

Preliminary study on physicochemical and biochemical stress markers at poultry slaughterhouse

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Abstract

Pre-slaughter stress can result in variations in the glycogen storage and metabolic changes of muscle, responsible for quality poultry meat. Aim of this study was to investigate, as pre-slaughter stress markers and quality meat, physicochemical (pH), biochemical (muscle glycogen content), and chemical (super oxides free radicals) parameters. The carcass quality, as incidence of individual carcass defects, was also evaluated. Twenty broilers were processed with two different electrical stunning: high (250 Hz; 640 mA; 60V) (Lot C or control) and low (150 Hz; 360 mA; 60 V) (Lot A) frequency and intensity, using sinusoidal alternating current. As preliminary results, the use of low frequency and intensity induced faster pH decline *post mortem* and adequate acidification of pH at 3 hours (6.49 Lot C; 6.37 Lot A), better muscle glycogen reserve (0.770 $\mu\text{L}/50\text{ mL}$ Lot C; 1.497 $\mu\text{L}/50\text{mL}$ Lot A), and lightly more rapid muscle oxidation (IDF: 0.109 Lot C; 0.122 Lot A), (FOX: 0.131 MeqO_2/kg Lot C; 0.140 MeqO_2/kg Lot A). The incidence of individual carcass defects sufficient to cause downgrading or rejection, both in Lot C and Lot A, was generally low. In a multi-disciplinary approach, to assess animal welfare and quality poultry meat, additional and feasible parameters should be implemented. Monitoring of pH, muscle glycogen reserve and superoxide free radical production measurements might be markers easier to use, routinely, in practice at abattoir. Further studies are needed to evaluate the usefulness of these parameters.

Introduction

Council Regulation 1099/2009/EC (European Commission, 2009) on the protection of animals at the time of killing sets

out rules governing the killing of animals kept for the production of food. The electrical stunning induce a lack of consciousness and sensibility before the animals are killed, and is the most accepted method to immobilize the poultry before slaughter (Bilgili, 1999). The use of a minimum current of 120 mA causes cardiac arrest in 90% of the birds and instantaneous and irreversible stunning. Nevertheless, the electrical stunning may represent serious concern about the welfare of broilers and quality meat (Gregory, 2008; Grandin, 2010). Intensity of electric current used for stunning can vary among slaughterhouses (Kissel *et al.*, 2015). The time of unconsciousness increases with increasing stunning voltage, but at the same time the extent of carcass damage may be aggravated, while the incidence of ventricular fibrillation and death increase (Gregory and Wotton, 1990; EFSA, 2004). On the other hand, a high frequency can increase the depth of unconsciousness, but the duration of unconsciousness decreases as the stunning frequency increases (Huang *et al.*, 2014; Kannan *et al.*, 1997; Mouchoniere *et al.*, 1999). The final quality of meat depends on the voltage, frequency, and duration of the electrical stunning. As pre-slaughter stress conditions, hanging operations, exposure to heat during the pre-slaughter period, and stunning can lead to rapid glycolysis (pH drop) (Petracci *et al.*, 2010), low pH (Rammouz *et al.*, 2004; Van Hoff, 1979) and muscle cytotoxicity in chicken broilers (Debut *et al.*, 2005; Kannan *et al.*, 1997; Loschi *et al.*, 2004; Petracci *et al.*, 2010). At the slaughterhouse, a potential indicator of animal welfare is the absence of stress, but there is no standard definition of stress and no single biochemical assay system to measure stress (Velarde *et al.*, 2010). The return of eye reflexes and rhythmic breathing are clinic indicators of the early return of the functions of the brain after electrical stunning (von Holleben *et al.*, 2010). The high level of plasma corticosterone is a biochemical indicator of stress in birds (McFarlane and Curtis, 1989; Xu *et al.*, 2011). Presently, Electrocardiogram (ECG) and Electroencephalogram (EEG) are considered indicators of unconsciousness and insensibility, nevertheless may not be practical methods to ascertain, routinely, the effectiveness of the stun at the abattoir (EFSA, 2012). For these reasons, studies on effective stunning should be validated by measures easier to use practically at poultry abattoir to assess animal welfare and quality meat (Gregory, 2008; Mercogliano *et al.*, 2016).

Aim of the study was to investigate the feasibility of physicochemical, chemical and biochemical parameters to evaluate pre-

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slaughter stress and quality meat in broilers processed using two electrical stunning treatments.

Materials and Methods

Two experiments were conducted, using N. 20 *Ross* commercial broilers (aged 56 days, with an average weight of 2.5 kg), obtained from a conventional poultry farm. The broilers were given *ad libitum* access to a standard diet throughout the period of growth (Council Directive 2007/43/EC; European Commission, 2007).

The animals were subjected to a 12-hour fasting period, prior to their slaughter (Council Directive 2007/43/EC; European Commission, 2007). They were transported to an authorized EC slaughterhouse within a 30 minute, to prevent the detrimental effects of a long journey on the welfare state (Council Regulation 1/2005/EC; European Commission, 2005). The birds were randomly divided into 4 groups of 5 animals. For each slaughter N. 5 of birds of Lot C (or control) were subjected to a stunning treatment at 250 Hz; 640 mA; 60 V (high frequency and intensity), and N. 5 of birds of Lot A at 150 Hz; 360 mA; 60 V (low frequency and intensity current). At the slaughterhouse *ante-mortem* inspection was carried out by an official veterinarian to evaluate the welfare conditions and health status in birds (Council Regulation 854/2004/EC; European Commission, 2004). The electrical stunning was conduct-

ed using sinusoidal alternating current (AC) in accordance with the minimum currents laid down (Reg. 1099/09/EC, Annex I, Chapter II, point 6.3), and the total stunning duration was 4 seconds.

The broilers were individually shackled from head to feet. The duration of shackling before stunning was kept to 2 minutes, and a blue light intensity (50 lux) was used to calm the animals. In a multi-bird water bath stunning (Cattaruzzi S02POL, Italy) the head of broilers of Lots C and A was brought in contact with an electric grid submerged in a saturated brine solution.

According to the experimental design all the birds were killed by the same operator, using a conventional unilateral neck cut, severing the carotid artery and jugular vein, and is allowed to bleed for 150 seconds, based on the following sequence: -birds of Lot C; -birds of Lot A.

The pH of *Pectoralis major* was measured immediately after the slaughter at 0.25 hours (pH0), 3 hours (pH) and 24h (pHu). Shortly after for chemical and biochemical analysis a sample of carcass each from *Pectoralis major* and from *Quadriceps femoris* was chilled in a static ice, and then held packed.

The eviscerated birds were packed in polyethylene bags and placed in insulated boxes filled with ice for transport to the laboratory, within 3 hours from the time of slaughter. They were then refrigerated at 3 to 5°C for 24 hours, when the evaluations of carcass and meat defects were conducted. Appearance quality attributes, such as skin colour, meat colour, broken bones, appearance defects like bruises and haemorrhages were evaluated in each carcass.

The chemicals and solvents used in the study were obtained from Sigma-Aldrich, Germany. All solutions were prepared from reagent-grade chemicals. In each carcass, the pH of *Pectoralis major* was measured using a pH meter (Hanna pH 211, Hanna Instruments, Woonsocket, RI, USA). In particular, the pH at 3 hours was investigated as a marker of acute pre-slaughter stress in poultry, because it may correspond to the start of shelf life of poultry meat.

Muscle oxidation was evaluated accord-

ing to Shantha and Decker (1994), homogenizing together a sample of *Pectoralis major* and *Quadriceps femoris* from each carcass. Superoxide free radical analysis was carried out following both the International Dairy Federation's method (IDF 74A, 1991), and the Ferrous Oxidation-Xylenol orange's (FOX) method. These methods are based on the oxidation of iron. In IDF method, the sample ($\leq 0.01-0.30$ g) was mixed in a disposable glass tube with 9.8 mL chloroform-methanol (7+3, v/v) in a vortex mixer for 2-4 seconds. After that, ammonium thiocyanate solution (50 μ L) was added, and the sample was mixed in a vortex mixer for 2-4 seconds. Then, 50 μ L iron (II) solution was added and the sample was mixed in a vortex mixer for 2-4 seconds. After 5 minutes of incubation at room temperature, the absorbance of the sample was determined at 500 nm against a blank that contained all the reagents, except the sample, by using a spectrophotometer. The entire procedure was conducted in subdued light and completed within 10 min. The FOX method (Shantha and Decker, 1994) was similar to the IDF method, except that, 0.01 mol/L xylenol orange sodium salt solution in water was used as the complexing dye, instead of ammonium thiocyanate. Absorbance was determined at 560 nm after 5 minutes of incubation at room temperature. To construct the curve of Fe^{3+} concentration v/s absorbance, a standard solution of iron (III) chloride (10 μ g Fe/mL) was prepared for both methods (Mehta *et al.*, 2015). Glycogen concentration was determined by a coupled enzyme assay (Sigma-Aldrich, Saint Louis, MO, USA) which produces a colorimetric (570 nm)/fluorometric ($\lambda_{ex}=535/\lambda_{em}=587$ nm) product, proportional to the glycogen present.

To prepare the standard for colorimetric detection 10 μ L of the 2.0 mg/mL Glycogen

Standard were diluted with 90 μ L of distilled water to prepare a 0.2 mg/mL standard solution. After that 0, 2, 4, 6, 8, and 10 μ L of 0.2 mg/mL standard solution were added into a 96 well plate, generating 0 (assay blank), 0.4, 0.8, 1.2, 1.6, and 2.0 μ g/well standards. Hydrolysis Assay Buffer was added to each well to bring the volume to 50 μ L. For colorimetric assays, the absorbance was measured at 570 nm (A_{570}).

To prepare the standard for fluorimetric detection 10 μ L of the 2.0 mg/mL Glycogen Standard were diluted with 990 μ L of distilled water to prepare a 0.02 mg/mL standard solution. After that 0, 2, 4, 6, 8, and 10 μ L of the 0.02 mg/mL standard solution were added into a 96 well plate, generating 0 (assay blank), 0.04, 0.08, 0.12, 0.16, and 0.20 μ g/well standards. Hydrolysis Assay Buffer was added to each well to bring the volume to 50 μ L. Fluorescence intensity was measured at $\lambda_{ex}=535/\lambda_{em}=587$ nm

Sample preparation

Ten mg of tissue were homogenized in 100 μ L of water on ice. Homogenates were boiled for 5 minutes to inactivate enzymes. After that the samples were centrifuged at 13000'g for 5 minutes to remove insoluble material. Hydrolysis Buffer was added to a final volume of 50 μ L. For colorimetric assays, the absorbance was measured at 570 nm (A_{570}). Fluorescence intensity was measured at $\lambda_{ex}=535/\lambda_{em}=587$ nm. The sample blank value was subtracted from the sample readings to obtain the corrected measurement:

$$Sa/Sv=C$$

where Sa is the amount of glycogen in unknown sample (μ g) from standard curve; Sv is the sample volume (μ L) added into the wells; and C is the concentration of glycogen

Table 1. Poultry slaughter: carcass quality evaluation defects (%) of Lot C (high frequency and intensity current) and Lot A (low frequency and intensity current) carcasses.

