Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

Scienze Veterinarie

Curriculum Sicurezza Alimentare

Ciclo XXIX

Settore Concorsuale di afferenza: 07/G1 – SCIENZE E TECNOLOGIE ANIMALI

Settore Scientifico disciplinare: AGR/19 – ZOOTECNIA SPECIALE

STUDIES ON ANTE- AND POST- MORTEM LESIONS AS ANIMAL-BASED CRITERIA TO IMPROVE WELFARE AND MEAT QUALITY IN SWINE

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DISSERTATION

STUDIES ON ANTE- AND POST- MORTEM LESIONS AS ANIMAL-BASED CRITERIA TO IMPROVE WELFARE AND MEAT QUALITY IN SWINE

By

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Thesis submitted to the Doctoral Studies Office in partial fulfilment of the requirements for the degree of Doctor of Philosophy

March 2017

ABSTRACT

The study of lesions as animal-based criteria on pigs is of increasing interest at both research and industry level. Lesions are important outcome measures, able to detect when animal welfare is suboptimal. The presence of lesions is also correlated to a lower quality, and thus to a loss of profits, in the derived products.

This manuscript is composed by two studies investigating the use of lesions as suitable indicators of welfare level and meat quality at both farm and slaughter level.

The first study, performed in Italy, studied the effect of different environmental enrichment devices provided to Italian heavy pigs (intended for the production of Parma Ham PDO) on the occurrence of skin, tail and anatomopathological (e.g. oesophageal gastric lesion) lesions, on carcass traits, meat quality and long-dried products. The tested devices were: hanging chains, wood-log inside a metal racket, edible block inside a metal racket. Results showed an increased tail score in the wooden enrichment, united to a higher F-o-M and a lower backfat thickness in the carcass, and to a lower water holding capacity of the meat. The edible block has not presented changes in skin and tail score, while an increased number of oesophageal lesion score was observed, which did not affect carcass and derived products.

The second study was conducted in Canada. It aimed at assessing the age of the lesion on pig carcass at slaughter through the use of a spectrophotometer and biological indicators (i.e. gene expression, histochemistry, histology) on skin samples analyzed in the laboratory. Results demonstrated that spectrophotometric color assessment is a suitable method which allows to discriminate between fresh lesions (occurred pre-slaughter) and older lesions (on-farm). The results were also comparable with what was obtained from the expression of some tested genes and from inflammation scores assessed through histology.

RIASSUNTO

Lo studio delle lesioni nel suino è di crescente interesse nel panorama scientifico e industriale in quanto la presenza di lesioni è un importante indicatore per evidenziare uno scarso livello di benessere animale che si può tradurre in un deterioramento e in una perdita di valore dei prodotti derivati.

L'elaborato si compone di studi che utilizzano le lesioni come indicatori di problematiche presenti all'interno della filiera suinicola.

Il primo studio, svoltosi in Italia, considera come diversi tipi di arricchimenti ambientali impiegati nel suino pesante italiano (destinato alla produzione di Prosciutto di Parma DOP) influenzino l'insorgenza di lesioni cutanee, della coda e anatomopatologiche (con particolare riferimento alle lesioni della pars oesophagea), nonché gli effetti sulla qualità delle carcasse, della carne ottenuta e del prosciutto stagionato. Gli arricchimenti testati erano: catena, tondelli di legno inseriti in una rastrelliera, substrato edibile inserito in una rastrelliera. I risultati hanno dimostrato che l'arricchimento legnoso ha provocato un incremento di lesioni della coda e un tenore di carne magra superiore e di spessore del lardo dorsale inferiore nella carcassa, nonché una minore capacità di ritenzione idrica nella carne. Il substrato edibile non ha prodotto effetti indesiderati sulle lesioni cutanee e della coda mentre è stato riscontrato un incremento di lesioni dello stomaco, che non ha influenzato la qualità della carcassa e dei prodotti ottenuti.

Il secondo studio, svoltosi in Canada, si è occupato di determinare l'età delle lesioni cutanee presenti sulla carcassa suina attraverso l'utilizzo di uno spettrofotometro in sede di macellazione e di indicatori biologici (espressione genica, istochimica, istologia) sui campioni prelevati e analizzati in laboratorio. I risultati hanno dimostrato che è possibile, attraverso l'utilizzo dello spettrofotometro, differenziare tra lesioni recenti (pre-macellazione) o lesioni più vecchie in allevamento. I risultati sono stati confermati da quanto ottenuto dall'espressione di alcuni geni testati e dalla risposta infiammatoria.

“In a world full of people who couldn’t care less,

be the one who couldn’t care more.”

Anonymous

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PREFACE

Maintain livestock animals in a good animal welfare is a responsibility which involves the entire human society. According to Broom (2016), despite a utilitarian approach, a deontological approach is also necessary because in some cases a poor welfare cannot be justified by benefits to others. The term sentient has become used in legislation about animals since the Treaty of Lisbon (European Union, 2007) unless the concept that animals used by people should be protected from actions that might cause suffering was widespread so far. So animal welfare becomes a scientific concept and being able to assess how good the welfare is as well as to evaluate poor welfare one of the major challenges during the last 30 years (Broom, 2016). Faster, animal welfare becomes an important component in the sustainability of systems and the quality of animal products (Velarde et al., 2015), indeed a part of consumers' concern (Di Pasquale et al., 2014). Welfare outcome indicators have been developed by many scientists, with the purpose to be used by veterinary, inspectors and, generally, by those who work with animals. Animal-based measures are now considered best reliable criteria to identify animals whose welfare is poor or to identify where welfare is deteriorating in order to prevent the risks and to maximize benefits (EFSA Panel on Animal Health and Welfare, 2012). An example of such indicator in pigs is tail lesion assessment. Although many animal-based measures are simple and easy to use even under commercial conditions, in some cases the measure may require further analysis or further studies to be correctly interpreted. A prospective for those last ones, with continued technical developments, is to improve automatic recording and precision livestock farming techniques, to let currently unfeasible animal-based measures become suitable in the future (Wathes, 2009).

Talking about swine, among the others, the study of lesions in the pig skin, tail lesion and anatomopathological lesions, appears to be interesting indicators which may not only provide important information on the level of animal welfare and of a given pig or batch of pigs, it may provide information on the critical points in pig marketing, both in rearing system and in pre-

slaughter practices. To a better understanding of the information and opportunities on the utilization of this welfare indicators in risk assessment, deeper investigations both at basic and applied science level are required, with the purpose to improve sustainability and quality of the entire production system and derived products.

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1.
LITERATURE REVIEW

1.1. Animal-based measures

Animal Welfare can be assessed through wide variety of parameters which measure behavior, physiological changes, clinical condition, productivity parameters and others. Nowadays, the trend in writing animal welfare protocols are more focused on the adoption of animal-based measures instead of considering resource-based or managed-based measure (Grandin, 2014). An example of this protocols is the European Welfare Quality® (Welfare Quality®, 2009) (Fig. 1.1).

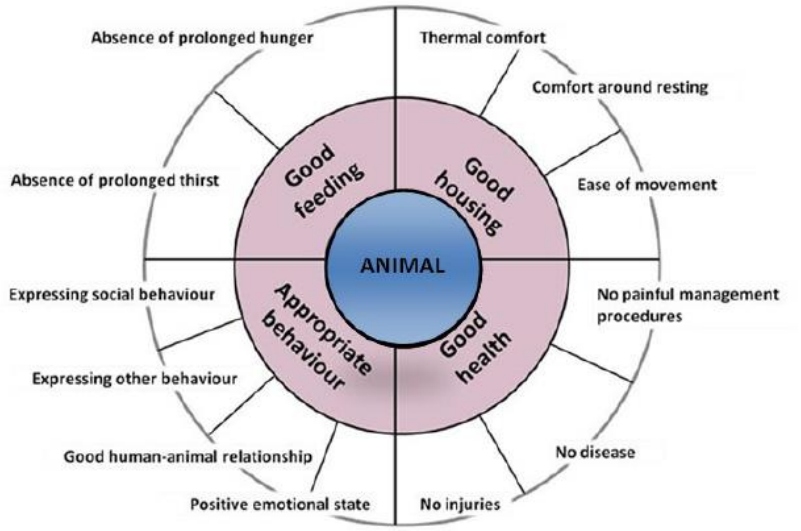


Figure 1. 1 The 4 principles and 12 animal-based criteria used in the Welfare Quality® project. Animal-based measures could predict the risk of poor welfare if no change or intervention will made and help to identify hazards in a risk assessment prospective.
(Source: EFSA, Panel on Animal Health and Welfare, 2012).

Animal-based measure can be assessed directly on the animals themselves, regardless of their keeping conditions (Pandolfi et al., 2017) and it considers how an individual will react, according to its physiological characteristic, to the many factors which may affect animal welfare, such as management practices, rearing conditions, environment etc.

These outcome measures make possible to assess and compare animal welfare across different environments. Animal-based measures can be collected during all the phases of the production

marketing in farms or at the abattoir. Indeed, they can be obtained directly from the animals or through the use of reporting systems (*e.g.* sanitary surveillance), recording production parameters, or after laboratory analyses (EFSA Panel on Animal Health and Welfare, 2012).

Unless animal-based indicators are considered the most appropriate to effectively measure animal welfare in animals, being able to select which measure or combinations of measures are the most appropriated to monitor a given issue on animal welfare may become very challenging.

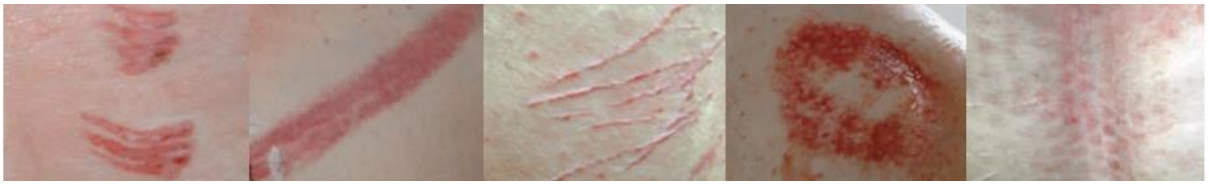
There are many factors which should be taken in account such as practical feasibility, economical aspect, required technology if the output is easy to be interpreted or further study are required. For this reason, the research of so-called iceberg-indicators, with the purpose to reduce the large amount of existent outcomes through the use of multivariate analysis, seems very promising (Pandolfi et al., 2017). Also, the developing of precision livestock-farming technologies for monitoring animal welfare may be greatly supportive. It might allow checking routinely a lot of animals, through the development of specific technology for monitoring specific animal welfare criteria or integrating the existing technology with more specific data, helping to better integrate animal welfare in the risk-assessment perspective (Wathes et al., 2008).

1.2. Lesion as animal-based measures in pigs

1.2.1. Skin lesions

Also if may be plenty of sources of skin lesions in pigs (Fig.1.2), the most frequent derive from fighting (ITP, 1996; Varón-Álvarez et al., 2014).

Figure 1. 2 Shapes of frequent lesions detected on the carcasses.
1= comma; 2= rectangular; 3=linear; 4=diffuse; 5= rhomboidal (Modified from: Varón-Álvarez et al., 2014)



Fighting-type lesion is usually up to 10 cm length, linear and tramline, mostly concentrated in high number in the front and hindquarter part (Teixeira and Boyle, 2014), presenting a typical comma shape (Fig. 1.3). According to Stewart et al., (2008) fighting occurs between 2 pigs placed with the body parallel or perpendicular to each other, ramming or pushing with the head and often biting, in rapid succession, the opponent (Fig 1.3). The number of skin lesions in the upper-front region of pigs' body is positively correlated with the duration of the fights (Turner et al., 2006) unless, according to the same author, pigs that refuse to fight presents more lesion located in the rear part of the body.

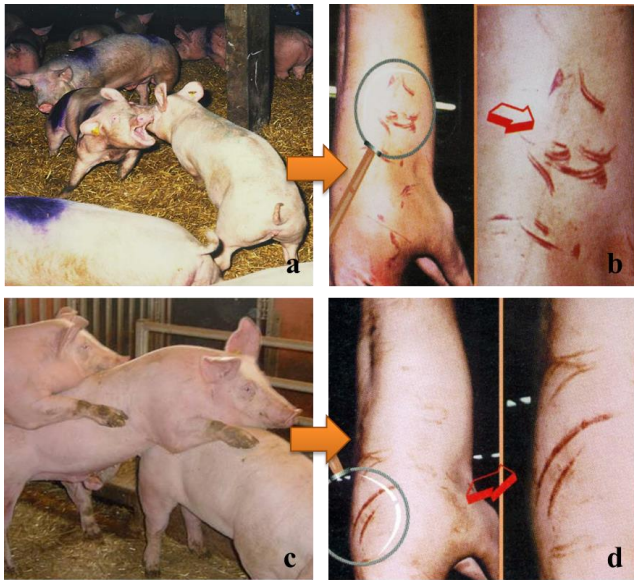


Figure 1. 3 Comma-shape lesions (b) and long linear lesion (d) deriving from biting during fighting (a) and mounting behavior (c) respectively. (Sources: ITP, 1996; www.welfarequality.net; qpc.adm.slu.se. Last access: 26/03/2017)

The main risk factor for the expression of this aggressive behavior consists in when unfamiliar pigs are mixed. Mixing is considered the major source of social stress in pigs (van de Weerd and Day, 2009) and it may occur many times during the pig marketing chain: on farm, at loading, in the truck during transportation and at lairage. A study from Aaslyng et al. (2013) demonstrated that the number of skin lesions increased significantly during all these phases (Fig 1.4.).

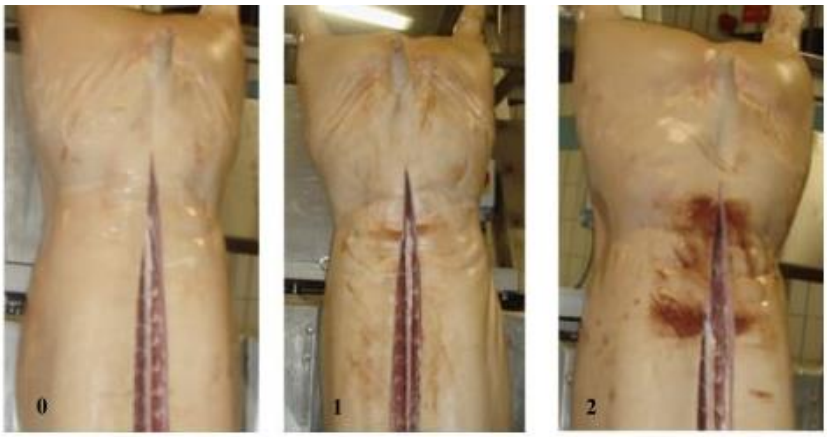
Particularly the duration of lairage has been largely investigated. In a study from (Geverink et al., 1996), skin damage was found to increase with the increasing between 0 and 3h, and from 1 to 15h (Guàrdia et al., 2009). A longer lairage appears to allow the pig to rest and then increase the fight and thereby skin damage (Faucitano, 2010). Also keeping a pig in big groups during lairage and increasing stocking density from 1.0 to 2.7 pig/m^2 was found to increase the risk of fighting and

skin lesion appearance (Geverink et al., 1996; Rabaste et al., 2007).

Other factors may influence the incidence of fighting. One of them is imputable to the poor environment, intended as a lack of space and to the absence of stimulus, due to a barren environment or to the provision of ineffective environmental enrichment (Thomsen et al., 2012).

Figure 1. 4 Example of a severe damaged carcass. Lesions were mostly due to fighting

Figure 1. 5 Loin bruising score for carcasses:
0= no evidence of bruising; 1= moderate loin bruising; 2=severe or extensive loin bruising (Modified from: Teixeira and Boyle, 2014)



Also and increased incidence of fighting was found in groups composed of only male (Teixeira and Boyle, 2014), and a partial correlation with faster-growing pig was noticed by Pitts et al., (2000).

Another common-type of lesions is the result of mounting behavior, which is a part of a normal pigs' behavior repertoire, consisting of placing hooves on the back of a standing pen-mate (Scott et al., 2006). This behavior was found common in both sexes and in barrows, with and increased incidence groups constituted only by entire males (Van Staaveren et al., 2015). Lesions, in this case, are more than 10 cm length, linear and mostly placed in the back region (Teixeira and Boyle, 2014). An increase occurrence of this behavior was also correlated with aggressive behaviors and to an increased number of lesion assessed in finishing pigs prior to slaughter (Brandt et al., 2015). Mounting behavior may also result in loin bruises (Teixeira and Boyle, 2014) (Fig. 1.5).

Another source of skin blemishes may be imputable to rough handling, due to the use of electric

goads and sticks and as a consequence of a blunting trauma (Fig. 1.6) (Barington and Jensen, 2013). The assessment of skin lesion may be done using pictorial standard (ITP, 1996) or counting the lesion, per each region of the body as proposed from some protocols such as the Welfare Quality® (Welfare Quality®, 2009) and the Canadian Animal Care Assessment, and American Meat Institute audit guide criteria (Rocha et al., 2016).

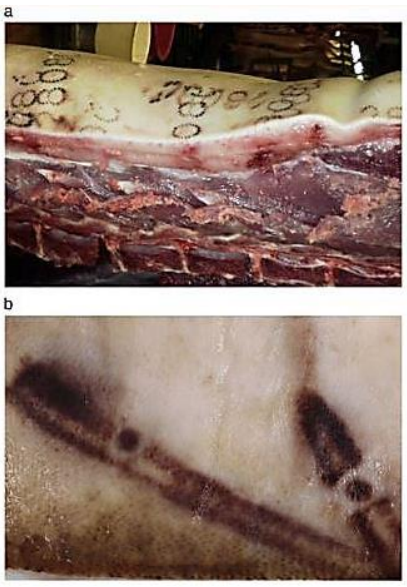


Figure 1. 6 Examples of lesion inflicted by humans: skin of a finishing pigs with lesions due to the excessive use of a tatoo hammer which has caused haemorragia in the subcutaneous fat tissue (a); skin of a finishing pig whit bruises due to a blunt trauma (Source: Nielsen et al., 2014).

Skin lesion may be measured both at farm and at slaughter level (and in the last case they may be measured on the animal at lairage, in the stunning cage, or post-mortem on the carcass). Studies between the correspondence of lesions assessed ante- and post- mortem presented contrasting results. Some researchers argue that carcass treatments (i.e. dehairing and singeing) could make welfare-related carcass damage difficult to detect (Aaslyng et al., 2013). Conversely, Carroll et al., (2016) stated that skin lesion were more detectable after carcass deharing and singeing and Brandt et al. (2015) have found that assessing lesion in the carcass was most correlated with aggressive behaviors and welfare of pigs at farm, while (Van Staaveren et al., 2015) have found correlation between skin lesions scores recorded at farm and in the carcass, declaring that both methods are accurate indicators of pigs' welfare. Overall, it is important to consider that on-farm assessment is more dependent from the occurrence of some on-field conditions, such as a big group of animals, lack of space, light conditions during the assessment and pigs' body dirtiness. Also the presence of bristles, especially in some pigs, may hide some lesions from the count. A correct choice about

where is more appropriate to assess skin lesions needs to be evaluated in accordance with the purpose of the study and to both farm and slaughter conditions.

1.2.2. Tail lesions

Tail biting is deeply important in swine production, due to the high prevalence of the pathology and to the difficulty to prevent and to eradicate its occurrence in intensive barren husbandry systems (EFSA, 2007). Hothersal et al., (2016) survey suggested that an intact tail indicates a good level and quality of animal welfare related to pig's husbandry and management. Moreover, reducing the number and severity of tail biting behavior and so tail lesions has been associated with a higher incidence of carcass condemnation (Harley et al., 2014).

Tail biting is an abnormal behavior, which arises when a pig (biter) bites the tail of another pig (victim). Motivationals basis are mostly related to the frustration of the exploratory and oral needs in pigs. In fact, in intensive husbandry, the poor environment conditions don't allow them to manifest rooting, chewing and manipulate behaviors (Studnitz et al., 2007).

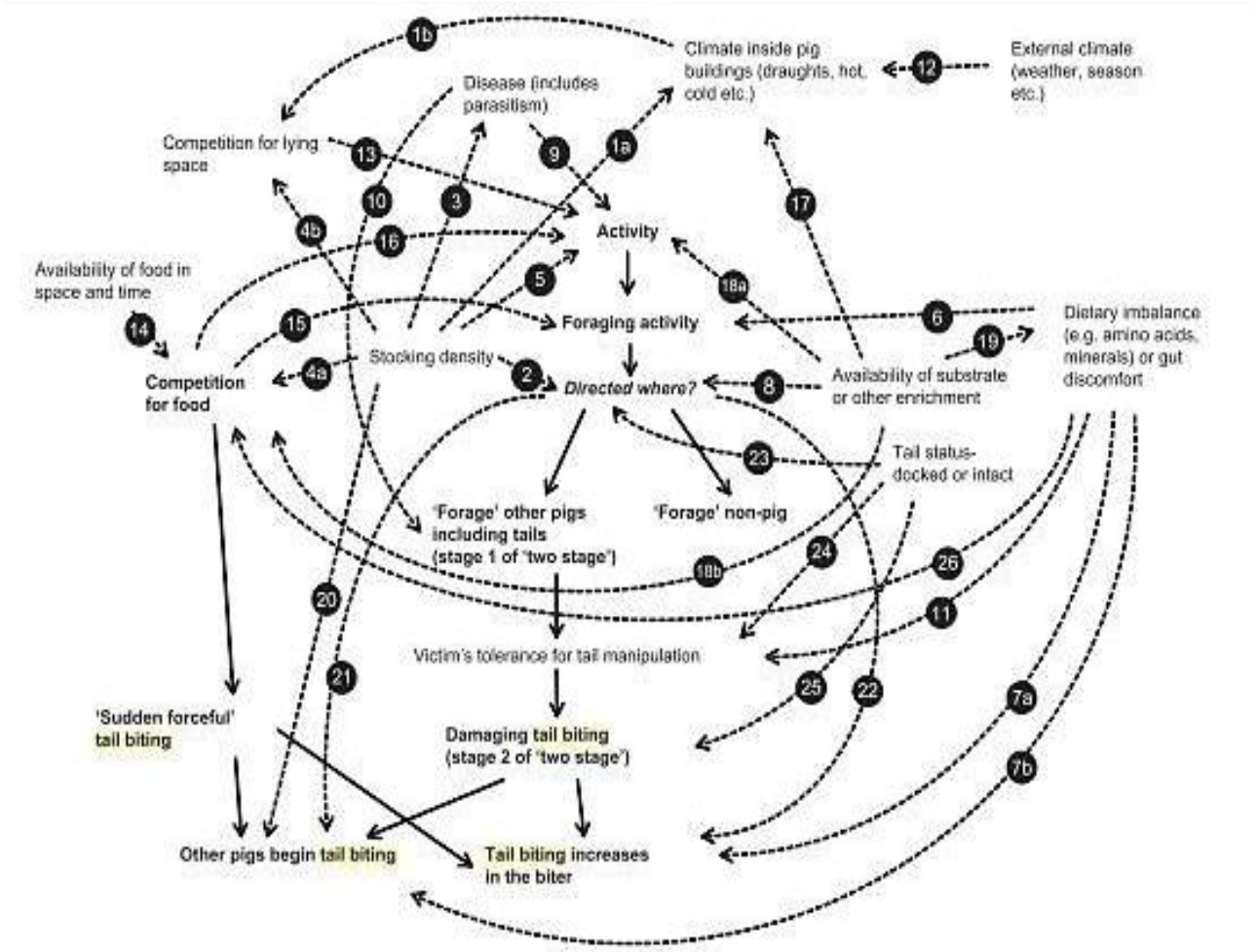
A study from Schrøder-Petersen and Simonsen (2001) identified two phases on the development of the pathology. The first one called "the pre-injury stage", is characterized by a pig chewing lightly the tail of another, which usually tolerates it. The second one, "the injury stage", follows when the tail is injured and bleeding. Usually, the first stage is not detectable from the farmer, and when the second stage occurs, it is already difficult to solve the problem. The frequency and intensity of tail biting may results in tail skin damage, bleeding, and even the complete loss of the tail. Subsequently, infections, abscesses, paralysis, pyaemia and death may occur (Moinard et al., 2003).

When tail biting occurs, the sign that welfare is poor is clear in both victims and biters. The victim will suffer pain and fear, being unable to escape from the attacks because of the limited space; the taste of blood and other stimulations (e.g. an increased activity) make biters more excited, causing an escalation of tail biting behavior, involving more pigs in the pen (EFSA, 2007).

Tail biting is considered as the main "iceberg indicator" of animal welfare in finishing pigs (D'Eath

et al., 2014). In fact, many factors may interact to increase the incidence of tail biting behavior (Fig. 1.7). Anyway, barren environment, lack of space, poor air quality, rationed feeding and mixing of animals are considered the most important factors in increasing tail biting in growing-finishing pigs. Environmental enrichments are proposed as one way to reduce tail lesion to furnish pigs with rootable, chewing and destructible substrates.

Figure 1. 7 The scheme shows how many underlying processes may interact and outbreak as tail biting. In bold, factors connected by solid arrows, while the others are suspected risk factors. The numbers in the arrows show how some of the risk factors might influence each other or the process of tail biting. (Source: D'Eath et al., 2014)



Environmental enrichment for pigs is mandatory in the EU since the Directive 2008/120/EC (European Council, 2008), and it usually consists in alternative-enriched husbandry systems, straw-based housing systems or point-source-enrichments objects (van de Weerd and Day, 2009). However, because of its feasibility with the slatted floor, which is the most common husbandry

system in EU (EFSA, 2007), the use of point-source enrichment objects is the most common, unless it presents some critical points. In fact, providing pigs with enrichment devices was found to be barely effective on the reduction of tail lesions , because most of the objects tested were found to be less attractive to pigs, or were not able to maintain interest all along the rearing time (van de Weerd and Day, 2009) . Other factors limiting the effectiveness of point-source enrichment objects may have occurred where the toys provision alone was not enough to guarantee a good welfare status (Nannoni et al., 2016). The provision of straw was found to considerably reduce tail lesion score in pigs (EFSA, 2007a). This may be related to its attitude to reduce aggressive behavior because of its being a highly manipulative substrate (EFSA, 2007a) as well as a functional nutrient: it increases fiber content in high-rationed pigs' diet (Di Martino et al., 2013) and, if correctly managed, it improves the comfort of bedding and the quality of the air. Conversely, problems concerning its application in slatted-floor husbandry systems, waste disposal and economic costs, have limited its application in modern industrial farming (EFSA, 2007a).

Differently than skin lesions, tail lesions are less influenced by animal fighting or pre-slaughter handling, unless mixing of animals and unstable group hierarchy increase the incidence of tail lesions (Van Staaveren et al., 2015). Indeed, other factors were found to be effective in the reduction of tail lesion such as: mixed-sex groups, weight homogeneity between pen-mates., and keep pigs in small groups instead of large groups (Schrøder-Petersen and Simonsen, 2001; Zonderland et al., 2008; D'Eath et al., 2014; A. Scollo et al., 2016)

The role of high stocking density in the development of tail biting cannot be underestimated: the reduction in the available space per pig increases the social tension within the pen and compromises pigs' freedom to perform normal activities such as locomotion, resting synchronization, and generally the ability to reach resources, increasing frustration (Schrøder-Petersen and Simonsen, 2001; Moinard et al., 2003; A Scollo et al., 2016). Moreover, some production systems may increase the risk of high stock density. In fact, the current directive provides indication on space allowance for pigs up to 110 kg (1.0 m^2/pig), but in some productive system (i.e. Italian heavy-pig

production) pigs must reach a weight range of 160-170 kg to be used for PDO (protective designation of origin) products, unless they are reared in the same design-environment common throughout the EU (Scollo et al., 2016).

Additional risk factors for tail biting is the poor air quality, because sub-optimal microclimate conditions may be a source of chronic stress (Hunter et al., 2001). Particularly, ammonia level was considered as the primary noxious gas able to induce aversive behavior (Wathes et al., 2002). However, it could also be assumed that an adverse combination of environmental parameters (e.g. temperature, humidity, ammonia, carbon monoxide, dust) may influence the occurrence of tail biting (Wathes et al., 2000).

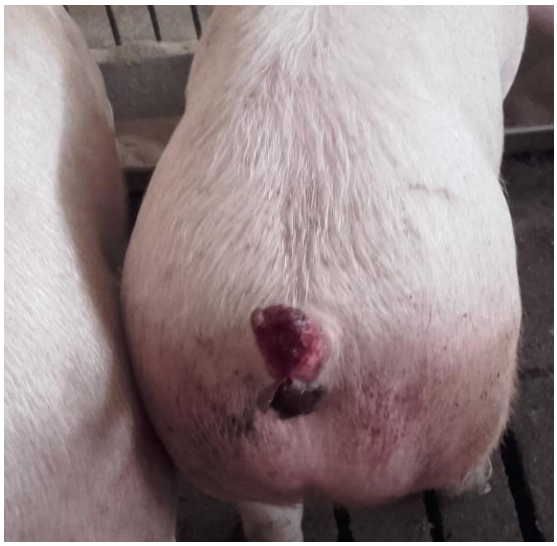
Feeding practices are also very important. Studies have demonstrated that an appropriated design of the feeder, e.g. number, types, position, length (Hunter et al., 2001; Moinard et al., 2003), and constancy in feeding time (Scollo et al., 2016) are factors able to limit the incidence of tail biting. Survey data showed that pellet or dry feed increased tail biting outbreaks, while liquid meal feeding didn't (Van Putten, 1969; Hunter et al., 2001; Moinard et al., 2003).

Despite the factors mentioned above, the main risk factor is still the pig's tail, hence the main "preventive" measure usually adopted by pig farmers: tail docking. Tail docking is now prohibited in EU from the Council Directive 2008/120/EC because of welfare and ethical issues concerning the acute and chronic pain deriving from the amputation (Nannoni et al., 2014), with some exception in the case of effective needs. Despite that, tail-docking is still a widespread practice throughout the Union (Nannoni et al., 2014). Indeed, studies on the effectiveness of this practice on preventing tail biting are contrasting. Hunter et al. (2001) and Scollo et. al. (2016) observed that animals with longer tails were the recipients of more tail-directed behaviors, showing an increased tail score; while Moinard et al., (2003) observed that tail-docking increased the risk of tail biting. Differences in the experimental design and tail lesion score method may have been the cause of this discrepancy.

Behavioral analysis presents a good perspective as a preventing tool to detect tail biting before second-phase symptoms come out (Schrøder-Petersen and Simonsen, 2001), but studies in this direction have not obtained suitable results so far (Zonderland et al., 2008b). Conversely, the behavioral analysis was found to be not correlated with tail lesion score assessed at slaughter (Teixeira and Boyle, 2014). A limit to behavioral assessment may be related to the long time needed to process video analysis (Fig. 1.8), or to the scan sampling used, or again to the loss of some information (*e.g.* a pig may be hidden by another, light condition, dust).

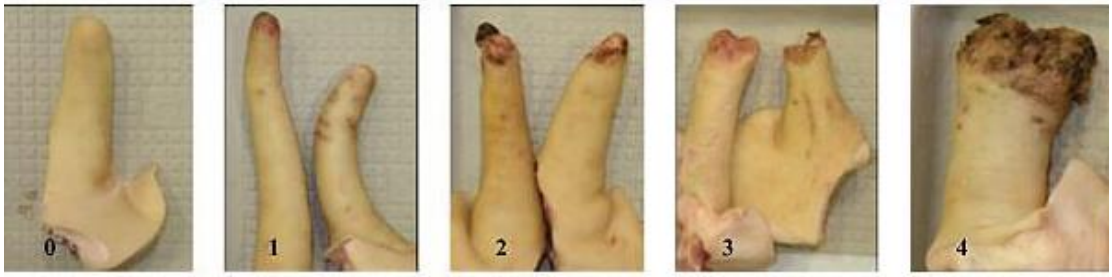
Figure 1. 9 Tail biting behavior assessed through video analysis

Figure 1. 8 Severe tail damage noticed during on-farm assessment. The lesion present a partial loss of the tail which is also bleeding and swelling.



Indeed, tail lesion scoring provides a feasible indicator of tail biting occurrence in the pig batch, also measuring the severity of the damage. The score can be recorded both at the farm (Fig. 1.9) and at slaughter, and it is usually based on a score system assessing the severity of the damage (Fig. 1.10).

Figure 1. 10 Tail lesion score for the carcasses: 0= no evidence of tail biting; 1= healed or mild lesions; 2= evidence of chewing or puncture wounds, but noevidence of swelling; 3=evidence of chewing or puncture wounds with swelling and signs of possible infection; 4= partial or total loss of the tail. (Source: Teixeira and Boyle, 2014)



1.2.3. Oesophageal gastric lesions

Ulceration of the pars oesophagea is a common widespread condition in growing-finishing pigs worldwide, reared in the intensive production system (EFSA Panel on Animal Health and Welfare, 2012). Surveys at the abattoir reveal that 20% of pigs present erosive lesion and 60% have pre-ulcerative parakeratosis lesion (Friendship, 2004). The major concern associated with gastric ulceration is represented by mortality. A survey from Melnichouk (2002) evidenced at necroscopy that approximately 27% of pigs died because of severe blood losses, becoming OGU the first cause of death, followed by pneumonia (19%).

The pars oesophagea is a non-glandular region of the stomach and does not secrete mucus. Is covered with stratified squamous epithelium and this layer offers limited protection against low pH in the lumen and gastric enzymes (Amory et al., 2006).

When the epithelium is damaged, hyperplasia results in thickening hyperkeratosis, and, if left to spread, it prevents the nutrients to reach the cells. Subsequently, the weakening of the junctions between the epithelial cells allows gastric juices into the underlying tissue, causing the development of erosion and ulcers (Robertson et al., 2002). Oesophageal gastric ulceration (OGU) is a multifactorial condition arising from one or more factors (Tab. 1.1), involving feeding, managing and welfare practices.

Factors which are able to maintain firmness in the stomach instead of a rapid transit were considered able to prevent OGU (Herskin et al., 2016). Because of that, the presence of liquid content in the stomach was associated with an higher score in OGU assessment (Baustad and

Nafstad, 1969). Factors as providing the pigs with straw, also in small quantity (Nielsen and Ingvartsen, 2000; Di Martino et al., 2013; Herskin et al., 2016), and a generally coarse ground diet were found to be able to give partial or complete protection against OGU in relation to the percentage of inclusion in pigs' diet (Baustad and Nafstad, 1969; Bubenik et al., 1998).

Table 1. 1 Risk factors associated with oesophageal gastric ulceration in pigs

Nutricion	Housing/Managemnt	Other
Absence of straw providing [1,2]	Confinement rearing [4]	Concurrent disease [4]
Ad libitum feeding [11]	Feeding regiment [4]	Genetic [3]
Automated feeding system [11]	Herd size [4]	Helicobacter infection [4]
Changing in diet [11]	Holding and transport [4]	Helicobacter spp. [8]
Dam water [11]	Lairage duration [5]	Heredity [4]
Fasting [6,7,9]	Mixing pigs [4]	Histamine [4]
Feed particle size [4,5]	Overcrowding [4]	Parturition [4]
Finely ground feed [3]	Slatted floor [1]	PCV2 [4]
Grinding vs. rolling [4]	Starving [12]	Season [4]
Hammer-mill grain ground [3]		Somatotrophin [4]
Heat processing [4]		Stress [3]
Lack of fiber [4]		
Maize [10]		
Milling [4]		
Pellet feeding [1,11]		
Pelleting [4]		
Rancid fat [4]		
Type of grain [4]		
Vitamine E/Se deficiency [4]		
Withdrawal of feed [4]		

[1] Amory et al., 2006; [2] Di Martino, 2013; [3] EFSA, 2007; [4] Friendship 2006 in: EFSA, 2007; [5] Friendship, 2004; [6] Lang et al., 1998; [7] Lawrence et al., 1998; [8] Maghalha and Miranda, 1996; [9] Makinde and Gous, 1998; [10] Nielsen and Ingvartsen, 2000; [11] Robertson, 2002; [12] Straw et al., 1994

Type and frequency of antibiotics' administering was found to have no effect on OGU in a study from Robertson et al. (2000). The same author observed that the quality and the source of the drinking water provided might have an impact on gastric lesion development, due to pH values, buffering abilities (e.g. the presence of bicarbonate anions) and microbiological quality. A study from Wondra et al., (1995) reported that adding sodium bicarbonate and potassium bicarbonate to a finely ground diet limited the incidence of ulcers. The role played by stress in the development of

gastric lesion was hypothesized in some studies (Bubenik et al., 1998; Robertson et al., 2002; Herskin et al., 2016), but a lack of evidence is attested, due to the multifactorial source of the pathology (Amory et al., 2006). Still, it appears logical that the presence of ulcers or erosions on the pars oesophagea represent a source of pain for the animals, requiring to consider this indicator on the overall evaluation of animal welfare of a given pig or batch of pigs.

The first step on OGU assessment should be done on living animals by detecting the clinical signs of gastric ulceration. In fact, according to Friendship (2004), blood loss into the gastrointestinal tract is the main clinical event in this condition and a source of anemia and melena. Severe anemia results in a very pale, weak pig, with rapid breathing. The feces are scant, black and tarry. Death may occur, also as a secondary consequence to another clinical problem (e.g. an outbreaking respiratory disease, or anorexia by infectious disease). Nevertheless, in many cases symptoms are subclinical and pigs may appear healthy if blood loss is minimal. In some cases, a reduction of average daily gain was detected also in pigs with lesions of a medium level (Ayles et al., 1996).

Endoscopic examination may be useful to detect subclinical OGU. The technique is simple and easy to master. The advantage is that erosions are easily detectable, while a disadvantage is that the visualization needs an empty stomach and, because of starving, gastric acids and bile may worsen damages on the proximal part of the stomach, increasing the risk of ulcer development (Ayles et al., 1996). Moreover, endoscopic examination requires anesthesia, which increases the cost of the practice. The usual method for monitoring the prevalence of oesophageal gastric lesion in a large group of animals is to record gross lesions at slaughter (Ayles et al., 1996) (Fig. 1.11).

Monitoring the prevalence of OGU at slaughter may help to identify issues regarding the management/feeding practices and to improve the quality of pig marketing, therefore preventing OGU. It is important to consider that, because ulcer may occur rapidly (24 h in total for the entire progression from normal pars oesophagea to complete ulceration) and heal quickly as well, in some cases it is difficult to relate lesions at slaughter with farm-related issues (EFSA 2007b). From another point of view, if serious occurrence of OGU is noticed (i.e. high mortality), a change in diet

to a coarsely ground ration, also from a short period, can quickly resolve the problem (Ayles et al., 1996). Lastly, because many of the factors associated with increased risk of OGU development are also associated with economic competitiveness (*e.g.* finely ground feed and fast-growing pigs), a cost-benefits analysis applied to the prevention program is recommended for each farm, in order to best balance economic and productive traits with animal welfare concerns (EFSA, 2007).

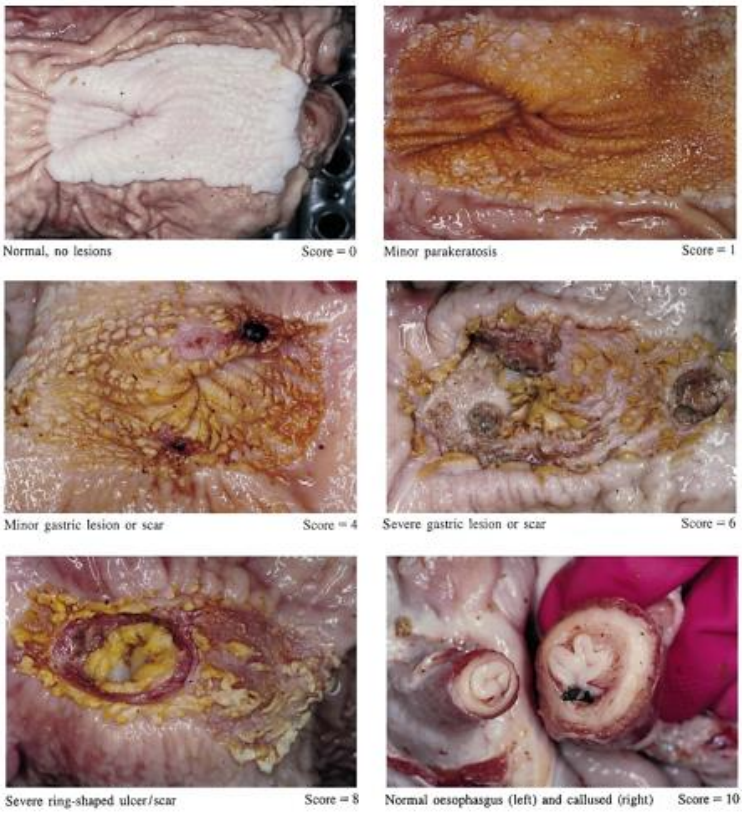


Figure 1. 11 Examples of oesophageal gastric lesion. (Source: Nielsen and Ingvartsen, 2000)

1.3. Impact of pre-slaughter lesions on carcass condemnation and poor meat quality

Consequences deriving from a poor animal welfare during all the pre-slaughter phases are actually well recognized from both researchers involved in animal science, veterinaries and stakeholders involved in pigs' marketing (Grandin, 2014) so far.

For example, increasing animal welfare may have an impact on reducing carcass damages and waste by reducing the occurrence of injuries; decreasing pre-slaughter mortality; improving the quality of the meat by reducing stress in animals (Chambers and Grandin, 2001).

According to Guàrdia et al. (2009), skin damage affects the risk of obtaining PSE (pale soft and

exudative) and DFD (dark firm and dry) pork meat. The risk of PSE occurrence doubled in skin damaged carcasses than in unblemished carcasses (6.9% vs 3.3% respectively). Also, the incidence of PSE was higher in pigs with the *nn* genotypes for the gene RYR1 and skin damages than in pigs with the same genotype but with unblemished skin or in *NN* genotype pigs with skin lesions. Also, DFD pork was found to be positively influenced by skin damage, and the risk was estimated to be almost 4 times higher than the risk between the same categories of damage (11.7% vs 3.3% respectively). The same result on the correlation between the incidence of DFD meat and skin lesion was also obtained from Guàrdia et al. (2009). This result is interesting because as DFD pork is more related to early stress (*e.g.* at loading) and PSE more to acute stress (*e.g.* at lairage) it suggests that the skin damage score may be a valid indicator to better understand the effect that some underlying source of stress may have on meat quality (Guàrdia et al., 2009). For example, because mixing is the main source of skin lesion and results moslty in fighting-type lesions (as described above), if there were a greater incidence of a higher pH24 value, these aggressive behaviors were more related to on-farm practices, loading or transport practices (depending on the length of the transport). Contrarily, if aggressive behavior and fighting take place just prior to slaughtering (at unloading or lairage), then skin blemishes are expected to be more related to a very low pH24 (Guàrdia et. al., 2009; Warriss and Brown, 1985).

Harley et al. (2014) have found that 16% of carcasses were affected by severe loin bruising, and that among that, 2.5% were condemned mainly because of abscesses, and 3.3% were trimmed.

Skin lesions and bruises were also associated with pre-slaughter losses (Nannoni et al., 2016) and carcass condemnation (Carroll et al., 2016). Also, along with the increasing number and severity of skin damages, the average cold carcass weight decreased (Carrol et al., 2016).

Lee and Veary (1993) found that tail-biting caused 94% of cases of carcasses condemned because of pyaemia. Pyemia was found to lead to partial or full condemnation of carcasses (Schrøder-Petersen and Simonsen, 2001). Conversely, Harley et al., 2014 found that tail lesion was detectable in 72% of pigs, but didn't increase the risk of abscess, unless it was significantly associated with

carcass condemnation. Also, male pigs were found to have a higher risk of tail lesions and subsequent carcass condemnation. According to Moinard et al. (2003) a higher tail score corresponded to a reduced thickness of P2 back-fat in the carcass. This concords with the results obtained by Harley et al. (2014), in which severe tail lesions were associated with an estimated 12 kg loss in carcass weight, while mild lesions were associated with a -1.2 kg loss.

Oesophageal gastric ulceration is also an important cause of pre-slaughter losses, as previously described. Melnichouk (2002) assessed that 27% out of 147 pigs died because of hemorrhage due to gastric ulceration in one month.

To conclude, information on partial or total carcass condemnation in pigs is still limited (Garcia-Diez and Coelho, 2014), just like the effect of lesions in carcass trait and meat quality. It is worth noting that investigations aiming at a deeper knowledge of the risk factors are strongly recommended.

1.4. Consideration on the economic impact of lesions

The quantification of farm-to-fork losses in pig chain is an inaccurate science (Faucitano, 2010, Harley et al., 2012). In fact, disease, injury or chronic stress may occur at numerous stages and result in a loss of value by the production. Being able to determine the stage at which rejection of the products occurs can define which stakeholders must bear such losses (Harley et al., 2012; Grandin, 2014) (Tab 1.2.).

The main sources of loss of value are pre-slaughter losses during transport and lairage (Nannoni et al., 2016), or carcass condemnation at meat inspection (Faucitano, 2010). Anyway, the financial losses by producers are indicative, since the real value of meat depends on the carcass's traits (e.g. meat and fat content, carcass classification), anatomical location of the rejected parts, production target and seasonal price variation (Faucitano, 2001)

Growth-retarded pigs are an underappreciated source of loss for the producer, and it is related to poor welfare on farm. Martinez (2007) has estimated a loss of 30,000 € (according to the market

value) related to carcass condemnation in a 3-year survey conducted on 6017 pigs in an abattoir in Spain, showing a correlation between carcass condemnations and grow-retarded pig carcasses. Disease and welfare problems, such as tail biting, oesophageal gastric ulcers, pleurisy and other disease conditions are correlated with decreased carcass weight (Martínez et al., 2007).

In a study from Harley et al. (2014), tail lesions were considered responsible for financial losses due to carcass condemnation and trimming, and also to minor carcass weight. Losses from carcass condemnation and trimmings were estimated at 1.10 €/pig, while potential reduction of carcass weight was estimated to be 0.59 €/pig. On the whole, a loss of 43% of the profit margin per pig was attributable to tail biting.

In addition, the efficiency of the slaughter line depends from the number of pigs processed daily. According to Faucitano (2001), reducing the line speed for trimming or more detailed inspection processes decreases the efficiency and profitability of the business. Moreover, cuts deriving from a trimmed carcass were usually destined to lower value products with a lower margin of profit. With regards to that, it is important to considered that some high-quality meat-derived products are composed from an whole cut. The presence of blemished skin (usually correlated with damages on the subcutaneous fat or muscular tissue) in these products will cause their exclusion from the disciplinary with a considerable loss of value (Faucitano, 2001). One example of this kind of products is represented by the Italian Parma Ham (PDO). According to the production disciplinary (Consortium for Parma Ham, 1992), skin must be unblemished, otherwise it will cause the exclusion from the PDO production.

According to Harley et al. (2014): “These findings illustrate the magnitude of the impact of the presence of animal welfare for the profitability of the pig industry. They also emphasize the potential contribution that the inclusion of welfare parameters at meat inspection could make to pig producers in informing herd health and welfare management plans”.

Table 1. 2 Stages at which losses may occur in pig marketing chain (modified from: Harley et al., 2012)

Stage of production	Reason for loss	Resource in which losses occur	Stage loss is incurred	Stakeholder incurring losses
Farm	Mortality	Carcass	Farm	Producer
	Clinical illness	Medicines	Farm	Producer
	Subclinical illness	Carcass	Abattoir	Producer
	Injury	Carcass	Farm	Producer
Transport/ unloading	Mortality (dead on arrival)	Carcass	Transit	Producer unless very hight numbers indicate transporter responsible
	Injuries: fracture/bruise	Carcass	Abattoir	
	Stress	Meat quality	Retail	
	Dehydration	Decreased carcass weight	Abattoir	Producer
Ante-mortem inspection	Mortality (dead in lairage)	Carcass	Abattoir	Abattoir
	Injuries	Carcass	Abattoir	Abattoir
Post-mortem inspection	Desease conditions	Carcass	Abattoir	Producer
	Injuries	Carcass	Abattoir	Producer
	Welfare conditions	Carcass	Abattoir	Producer
	Factory Loss (mangling/contamination)	Carcass	Abattoir	Abattoir
Carcass granding	Weight too hight/low	Penalty c/kg	Abattoir	Producer
	Poor learn meat %	Penalty c/kg		Producer
Processor	Trimmed cuts can't go for premium products	Decreased value	Retail	Abattoir
Retailer	Pale, soft and exudative Dark, firm and dry Trimmed cuts	Decreased retail potential	Retail	Retailer

1.5. The prospective of assessing lesions post-mortem at abattoer

The perspective of developing an animal-welfare surveillance at the slaughter plant is of concrete interest for the industries due to the provision of valuable information about conditions all along the pig marketing chain (Van Staaveren et al., 2015). Meat inspection in particular has the potential to contribute to the surveillance of animal welfare, and currently meat inspection data are under-utilized in the EU, even as a means to inform herd health programs (Harley et al., 2012).

Being animal-based, the welfare indicators may reflect conditions during on-farm, transport, lairage and pre-slaughter. Moreover, since animal health is a component of animal welfare, meat inspection data can be used to inform herd health programs, thereby reducing the risk of injury and disease and improving production efficiency (Harley et al., 2012). Although certain measures on the carcass can be reliably used to detect injuries and disease, for most indicators research is needed to validate them as welfare indicators (Brandt et al., 2013, 2015).

Ante- and post-mortem carcass condemnation records could allow to create a database for swine issues. This database could be used to monitor swine disease as well as to improve animal health, food safety and animal welfare (Garcia-Diez and Coelho, 2014). A positive correlation was found between the percentage of carcasses condemned and the percentage of pigs with skin or tail lesion and loin bruising (Carroll et al., 2016).

Surveillance of the type and quantity of lesions on the carcass may be helpful to establish the degree to which they are correlated to aggressive behaviors – for example, the relationship between tail lesion at slaughter, and tail biting behavior at the farm is unknown (Van Staaveren et al., 2015). Teixeira and Boyle (2014) have found that skin lesion assessment on the carcass was a more sensitive indicator of aggressiveness and animal welfare assessment than those involving living animals.

Many lesions (e.g. fractures, skin lesion, tail lesion, pulmonary lesions) detected at meat inspections were considered a direct consequence of suboptimal production systems (Harley et al., 2012). Some studies have demonstrated how adopting farm-level measures have decreased the incidence of this

disease (Martínez et al., 2009; Meyns et al., 2011). Likewise, Belk et al. (2002) argued how also the prevalence of disease conditions which may occur during transportation and slaughter (e.g. Porcine Stress Syndrome) may be reduced by changes at the farm-level. On the other hand, a recent study from Rocha et al. (2016), evidenced that the debate is still been open.

As previously described, monitoring the presence of gastric lesion at slaughter is presently the best method to notice hazards in farms, these being mostly related to feeding and on-farm management practices.

Among meat inspection, there is a great variability on the utilization and recording of information all along the European Union. Carcass condemnation, lesions and a classification of each area of the body presenting lesions is recorded in countries such as Denmark, Netherlands, Norther Ireland and United Kingdom. The Danish pig health scheme aims to identify farms with particularly high prevalence of carcass condemnations and provide them with expert veterinary assistance, while in Northern Ireland data from sanitary surveillance are directly uploaded and accessible for producers via an online platform (Harley et al., 2012). Nevertheless, the EFSA opinion (EFSA, 2011) stated that the potential for meat inspection all along Europe is "greatly underutilized".

When developing diagnostic tools to be used at the abattoir, many factors should be taken into account. Cleveland-Nielsen (Cleveland-Nielsen et al., 2004) said that meat inspection might be a "cheap diagnostic tool" in herd welfare classification. Diagnostic tools should be created as to be applicable in every abattoir, reducing the actual variation existing in the sensitivity, specificity and prevalence of the inspection (EFSA, 2011). Post-mortem outputs need to be applicable under dressing-line conditions, these being throughput, line-speed and working conditions. Outputs also need to be compatible with compatible with the recording methods already in use in the abattoir (Elbers et al., 1992). The variation and inconsistency of data should also be reduced by using a standard terminology – variation in the description of identical conditions or a single cause of condemnation, or recoding a single cause of condemnation when more conditions may exist is a source of inconsistency in data capture (Harley et al., 2012). Validation of the results is also

essential to have reliable data on the prevalence of the output (Bonde et al., 2010). Finally, data should be of practical value and must be made available to decision-makers (EFSA, 2011) and at national or local level (Fig. 1.12, 1.13).

Figure 1. 12 Example of key steps for data utilization. OV= Official Veterinarian; PVP= Private Veterinary Practitioner. (Modified from: Harley et al., 2012)

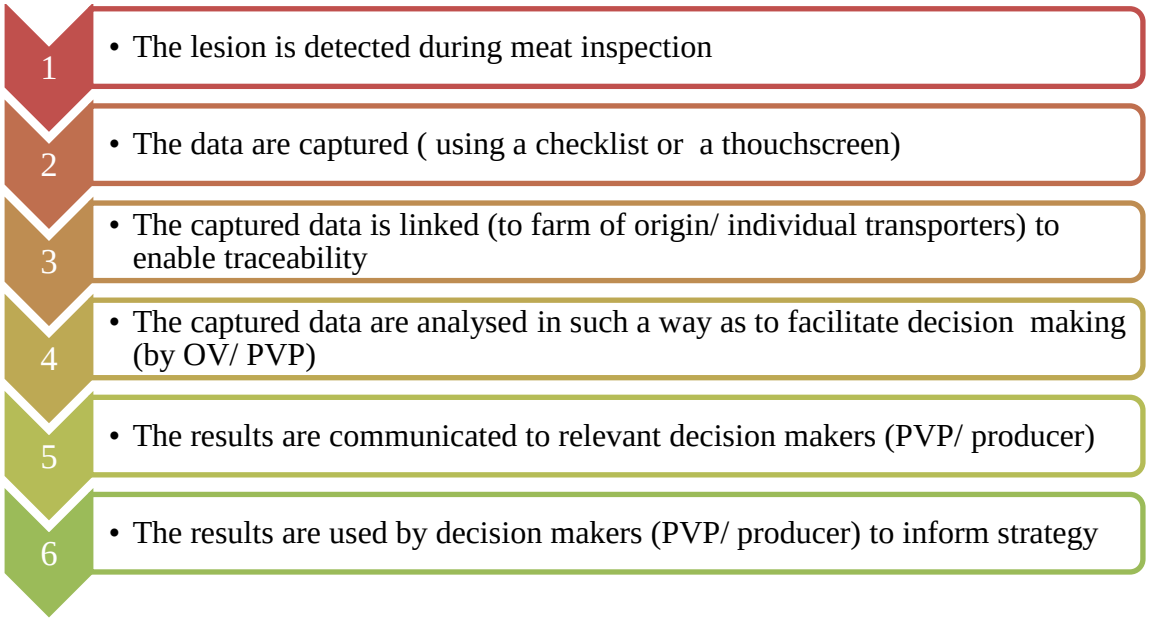
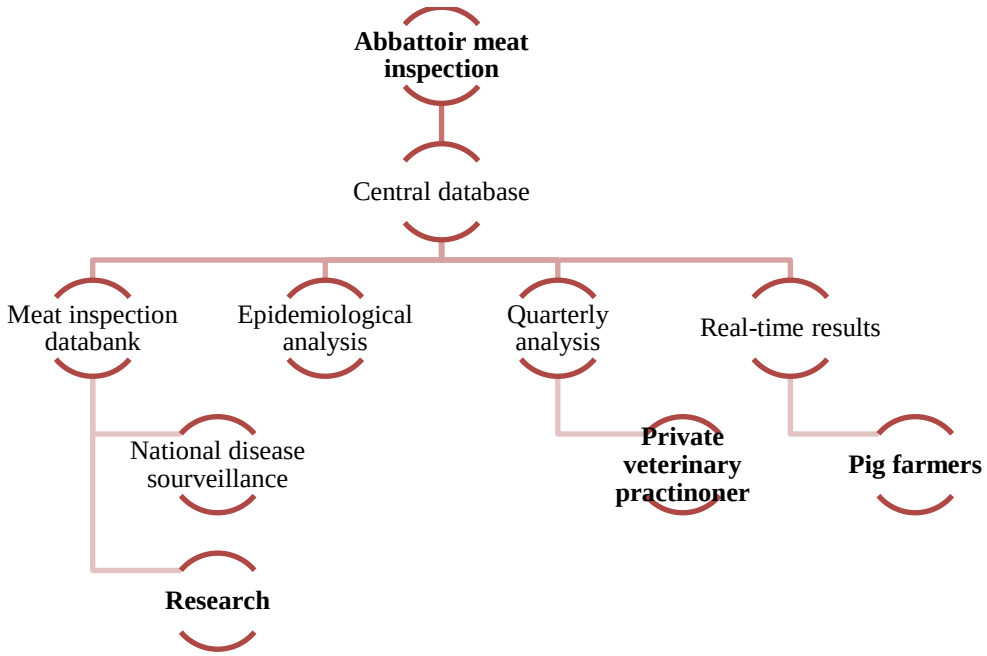


Figure 1. 13 Diagram of the information flow in the UK pig health schemes. The collection of electronic records of the gross pathology in the abattoirs will result in the summary reports sent to producers and veterinary advisers. (Modified from: Harley et al., 2012)



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PREFACE TO CHAPTER 2

The presence of environmental enrichment for pigs is mandatory in Europe, but still exist an open debate on their effectiveness, mostly on the prevention of aggressive behavior which results in increasing body lesions, and a lack of knowledge on their potentially effect on carcass traits and meat quality.

The study aims to evaluate the effect of providing pigs with three different environmental enrichment devices on skin, tail and anatomopathological lesions occurrence, and their influence on meat quality.

This study was carried out in the piggery and laboratory at the Department of Veterinary Medical Science of the University of Bologna during a two-year period.

Preliminary results based on this study were presented at the WAFL 2014 conference in Clermont-Ferrand (FR):

Nannoni E., Vitali M., Bassi P., Sardi L., Militerno G., Barbieri S., Martelli G. 2014. Study on gastric ulcers in heavy pigs receiving different environmental enrichment materials, in: Proceedings of the 6th International Conference on the Assessment of Animal Welfare at Farm and Group Level, Wageningen, Wageningen Academic Publishers, 2014, pp. 105 - 105 [Poster].

2.

EFFECTS OF DIFFERENT ENRICHMENT DEVICES ON ON-FARM AND POST- MORTEM LESIONS AND MEAT QUALITY OF ITALIAN HEAVY PIGS

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Abstract: the presence of environmental enrichment devices in pig production is mandatory in Europe. Although straw is considered as the gold enrichment for pigs, it is mostly unsuitable in slatted floor rearing systems. Hanging chains are presently the most widespread enrichment device but they are considered not sufficient to satisfy pig's needs and to prevent adverse behaviors. Two separate and independent trials were carried out in order to test the influence of two innovative environmental devices on the prevalence of skin and tail lesions on-farm, on oesophago-gastric lesions (OGL) and other anatomopathological lesions (pleuritis, pericarditis, pneumonia, white spots on the liver) and on carcass and meat quality traits of Italian heavy pig. A total of 80 undocked barrows was used. In Trial 1, one group received a metal chain (C1) as a positive control, and the other group received a wooden log placed inside a metal rack (WL). In Trial 2, the control group was provided with hanging chain (C2), and the other group with an edible block placed inside the metal rack (EB). In both trials, no differences were observed in the incidence of skin lesions and anatomopathological lesion. Besides, most of the carcass and meat quality traits were not affected by the type of enrichment provided. In Trial 1, WL group presented a higher overall tail lesion score, higher F-o-M and low backfat thickness compared to C1 ($P < 0.05$ for all the parameters). In Trial 2, EB group presented a higher incidence of severe OGL (25% vs 0%, $P < 0.001$) and a lower loin thickness ($P < 0.05$) when compared to C2 group. Our results indicate that wood logs are a less

effective device than hanging chains to prevent tail biting and, considering the urge to reduce this issue in pigs, it may not be suitable as environmental enrichment device.

Conversely, edible blocks seem to be suitable for pigs and do not alter meat quality and carcass traits, but further studies are required in order to better understand the reason for the higher OGL prevalence in this group.

Highlights:

- Enrichment devices (wood-logs, edible blocks, hanging chains) were tested in pigs;
- Wood-logs increased tail lesions and affected carcass quality;
- Edible blocks increased the risk of gastric lesions;
- Further studies on block formulation are deemed necessary.

Keywords: Animal welfare, environmental enrichment, gastric ulcers, intensive pig husbandry, skin lesions, tail biting.

Funding: This research was supported by Progetto AGER, grant number 2011-0280.

2.1. Introduction

Environmental enrichment has been defined as "an improvement in the biological functioning of captive animals resulting from modifications to their environment" (Newberry, 1995). In swine, the emphasis is put on the satisfaction of some species-specific behaviors, such as positive social interaction, exploration and rooting, and on the prevention of abnormal behaviors, mostly redirected towards penmates, which results in an increased risk of lesions on pigs' body (EFSA, 2007).

Since 2013, in the European Union the presence of environmental enrichment is mandatory by the Directive 2008/120/EC (European Council, 2008). Although the legislation provides some indication of the characteristics of manipulable materials, the final choice is on the farmer and a lack of ability to identify the effectiveness of an enrichment was evidenced at both farm and

inspection level, as reported from Hothersal et al. (2016). Nowadays in Europe, metal chains are widespread in intensive production, but their use is a source of concern from the scientist because they are deemed to be incapable of satisfying rooting, manipulating and exploring behavioural needs in pigs (Bracke, 2006).

Despite a large body of literature defining straw as the gold standard enrichment for pigs (Beattie et al., 2000) its use is unsuitable when pigs are raised on slatted floors (Zonderland et al., 2008). Other devices were largely investigated but with conflicting results (Bracke et al., 2006; Studnitz et al., 2007).

Among the manipulable materials, wood materials and edible enrichments are relatively less investigated, although they meet some important requirements for enrichment devices, i.e., they are rootable and chewable (Bracke et al., 2006), and, in the case of edible substrates, they may be able to sustain interest all along the rearing time (van de Weerd and Day, 2009). One of the main welfare outcome measures in pigs is the lesion assessment (EFSA, 2012).

Effective enrichment devices were proved to help preventing biting lesions, particularly in tails and ears, and they may reduce overall aggressiveness in pigs and decrease the incidence of lesions on the surface of pigs' body (Studnitz et al., 2007). Nowadays, one of the most important welfare challenges in pig production is the reduction of tail biting, and the abandon of the tail docking practice, which is actually regulated in Europe from the Directive 2008/120/EC but is still a current practice across the EU (Nannoni et al., 2014). Tail biting was also positively correlated with the number of lesions on the surface of pigs and with carcass downgrading and condemnations (Carroll et al., 2016). Moreover, poor rearing conditions may be responsible for acute or chronic stress, which may results in an augmented incidence of gastric lesions, particularly involving the pars oesophagea, which may cause losses in the growth ratio and be a source of pain, poor welfare and death of the animals (Di Martino et al., 2013; Melnichouk, 2002; Robertson et al., 2002).

Moreover, another important aspect to consider when introducing an enrichment device is whether it affects production traits (Weerd et al., 2006). Studies investigating the effects on carcass and meat

quality of an enrichment object have found contrasting results (Beattie et al., 2000; Foury et al., 2011; Hill et al., 1998; Klont et al., 2001). This study assessed the impact of two innovative environmental enrichments (wood logs and a block of edible material), compared to a positive control group (provided with hanging chains).

The enrichment objects were tested for their effect on on-farm lesion (skin and tail lesion) and post-mortem lesions (OGL and anatomopathological lesions) and on the main carcass and meat quality traits of Italian heavy pigs. It is important to mention that these pigs are intended for the production PDO (protected designation of origin) dry-cured ham (Parma Ham). The product specification require pigs at least nine months old and weighing approximately 160 kg at slaughter and reared under specific feeding strategies (Consortium for Parma Ham, 1992). Because of all the reasons above, these pigs are more exposed to stress factors such as frustration for hunger, poor environment, space limitation and competition for the resources.

2.2. Material and Methods

The study was carried out at the experimental farm of the Department of Veterinary Medical Sciences (DIMEVET) of the University of Bologna, Italy, in compliance with the Italian legislation implementing European Council Directive 2008/120 on swine protection. The experimental protocol was approved by the institutional Ethics Committee of the University of Bologna (Authorization Prot. n. 2-IX/9 – 27.02.2012). In accordance with the mentioned Directive, and so to mimic farm conditions (i.e., to provide environmental enrichment materials to all categories of pigs), the experimental protocol did not include a negative control (i.e., without enrichment) group.

2.2.1. Animals

A total of 80 barrows (Large White/Landrace crossbred) were used in two independent and separate trials (40 animals per trial). The trials started when animals were 80 days old the trials started when animals were 80 days old, at an average body weight (BW) of 27.1 kg (24.2 kg [±3.2kg] and 29.9

kg [± 3.9kg] respectively in Trial 1 and 2) and lasted until they reached the average BW of 163.7kg (159.0 kg [±12.1 kg] and 157.72 [±14.0 kg], respectively), for a total period of 205 days in Trial 1 and 200 days in Trial 2.Tails were left undocked. Sanitary status of the animals was constantly monitored. Pigs in collective pens (5 pigs/pen, 4 replications per group), balanced for body weight. Animals were allowed a floor space of 1.3 m^2 per pig and kept on a slatted floor. Groups were maintained the same during all the trial period (pigs were never regrouped or mixed during the trials). Each pen was provided with a nipple drinker (installed on the opposite side than the enrichment device) and a metal trough feeder. Feed was provided in a liquid form (feed to water ratio = 1:3), and distributed twice a day. The temperature in the room was kept at 22°C and humidity at 65% during the whole study, thanks to a forced-air ventilation system.

2.2.2. Enrichment devices

TRIAL 1:

In the first trial the two experimental groups were provided with the following enrichment devices:

- Chain (C1), (control) consist of a steel metal chain, hanging from a side of the pen;
- Wood Logs (WL), 3 poplar logs (10 cm in diameter, 1 m long) were put horizontally inside a metal rack. The rack was attached to the pen wall approximately 10 cm above the ground, and its overall height was approximately 70 cm as illustrated in Fig. 2.1. Pigs were able to rotate or chew the poplar logs between the metal bars of the rack.

TRIAL 2:

The two groups were provided with the following enrichment tools:

- Chain (C2) group (control), as previously described in trial 1;
- Edible Block (EB) group: these pens were enriched by providing an edible block (approximately 10 cm thick, 1m long and 30 cm wide) inside the same metal rack described above (Fig.2.1). The block was specifically formulated for the study and consisted of a mixture of corn feed, alfalfa meal, sugar beet molasses, and minerals.

Animals were checked at least once a day, and if necessary the enrichment devices were topped up in order to ensure that the manipulable materials were always available to the animals.

Figure 2. 1 Enrichment devices used in the study.
C= hanging chain; WL= wood logs placed inside a metal rack; ED= edible block placed inside a metal rack.

2.2.3. On-farm lesion assessment

The presence of skin and tail biting lesions was assessed according to the Welfare Quality® protocol for growing and finishing pigs (Welfare Quality®, 2009), with some slight modification. Skin lesions were assessed on both sides of the body and the result was evaluated following the scale described in the Welfare Quality® protocol. In the protocol, the body was divided into 5 regions (ears, front, middle, hindquarters, and legs) and scored as "a" (up to 4 lesions), "b" (5 to 10 lesions) or "c" (11 to 15 lesions). Each pig was then scored using a 0-to-2 scale, where 0 corresponded to a pig having the full body classified as "a" and 2 to a pig having at least two body regions or more classified as "c", or at least one body region with more than 15 lesions.

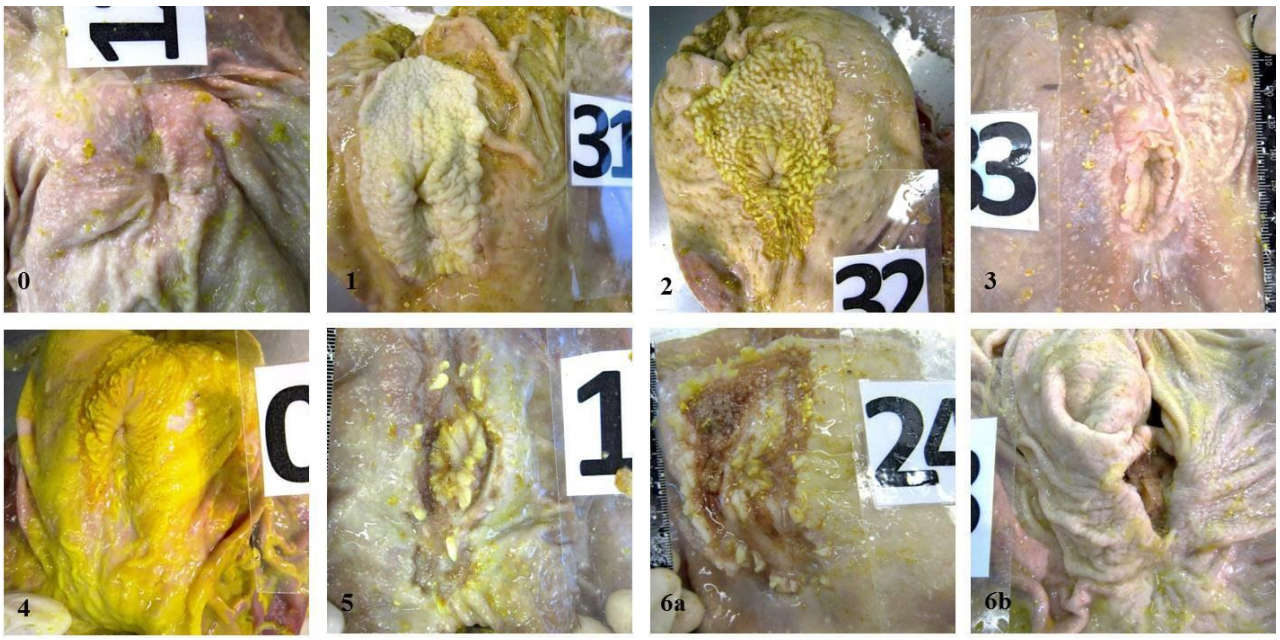
Tail biting was assessed according to the following scale: 0 (intact tail, no evidence of tail biting), 1 (superficial biting but no evidence of fresh blood or swelling) and 2 (fresh blood, evidence of swelling or infection, or tissue missing with the formation of a crust).

2.2.4. Post- mortem lesion assessment

Pericarditis, pleurisy, pneumonia and white spots on the liver were assessed in the abattoir during the veterinary inspection. The presence of pericarditis and white spots on the liver was scored with a yes/no scoring system (presence/absence). Pneumonia was scored on each of the lung lobes according to a scale proposed by Madec and Kobish (1982). The scale considers the percentage in which each lobe was damaged, and it can be summarized as follows: 0 (absence of lesions), 1 (<25% of the lobe damaged), 2 (25-50%), 3 (51-75%), 4 (75-100%). The score for each lobe was summed up. Pleuritis was scored as 0 (absence), 1 (visceral focal lesion), 2 (dorso-caudal focal lesion), 3 (deep lesions and adherence of the lung to the rib cage - lung partially ripped), 4 (diffuse inflammation and seriously ripped lung) according to Madec and Kobish (1982).

Figure 2. 2 Examples of oesophago-gastric lesions (OGL) observed during the study:(the numbers in the lower left corner of each picture indicate the score attributed to the lesion)

- 0 = intact epithelium;
- 1 = small degree of hyperkeratosis;
- 2 = distinct hyperkeratosis
- 3 = distinct hyperkeratosis and erosion at an early stage
- 4 = hyperkeratosis, mucus and one erosion
- 5 = hyperkeratosis plus more than five erosions and/or erosions > 2.5 cm in diameter
- 6a = hyperkeratosis and ulcers
- 6b = chronic ulcer



Stomachs were collected at slaughter and transferred to the Department of Veterinary Medical Sciences at the University of Bologna. There the stomachs were opened along the greater curvature, emptied and gently washed removing stomach content, in order to evaluate the lesion on the pars oesophagea (Fig.2.2). A picture of each stomach was taken, and lesions on the pars oesophagea

were scored according to Hessing et al. (1992, in Amory et al., 2006) using the following scale: 0 (intact epithelium); 1 (small degree of hyperkeratosis occupying <50 % of total surface); 2 (distinct hyperkeratosis at stage 1: > 50 % of total surface but thickness <1mm); 3 (distinct hyperkeratosis at stage 2: > 50 % of total surface but > 1mm thickness); 4 (hyperkeratosis plus less than five erosions < 2.5 cm in diameter); 5 (hyperkeratosis plus more than five erosions and/or erosions > 2.5 cm in diameter); 6 (hyperkeratosis plus more than 10 erosions and/or erosions > 5 cm in diameter, and/or ulcers, with or without bleeding, or stenosis of the esophagus towards the stomach). Scores were then regrouped in 3 classes of damage: absent or slight (0-1), medium (2-3), severe (5-6).

2.2.5. Meat Quality

Pigs were slaughtered at the average weight of 158 kg (±14 kg), according to the required body weight for Parma Ham production (Consortium for Parma Ham, 1992). The weight of each carcass was recorded. The lean meat percentage and back-fat thickness per each carcass were measured by means of a Fat-o-Meater (FOM-SFK, Copenhagen, Denmark). The pH was measured using a pHmeter (model 250A; Orion Research, Boston, MA) at 45 minutes post-mortem in the *semimembranosus* muscle. Then the carcasses were dissected and the weights of the main commercial cuts (thigh, loin and shoulder) were recorded. Dressing out percentage and the yield of the main commercial cuts was calculated from the carcass weight. At 24h post-mortem, pH was measured for the second time in the *semimembranosus* muscle and the color of the lean portion of the thighs (*Biceps femoris* muscle) was assessed using a Minolta Chromameter CR-400 (Konica Minolta optics INC., Japan), set with the D65 illuminant, and according to the CIELab (L*, a*, b*) color space (CIE, 1976). A sample was taken from the *Longissimus dorsi* (LD) muscle of each pig and used to assess meat quality. Drip and cooking loss analysis were carried out on the LD samples, according to the method proposed by Honikel (1998). Shear Force was measured on six cores from the cooked samples using an Instron Universal Testing Machine, model 1011 (Instron Ltd.,

England) fitted with a Warner-Bratzler (WB) device at a cross-head speed of 200 mm/min (Fig. 2.3). Hams were followed during the entire dry-curing process. They were weighted after dissection from the carcass, and at the end of the dry-curing period (18 months), then weight losses were calculated.

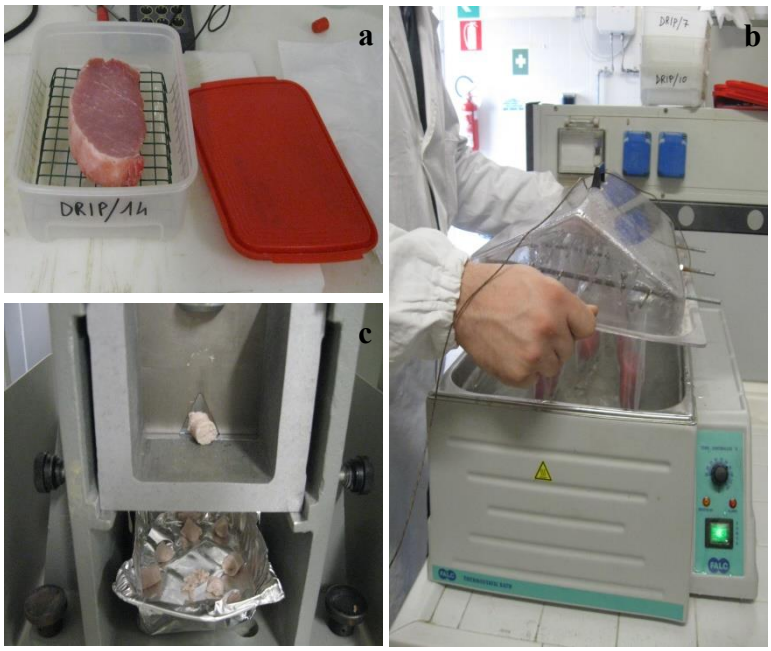


Figure 2. 3 Determination of meat quality.
a= drip loss; b= cooking loss; c= shear force.

2.2.6. Statistical Analysis

Statistical analysis was carried out for each trial separately using the STATISTICA 10 software (StatSoft, 2011). For all the on-farm and post-mortem lesions the Mann-Whitney test was used. Chi-squared test was used to evaluate the distribution of skin lesion and tail lesions in the severity classes. Data from post-mortem lesions were analyzed using the Mann-Whithney test. Carcass traits (F-o-M and weight of the main carcass cuts) and meat quality (drip and cooking loss, shear force, pH and color) were analyzed using the Kolmogorov–Smirnov test, in order to assess their normal distribution, then submitted to one-way ANOVA using the type of environmental enrichment as the main effect. The significance level was set at $P < 0.05$ for all the statistical tests.

2.3. Results

On- farm lesions

In both trials. skin lesion score presented no statistically significant differences between the experimental groups (Table 2.1). In Trial 1, tail lesions were significantly more severe in WL than in C1 group (P<0.01). In Trial 2, no differences were found between the experimental groups for tail lesions.

Table 2. 1 Skin and tail lesion assessment of pigs receiving different environmental enrichment materials. (C1 and C2 = hanging chains; WL = Wood Log; EB = Edible Block)

		Trial 1		Trial 2	
		C1	WL	C2	EB
Pigs	n	20	20	20	20
1st assessment					
Skin lesions[1]	pt	0.38	0.43	0.40	0.35
Tail lesions[2]	pt	2.00	1.75	1.37	1.50
2nd assessment					
Skin lesions	pt	0.41	0.39	0.40	0.60
Tail lesions	pt	1.14	1.41	1.31	1.32
3rd assessment					
Skin lesions	pt	0.64	0.66	0.55	0.80
Tail lesions	pt	1.11	1.12	1.10	1.21
Overall skin score distribution	pt	0.50	0.50	0.58	0.64
None or slight lesion (0)	%	53.1	52.9	44.54	39.50
Moderate lesion (1)	%	45.8	46.0	52.94	57.14
Severe lesion (2)	%	1.1	1.1	2.52	3.36
Overall tail score distribution[2]	pt	1.11^{A}	1.24^{B}	1.26	1.27
Intact tail	%	79.2	73.2	76.72	76.27
Moderate tail lesion	%	15.5	22.0	20.69	20.34
Severe tail damage	%	5.4	4.8	2.59	3.39

Values with different superscripts within the same row are significantly different (A, B= P < 0.01)

[1] Skin scoring system: 0=a pig having the full body regions with up to 4 lesions; 1=all body region with at least 5 lesion and up to one region with more than 11 lesion; 2 = a pig having two body regions with more than 11 lesions or at least 15 lesions in one on the considered body regions (Welfare Quality ®, 2009).

[2] Tail scoring system: 0= intact tail, no evidence of tail biting; 1=superficial biting but no evidence of fresh blood or swelling; 2=fresh blood, evidence of swelling or infection, or tissue missing with the formation of a crust (Welfare Quality ®, 2009)

Post- mortem lesions

The incidence of pneumonia, pleurisy, pericarditis and white spot on the liver were almost absent in both trials and has shown no statistical differences between the tested groups (P>0.05, data not shown). Results from the *pars oesophagea* are presented in Table 2.2. In Trial 1, the C1 group presented more severe lesions than WL group (P < 0.01), although WL had the 60% of the lesions in the middle class of severity, compared to the 35% in the C1 group (P < 0.01). Anyway, the average score did not statistically differ between the two groups (P > 0.05). In trial 2 the overall score of gastric lesions is significantly different (P < 0.001) between the 2 groups, with a lower average score in C2 than in EB. The class distribution also confirms that EB group presented an increased incidence of severely damaged stomachs, stated at 25% versus 0% of the C2 group (P < 0.001) and an opposite pattern in the absent-slight damage class (P < 0.001).

Table 2. 2 Oesophago-gastric lesion (OGL) score of pigs receiving different environmental enrichment materials. (C1 and C2 = hanging chains; WL = Wood Log; EB = Edible block)

		Trial 1		Trial 2	
		C1	WL	C2	EB
Pigs	n	20	20	20	20
OGL score[1]	pt	3.0	3.4	2.7	3.6
Severity					
Absent-slights (0-1)	%	35^{a}	20^{b}	25^{A}	0^{B}
Medium (2-4)	%	35^{a}	60^{b}	75	75
Severe (5-6)	%	30^{a}	20^{b}	0^{A}	25^{B}

Values with different superscripts within the same row are significantly different (a, b= P < 0.01; A,B= P < 0.001)

[1] Oesophago- gastric lesion scoring system (From Hessing et al. (1992, in Amory et al., 2006)):

- 0-1= intact epithelium or small degree of hyperkeratosis occupying <50 % of total surface;
- 2-4= distinct hyperkeratosis and up to five erosions < 2.5 cm in diameter;
- 5-6= more than five erosions and/or erosions > 2.5 cm in diameter and/or ulcers.

Carcass trait and meat quality

Results from carcass and meat quality traits are shown in Table 2.3. In Trial 1, WL group presented higher F-o-M value and lower backfat thickness, compared to C1 group (P < 0.05). In Trial 2, carcasses from EB group had lower loin thickness than C2 group (P < 0.05). All the other carcass

traits did not differ between the experimental groups. Meat quality parameters showed differences in trial one for the parameter drip loss which was increased in WL if compared to C1 ($P < 0.05$). In Trial 2, meat quality parameters did not differ between the experimental groups. In both trials, instrumental color and hams weight losses during dry-curing showed no differences between the experimental groups (Tables 2.4 and 2.5).

Table 2. 3 Carcass traits and meat quality variations of pigs receiving different environmental enrichment materials. (C1 and C2 = hanging chains; WL = Wood Log; EB = Edible block)

		Trial 1			Trial 2		
		C1	WL	RMSE[1]	C2	EB	RMSE
Pigs	n	20	20		20	20	
Live weight	kg	164.90	165.00	4.08	167.34	169.58	7,52
Cold carcass weight (CW)	kg	140.58	140.68	3.95	143.14	145.37	6,33
Dressing out	%	85.24	85.24	1.09	85.55	85.73	1,06
Fat-o-Meater	%	49.86^{a}	51.06^{b}	1.41	51.27	49.54	3,07
Backfat thickness	mm	25.15^{a}	22.55^{b}	2.10	23.25	24.85	4,98
Loin thickness	mm	63.15	62.55	2.63	64.50^{a}	59.20^{b}	7,62
Shoulder	%CW	13.48	13.39	0.75	13.86	13.97	1,17
Loin	%CW	24.09	24.97	1.05	22.93	23.23	0,77
Thigh	%CW	24.10	24.04	0.96	23.64	23.50	0,83
Lean cuts	%CW	61.67	62.40	1.50	60.43	60.70	1,87
pH_{1h} Loin		6.54	6.46	0.34	6.54	6.43	0,24
pH_{1h} Ham		6.53	6.35	0.67	6.43	6.46	0,24
pH_{24h} Ham		5.59	5.66	0.22	5.73	5.79	0,14
Drip loss	%	1.00^{a}	1.23^{b}	0.31	1.55	1.52	0,30
Cooking loss	%	23.13	22.08	1.91	23.92	24.67	2,54
Shear force	kg/cm^2	5.61	4.84	0.89	3.52	4.02	1,06

Values with different superscripts within the same row are significantly different (a, b= $P < 0.05$).
[1]Root mean square error

Table 2. 4 Meat color of pigs receiving different environmental enrichment materials. (C1 and C2 = hanging chains; WL = Wood Log; EB = Edible block)

	Trial 1			Trial 2		
	C1	WB	RMSE[1]	C2	EB	RMSE
Pigs (*n*)	20	20	-	20	20	-
Color (Longissimus dorsi muscle)						
L*	48.05	49.86	2.43	49.11	50.43	6,35
a*	9.35	9.77	2.01	10.25	11.20	2,98
b*	7.91	8.30	1.28	7.10	8.00	1,97
Hue	0.73	0.72	0.27	34.80	35.84	5,66
Croma	12.39	12.92	1.91	12.51	13.84	3,35
Color (Biceps femoris muscle)						
L*	46.44	47.56	2.07	45.76	45.02	3,50
a*	6.11	6.76	1.17	7.85	8.39	1,73
b*	5.92	7.49	1.07	4.90	4.76	1,39
Hue	43.89	47.21	5.35	31.56	29.58	4,94
Croma	8.73	9.93	1.26	9.29	9.67	2,06

Data analysis evidenced no statistically significant difference ($P > 0.05$) between the experimental groups.
[1]Root mean square error

Table 2. 5 Hams weight losses during dry-curing (C1 and C2 = hanging chains; WL = Wood Log; EB = Edible block)

		Trial 1			Trial 2		
		C1	WL	RMSE[1]	C2	EB	RMSE
Pigs	n	20	20	-	20	20	-
Pre-trimming weight	kg	16.87	16.77	-	17.05	17.19	-
Final weight after 18 months	kg	9.08	8.89	-	9.49	9.66	-
Weight loss after 18 months	%	35.74	36.86	1.40	34.58	33.94	1.49

Data analysis evidenced no statistically significant difference ($P > 0.05$) between the experimental groups.
[1]Root mean square error

2.4. Discussion

No sanitary problems occurred during the experimental trials. Behavioral results from the companion paper (Nannoni et al., 2017, *submitted*) on the same trials have showed that pigs receiving WL enrichment, interacted the 0.64% of the time with it, while C1 group spent 1.84% of the time with chain ($P < 0.01$). In Trial 2, the time spent in the interaction was the 2.85% in EB group and 1.12% in C2 group ($P < 0.0001$).

On-farm lesions

No differences were found in the lesion score parameter. Furthermore, the incidence of severe skin lesions was very low during all the rearing period in all the experimental groups. Also, the behavioral analysis (Nannoni et al., *submitted*) saw that the incidence of aggressive behaviors was very low (below 0.60% of the diurnal hours) and showed no differences between the experimental groups . In this trial, pigs were grouped at the beginning of the trial (according to their weight) and never mixed again. Because mixing unfamiliar pigs was found to be the main risk factor for skin lesions in pigs (Aaslyng et al., 2013; Beattie et al., 2000; Fàbrega et al., 2013; Olsson et al., 1999; Turner et al., 2006; Van Staaveren et al., 2015), this result is not surprising. Instead, tail lesions presented an increase incidence only in the WL group, and this result is due to the higher number of lesions classified as moderate in that group. It may suggest that wood logs placed in the rack were not enough accessible and, therefore, rootable for the pigs. According to Moinard et al. (2003), providing an enrichment which is not manipulable may increase the incidence of tail biting. Behavioral observation in the companion study has shown no differences in the frequency of tail biting behavior between the groups, but it may be due to the scan sampling method used for video analysis, which may not have been representative in this case. Indeed an increased amount of time spent in lateral recumbency and a reduced time spent walking and interacting with the environment was observed in WL group. According to Schrøder-Petersen and Simonsen (2001) pigs which spend time laying down on the floor are likely to be either victims of tail biting or biters. Overall, the

presence of severe damages on the tail was low in all the groups. It is important to consider also that the tail was left undocked, and other factors may have helped preventing tail lesions, such as the low number of pigs per pen (5 pigs/pen), the liquid meal, the presence of environmental enrichments and the stability of the groups, according with other studies in this field (Moinard et al., 2003; Scollo et al., 2016; Taylor et al., 2010).

Post-mortem lesions

Results from Trial 2 showed a higher incidence of severe lesions of the pars oesophagea in EB compared to C2 group. However, the worse average gastric lesion score didn't affect the average daily gain (ADG) and feed conversion ratio (FCR) (Nannoni et al., *submitted*), and did not result in disease signs. This is in contrast with a study from Ayles et al. (1996) which observed a lower ADG in pigs with at least moderate damages in the pars oesophagea (assessed through endoscopic examination in alive animals). Moreover, none of the pigs has showed clinical signs of oesophageal gastric ulceration (OGU), described in Friendship (2004). OGU is a serious multifactorial pathology in intensive pig farming and it is a source of animal welfare and economic concern worldwide (Amory et al., 2006b; EFSA, 2007; Melnichouk, 2002). Many factors were found to play a role in the incidence and severity of OGU such as feeding practices, fineness of feed particles, feed nutrient content, infections with bacteria and stress (Robertson et al., 2002). Perhaps the difference between the 2 groups in Trial 2 may be explained by the composition of the edible block. Many studies evidenced that small particle-size (Ayles et al., 1996; Nielsen and Ingvartsen, 2000); low content of fiber (Amory et al., 2006a; Di Martino et al., 2013; Fuller, Dale E., and Boenker, 1968; Herskin et al., 2016); maize (Nielsen and Ingvartsen, 2000) and a high content of sugar (Fuller, Dale E., and Boenker, 1968) have increased the incidence of OGU incidence in pigs. Question is if the intake of the enrichment from the animals was really able to affect gastric physiology or if other unknown factors may have occurred. In fact, according to the companion study (Nannoni et al., submitted), pigs from the EB group have spent 2.85% of their time interacting with the environment

and, based on piggery recording, the rack was refilled with a new block on average once a month during the 6 months of the trial. Other factors that should be investigated are the stress or frustration derived from reaching the enrichment material, the possible competition to access the material (Docking et al., 2008), the individual pig intake, and bacterial infection, especially from Helicobacter spp. (Magalha et al., 1996).

Carcass traits and meat quality

In Trial 1, results showed that pigs which received the wood-log device had lower backfat thickness, higher value at the F-O-M, and higher drip loss if compared with the control group with hanging chains. A study from Moinard et al. (2003) which considered the risk factors for tail biting on 92 pig farms in England, observed that farms which low backfat were more likely to report tail biting, which happened also in this study. An increased drip loss was evidenced also from Klont et al. (2001), in pigs kept in a barren environment, while Beattie et al. (2000), evidenced also an increased cooking loss (but not drip loss) and reduced backfat thickness in pigs kept in a barren environment compared to pigs with a fully enriched environment (extra space, plus peat and straw in a rack). Similarly, Lebret et al. (2006) measured lower backfat thickness, higher F-o-M value and increased drip loss in pigs kept in a barren environment compared to pigs kept in an enriched environment (access to outdoor space).

In Trial 2, the group EB presented a lower loin thickness. However, the parameters backfat thickness and F-o-M did not differ between the two groups. To the author's knowledge, no studies on environmental enrichment provision have considered this parameter before.

General remarks

Surprisingly, the results obtained from WL group in both this and the companion study, suggest that the level of enrichment provided by wood log placed inside a rack, is more similar to a barren environment than to an enriched one, and also that its effectiveness is lower than what has been

obtained in the present study by supplying pigs with plain hanging chains as enrichment. In fact, to resume, pigs in WL group showed less interaction with the environment, less activity and more time spent in lateral recumbency (Nannoni et al., *submitted*) united to higher tail lesion score and differences in backfat content, F-o-M and drip loss. The reason might be searched in the design of the enrichment. In fact, WL was a mostly static and less rootable enrichment. Also if the design was similar to what provided for the edible block, we believe that the block, conversely to the wood logs, might compensate with flavor and odor stimulus and might also create a positive reinforcement, which motivates pigs to reach and manipulate it, according to Van De Weerd et al., (2003) and van de Weerd and Day (2009).

2.5. Conclusion

The results obtained from the present research assessed that point-source environmental device may have a slight effect on tail lesion, OGL and carcass and meat quality variation. Particularly, wood-logs resulted to have lower efficacy than hanging chain enrichments, due to the higher prevalence of tail biting in the pigs of the wood-log group and to a worse water-holding capacity of the meat. Edible blocks appear to be a promising device for pigs, although they presents a critical point, due to the increased incidence of OGL in the group, which may suggest further studies to increase the fiber content in the formulation.

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PREFACE TO CHAPTER 3 AND 4

The perspective of developing a diagnostic tool suitable at slaughter which allows assessing the age of the lesion in the carcass is of importance today because it will respond to both industries and animal welfare needs in term of risk assessment. Being able to estimate the age of lesions in the carcass can allow knowing at which point of the pig marketing problems may occur, and therefore to prevent it.

This study tested the use of spectrophotometry as a method for skin age lesion assessment and compared it with biological indicators such are gene expression, histology and histochemistry.

The study has been anticipated from a preliminary study in where potential genes indicators of the time of injury were selected.

The research was carried out in the facilities of Agriculture and Agri-food Canada, Research and Development Centre in Sherbrooke (Canada).

Preliminary results based on this study were presented at the Joint annual meeting ASAS 2016 conference in Salt Lake City (UT):

Vitali M., Conte S., Lessard M., Martelli G., Guay F., Faucitano L. 2016. Assessment of the age of lesions on the pig carcass at the abattoir through spectrophotometric color assessment and gene expression analysis. Journal Of Animal Science, 94, pp. 845 - 845 [oral presentation].

3.

CAN THE AGE OF LESIONS ON THE PIG BODY AND CARCASS BE DETERMINED BY GENE EXPRESSION? A PRELIMINARY STUDY

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3.1. Introduction

Besides being an economic issue as it downgrades the carcass value, the presence of skin damages on the carcass is an indicator of poor animal welfare (Faucitano, 2001). In pigs, the most frequently observed lesions (71.8%) are the coma-shaped (2 to 10 cm long; Varón-Álvarez et al., 2014), which are mostly located on the head, neck and shoulder, because they are caused by biting during aggressive acts (Turner et al., 2006).

The second most common lesion is the mounting-type (> 10 cm long), which is usually located on the back (Teixeira and Boyle, 2014). However, to better monitor the welfare of animals from the farm to slaughter and identify the critical areas through the preslaughter process, the assessment of the age of skin lesions is also required as it provides useful information on the time of infliction allowing the identification of the cause (Aaslyng et al., 2013). According to Epstein et al. (1999) and Vanezis (2001), in the assessment of the age of a skin lesion it would be appropriate to consider the inflammatory, proliferation and remodeling phases, and related biochemical changes occurring in the affected skin. Objective methods for this assessment, such as the analysis of the expression of genes involved in inflammation and tissue repair in a skin wound, have been studied in human and animal models (Gallant-Behm and Hart, 2006; Kubo et al., 2014; Wang et al., 2015), but few

studies were done in pigs (Barington and Jensen, 2013). Moreover, promising method, such as quantitative PCR analysis of cytokines, chemokines growth factors and other genes involved in tissue repair (Cecchi, 2010), have been generally used for determining the age of deep wounds (Sato et al., 1999; Wang et al., 2015; Wang et al., 2016) and thus they must be validated in superficial lesions such as those commonly observed on the pig carcass at the slaughterhouse.

The objectives of this preliminary trial have been to determine which genes are the most suitable for the assessment of the lesion age in pigs and to evaluate the effects of *post-mortem* carcass handling (i.e. dehairing and singeing) on the gene expression in skin samples collected on the slaughter line in order to identify the most reliable genes to use for the validation of on-line objective methods (i.e. spectrophotometric color evaluation).

3.2. Materials and Methods

All experimental procedures performed in this study were approved by the institutional animal care committee at the Agriculture and Agri-Food Canada Research and Development Centre in Sherbrooke (AAFC, QC, Canada), based on the current guidelines of the Canadian Council on Animal Care (2009).

3.2.1. Animals

For this study, 10 crossbred pigs (7 gilts and 3 boars; Large White x Landrace x Piétrain) weighing 100 kg (±10 kg) on average were raised individually at the AAFC swine unit in Sherbrooke (QC, Canada). After one week of habituation, a total 5 lesions (or scratches) were inflicted on each pig's shoulders using a piglet's lower jaw. Scratches were 4 mm large and 2 cm long and separated by 3 cm (Fig. 3.1). On each lesion 5 successive skin biopsies were done using a 4 mm biopsy punch (Integra™ Miltex®, York, PA) at 1, 4, 8, 24 and 48 h after the infliction of the lesion (1 time = 1 lesion). A biopsy of the non-lesioned skin (control) was also done in the area surrounding the

lesions. After collection, samples were immediately frozen in liquid nitrogen and later stored at -80°C until RNA extraction analysis. To prevent pain, the animals were treated with an anesthetic cream (EMLA®, Akorn pharmaceutical, Forest lake, IL) spread on the shoulders for 90 min before the infliction of each lesion and the biopsy sampling (Fig. 3.2), and with an analgesic (Torbugesic®, Zoetis inc. Kalamazoo, Milan, IT) that was injected three times/day (0.3mg/kg). In order to assess the effects of the post-mortem carcass handling (i.e. dehairing and singeing) on gene expression, other skin biopsies were performed on 2 lesions inflicted on the hams of 5 pigs at 24 h before slaughter (BS), after exsanguination (AE) and after singing (AS).

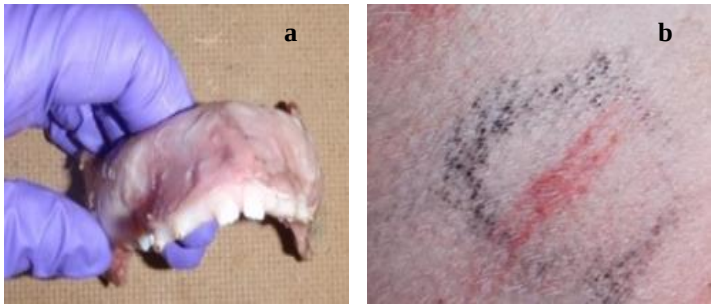


Figure 3. 2 The instrument used to injury the animals was a piglet's lower jaw (*a*), in order to reproduce as much as possible the bites which happen in the reality during fighting. Obtained lesions were long around 2 cm and constituted by two parallel scratches (*b*), being large approximatively 4mm. Lesions were inflicted from the same trained person in all the pigs, after the administration of a local anesthetic (EMLA® cream) on pigs' skin.

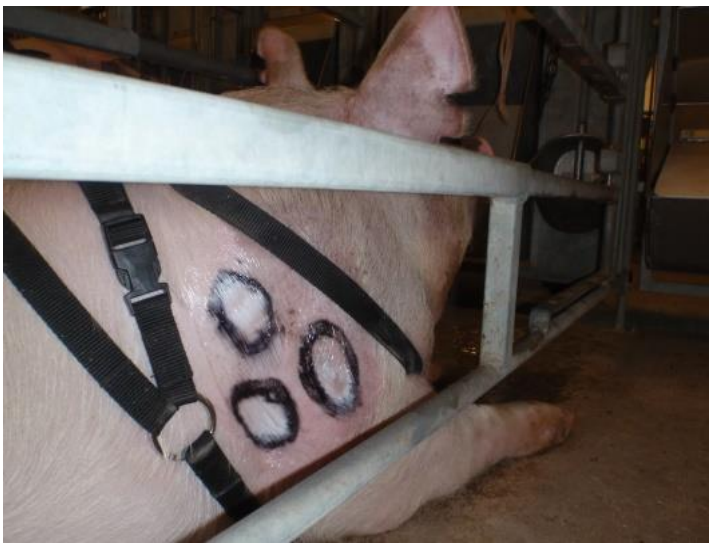


Figure 3. 1 Application of a topic anesthetic (EMLA® cream) in a pig 90 minutes before sampling

3.2.2. Gene expression analysis

From the skin punches, 30 mg of sample was homogenized in RLT buffer and mercaptoethanol. Total RNAs was extracted following the RNA Extraction Fibrous Mini® Kit, (Qiagen, Hamburg, Germany) protocol and diluted in 30 µl of nuclease-free water. All samples were quantified through

a NanoDrop Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) at a wavelength of 260 nm; the accepted value was ≥ 62 ng/µl. RNA quality was assessed through bioanalyzer with Agilent RNA 6000 Nano Kit (Agilent technologies, Germany). Obtained values of RIN >8 confirmed that the RNA quality was adequate for proceeding with Retro-transcription PCR. cDNA synthesis was performed from 1µg of RNA using the RT2 First Strand Kit (Qiagen, Hamburg, Germany) obtaining 20 µl of cDNA solution than diluted to a concentration of 1:15 µl in nuclease-free water. Real-time qPCR was performed using Step One Plus Real-Time PCR System (Applied Biosystems, MA). The qPCR analysis was initially performed using the Pig Wound Healing RT2 Profiler PCR Array by Qiagen (Qiagen, Hamburg, Germany), in 5 pigs for the following categories of samples: controls; 1h, 4h, 8h, 24h and 48h (age categories); BS; AE (40 samples in total). The genes tested in this phase are listed in Fig. 3.3.

Extracellular Matrix (ECM) & Cell Adhesion Molecules
Extracellular Matrix (ECM) Structural Constituents: COL14A1, LOC397571, COL1A2, COL3A1, LOC397502, COL5A2, COL5A3, DCN, EDN1, VTN.
Extracellular Matrix (ECM) Remodeling Enzymes: CTSK, F13A1, FGA (FGA), LOC100154047, PLAUR, F3, MMP1, MMP2, MMP3, MMP7, MMP9, PLAT (tPA), PLAU (uPA), PLG, SERPINE1 (PAI-1).
Cell Adhesion Molecules: CDH1 (E-cadherin), ITGA2, LOC100517053, ITGA4, ITGA5, ITGA6, ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, TNC.
Cytoskeleton Regulators: ACTA2 (ACT-4), ACTC1, RAC1, TAGLN (SM22A).

Inflammatory Cytokines & Chemokines
CCL2 (MCP-1), CD40LG (TNFSF5), CXCL11 (I-TAC, IP-9), CXCL12 (SDF1), CXCL2, IFNG, IL1A, IL10, IL1B1 (IL1B), IL2, IL4.

Growth Factors
ANGPT1, CTGF (CTGF), CSF2 (GM-CSF), CSF3 (GCSF), EGF, FGF2, FGF7, HBEGF (DTR), IGF1, LOC100525086, MIF, TGFA, TGFB1, TNF, VEGFA.

Signal Transduction
TGFβ / BMP Signaling: SMAD3 (MADH3), TGFB1, TGFB2, TGFB3, TGFBR3.
WNT Signaling: CTNNB1, LOC100627044 (WISP1), WNT5A.
Kinases: AKT1, EPHB2, MET, MYLK, PTEN.
Cell Surface Receptors: EGFR (ERBB1), EPHB2, IL6ST (GP130), MET, TGFBR3.
Other Signal Transduction Genes: PTGS2 (PGHS-2, COX2), PTGS1, STAT3.

Figure 3. 3 Genes tested using the Pig Wound Healing RT2 Profiler PCR Array (Qiagen, Hamburg, Germany). Modified from: https://www.qiagen.com (last access: March 12, 2017).

Later, the same procedure was used to test 13 genes selected based on the literature and whose primers were designed (Table 3.1a). Genes from the Kit and from other studies whose expression was not influenced by the exsanguination process and which presented a significant fold change (threshold was set at ±1.5 fold change) were selected to be tested on 10 animals in the 5 age classes and in the controls. Second-time designed primers are listed in the Table 3.1b. In all cases qPCR amplification was started with an initial 10 min of denaturation stage at 95°C, followed by 40 cycles of 15 s at 95°C of denaturation and 1 min at 60°C of annealing and elongation. For the genes home-

designed, the fold change in the expression was calculated using the $2^{-\Delta\Delta}$CT method (Livak and Schmittgen, 2001), using B2M, HPRT, RPS18 as housekeeping genes (with the exception of the genes in the kit which have already their housekeeping genes) and non-lesioned skin samples as a control. When the effect of exsanguination was tested, the control was represented by BS skin samples.

Table 3. 1 List of genes and primers used in the study.

Gene	Primer	Dilution (nM)	Sequence number
a			
IFNγ	F AGGTTCCTAAATGGTAGCTCTGGG	300	NM_213948.1
	R AGTTCACTGATGGCTTTGCGCT	300	
IL12 sub p35	F GGCTGCCAGAGAGACCTCTTTAAT	900	NM_213993.1
	R ATACTGCTAAGGCACAGGGTTGTC	900	
IL12 sub p40	F GCCAAGGTTACATGCCACAA	300	NM_214013.1
	R TAGAACCTAATTGCAGGACACAGATG	900	
IL17	F CCAGACGGCCCTCAGATTAC	300	NM_001005729.1
	R CACTTGGCCTCCCAGATCAC	300	
IL6	F GGAAATGTCGAGGCTGTGCAGATT	300	NM_214399.1
	R GGTGGTGGCTTTGTCTGGATTCTT	300	
IL2	F TGGAGCCATTGCTGCTGGATTTAC	900	NM_213861
	R TTCTGTAGCCTGCTTGGGCATGTA	900	
IL4	F GTGACGGACGTCTTTGCTGC	900	NM_214123.1
	R TGCTGCTCAGGTTCCTGTCAAG	900	
IL8	F AGAACTGAGAAGCAACAACAACAG	300	NM_213867.1
	R CACAGGAATGAGGCATAGATGTAG	300	
CCL5	F GAAGGTCTCCACCGCTGCCC	50	NM_001129946.1
	R ACAGCAGGGTGTGGTGTCCGA	300	
ANXA1	F GCAGTGAGCCCCTATCCTACCTTC	300	NM_001163998
	R GCAAAGCCAAAGCAACTTCCTC	300	
TIMP1	F ACCAAGATGTTCAAAGGGTTCAAT	300	NM_213857
	R TCCGCAGACGCTCTCCAT	300	
MMP14	F GCCTACTGACAAGATTGATGCTGC	300	NM_214239
	R CATCGCTGCCCATGAATGACCC	300	
HPRT1	F TTGTGGTAGGCTATGCCCTTGACT	300	NM_001032376.2
	R CTCAACTTGAACTCTCCTCTTAGG	300	
RPS18	F CATGTGGTGTTGAGGAAAGCA	300	NM_213940.1
	R TTGGCGAGGATTCTGCATAAT	150	
B2M	F TCACTCCTAACGCTGTGGATCAGT	300	NM_213978.1
	R TGATGCCGGTTAGTGGTCTCGAT	300	
b			
CCL2	F CTGCACCCAGGTCCTTGCCC	300	NM_214214
	R CTGCTGGTGACTCTTCTGTAG	300	
COX2	F AAGCGAGGACCAGCTTTCACCAAA	300	NM_214321.1
	R GCGCAGTTTATGCTGTCTCTCCAA	300	
EGF	F GAGGCAGTTCTCGTATTTGTTCCTG	50	NM_214020
	R CACCAAAAAGGGACATTGCAAACAC	900	
HBEGF	F CTGGACTTGGAAGAGGCAGAC	300	NM_214299
	R CACGTACTTGCACTCTCCGTGG	300	
IL10	F GATATCAAGGAGCACGTGAACTC	300	NM_214041.1

	R GAGCTTGCTAAAGGCACTCTTC	300	
ITGA6	F TTCTTGCCAGCAAGGTGTAGC	300	XM_005657544
	R CTCCACCAACTTCATAAGGCCC	300	
MMP1	F TGCCAAATGGACTTCAAGCTGC	300	NM_001166229
	R GATCTGTGGATGTCCTTGGGG	300	
MMP3	F TGGAGCCAGGTTTTCCCAAGC	300	NM_001166308
	R GGGTCAAACTCGAACTGCGAAG	300	
SERPINE1	F CCACCCCCGACGGCCATTAC	300	NM_213910.1
	R TGGTGAGGGCGGAGAGAGGC	300	
TNFα	F CACTGACCACCACCAAGAATTGGA	300	NM_214022.1
	R CATTCCAGATGTCCCAGGTTGCAT	300	

3.2.3. Statistical analysis

For all analyses, the experimental unit was the lesion. The fold change value for each cytokine was log transformed and analyzed using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) with the age categories in the model. Multiple comparisons were made using the Tukey-Kramer adjustment. Back-transformed values are reported as means with lower and upper limits in squared brackets.

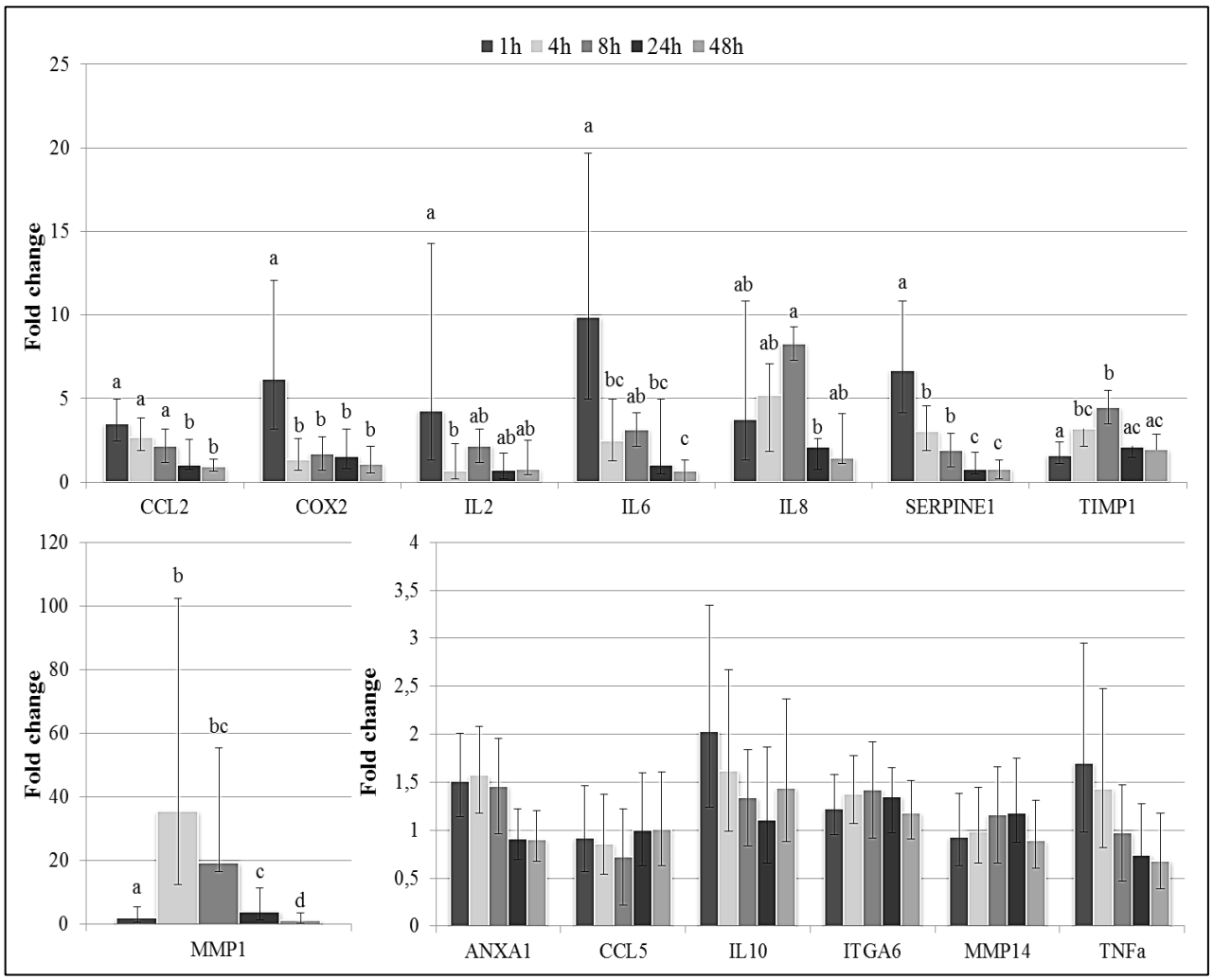
A probability level of $P < 0.05$ was chosen as the limit for statistical significance in all tests, whereas probability levels of $P \leq 0.10$ were considered to be a tendency.

3.3. Results and Discussion

All AS samples presented a very low content of RNA extracted (6.6 ±6.2 ng/μl), which is likely due to the high temperature carcass skin was exposed to during singeing resulting in RNA denaturation. No difference was found in the RNA content between BS and AE samples (106.6 ± 26.3 ng/μl and 126.9 ± 29.5 ng/μl, respectively; results not shown), which confirms that collection of skin samples immediately after exsanguination ensures the availability of good quality samples for the analysis. Of the 98 tested genes, 14 (ANXA1, CCL2, CCL5, COX2, IL2, IL6, IL8, IL10, ITGA6, MMP1, MMP14, SERPINE1, TIMP1 and TNFα) did not present a different expression after exsanguination and were also differently expressed between lesions and controls. As showed in Fig. 3.4, the expression pattern of CCL2, COX2, IL2, IL6, IL8, MMP1, SERPINE1 and TIMP1 varied with the

time of the lesion (P<0.05). Particularly, a greater (P < 0.01) expression was found for COX2 in 1 h old lesions (1 h = 6.2 [3.2-12.1], 4 h = 1.4 [0.7-2.6], 8 h = 1.7 [0.9-3.4], 24 h = 1.6 [0.8-3.1], 48 h = 1.1 [0.6-2.2], for SERPINE1 may allow classifying lesions in 3 groups: 1h, 4-8h and 24-48h (1h= 6.70 [4.15-10.81], 4h= 3.05 [1.89-4.92], 8h= 1.92 [1.19-3.09], 24h = 0.77 [0.48-1.24], 48h= 0.80 [0.5-1.3], P < 0.05), for MMP1 in 4-8 h old lesions (1 h = 1.9 [0.7-5.5], 4 h = 35.6 [12.4-102.3], 8 h = 19.2 [6.7-55.3], 24 h = 4.0 [1.4-11.4], 48 h = 1.3 [0.4-3.6]; P < 0.001) and for CCL2 and IL8 in < or > 8 h old lesions (1h= 3.49 [2.45-4.97], 4h= 2.71 [1.90-3.86], 8h= 2.17 [1.52-3.10], 24h= 1.05 [0.74-1.50], 48h= .95 [0.67-1.36], P < 0.05).

Figure 3. 4 Fold change in gene expression for 14 genes. The legend on the top of the graphs indicates the 5 age categories of the skin lesions. Means within a row with different superscripts differ (P <0.05). The error bars represents the maximum and minimum values in fold change.



3.4. Conclusions

The analysis of the expression of genes involved in inflammation and tissue repair is useful in the determination of the age of skin lesions. However, this study showed that sampling time should be considered to ensure the suitability of samples for the analysis and the reliability of the results. Based on the expression of the genes tested in this study, COX2 and SERPINE1 were the best indicators of 1 h old lesions, while the MMP1 gene is able to identify 4-8 h old lesions. To confirm the usefulness of CCL2, IL2, IL6, IL8 and TIMP1 genes for the assessment of the age of lesions in pigs, further larger scale studies are needed.

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4.

USE OF THE SPECTROPHOTOMETRIC COLOR METHOD FOR THE DETERMINATION OF THE AGE OF SKIN LESIONS ON THE PIG CARCASS AND ITS RELATIONSHIP WITH GENE EXPRESSION AND HISTOCHEMICAL AND HISTOLOGICAL PARAMETERS

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ABSTRACT: The presence of lesions on the pig carcass is both an indicator of poor animal welfare and economical issue as it downgrades the carcass value. The assessment of the age of lesions on the carcass may help identify and prevent the risk factors. The aim of this study was to assess the age of lesions on the pig carcass through spectrophotometric color evaluation and to relate the results with gene expression and histochemical and histological parameters. A total of 96 barrows were mixed 4 times during 3 d before slaughter and 80 lesions were selected after skin lesion observations to define 4 age categories: < 7 h (T1), 7-25 h (T2), 25-30 h (T3) and 49-54 h (T4). A non-lesioned skin area was used as a control. At slaughter, 3 biopsies per lesion and control skin were taken immediately after bleeding for analyses of gene expression (CCL2, COX2, IL6, IL8,

IL10, ITGA3, MMP1, TNFα, TIMP1, SERPINE1), skin histological characteristics (inflammation, erosion or ulceration and necrosis) and enzyme activity (alkaline phosphatase and adenosine triphosphatase). The number of lesions was counted on each carcass and the color was assessed visually by a pictorial chart and instrumentally through a spectrophotometer. Delta values (Δ) were calculated as the difference between the value of the lesion and the value of the control for all measures, except for the histological analysis. Results indicated that visual color observation was not sufficiently accurate to discriminate lesions by time of infliction ($P > 0.10$), while the spectrophotometer ΔL* and Δa* values variation allowed identify < 7 h or > 25 h old lesions ($P < 0.05$). Similarly, the expression of CCL2, IL6, ITGA3, MMP1 and SERPINE1 genes increased in < 7 h old lesions ($P < 0.05$), while TIMP1 gene expression was greater ($P < 0.05$) in < 25 h old lesions. As for the histological analysis, only the inflammation parameter was able to detect a difference between < 7 h and > 25 h old lesions ($P < 0.05$). To conclude, the spectrophotometric color assessment of the carcass lesions at slaughter appears to be a reliable method to discriminate between fresh and older lesions at the abattoir.

Key words: carcass, color assessment, enzyme histochemistry, gene expression, histology, lesion age, pig, skin

4.1. Introduction

The presence of skin lesions is both an animal-based criterion in the animal welfare assessment (Welfare Quality®, 2009; EFSA, 2012) and an economical issue related to the loss of profits due to carcass downgrading (MLC, 1985) and additional handling for carcass inspection (Faucitano, 2001). Common causes of skin lesions on the pig carcass are poor housing, mixing of unfamiliar pigs and rough handling (Faucitano, 2001). Lesions are commonly assessed on the carcass using pictorial standards (MLC, 1985) or by giving a score based on their number and type (ITP, 1996; Welfare Quality®, 2009). However, no reliable technique exists for the determination of the time of infliction of a lesion in pigs. As injuries can occur at any moment during the marketing process

(Dalla Costa et al., 2007; Aaslying et al., 2013), knowing the time of infliction may be very helpful to prevent their occurrence by limiting the impact of risk factors. The most common method to assess lesion age at the abattoir is the visual color assessment (Rocha et al., 2013), which, however, may be biased by human error (Scafide, 2012). Objective evaluation of lesion color using spectrophotometry, molecular biology, histochemical and histological techniques has been studied in different livestock species (Hamdy et al., 1957; Strappini et al., 2009; Munro and Munro, 2013) and in forensic medicine using human and animal models (Wang et al., 2001, Gallant-Behm and Hart, 2006; Kondo and Ishida, 2010; Barington and Jensen, 2016a,b). However, despite the need for a reliable assessment of the age of lesions on the pig carcass at slaughter (Barington and Jensen, 2013), to our knowledge, an objective technique for this determination on the pig carcass under marketing conditions is still missing.

The objective of the study was to provide a reliable determination of the age of the lesion on the pig carcass at the abattoir through objective color assessment and to relate the results with biological indicators, such as expression of genes, enzyme histochemistry and histological parameters.

4.2. Materials and Methods

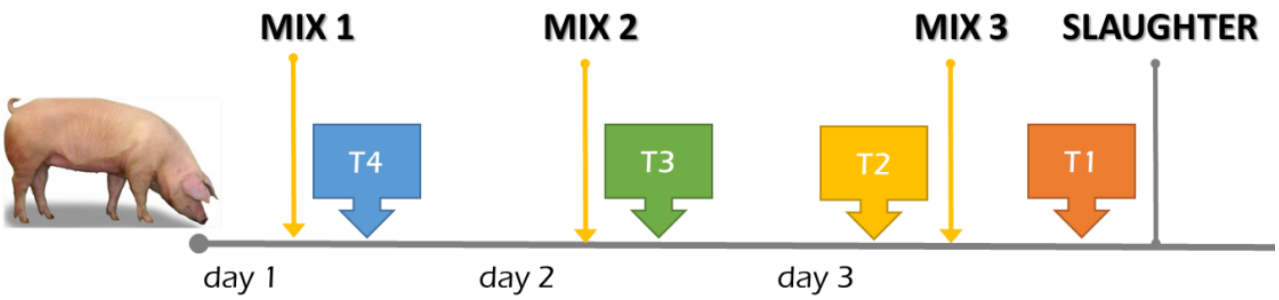
All experimental procedures performed in this study were approved by the institutional animal care committee at the Agriculture and Agri-Food Canada (AAFC) Sherbrooke Research and Development Centre (Sherbrooke, QC, Canada) based on the current guidelines of the Canadian Council on Animal Care (2009).

4.2.1. Animals and Handling

A total of 96 crossbred barrows (Large White x Landrace x Piétrain) were distributed into 8 groups of 12 pigs (4 replicates of 2 groups; 124.2 ± 1.7 kg body weight) at the AAFC swine unit in Sherbrooke (QC). Pigs from 2 groups were mixed 3 times over 3 d before slaughter: twice at the farm (2 d before slaughter and the day before slaughter) and once at loading. The first mixing

consisted in grouping 6 pigs from one pen with 6 pigs from the second pen to create two new groups of 12 pigs. The second mixing involved the remaining 6 pigs left in each pen and that had never met before. For the 3rd mixing, all pigs (24) were grouped at loading and in lairage (Fig. 4.1).

Figure 4. 1 Scheme of the experimental protocol.



Before and after each mixing event, skin lesions were scored according to the Welfare Quality® Protocol for Pigs (Welfare Quality®, 2009) by a trained observer. At the same time, lesions on all pigs were mapped on both sides of a pig body image printed on a paper and marked with different colors according to the observational times (6) in order to trace-back the exact time of the lesion's appearance. A video of each pig body was also recorded to validate the visual observation (Fig. 4.2). Biting- and mounting-type lesions, as defined by the comma short or long scratch shape, respectively, were only considered in this study as they are the most commonly observed on the pig carcass at slaughter (Varón-Álvarez et al., 2014). Lesions on the head, ears, abdominal part, legs and tail were not considered because of the risk of being affected by the *post-mortem* carcass handling procedures. Two parallel scratches of at least 2.5 cm length and 4 mm width were considered as one lesion. Just before slaughter, the lesions were selected and distributed into 4 age categories of 20 lesions each, based on the time of their appearance on the pig body and slaughter time (time 0), as follows: T1 (< 7 h old), T2 (7-25 h old), T3 (25-30 h old) and T4 (49-54 h old). The T1 lesions corresponded to fresh lesions inflicted during loading, transport and/or lairage, while the other lesions (T2, T3, and T4) corresponded to lesions inflicted during mixing at the farm.

To ease skin sampling, body areas with a selected lesion were carefully hair clipped and individually identified by a colored circle right before slaughter.

Pigs were subjected to a 12 h fasting before shipment. On the day of slaughter, pigs were loaded on the truck in groups of 4 or 5 pigs using boards and paddles and transported to the slaughterhouse (45 min). At the slaughterhouse, pigs were kept in lairage for approximately 3.5 h (excluding 1 h of skin lesion observation in the pen) and slaughtered by exsanguination in the prone position after head-only electrical stunning.

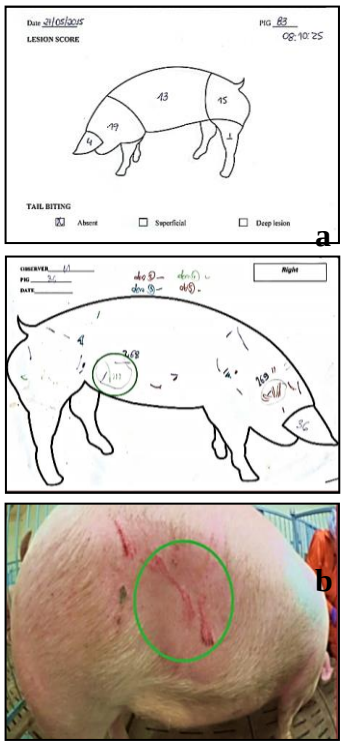


Figure 4. 2 Assessment and selection of the lesion performed at the farm before and after each mixing event and at lairage, before slaughter.
The letters in the right corner of the pictures meaning: a= lesion score; b= lesion mapping; c=video validation.

4.2.2. Sample Collection and Measurements

Immediately after exsanguination, 3 biopsies per lesion were collected on the 80 selected lesions and on an equal number of non-lesioned skin (control) using 4 mm diameter disposable biopsy punches (Integra™ Miltex®, York, PA) (Fig. 4.3). A sample for histological analysis was fixed in a 10% buffered formalin solution and one sample was embedded in O.C.T. (Optimum Cutting Temperature) compound for histochemical enzyme analysis, then snap-frozen in liquid nitrogen and

later stored at -80°C. A third sample was snap-frozen in liquid nitrogen and stored at -80°C pending Real-Time PCR mRNA analysis.

Figure 4. 4 Skin sampling at the abattoir. Biopsies were taken immediately after exsanguination.

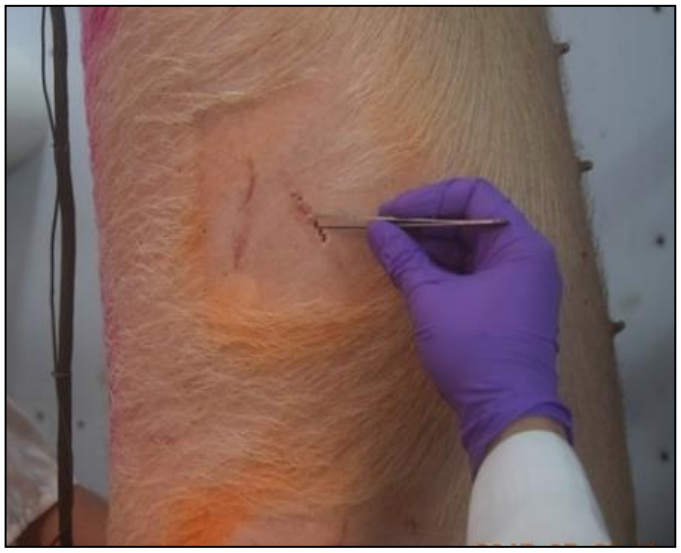
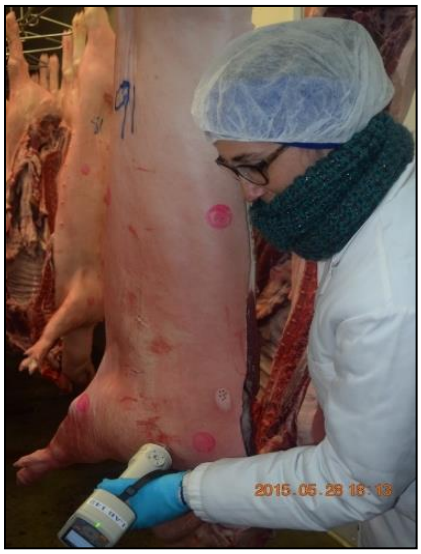


Figure 4. 3 Color assessment, performed in the cooler, after carcass deharing and singing.



In the cooler, the number of lesions was counted on each carcass using an adapted version of the Welfare Quality® Protocol for Pigs (Welfare Quality®, 2009), excluding the ears from the count as they were removed during the carcass processing. Skin damages that may have been caused by carcass processing (i.e. dehairing) were also excluded from the count. Carcasses were classified using a scale from 0 (up to 4 lesions on the whole body) to 2 (> 11 lesions in two body regions or at least 15 lesions in one body region) according to the Welfare Quality® Protocol (Welfare Quality®, 2009). The lesions and non-lesioned skin color was first assessed visually using a scale of hue and brightness values (Fig. 4.4) and a picture of each lesion was taken for further validation. Color was then measured 3 times at 3 different positions along each lesion and on the non-lesioned skin using a CM 700d Spectrophotometer (Konica Minolta Sensing Inc., Osaka, Japan). The measure were taken using a 8° viewing angle, a 2° observer angle, D65 illuminant, SCI (specular component included) mode, with an illumination measurement area of 3 mm in diameter. CIE L*a*b* color coordinates were used (Fig. 4.5).

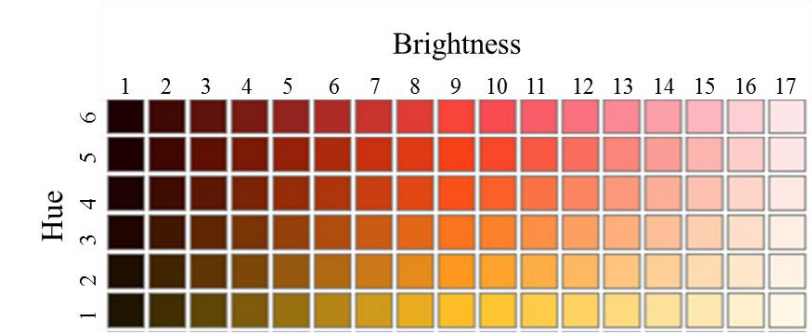


Figure 4. 5 Visual color assessment scale used to describe the color of both lesion and intact skin (control)

4.2.3. Histological and Histochemical Analysis

Histology. Samples were cut into two, routinely processed and embedded in paraffin (TissuePrep™ 2 Micron, Fisher Scientific). Sections of 4 μm thickness were deparaffinized and then stained with hematoxylin-eosin-phloxin-saffron (HEPS).

Alkaline phosphatase (ALP). Samples were cut into 4 μm sections in a Leica CM3050S research cryostat (Leica Biosystems, Concord, Canada) at -24°C. Alkaline Phosphatase Staining (Red) Kit (Cell Biolabs Inc., San Diego, CA) was used. The specimens were stained with StemTAGTM AP Staining solution and incubated at room temperature for 15 to 30 min, protected from light. Alkaline phosphatase activity appeared as a reddish stain.

Adenosine triphosphatase (ATP). Samples were cut into 4 μm sections in cryostat at -24°C and stained at pH 9.4 following the method described by Bancroft (1975). Slides were counter-stained with Harris hematoxylin for 25 s, then rinsed with distilled water. Dehydration was performed as follows: 80% alcohol for 2 min, 95% alcohol for 2 min, 100% alcohol for 2 min, and toluol for 5 min. Slides were mounted with a Leica CV 5000 automated coverslipper (Leica Biosystems, Concord, Canada).

Microscopic examination. Microscopic examination for HEPS and histochemistry stains was performed with an Olympus BX41 optical microscope (Olympus America Inc., Melville, NY).

Magnification of 25 to 400 X was used as appropriate. For inflammation, hemostasis and enzyme histochemistry, the following grading system was used to describe lesions: 0 = absent, 1 = minimal to mild, 2 = from moderate to severe. For epidermal erosion/ulceration and necrosis, this grading was modified and expressed as the percentage of epidermal surface of the sample section being affected: 0 = 0% of the epidermis, 1 = < 25% - < 50%, 2 = 50 -100%.

4.2.4. Gene Expression Analysis

Thirty (30) mg of sample was collected from the thawed skin punches and homogenized in RLT buffer and mercaptoethanol. Total RNAs was extracted following the RNA Extraction Fibrous Mini® Kit (Qiagen, Hamburg, Germany) protocol and eluate in 30-µl of nuclease-free water. All samples were quantified through a NanoDrop Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) at a wavelength of 260 nm; the accepted value to proceed with Retro-Transcription was ≥ 62 ng/µl. The quality of RNA was assessed through bioanalyzer with Agilent RNA quality 6000 Nano Kit (Agilent Technologies, Waldbronn, Germany). Obtained values of RNA integrity number (RIN) > 8 confirmed that the RNA quality was adequate for proceeding with the real-time PCR analysis. The synthesis of cDNA was performed from 1 µg of RNA using the RT2 First Strand Kit (Qiagen, Hamburg, Germany) obtaining 20 µl of cDNA solution, which was later diluted to a concentration of 1:15 µl in nuclease-free water. Real-time PCR was performed using Step One Plus Real-Time PCR System (Applied Biosystems, Framingham, MA) to evaluate the mRNA modulation for the 12 genes listed in Table 1. Of these genes, 10 were the target genes (CCL2, IL6, ITGA3, SERPINE1, MMP1, T1MP1, TNFα, IL8, IL10 and COX2) that were previously selected out of 94 genes analysed for their efficiency in skin lesion age determination in a preliminary study on live pigs (unpublished results). Primers were designed using known pig sequences for the genes of interest (Table 4.1). Real-time PCR amplification was started with an initial 10-min denaturation stage at 95°C, followed by 40 cycles of 15 s denaturation at 95°C and 1 min annealing and elongation at 60°C. Each sample was analysed in triplicate. The fold change in

the expression of the 10 target genes was calculated using the $2^{-\Delta\Delta}$CT method (Livak and Schmittgen, 2001), using B2M and RPS18 as housekeeping genes and non-lesioned skin samples as a control.

Table 4. 1 PCR primer sequences of genes used in this study

Gene	Primer	Dilution, nM	Sequence number
CCL2	F CTGCACCCAGGTCCTTGCCC	300	NM_214214
	R CTGCTGGTGACTCTTCTGTAG	300	
IL6	F GGAAATGTCGAGGCTGTGCAGATT	300	NM_214399.1
	R GGTGGTGGCTTTGTCTGGATTCTT	300	
COX2	F AAGCGAGGACCAGCTTTCACCAAA	300	NM_214321.1
	R GCGCAGTTTATGCTGTCTCTCCAA	300	
ITGA3	F ATCATTTGCGAGCTGGGGAAC	50	XM_005668880
	R CAGCTGCGCCTGGAGTTC	50	
MMP1	F TGCCAAATGGACTTCAAGCTGC	300	NM_001166229
	R GATCTGTGGATGTCCTTGGGG	300	
TIMP1	F ACCAAGATGTTCAAAGGGTTCAAT	300	NM_213857
	R TCCGCAGACGCTCTCCAT	300	
IL8	F AGAACTGAGAAGCAACAACAACAG	300	NM_213867.1
	R CACAGGAATGAGGCATAGATGTAG	300	
TNFα	F CACTGACCACCACCAAGAATTGGA	300	NM_214022.1
	R CATTCCAGATGTCCCAGGTTGCAT	300	
SERPINE1	F CCACCCCCGACGGCCATTAC	300	NM_213910.1
	R TGGTGAGGGCGGAGAGAGGC	300	
IL10	F GATATCAAGGAGCACGTGAACTC	300	NM_214041.1
	R GAGCTTGCTAAAGGCACTCTTC	300	
B2M	F GGATCAGTATAGCTGCCGCG	300	NM_213978.1
	R TCTGTGATGCCGGTTAGTGG	300	
RPS18	F CATGTGGTGTTGAGGAAAGCA	300	NM_213940.1
	R TTGGCGAGGATTCTGCATAAT	150	

4.2.5. Statistical Analysis

Visual color assessment. Delta values (Δhue and Δbrightness) were calculated as the difference between the value of the lesion and the value of the control skin. Data were analyzed using Kruskal-Wallis test including the four age categories in the model. Values are reported as median (lower and upper quartiles in brackets).

Spectrophotometric color assessment. The three spectrophotometer L*,a*,b* color values of each lesion were averaged and a delta value (ΔL*, Δa* and Δb*) was obtained by calculating the difference between the color measure taken on the lesion and taken on the control skin. Delta values were analyzed using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) including the lesion age categories in the model. Multiple comparisons were made using the Tukey adjustment. Data are presented as LSM ± SEM. Moreover, the accuracy of the spectrophotometric measures for the determination of the age of a lesion was evaluated as described by Parikh et al. (2008). Briefly, this method consisted in comparing the classification of the lesion age obtained with a measure (e.g. ΔL*) with the real age of the lesion (based on direct observations of the lesion appearance on live pigs after each mixing event). To select the best threshold value above or below which the lesion would be correctly classified as fresh or old, sensitivity, specificity and the positive predictive value (PPV: the probability that the lesion is actually fresh when the indicator is above a certain value) were calculated for several threshold values. The more the sum of sensitivity, specificity and PPV was high, the more accurate a threshold was for the lesion age classification. The negative predictive value (NPV: the probability that the lesion is old when the indicator is below the threshold value) of the indicators was also calculated (Parikh et al., 2008).

Histology. The relationship between necrosis, erosion, inflammation and hemorrhage score and age categories was assessed using the Cochran–Mantel–Haenszel test of association for ordinal

categories (row mean score statistic). In the case of a significant association, multiple comparisons (6 comparisons) were made using a Bonferroni adjusted P-value ($P < 0.008$).

Enzyme histochemistry. Delta values were calculated as the difference between the scores of the lesion and of the control. Delta values were then re-categorised into score 0 if the delta was 0, score 1 if the delta was 1 or 2, and score 2 if the delta was 3 or 4. The relationship between the score for each enzyme and the age categories was assessed using the Cochran–Mantel–Haenszel test of association for ordinal categories (row mean score statistic).

Gene expression. For technical reasons, gene expression data from one lesion in the T3 age category were missing. The fold change value for each cytokine was log-transformed and analyzed using the MIXED procedure of SAS including the lesion age categories in the model. Multiple comparisons were made using the Tukey-Kramer adjustment. Back-transformed values are reported as means with lower and upper limits in squared brackets.

Multivariate analysis. A Factorial Analysis of Mixed Data (FAMD) was performed with the PRINCOMP procedure of SAS on selected variables related to the lesion age categories in order to analyze relationships among these variables and the lesion age category. The categorical variables were transformed into a complete disjunctive table and included in the PCA analysis along with the centered and scaled matrix of numerical variables.

For all analyses, the experimental unit was the skin lesion (n = 80). A probability level of $P < 0.05$ was chosen as the limit for statistical significance in all tests, whereas probability levels of $P \leq 0.10$ were considered to be a tendency.

4.3. Results and Discussion

4.3.1. Lesion Score

According to Hamdy et al. (1957), the number of lesions on the body can influence the speed of physiological responses in the healing process. This was not the case in this study as all carcasses that were selected for the lesion assessment presented the same high score (score 2) for the number of lesions according to the Welfare Quality® Protocol (2009), meaning that in this study this factor influenced the healing process of the lesions in all pigs indifferently and did not bias the results.

4.3.2. Color Assessment

In this study, overall the visual color assessment showed that the lesions were darker (- Δbrightness; $P|t| < 0.001$) with a redder hue (-Δhue; $P|t| < 0.001$) than the control/non-lesioned skin (Table 4.2).

Table 4. 2 Effect of the age categories on the delta color values (difference between the skin lesion and the control) as assessed by visual and spectrophotometric color methods

	Age category[1]					
Color assessment	T1	T2	T3	T4	se	*P*-value
Visual						
Δ brightness	-4.50 (-6.50, -3.00)	-4.00 (-5.00, -3.00)	-3.00 (-4.00, -2.50)	-4.00 (-5.00, -3.00)	-	0.34
Δ hue	-1.50 (-2.00, -1.00)	-1.00 (-2.00, -1.00)	-1.00 (-2.00, -1.00)	-1.00 (-2.00, -1.00)	-	0.87
Spectrophotometric						
ΔL*	-13.16 ^{aA}	-9.05 ^{abB}	-8.22 ^{b}	-6.63^{b}	1.141	0.001
Δa*	15.11^{aA}	10.62 ^{abB}	9.34^{b}	8.43^{b}	1.317	0.003
Δb*	5.85	4.99	5.38	5.04	0.589	0.71

[1]Age categories: T1= < 7 h; T2= 7-25 h; T3=25-30 h; T4= 49-54 h
$^{a–c}$ Means within a row with different superscripts differ ($P < 0.05$)
$^{A–B}$ Means within a row with different superscripts tend to differ ($P < 0.10$)

However, the visual assessment failed to find differences in these color parameters between age categories ($P > 0.10$) confirming the inaccuracy of this method for lesion age determination as already reported in previous studies (Hughes et al., 2004a; