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Effect of transport length and genotype on tonic immobility, blood parameters and carcass contamination of free-range reared chickens

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ABSTRACT

The aim of the present study was to investigate the effect of transport on welfare traits, several haematological parameters and carcass hygiene in two different chicken genotypes (fast- and slow-growing strains) reared under free-range conditions. For this aim, two hundred male chicks, 100 from fast-growing (Ross 308, R) and 100 from slow-growing (Naked Neck, NN) strain were farmed. At the end of the rearing period, at 81 days of age, 56 birds/strain were randomly selected for slaughtering and submitted to two different pre-slaughter conditions: no transport (0h) or 4 hours of transport (4h). Tonic immobility (TI), blood parameters and carcass hygiene traits were determined. Strain and transport significantly affected TI of birds. Both experimental factors and their interaction significantly affected plasma creatine kinase, alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase. Cholesterol and triglycerides were not different between the experimental groups, whereas glucose decreased after 4 hours of transport in both strains. A significant difference between groups for the heterophils/lymphocytes ratio after transport was also observed, with NN being higher than Ross. Concerning the oxidative stress, we observed a higher ROS production in NN chickens. The carcass microbial characteristics showed a higher level of contamination after the transport (total viable counts), but not concerning the Enterobacteriaceae counts.

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Introduction

Poultry transport to the slaughterhouse is one of the critical factors that can affect animal welfare and quality and hygiene of meat. Chickens are caught and placed in crates to reach the slaughterhouse and during the transport they are deprived of feed and water, exposed to environmental changes (i.e. movement, noise, vibration), subject to even extreme conditions of temperature and humidity, forced to counteract the track movement; at the end they are unloaded and hanged on the shackle line. These different stressing situations can reduce bird welfare and increases the risk of body injuries and mortality (Ali et al. 2008).

Under hygienic point of view, the pre-slaughtering stress increases the spread of infectious diseases (Mulder 1995). Birds catching and crating influences the rate of *Campylobacter spp.* recovery and also

transport vehicles and crates could be considered a source of *Campylobacter* contamination (Slader et al. 2002). The presence of faecal shedding of different pathogens during transport could contaminate birds and spread microorganisms within and between crates (Heyndrickx et al. 2002). However, reports on the effect of different poultry processing procedures are scarce (e.g. mobile slaughterhouse; Ka Wang et al. 2017).

European rules set several points that have to be respected when poultry are transferred to the slaughterhouse: density in the crates; drinking and feeding if more than 12 hours are needed to reach the abattoir; limit faecal falling from upper animal to the underlying crates; temperature and ventilation in the vehicles during transport. These rules are mainly focussed on fast-growing broilers produced in intensive farms and few

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data are available on birds of different genetic strains *free-range* reared (Castellini, Mugnai, et al. 2016).

Studies on *Campylobacter* prevalence of broilers based on faecal shedding are available on chicken reared under *free-range* conditions (Economou et al. 2015). Furthermore, chicken genetic strain seems to affect the caecal colonisation of *Campylobacter jejuni* (Stern et al. 1995).

As reported in a previous paper (Castellini, Mattioli, et al. 2016), slow-growing strains seem more sensible to stress transport due to their higher activity before slaughter; accordingly, the effect of stressful conditions could be different in slow-growing versus fast-growing broilers.

The aim of the present study was to investigate the effect of the transport on welfare traits and several haematological parameters in two different chicken genotypes (fast- and slow-growing) reared in free-range conditions; the hypothesis that the transport could affect the hygiene of carcasses was also investigated.

The experimental protocol was devised according to the Italian directives (Gazzetta Ufficiale 1992) on animal welfare for experimental and other scientific purposes.

Materials and methods

Pre-slaughter and slaughtering conditions

This trial was carried out at the experimental section of University of Perugia (Italy) during Summer of 2015, using two hundred male chicks, 100 from fast-growing (Ross 308, R) and 100 from slow-growing (Naked Neck, NN) strain, furnished by a commercial poultry farm (Avicola Berlanda, Italy). Until 20 days of age, birds were raised separately in an environmentally controlled poultry house (0.12 m²/bird). At 21 days of age, chicks were transferred to straw-bedded indoor pens (0.10 m²/bird), each equipped with feeders and drinkers and with free access to forage paddock (4 m²/bird). Each strain was replicated in four pens containing 25 chicks each. Birds were confined to indoor pens during night. The chicks were vaccinated at hatch against Marek's disease and Newcastle disease. Chicks were fed starter (1 to 21 d) and finisher (22 d to slaughter: 81 d) diets. Access to feed and water was freely available complete starter and finisher diets containing 20, and 18.5% crude protein and 3000 and 3100 ME/kg, respectively.

At the end of the rearing period, at the age of 81 days, 56 birds/strain were randomly selected for slaughter. Prior to slaughter, chickens were subjected

to a 12-h feed withdrawal. Before slaughter, chickens were taken and putted randomly in crates (7 birds per crate 96 × 56 × 26 cm) and allocated to different pre-slaughter conditions:

- absence of transport (0h) – birds were catch and placed in crates and immediately brought to the mobile slaughterhouse (placed in the farm) and sacrificed within 30 min from the catching;
- 4 hours of transport (4h) – this lag time was chosen as standard pre-slaughter conditions because it is commonly observed in commercial practice. Bird crates were placed and driven for 4h in a truck (temperature inside the crate ~25 °C to 28 °C). The procedure included 1-h loading, 2.5-h transport and 0.5-h unloading. Transport was performed with an opened truck during a single day, separating the crates of each genotype to avoid cross contaminations.

All animals were slaughtered in the same mobile slaughterhouse approved by EU, which was completely cleaned and disinfected before the slaughtering of each group. The slaughtering procedure provides the following steps: stunning by electro-narcosis (3 sec.; 1.0 A); bleeding by incision of the major blood vessels of the neck; dipping the birds in hot water in a scalding tank (56.5 °C for 1 min); removal of the feathers by a rotating picker equipment provided by plastic digits; hanging of the carcass on a processing line and manual evisceration (non-edible viscera: intestines, proventriculus, gall bladder, spleen, oesophagus and full crop); carcass placing in plastic trays and refrigeration in a force air chiller (2 °C), till the temperature of 4 °C was reached.

Quantitative evaluation of the carcass hygiene was performed on carcass surface; swabs were collected just before chilling at breast level and sent to the laboratory under refrigerate condition (4 ± 1 °C) for *Campylobacter spp.* and *Salmonella* detection was also performed by excision of a skin fragment at neck level (EC Reg. 2073/2005), promptly stored in sterilised plastic bags and send, under the above-mentioned conditions, to the laboratory.

Tonic immobility and blood parameters of birds

The Tonic Immobility (TI) test was carried out, for all birds at crating, prior to slaughter, to estimate their fearfulness. As described by Jones (1987), each bird was placed on its back in an unshaped cradle and restrained by maintaining a light pressure on the sternum and neck for 10 s. The duration of TI was defined

as the interval between the moments when the bird entered into TI until it righted itself. If TI was not induced after 5 trials, the bird was given a score of 0 for TI. A maximum duration of 10 min was imposed for the test.

Blood samples for analytical determination were collected during bleeding, collected in heparinised vacutainers and centrifuged at $1500 \times g$ for 10 min at $+4^\circ\text{C}$, to measure the *in vivo* oxidative status. After collection, blood samples were immediately sent to the laboratory of Department of Agricultural, Food and Environmental Science where they were centrifuged and frozen at -80°C until analysis. The analysis of serum levels of calcium, phosphorus, total protein, albumin, glucose, triglycerides, cholesterol, and the enzyme activities of alkaline phosphatase (AP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), (CGT) gamma-glutamyl transpeptidase and creatine kinase (CK) were measured using the Express Plus (Ciba-Corning Diagnostics Corp., Medfield, MA) automated clinical chemistry analyser according to the manufacturer's directions.