Score	Lot C	Lot A
0	80.2	84.5
1	12.4	15.5
2	7.4	-

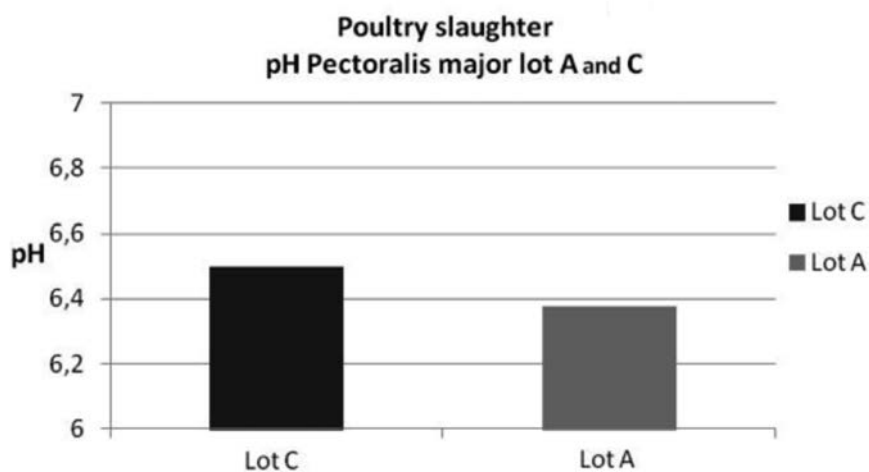


Figure 1. Poultry slaughter: pH values (mean) of *Pectoralis major* at 3 h of Lot A (low frequency and intensity current) and Lot C (high frequency and intensity stunning).

in sample. To evaluate the carcass quality, the skin on the breast area was removed and several incisions were made along the breast, thus, the muscles were examined for superficial and internal (deep) defects. As appearance defects, i) skin colour, ii) meat colour, iii) broken bones, iv) bruises and v) hemorrhages in each carcasses were evaluated. The carcasses were scored from 0 to 2 for all conditions: a score of 0 indicated no defects; 1 slight to moderate defects; 2 severe defects. The percentage of carcasses with defects was calculated, using the ratio between the number of birds, showing specific defect, to the total number of birds examined.

Results

Data on physicochemical, chemical, biochemical parameters and evaluation of appearance carcass quality attributes are shown in Figures 1-3 and Table 1.

Discussion

At the end of the slaughter, pre-slaughter stress increases the concentration of lactate and lead to a rapid pH drop in Pectoralis major at 15 minutes until 6 hours post-mortem, after which no difference is observed after 24 hours (Craig and Fletcher, 1997; Papinaho and Fletcher, 1996; Petracci *et al.*, 2010).

According to literature, carcasses of Lots C and A stunned at high and low frequency and intensity showed a rapid pH drop in Pectoralis major at 15 minutes and up 3 hours post-mortem (pH 6.49 and 6.37, respectively). After 3 hours, and until 24 hours, no important differences were observed, even if pH values were slightly lower in the carcasses stunned at lower intensity and frequency conditions. The monitoring of pH, in particular at 3 hours, might be considered a rapid and feasible measurement of pre-slaughter stress at slaughterhouse.

Pre-slaughter stress can lead to increase of oxidative activity and production of superoxides free radicals in the muscle of poultry (Shantha and Decker, 1994). Concentration of peroxide provides useful data on the degree of the muscle oxidation (Gregory, 1994; Mujahid *et al.*, 2005; Shantha and Decker, 1994). The IDF and FOX data showed a slightly higher superoxide free radicals production in Lot A, if compared to Lot C carcasses (IDF: Lot C 0.109 MeqO₂/kg; Lot A 0.122 Meq O₂/kg) (FOX: Lot C 0.131 MeqO₂/kg; Lot A 0.140 MeqO₂/kg). Under the experimental electric

conditions, a more intense muscle oxidation was observed. In poultry muscle the measurement of superoxide free radicals production seems to be a sensible indicator of pre-slaughter stress related to stunning.

Glycogen concentration of *Pectoralis major* decreased in carcasses stunned with high frequencies showing a fast exhaustion of glycogen at the early post mortem examination, according to Xu *et al.* (2011). Preliminary results of the study showed that the use of lower intensity and frequency electrical conditions led to a major muscle glycogen reserve in Lot A carcasses than Lot control (Lot C 0.770 μ L/50mL; Lot A 1.497 μ L/50mL). For this reason, the lactate production increased and lower pH values were observed in Lot A carcasses.

High stunning frequencies may improve meat quality without aggregating stress,

when the current is not too low, with a considerable commercial advantage (Xu *et al.*, 2011). Incidence of birds with carcass defects sufficient to cause downgrading or rejection, was generally low. Using 150 Hz/60 V electric conditions, the Lot A carcasses showed a lower incidence of defects (84.5% scored 0; 15.5 % scored 1) than Lot C (80.2% scored 0; 12.4% scored 1; 7.4% scored 2) using 250 Hz/ 60 V stunning. According to literature the current stunning conditions above 60V produced the best carcass quality, than lower (until 23 V) or high (until 193 V) voltage (Ali *et al.*, 2007).

