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Original Article

Canonical discriminant analysis and meat quality analysis as complementary tools to detect the illicit use of dexamethasone as a growth promoter in Friesian bulls

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Highlights

- Methods for the detection of illicit dexamethasone treatment in cattle were evaluated.
- Canonical discriminant analysis and meat quality analysis were applied as complementary screening tools.
- Friesian bulls treated orally with dexamethasone at a growth-promoting dose regimen were compared with untreated controls.
- A portable electronic nose alone was not fully successful in detecting treated meat.
- In contrast, a multivariablee approach was suitable for meat screening purposes.
- Meat quality (including aroma) and weight gain were the most useful parameters.

Abstract

A screening method based on meat quality parameters and production traits for detecting the effects of illegal administration of dexamethasone in Friesian bulls was assessed. Twenty finishing bulls were divided into an untreated control group (n = 8) and two treatment groups receiving dexamethasone orally at dosages of 1.4 (n = 6) or 0.7 (n = 6) mg per head per day for 60 days. The animals were slaughtered 26 days after cessation of treatment. Thirty-six parameters were measured on live animals, carcasses and samples of the longissimus thoracis muscle. The production traits were similar between groups, but there were significant differences in meat quality between treatment groups. The higher dosage of dexamethasone improved meat tenderness, while the lower dosage resulted in more saturated red meat, with increased meat cooking shrinkage and cooking loss. The use of a portable 'electronic nose' as a screening tool was not successful in discriminating between treated and untreated meat. These results indicate that a multivariable approach using canonical discriminant analysis may be a complementary tool to identify meat from animals illegally treated with dexamethasone, based on several parameters (meat flavour, cooking and thawing

loss, tenderness, colour and live weight gain), which are part of the normal analysis of meat quality.

Keywords: Bovine; Friesian bulls; Dexamethasone; Illicit growth promoters; Meat quality

Introduction

According to European Union (EU) Council Directive 96/22/EC[1], the use of certain compounds capable of manipulating the growth of food producing animals is banned in the EU (Stephany, 2010; Valladares-Carranza et al., 2015). Synthetic glucocorticoids, including dexamethasone, have a wide range of therapeutic applications in veterinary clinical practice (Corah et al., 1995; Ferguson and Hoenig, 1995), but are also used illegally as growth-promoters in veal calves and finishing bulls, either alone or in combination with other banned substances (Courtheyn et al., 2002; Tarantola et al., 2004; Cannizzo et al., 2008; Gottardo et al., 2008; Cannizzo et al., 2010; Girolami et al., 2010). The low dosages of glucocorticoids used for promotion of growth often result in urine concentrations below the limits of detection of the current screening methods (Vincenti et al., 2009).

Italy has the highest detected frequency of dexamethasone misuse in cattle in the EU, with 12/24 non-compliant results in 2013[2]. A pilot study conducted in Italy and involving 295 veal calves and 1035 finishing bulls, reported typical thymic lesions (i.e. parenchymal atrophy) due to the misuse of glucocorticoids in 17.7 % of cases examined[3]. Several biomarkers for exposure to dexamethasone or other glucocorticoids have been identified in cattle (Girolami et al., 2010; Rijk et al., 2010; Nebbia et al., 2011; Ludwig et al., 2013; Guglielmetti et al., 2014; Pegolo et al., 2014).

Changes in the physical traits of retail cuts of meat from cattle treated with glucocorticoids include improvement in warm carcass dressing percentage, meat colour and tenderness (Tarantola et al., 2004; Gottardo et al., 2008); producers and consumers consider

[1] See: http://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:31996L0022 (accessed 12 March 2016).
[2] See: http://www.efsa.europa.eu/it/supporting/pub/723e (accessed 5 March 2016).
[3] See: http://www.salute.gov.it/imgs/C_17_pubblicazioni_1146_allegato.pdf (accessed 13 May 2016).

these traits to be indicators of high quality meat. Several variables assessed routinely in the meat industry, such as tenderness, colour and yield, may be useful for establishing a protocol for the detection of animals illegally treated with glucocorticoids.

Meat aroma measured by a portable 'electronic nose' (EN) has been used to evaluate the shelf life of livestock products (Tikk et al., 2008; Berna, 2010; Narsaiah and Jha, 2012) and to analyse meat quality (Cornale and Barbera, 2009; Isoppo et al., 2009); we hypothesised that this variable could be used to identify meat from animals treated with dexamethasone.

