Biolatti et al., 2005; Zanardello et al., 2018) hypothesized that the apoptotic processes may increase in the early phase of treatment, followed by a drastic decrease of apoptotic activity in case of prolonged treatments and a partial or complete regeneration of the organ after an appropriate withdrawal period. Our data confirm this hypothesis. Moreover, recent literature underlines as both atrophy and regeneration are independent from changes in epithelial cell number, suggesting that the size of the thymus is regulated primarily by rate-limiting morphological changes in cortical stroma, rather than by their cell death or proliferation (Venables et al., 2019).

Currently, among the tests used to identify illicit treatments, the physicochemical traits of meat are not applied in official control activities, although differences between animals treated and controls were reported (Tarantola et al., 2004; Gottardo et al., 2008; Barbera et al., 2018 and 2019). It is well known that pH has a deep influence on meat quality, since variation in pH fall and ultimate pH during the conversion of muscle to meat cause protein denaturation and influence colour, water holding capacity and tenderness. In our study, for all treatments, the pH values were within the expected range (5.4–5.7) indicating the absence of stress factors and consequently a regular development of post-mortem glycolysis. These values were in agreement with those reported in calves and bulls treated with DEX (Tarantola et al., 2004; Gottardo et al., 2008; Barbera et al., 2019). Colour is an important quality trait of meat from calves considering the efforts to classify carcasses according to meat colour as early as possible at slaughterhouse, to evaluate the consumer's perception and acceptance and to control iron intake in order to maintain the typical pale pink colour.

Although not statistically significant, the DX group showed a darker colour of LTL muscle (lower $L^*$LTL and higher $a^*$LTL) in comparison with DT. The meat of DX animals had also a more ($P < 0.05$) vivid colour (the greater the chroma the more vivid the colour) than DT animals. Similarly, Tarantola et al. (2004) observed a more vivid colour in animals administrated dexamethasone by oral and intramuscular injection, and Barbera et al. (2018) found a more vivid colour in animals treated with two dosages of dexamethasone (low and high dose). The dark colour of meat of DX group may also be attributable to the high level in haem iron. Water holding capacity traits, such as drip loss and cooking loss, are important for both consumers and meat industry as they affect nutritional, palatability and economic traits.

In our study, the percentage of drip loss tended to be higher in DX group than in CO and DT groups. Similar results were reported by Tarantola et al. (2004) and Gottardo et al. (2008) in calves and bulls experimentally treated intramuscularly and *per os*, with DEX, respectively. On the contrary, Barbera et al. (2019) found low drip loss values in DEX treated Charolaise bulls.

As regards cooking loss, DX group lost a small amount of water during cooking, showing a value 26% and 32% lower than that observed in CO and DT group, respectively.

Previous reports concerning the effects of DEX on cooking loss provided conflicting evidence. Tarantola et al. (2004) and Barbera et al. (2019) found that the oral administration of DEX decreased cooking loss in calves and beef, respectively. Barbera et al. (2018), conversely, found that treatment with a low dose of DEX increased cooking loss in Friesian bulls.

Among all meat texture attributes, tenderness has been considered the major factor affecting consumer satisfaction and Warner-Bratzler shear force is the most widely used method for objective evaluation of beef tenderness, yielding the best correlation with consumer tenderness perception (Silva et al., 2017). Warner-Bratzler shear force values were not affected by the treatment, averaging 4.85 kg, indicating a moderate tender meat (Destefanis et al. 2008).

Similar results were obtained by Gottardo et al. (2008) and Barbera et al. (2019) while other authors reported that the administration of DEX decreased instrumental tenderness as compared to control group (Tarantola et al., 2004; Barbera et al., 2018).

On the contrary, the results for shear firmness were significant ($P = 0.02$) indicating that the meat of DT group showed more resistance to cutting than the meat of CO and DX groups. As regards proximate composition, the administration of DEX per *os* affected only the CP content, which was lower in DX group in comparison with CO group (20.76 vs 21.58; $P < 0.05$). Tarantola et al. (2004) observed a reduction in protein and an increase in water content in veal calves treated with DEX, but the differences were not statistically significant. Similar content of water but lower protein content and higher fat content were reported by Barbera et al. (2018) in Friesian bulls treated with two dosages of DEX.

The LDA was used to determine which physicochemical traits were the most useful for discriminating among groups. The analysis performed on 23 physicochemical traits allowed us to identify 11 of these as useful for discriminating calves from different treatments. Three variables ($P < 0.01$), namely, CL, SF and CP, had the better discriminating power (lower rank position) showing the greater value of F and the lower value for Wilks' Lambda. Looking at the standardized discriminant coefficients used to compare the relative importance of the independent variables, i.e., the physicochemical traits in predicting the treatment group, it was possible to assess that the most important discrimination variables were, respectively, CP, DMb, Fe, CL and $L^*_{REA}$ for the first discriminant function and SF and WB for the second.

The discriminant analysis was able to clearly discriminate DX and CO groups (Fig. 2), while CO and DT were closer to each other. Consequently, this set of physicochemical traits could be also efficiently used to identify calves treated with illegal administration of DEX.

Since the 11 physicochemical traits selected by the stepwise procedure were still too numerous for an accurate and practical discrimination of calves treated with illicit dose of DEX, a decision tree was designed to further reduce the number of the variables.

In DTA, there is a balance between accuracy (no classification errors) and robustness (lower number of leaves obtaining by

pruning) (Combes et al. 2007). The final decision tree was very simple with three remaining leaves. As shown in Fig. 3, the most important variable was CL, which means that had the highest power in division of observation into groups. This variable was also the same first variable selected by LDA. Being the first discriminator, it splits the root node into two groups presented as node 2 and node 3. A cut off value of 14.86% was set up for CL, with calves with CL under or equal to this value classified as DX (83% of the DX calves). For CL > 14.86%, the WB was defined as the second splitting variable. Among the animals whose CL was over 14.86%, WB $\leq$ 2. 39 kg classified one DX animal while WB > 2.39 kg classified correctly all CO and DT calves. Our results were in agreement with that of Briskey (1963), who found that water holding capacity of meat is associated with tenderness. In fact, muscles that had a low cooking loss were tender, whereas muscles that showed a high cooking loss lacked tenderness as determined by Warner-Bratzler shear values.

Comparing the results obtained by the two multivariate analyses, we found that both methods perform nearly equally. In fact, CL was selected as first variable in both discrimination methods, while SF by LDA and WB by DTA were selected as second variable. To reconcile these apparently contradictory results, it should be underlined that in our study, the two shear parameters were strongly positively correlated with each other ($r$ = 0.724; $P$ < 0.01) and similar findings ($r$ = 0.768; $P$ < 0.01) were reported by Brady and Hunecke (1985). In addition, these authors demonstrated that SF and WB were both strongly positively correlated with some important sensory characteristics such as hardness and chewiness and negatively with tenderness and concluded that these two shear parameters were measuring similar elements of texture. Therefore, from a practical point of view, the choice of one of the two variables to discriminate between therapy or illicitly corticosteroid treated animals is irrelevant because these two shear parameters could both be used as tenderness or toughness indicators.

In the present study, LDA and DTA were employed to discriminate calves from animals treated with illicit dose of DEX. In both discrimination methods, the most important variables were CL, SF or WB which can be assessed using two different analytical methods on the same meat sample. In this respect, portable shear force devices equipped with a semi-automatic sample loading and shearing process are already available. Therefore, apart from the initial purchase cost of the shear texturometer, these methods are relatively inexpensive to perform, do not require complicated instruments or skilled technical personnel and for these reasons can be easily applicable and resistant to utilization under the conditions of practical meat handling and processing.

## Conclusion

The analyses performed on thymus confirmed that this approach can be used to discriminate between therapy or illicitly corticosteroid treated animals. This strategy could be simple applied; in fact, thymus collection at the slaughterhouse is nowadays an already routinely practice evidencing the suspect of illegal administration of dexamethasone.

The preliminary results obtained with the physicochemical analyses gave also evidence that only two analytical methods, cooking loss and texture measurements, can be applied to identify any illegal dexamethasone treatments in the calves. Although the statistical analysis presents some limits, mainly related to the small size of the groups under study, this work suggests that it is possible to introduce some reliable analytical methods that could help both the meat industry and official controls for a rapid and easy meat evaluation. Nevertheless, additional validation tests are necessary before definitive conclusions can be drawn.

## Ethics approval

The experimental procedure was approved by the Italian Ministry of Health and the Ethics Committee of the University of Turin.

## Data and model availability statement

None of the data were deposited in an official repository. The data that support the study findings are confidential.

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## Declaration of interest

The authors declare no competing interests regarding this publication.

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