However, low frequency and intensity current conditions might led to an increased of peroxides production, as observed in carcasses of Lot A. For this reason, further reduction of the frequency and intensity current conditions is not recommended.

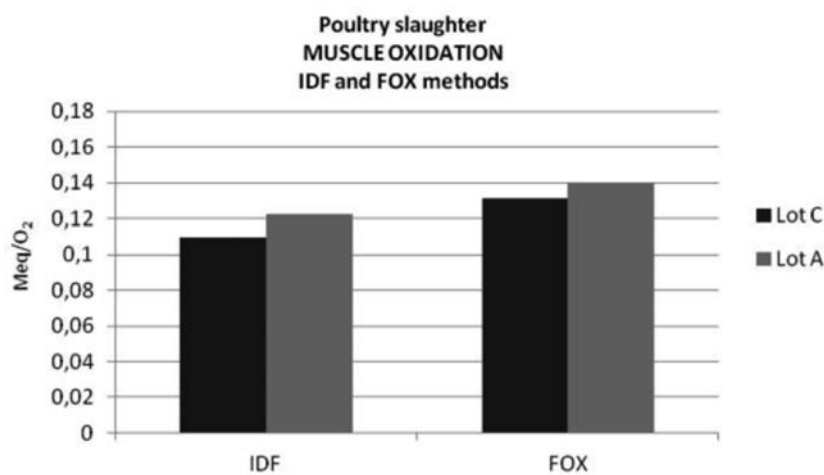


Figure 2. Poultry slaughter: superoxide free radicals values (MeqO₂/kg) of Lot A (low frequency and intensity current) and Lot C (high frequency and intensity current).

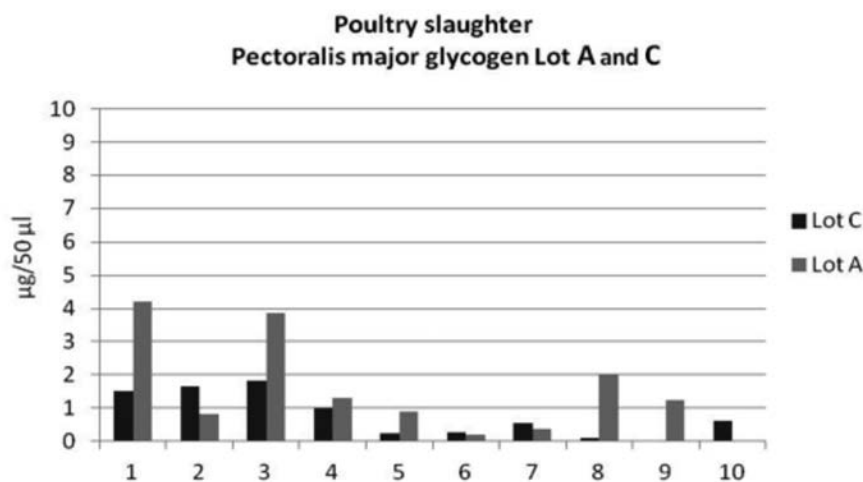


Figure 3. Poultry slaughter: muscle glycogen values (μ g/50 μ L) of Lot A (low frequency and intensity current) and Lot C (high frequency and intensity current).

Conclusions

At poultry slaughterhouse, monitoring of stunning efficiency through indicators should be carried out, to assure animal welfare and quality meat. Preliminary results of the study showed more acid values of pH, a higher values of muscle glycogen reserve and muscle peroxidation in carcasses obtained by using a 360 mA/150 Hz treatment. Using a multidisciplinary approach, the pH monitoring, measurement of superoxide radical production, and muscle glycogen evaluation might be additional and feasible measures, easier to use under practical conditions, to assess animal welfare and quality poultry meat.

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