Quantitative determination of total plasma glucose was determined using glucose oxidase method (Sigma G2133). The concentrations of serum glucose, triglycerides, total cholesterol (Pars Azmoon, Tehran, Iran) were measured using commercial kits.

Serum lysozyme was measured with a lysoplate assay (Osserman and Lawlor 1966), carried out in a moist incubator at 37°C for 18 min. The method is based on the lyses of *Micrococcus lysodeikticus* in 1% agarose. The diameter of the lysed zones was measured with a ruler and compared with the lysed zones of a standard lysozyme preparation (Sigma, Milan, Italy, M 3770). The value was expressed as $\mu\text{g}/\text{mL}$.

The assessment of the antioxidant capacity (AP) and reactive oxygen molecular substances (ROMs) in blood serum was carried out using the Oxy-adsorbent kit and the d-ROMs test produced by Diacron (Italy) (Cesarone et al. 1999), respectively.

Carcass hygiene determinations

Carcass hygiene was determined by sampling 25 cm^2 ($2.5 \times 10\text{ cm}$) of each carcass surfaces (at breast level) by swabs (wet and dry swabbing technique; Cenci-Goga et al. 2007). The swabs (wet and dry) from each carcass were immersed in isotonic diluents, sent to the laboratory in refrigerated conditions and promptly analysed within one hour.

The total viable count (TVC) was determined following the ISO 4833-1:2013 method while for Enterobacteriaceae count the swabs were decimally

diluted in buffered peptone water and plated in REBECCA Agar with EB supplement (BioMerieux Italia) aerobically incubated for 24 ± 2 hours at $37 \pm 1^\circ\text{C}$. The typical colony were counted and reported as colony-forming unit (CFU)/ cm^2 .

On the same samples collected for microbial loads, *Campylobacter* isolation was performed following the ISO 10272-1: 2006 method. Typical colonies were then selected and *Campylobacter* identification were performed by a multiplex PCR (Wang et al. 2002).

The *Salmonella* isolation on the poultry skin were performed by ISO method (see Reg. 2073/2005).

Statistical analyses

A linear model was used to assess the effects of strain and transport and their interactions. The significance of differences was estimated by the multiple Student's *t*-test. Differences were considered significant for $p \leq .01$ and $p \leq .05$.

Traditional statistical techniques were used for data analysis of *Campylobacter* spp. In particular, the effects of the two factors of interest (genotype and transportation time) on the probability of finding *Campylobacter* were estimated via logistic regression (Agresti 2003). For each marginal effect simple, logistic regression models were run. The two factors' joint effect was assessed fitting a multiple logistic regression model without interaction. The same analyses were repeated for the two species of the microorganisms (*C. coli* and *C. jejuni*) separately. The statistical significance of the effects under investigation was assessed by means of the standard errors provided by logistic regression models. Lower and upper 95% confidence intervals were also computed for *Campylobacter* prevalence.

However, some logistic models are associated to contingency tables with some low frequencies. For these cases, traditional standard errors might be inappropriate. Therefore, alternative measures were computed such as jack-knife standard errors (Efron and Efron 1982) and performed specific statistical tests like Fisher's exact test (Fisher 1935). Results are coherent with those obtained from logistic regression models. The whole analysis was implemented using the statistical software R (R Core Team 2016). No statistical determination was possible for *Salmonella* spp.

Results and discussion

The effect of strain and transport stress on TI and blood parameters is shown in Table 1.

Before to begin the discussion of the results, it is necessary to specify that, as expected, Ross 308 birds

Table 1. Effect of genetic strain and transport on tonic immobility, main plasmatic compounds and oxidative status of chicken.