A number of studies have addressed the effects of low dosages of dexamethasone on carcass characteristics and meat quality in cattle (Corah et al., 1995; Tarantola et al., 2004; Gottardo et al., 2008). However, none of these studies have included Friesian finishing bulls, for which the illegal use of glucocorticoids could be attractive, in view of their potential high carcass dressing percentages and meat quality.

The main aims of this study were: (1) to examine the effects of dexamethasone on production traits and meat quality in Friesian finishing bulls after a long withdrawal time; (2) to ascertain whether meat quality parameters, including analysis of aroma using a portable EN, could be a useful tool for identifying illegal treatment with glucocorticoids; and (3) to test the practical applicability of the abattoir as a suitable location to collect data and samples for the proposed screening test using a multivariable approach (canonical discriminant analysis).

Materials and methods

The study was approved by the Ministry of Health and the local committee for animal welfare on 20 September 2006. The trial used 20 Italian Friesian finishing bulls, with an

initial mean (± standard deviation) body weight of 439.7 ± 53.4 kg and an age range of 329-443 days. The number of experimental animals was selected according to the sample size test by the GLMPOWER procedure in SAS 9.4[4].

The animals were housed in outside pens with an overhead shelter on a farm in Piemonte, Italy. They were fed a total mixed ration with ad libitum access to fresh water. After an acclimatisation period of 4 weeks, the bulls were randomly divided into three groups based on convenience sampling. Treated animals received either 1.4 (high dose, HD; n = 6) or 0.7 mg (low dose, LD; n = 6) dexamethasone (sodium phosphate salt, Dexadreson, Intervet Italia) per animal per day orally for 60 days, mimicking a growth-promoting protocol (Gottardo et al., 2008), while animals from the control group (n = 8) were untreated. The commercial preparation was diluted with tap water to a volume of 10 mL and orally administered using a plastic syringe without a needle.

Cattle were slaughtered 26 days after cessation of treatment. The animals were weighed immediately prior to slaughter and muscle sampling and data recording at the abattoir were performed on slaughtered animals. Temperature, pH and hot carcass weight were recorded at the level of the twelfth rib on the left side, after 1 h in a cool chamber (0-4 °C), using a pH meter (pH 211) provided with an FC200B electrode and an automatic temperature compensator (HANNA Instruments).

Production traits examined were initial live weight (measured on farm), final live weight, 1 h carcass weight and yield, live weight gain and live percent gain, average daily gain, and 1 h carcass pH and temperature. Meat analysis was performed on both raw and

[4] See: http://support.sas.com/documentation (accessed 13 May 2016).

cooked meat samples, measuring commonly used parameters in meat science[5], as well as additional parameters (i.e. total cooking loss, cooling and cooking loss, and meat cooking shrinkage). A sample of longissimus thoracis muscle (between the ninth and eleventh ribs) was collected from each carcass, vacuum packaged, stored for 7 days at 2-4 °C and then frozen at -20 °C for 2 months. The sample was thawed for 48 h at 2-4 °C and thawing loss was measured. The raw meat chemical composition was determined by the oven method for dry matter and ash, the Kjeldhal method for crude protein and the Soxhlet extraction technique for ether extract (Helrich, 1990). Collagen content was assessed as hydroxyproline multiplied by 7.4 (Vázquez-Ortíz et al., 2005). Results are expressed as percentage of fresh meat.

Meat colour of raw meat was evaluated after 60 min of exposure to room temperature using a colourimeter (CR300; Konica Minolta Sensing), by determining lightness (L*), red index (a*), yellow index (b*), saturation index (chroma) and hue using the CIELab standard illuminant D65[5]. Three readings were taken for each sample, which consisted of a 2.5 cm thick raw meat slice.

Water holding capacity (WHC) was measured as drip loss (on raw meat) and cooking loss (on cooked meat)[5]. Meat cooking shrinkage (MCS) on cooked meat was measured with a video image analyser using the formula: (raw area - cooked area)/raw area. Fluid lost during cooking and after 20 min of cooling was designated ‘total cooking loss’, while fluid loss occurring during the 20 min of cooling was designated ‘cooling loss’. The ‘cooking loss’ was the difference between total cooking loss and cooling loss (Barbera and Tassone, 2006). The tenderness of cooked meat was evaluated by the Warner-Bratzler (WB) shear force (Instron

[5] See: http://www.meatscience.org/publications-resources/printed-publications (accessed 13 May 2016).

1011) using cylinders of meat (2.54 cm diameter), based on the highest load (WB peak) and the break force (WB break).

Aroma was measured on warmed meat samples using a portable EN (PEN 2; Airsense Analeptics) using 2 g sample per vial, according to a modified vial method (Haugen et al., 2006). Statistical analysis was performed on the 5 s average around the maximum value. The EN had 10 metal oxide sensors and analysed an air flow of 150 mL/min, providing output in a data matrix for 10 classes of chemical compounds (Table 3).

Results are expressed as least square means (LSmeans) ± standard error of the mean (SEM). A univariable model was applied to assess the effects of the treatments in SAS 9.4 STAT[4] using a general linear model (GLM) and multiple comparisons for unbalanced data using Tukey's test. After the application of a stepwise discriminant analysis (SDA), selected parameters were subjected to canonical discriminant analysis (CDA), a dimensional reduction technique performing a multivariable one-way analysis to derive canonical functions, i.e. linear combinations of the quantitative variables, summarising the variation among groups. Discriminant analysis was applied to validate the model[4].

Results

Animals in the HD group were significantly younger than those in the LD and CT groups (355 days vs. 406 and 396 days, respectively; $P = 0.03$) and also had a significantly lower initial live weight (389.5 kg vs. 453.8 and 466.8 kg for the HD, LD and CT groups, respectively; $P = 0.01$). To avoid a biased analysis[4], the initial live weight was used as a covariate for the analysis of production traits. At growth-promoting dosages in both HD and LD groups, dexamethasone was not effective in altering production traits upon univariable

analysis (Table 1). The multivariable approach may increase accuracy, power, and efficiency of data analysis. SDA was then applied and five parameters were selected among production traits. The CDA on the five selected parameters derived the first and second canonical variables (CAN1 and CAN2, respectively). CAN1 explained 99% of variability and separated the CT group from the treated groups (Fig. 1a). The largest contribution to the CAN1 was due to the live weight gain[4].

The effects of dexamethasone treatment on chemical and meat quality parameters are summarised in Table 2. Eight out of 18 tested parameters showed statistically significant differences following data analysis in the univariable model. Overall, dexamethasone-treated bulls exhibited higher raw meat colour values, water holding capacity and tenderness, as illustrated by a lower WB peak and break of cooked meat, compared to untreated bulls. HD-treated bulls had significantly lower WB peak and WB break values compared with controls. However, for other parameters, dose-related effects were not observed. The meat in the LD group differed significantly from that in the control group, the former presenting redder (a*) and more saturated (chroma) meat with a higher shrinkage upon cooking (MCS), total cooking loss and cooking loss. Meat collagen content did not show statistically significant differences between control and treated bulls; amongst treated bulls, there was a limited but statistically significant higher content in LD vs HD animals.

The application of SDA to chemical and meat parameters (Table 2) resulted in the selection of 13 parameters: thawing loss, L*, a*, b*, chroma, hue, WB break, cooling loss, cooking loss, dry matter, crude protein, ash and collagen. Using CDA, CAN1 was able to differentiate treated (LD and HD) from untreated (control) groups (Fig. 1b). Furthermore,

CAN2 was able to separate HD from LD treated groups, indicating that the selected variables had good discriminatory power.

When the portable EN was applied to discriminate between control and dexamethasone-treated bulls, statistically significant differences were detected only for W1S, W2S and W5S using univariable analysis (Table 3). W1S and W2S were able to differentiate CT from HD, while the W5S sensor could differentiate CT from LD but not from HD.

The SDA approach identified a subset of six out of 36 parameters (live weight gain %, cooking loss, WB break, hue, thawing loss and W1S) which discriminated dexamethasone treated and untreated cattle. CDA resulted in a clear separation among groups, in particular between the CT and HD groups. CAN1 explained 84% of the among-class variation and divided the HD from the CT group (Fig. 1c). CAN2 separated the CT and HD groups from the LD group. The original variables, which accounted mostly for this discrimination, were W1S (positively correlated) and hue (negatively correlated).

Discussion

This study showed that oral treatment of finishing Friesian bulls with dexamethasone did not result in substantial effects on production traits. Similarly, live weight and feed conversion ratio were not affected in Marchigiana finishing bulls treated with 0.75 mg dexamethasone per animal for 49 days (Gottardo et al., 2008), nor in purebred Brangus steers treated initially with a combination implant containing oestradiol and progesterone, and subsequently with another implant containing 100 mg of dexamethasone, for 30 days (Corah et al., 1995). In contrast, lower daily body weight gain and poorer feed conversion rate were reported by Tarantola et al. (2004) in crossbred veal calves administered dexamethasone at a

lower dosage (0.4 mg/per head) for a shorter period of time (25 days) compared with untreated controls. In the current study, CDA was able to distinguish treated from untreated bulls (Fig. 1a).

Our results show that collagen content was not associated with WB peak and WB break; however, Warner et al. (2010) suggested that total collagen content is of limited value in predicting tenderness. Our meat quality results are similar to those obtained by Tarantola et al. (2004) for veal calf meat treated with dexamethasone (0.4 mg/day), showing increased brightness and tenderness, but no alteration in WHC. Gottardo et al. (2008) treated bulls with a dosage similar to our LD group and could not detect effects on meat quality traits, except for a decreased red index. However, this variable was significantly increased in the LD group in our study. CDA for meat quality parameters was able to discriminate among the three groups (Fig. 1b), leading to the conclusion that this set of parameters could also be used to identify animals treated with dexamethasone. Our results only partially confirm the findings of other studies performed on beef cattle with similar administration protocols. The relatively long time between cessation of treatment and slaughter (26 days in the present study), along with the use of a dairy breed (Friesian), may at least partially explain these discrepancies.

The EN technique has been used for meat quality assessment (Wojnowski et al., 2017), but has not been applied previously for identifying illicit use of growth promoters. The rationale for using the EN was based on the complex modifications of meat components (e.g. lipids) caused by dexamethasone, possibly reflected by changes in meat aroma (Gonzalez-Martin et al., 2000). Only three sensors (W1S, W2S and W5S) measuring classes of compounds that are components of meat aroma (Calkins and Hodgen, 2007) were able to partially distinguish between HD and CT groups. Due to the broad range in sensitivity of

those sensors, it is difficult to identify which components are responsible for the recorded changes in meat aroma triggered by dexamethasone treatment.

Among the 36 measured parameters (eight production traits, 18 chemical and meat quality parameters, and 10 classes of chemical compounds measured by EN), only six parameters were selected by SDA (live weight gain %, cooking loss, WB break, hue, thawing loss and W1S). Discriminant analysis was applied to validate the model. The accuracy was 'very good', with all bulls being classified properly. Upon cross-validation, the accuracy was 'good', with 5% of bulls being misclassified. The CT and LD groups were adequately classified, while one bull in the HD group was misclassified as LD (17% cross-validation error), which was considered to be a minor error, since this still constituted a treatment group; no bulls were erroneously assigned to the CT group. It may be concluded that the multivariable approach could be used as a first screening of meat from suspected animals, before further analysis to confirm abuse. Similar statistical approaches have been applied successfully to a variety of meat science issues, such as a palatability prediction model (Cho et al., 2010) and meat adulteration (Alamprese et al., 2016).

Overall, the potential practical application of the proposed protocol could consist of collecting a convenience sample, mainly at the abattoir, of data and meat samples from a limited number of animals (5-6) per batch from a single farm and subjecting them to the described battery of tests. In the event of suspicious results, the veterinary authorities could then proceed with official controls on the farm of origin, possibly revealing animals under treatment. However, one should take into account that other confounding factors (e.g. long transport distance and prolonged lairage time) may affect some of the above parameters, including tenderness and hue (Chulayo et al., 2016), thereby limiting the potential usefulness

of this approach. Finally, the described protocol was tested in a particular production line of Friesian bulls fed a total mixed ration, under normal management conditions during transport and slaughter, potentially precluding extrapolation to other conditions.

Conclusions

The productive traits of dexamethasone-treated finishing Friesian bulls were similar to those from an untreated group, but the meat quality showed differences associated with the treatment dosages. The EN alone was not highly effective in discriminating between treated and untreated meat. In a production chain routinely applying meat quality controls, multivariable analysis could be a useful tool. According to the results of this study, the combination of a few parameters (meat flavour, cooking and thawing loss, tenderness, colour and live weight gain) could be useful as a first screening for identifying meat from animals illegally treated with dexamethasone. Further investigations could then be carried out at the farm, to eventually confirm the illicit treatment.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper. KWS Italia has no commercial interest in this technology.

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Table 1
Productive traits (least square means ± standard errors of the means) in untreated Friesian finishing bulls (Control) vs. bulls treated with two dosages of dexamethasone (Low dose: 0.7 mg dexamethasone/day; High dose: 1.4 mg dexamethasone/day).

Parameters	Control	Low dose	High dose
Final live weight (kg)	520.1 ± 6.62	504.2 ± 7.16	518.6 ± 8.52
Carcass weight 1 h (kg)	298.9 ± 5.66	285.8 ± 6.12	304.1 ± 7.29
Carcass yield 1 h (%)	57.2 ± 1.13	56.8 ± 1.22	58.8 ± 1.46
Live weight gain (kg)	80.4 ± 6.62	64.5 ± 7.16	78.9 ± 8.52
Live weight gain (%)	18.8 ± 1.56	14.8 ± 1.69	18.2 ± 2.01
Average daily gain (g)	874 ± 72.6	709 ± 78.5	867 ± 93.5
Carcass pH 1 h	6.8 ± 0.07	6.6 ± 0.07	6.8 ± 0.09
Carcass temp. 1 h (°C)	35.8 ± 0.63	35.5 ± 0.68	36.8 ± 0.81

Table 2
Effects of dexamethasone treatment on chemical and meat quality parameters (least square means ± standard errors of the means) in untreated Friesian finishing bulls (Control) vs. bulls treated with two dosages of dexamethasone (Low dose: 0.7 mg dexamethasone/day; High dose: 1.4 mg dexamethasone/day).

Parameter	Unit	Control	Low dose	High dose
Raw meat				
Dry matter	%	21.3 ± 0.43	22.4 ± 0.50	21.3 ± 0.50
Raw fat	%	2.0 ± 0.28	1.6 ± 0.32	1.8 ± 0.32
Crude protein	%	16.6 ± 0.43	15.8 ± 0.50	16.7 ± 0.50
Ash	%	0.8 ± 0.03	0.8 ± 0.04	0.8 ± 0.04
Collagen	%	0.7 ± 0.06 ^{ab}	0.9 ± 0.07 ^{a}	0.6 ± 0.07 ^{b}
Thawing loss	%	7.2 ± 0.45	5.6 ± 0.52	7.0 ± 0.52
Drip loss	%	4.1 ± 0.73	4.4 ± 0.85	3.0 ± 0.85
Lightness (L*)		35.6 ± 0.92	37.1 ± 1.06	38.5 ± 1.06
Red index (a*)		24.0 ± 0.31 ^{b}	25.4 ± 0.35 ^{a}	24.8 ± 0.35 ^{ab}
Yellow index (b*)		7.7 ± 0.31	8.7 ± 0.35	8.7 ± 0.35
Saturation index (Chroma)		25.2 ± 0.38 ^{b}	26.8 ± 0.43 ^{a}	26.3 ± 0.43 ^{ab}
Hue	°	17.7 ± 0.48	18.8 ± 0.55	19.2 ± 0.55
Cooked meat				
Total cooking loss	%	16.8 ± 1.10 ^{bB}	24.4 ± 1.28 ^{aA}	19.9 ± 1.28 ^{ab}
Cooling loss	%	3.9 ± 0.50	3.3 ± 0.57	3.3 ± 0.57
Cooking loss	%	12.9 ± 1.16 ^{bB}	21.1 ± 1.34 ^{aA}	16.6 ± 1.34 ^{ab}
Meat cooking shrinkage	%	14.0 ± 0.57 ^{b}	16.3 ± 0.65 ^{a}	15.4 ± 0.66 ^{ab}
Tenderness: Warner-Bratzler peak	N	114.8 ± 6.92 ^{A}	94.1 ± 8.00 ^{AB}	78.8 ± 8.00 ^{B}
Tenderness: Warner-Bratzler break	N	97.5 ± 5.27 ^{A}	83.9 ± 6.08 ^{AB}	66.4 ± 6.08 ^{B}

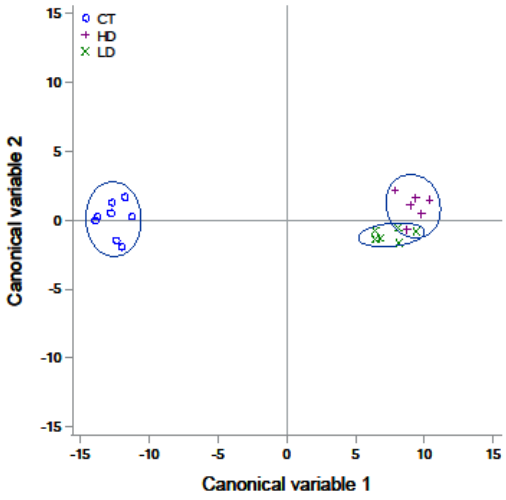
Superscript letters indicate significant differences between groups ($^{a, b, c}$ $P \leq 0.05$; $^{A, B, C}$ $P \leq 0.01$) based on univariable analysis and Tukey's test.

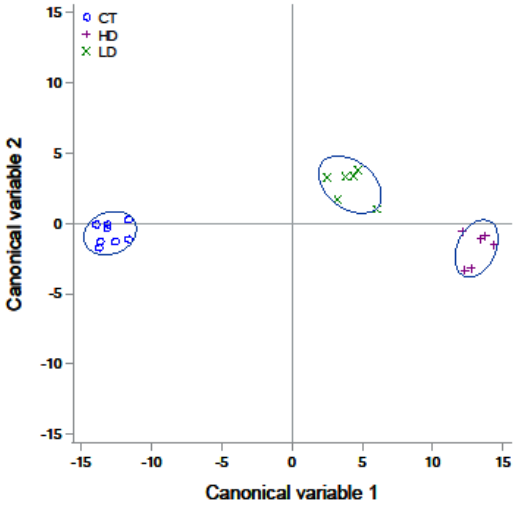
Table 3
Sensor outputs (least square means ± standard errors of the means) from different sensor arrays comparing components released from warmed meat core samples (vial method) collected from untreated Friesian finishing bulls (Control) vs. bulls treated with two dosages of dexamethasone (Low dose: 0.7 mg dexamethasone/day; High dose: 1.4 mg dexamethasone/day).

	Control	Low dose	High dose
Alcohols, partially aromatic compounds, ketones (W2S)	2.2 ± 0.05 ^{bB}	2.3 ± 0.05 ^{b}	2.5 ± 0.05 ^{aA}
Alkanes, aromatic compounds, less polar compounds (W5C)	1.3 ± 0.01	1.3 ± 0.02	1.3 ± 0.02
Ammonia and aromatic compounds, aldehydes and ketones (W3C)	1.3 ± 0.02	1.3 ± 0.02	1.3 ± 0.02
Aromatic compounds (W1C)	1.3 ± 0.02	1.3 ± 0.02	1.3 ± 0.02
Aromatic compounds, sulphur organic compounds (W2W)	1.2 ± 0.01	1.3 ± 0.01	1.3 ± 0.01
Broad range sensitivity for polar compounds (W5S)	15.8 ± 1.25 ^{a}	11.0 ± 1.45 ^{b}	17.1 ± 1.45 ^{a}
Mainly for hydrogen (W6S)	1.1 ± 0.00	1.1 ± 0.01	1.1 ± 0.01
Methane (W3S)	1.2 ± 0.001	1.3 ± 0.01	1.3 ± 0.01
Methane, hydrocarbon, broad range (W1S)	2.3 ± 0.04 ^{B}	2.3 ± 0.04 ^{B}	2.6 ± 0.04 ^{A}
Sulphur compounds, sensitive to many terpenes and sulphur organic compounds (pyrazine, terpene) (W1W)	1.0 ± 0.00	1.0 ± 0.00	1.0 ± 0.00

Superscript letters indicate significant differences between groups ($^{a, b, c}$ $P \leq 0.05$; $^{A, B, C}$ $P \leq 0.01$) based on univariable analysis and Tukey's test.

Figure legend





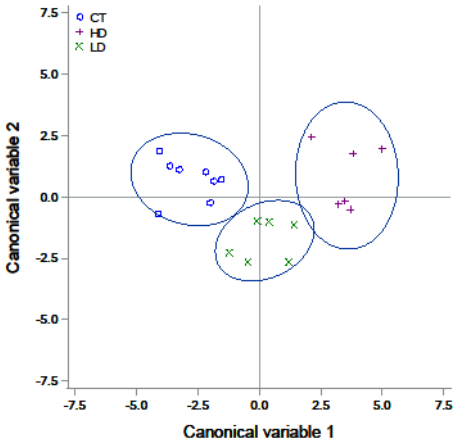


Fig. 1. Canonical discriminant analysis of the three different sets of measured parameters described by the first and second canonical variables which are based on productive traits (a), meat quality parameters (b) and the six parameters (c) selected by the stepwise procedure (HD, high dose, 1.4 mg dexamethasone/day; LD, low dose, 0.7 mg dexamethasone/day, CT, control, untreated).