Strain		Ross 308		Naked Neck		Significance			Pooled SE
Transport length (h)		0	4	0	4	S	T	S X T	
TI	sec	56.4	88.0	13.6	37.2	**	**	*	15.21
Ca	mg/dL	2.71	2.80	2.73	2.90	ns	ns	ns	0.53
P		1.92	1.80	1.83	1.72	ns	ns	ns	0.34
CK		11.0	12.2	10.0	11.8	*	*	*	0.82
ALT		9.8	10.4	10.2	10.8	ns	ns	ns	4.21
AST		310	327	300	316	ns	ns	ns	91.4
CGT		23.2	24.3	21.1	23.0	ns	ns	ns	5.08
Cholesterol	mg/dL	2.25	2.87	2.20	2.60	ns	ns	ns	0.52
Triglycerides	"	0.27	0.25	0.29	0.24	ns	ns	ns	0.17
Glucose	"	260	245	250	220	ns	*	*	22
Monocytes	%	5.40	4.87	5.32	4.99	ns	ns	ns	2.01
Eosinophils	"	2.17	2.14	2.18	2.14	ns	ns	ns	0.58
Basophils	"	0.44	0.32	0.41	0.38	ns	ns	ns	0.06
RBC	"	2.43	2.01	2.21	1.98	ns	ns	ns	0.35
Heterofils (H)	"	39.3	39.4	46.4	51.1	*	*	*	3.67
Lymphocytes (L)	"	55.1	47.6	44.4	36.1	*	*	*	4.58
H/L	"	0.71	0.82	1.04	1.42	*	*	*	0.11
Lysozyme	µg/mL	20.2	25.5	18.5	21.5	*	*	*	10.1
ROMs	mM H ₂ O ₂	0.14	0.16	0.16	0.21	*	*	*	0.14
AP	µM HClO ₄ /mL	75	70	121	100	*	*	*	51.8

N = 56 for each strain and transport length. T0: no transport; T4: 4 hours of transport; TI: tonic immobility. S: strain; T: transport; Ca: calcium; P: phosphorus; CK: creatine kinase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; CGT: gamma-glutamyl transpeptidase; RBC: red blood cell; H/L: heterophil/lymphocyte; ROMs: reactive oxygen molecular substances; AP: antioxidant capacity.

**p* < .05.

***p* < .01; ns: not significant.

reached a significant higher final live weight respect to NN ones (3811 vs. 2897 g, respectively; data not shown). This difference slightly modified the density within the crates, considering that the number of chickens was kept constant independently of the genotype.

Genetic strain and transport and its interaction significantly (*p* < .01) affected TI of birds. In agreement with our results, many Authors (Frazer and Brown 1990; Jones 1992) reported that fear levels and TI in birds are strongly correlated and TI could be impaired by transportation.

The effects of transport stress may be exacerbated by food and water withdrawal and by exposure to vibrations and accelerations of the truck. This fact can explain the increase of TI after 4h of transport observed in both the genetic strains.

The TI also has a genetic component and Ross 308 chickens showed longer TI compared to NN (Jones 1992). In laying hens, Mugnai et al. (2011) found that TI was affected by both genotype and rearing system (standard cage system vs. organic production); Gallup et al. (1976) also found strain-specific differences for TI.

Fast-growing strains, compared to less selected birds, may exhibit a reduced thermoregulatory capacity and may be more susceptible to heat stress during transport (Sandercock et al. 2001, 2006), especially if compared with NN birds, considering that featherless facilitates thermoregulation under hot conditions (Azoulay et al. 2011). It may be also

suggested that the occurrence of muscle myopathies of fast-growing strains may lead to behavioural changes and reduced welfare (Branciari et al. 2009) and the transport could have a further negative repercussion (Mitchell and Kettlewell 2009).

However, the TI value of NN strain after 4 h of transport, although lower than Ross, increased more than in fast-growing strain (2.8 fold vs. 1.5, respectively).

The serum levels of Ca, P, cholesterol and triglycerides did not show significant differences due to genotype and/or transport length.

On the contrary, both the experimental factors (strain and transport) and their interaction significantly affected plasma CK contents. In particular, CK after 4h transport increased in both fast- and slow-growing strains.

The CK level is an indicator of muscle damage and CK activity has been reported as being extremely sensitive to physical stress like catching and transport (broilers, Mitchell 1999; turkeys, Huff et al. 2008 and pigeons, Scope et al. 2002).

CK was also affected by genetic strain: CK values of different genetic lines of pig (Reddy et al. 1971) and indicate different susceptibility to stress with the fast-growing animals more sensible than slow-growing one.

In agreement with the wide body of literature, the present study confirms that fast-growing broilers have higher blood CK contents compared with the slow-growing line.

The levels of ALT, AST, CGT, cholesterol and triglycerides were not different between the experimental

groups, whereas glucose decreased after 4 hours of transport in both genetic strains.

Serum glucose decreased with the increase of transport length and NN showed a significantly lower level of glucose after transport. It is presumable that the higher activity of NN is responsible of the higher glucose depletion (12% vs. 5% of Ross 308) during 4h of transport.

Stress may alter energy metabolism and several Authors showed an increased reliance on glucose as an energy source; accordingly, hypoglycaemia (due to increased glucose utilization) is an indicator of stress level in birds (Freeman et al. 1984).

Huff et al. (2008) observed in turkey that transport decreased the levels of glucose, triglycerides, cholesterol, phosphorus, iron, albumin and alkaline phosphatase and increased the levels of uric acid, blood urea nitrogen, alanine aminotransferase, aspartate and aminotransferase.

The transport length, the genotype and even their interaction did not modify some haematic traits as monocytes, eosinophils, basophils and RBC. However, transport reduced RBC, even not significantly ($p = .10$), probably by destroying and removing red blood cells from bloodstream before their normal lifespan due to the higher oxidative stress, as confirmed by the higher ROMs values.

The haematic traits affected by the experimental factors were the heterophils (H) and the lymphocytes (L). It is widely known that stress increases H and decreases L; accordingly, the H/L ratio is a good index of response to a stressor (Zhang et al. 2009).

There was a significant difference between genetic strains for the H, L and H/L ratio, with NN being higher than Ross. Transport length significantly increased heterophils and decreased lymphocytes in both genetic strains (Borges et al. 2004). However, there were significant interactions ($p < .05$) for heterophils, lymphocytes and H/L trend. H/L ratio and H values were greater in the NN subjects, whereas they were smaller for the Ross 308, with the opposite being true for L.

There is a genetic component for heterophils and lymphocytes responses to stressors, as observed by Campo et al. (2008). Previous papers confirmed the same response of genotypes in term of H and L: Castellini, Mugnai, et al. (2016) showed that NN birds had higher H and lower L values than fast-growing strain. Genetic selection of poultry for growth rate may be unintentionally accompanied by a lower resistance to disease or reduced immunological response and changes in the main haematological traits (Qureshi and Havenstein 1994).

According to Gross and Siegel (1983), Ross showed H/L value close to high degrees of stress while NN

show values even higher. Moreover, it seems that NN birds suffered more stress during the 4h transport (H/L increase: 16% vs. 36%).

About lysozyme, it should be remembered that is a potent antibacterial enzyme, against certain viruses and parasites (Mugnai et al. 2011) able to carry out a synergic action with the humoral immune response and with complement factors.

Serum lysozyme allows to know the state of functionality of the monocyte-macrophage system and to identify the presence of inflammatory states. In our study, strain, transport, and there interaction (strain \times transport) showed significant effects on it. Accordingly, it could be argued that transport length enhances the inflammatory states of animals (lysozyme) mainly in fast-growing birds.

Our results are in agreement with Świerczewska et al. (2003) that reported the impact of genotype upon lysozyme of egg albumen.

Concerning the oxidative stress, we observed a higher ROS production in NN chickens, probably due to the higher activity before and after catching and placement in the crates. Accordingly, these chickens showed significantly higher levels of AP independently on transport length. These results confirmed our previous findings about genotype effect (Castellini, Mattioli, et al. 2016): Ross 308 presented lower plasmatic levels of antioxidants with respect to NN ones; accordingly, Ross 308 also showed a lower *in vivo* oxidative stability as demonstrated by the higher plasmatic and meat values of lipid oxidation. Indeed, antioxidant splay a major role in protecting cells from the actions of ROS by reducing chemical radicals and preventing the process of lipid peroxidation (Nishigaki et al. 1992). Some reports suggested that environmental stress diminishes *in vivo* antioxidant power of animals (Klasing 1998). Moreover, low plasma concentration of antioxidants such as vitamins C and E has been correlated with oxidative damages in stressed poultry (Sahin et al. 2002).

Concerning the transport effect, a significant reduction of AP with a consequent rise of ROS content was observed after 4 h of treatment.

Fast-growing strains have an elevated incidence of myopathy, both spontaneous and/or induced by stress (Sandercock et al. 2006). Genetic selection for growth rate and feed conversion may be associated with altered mitochondrial function (Bottje et al. 2006) and changes in ROS production. Mujahid et al. (2005) suggested that acute stress increases superoxide free radical production in skeletal muscle of broilers, which in turn may be responsible for the changes in muscle

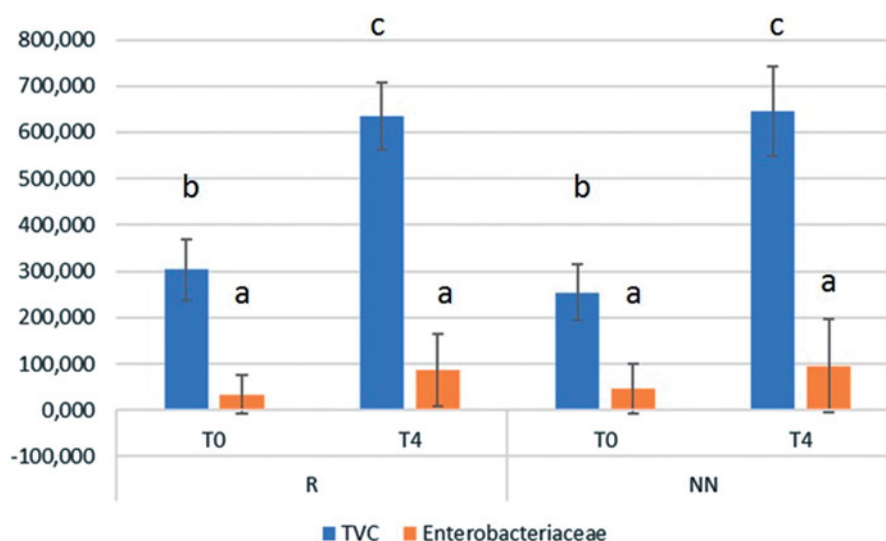


Figure 1. Interaction of genetic strain and transport length on bacterial counts (colony-forming unit/cm²) in free-range chicken carcasses. Legend R= Ross 308; NN= Naked Neck. For each strain and transport length $n = 14$. TVC = total viable counts, T0 = no transport; T4 = 4 hours of transport; different superscript letters are significantly different $p < .05$.

Table 2. Effect of genotype and transport on *Campylobacter spp.* prevalence in free range chicken carcasses.

	Naked Neck		Ross		p value genotype	p value transport
Transport length (h)	0	4	0	4		
<i>Campylobacter</i> (%)	100 (25/25)	96 (24/25)	80 (20/25)	96 (24/25)	ns	ns
<i>Campylobacter coli</i> (%)	92 (23/25) ^b	92 (23/25) ^b	16 (4/25) ^a	72 (18/25) ^b	**	**
<i>Campylobacter jejuni</i> (%)	12 (3/25)	40 (10/25)	64 (16/25)	24 (6/25)	ns	ns

For each strain and transport length $n = 14$. Within a row, means with different superscript letters are significantly different; ** = $p < .01$; ns = not significant.

and meat quality mainly observed in fast-growing broilers (Castellini, Mattioli, et al. 2016).

The results of carcass microbial loads are reported in Figure 1. The transport enhanced the TVC of carcass, but not the *Enterobacteriaceae*, after 4h transport. No difference was recorded between the two genotypes for both the considered parameters.

Few data are available on the carcass hygiene of slow-growing genotype reared in free range conditions. Cason et al. (2004) reported the effect of faecal contamination on broiler carcass hygiene before chilling on *Enterobacteriaceae* showing differences between the control and fecally contaminated carcasses (5.9 vs. 6.3 log CFU).

However, the focus of different papers is on the prevalence in broilers of *Campylobacter* and *Salmonella*, the main pathogens responsible for severe human diseases.

Salmonella detection in skin samples was always negative, as well as the faecal samples at farm before loading (data not shown). Accordingly, it could be underlined that the preventive control strategies adopted for *Salmonella* at farm were sufficient to prevent contamination of the carcass independently from the transport length and genotype.

The prevalence of *Campylobacter* positive carcasses is reported in Table 2. The overall prevalence was high and almost the same in both the strains (96%) and transport length confirming the high prevalence of *Campylobacter spp.* in free-range farms (Colles et al. 2008).

This fact affected the overall prevalence independently on the transport length; indeed, if high *Campylobacter spp.* prevalence occurs at the catching of the animals (up to 100%), the same prevalence is expected at the slaughterhouse. The effect of carcass chilling was not considered in this study, even if a total reduction of the prevalence could be expected (Economou et al. 2015).

As regarding the *Campylobacter* identification, *C. coli* and *C. jejuni* were detected in all the groups and difference due to bird genotype were recorded for *C. coli* only. The higher prevalence of *C. coli* was recorded in NN respect to R. However, in R genotype, where the prevalence with no transport was relatively lower, the increase of transport length increased the prevalence *C. coli* (72%; 54–89, lower and upper 95% confidence intervals).

The higher level of *C. coli* in slow-growing genotype confirms other previous work (Miraglia et al. 2007).

Even in this case, the prevalence was so high that the effects of transportation length could not be evaluated.

No effect of genotype and transport were recorded for *C. jejuni*, which is considered the major responsible for human infections.

Conclusions

In conclusion, the results of the present study suggest that transport for 4 h prior to slaughter negatively affect some animal welfare traits (TI, CK, H/L, glucose) and the carcass hygiene of outdoor reared chickens.

The NN birds showed the highest stress susceptibility, but on the contrary presented higher antioxidants defence probably connected with their higher foraging behaviour.

The significant modification observed in the transported birds for TI, CK, H/L, lysozyme, ROS glucose and AP, might be used for profiling flocks to determine their responses to transport stress and feed withdrawal and possibly more general stress responses.

These tools could be used as selection parameters to improve stress resistance to transport and, consequently, meat quality.

On the same time, a less stressful slaughter procedure should be developed with shorter take-up times in the farm, transport and animal storage at the slaughterhouse mainly for sustaining the high quality of SG birds reared in *free-range* conditions.

Sanitary strategy seemed effective for the reduction of *Salmonella* at farm level but no effects were evident for *Campylobacter spp.*, which was relatively high also without transport and affects the overall prevalence in poultry carcasses.

Further studies are needed to better understand the effects of genotype and transport on the different *Campylobacter spp.* evaluating in detail the microbial load at different steps of production.

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Disclosure statement

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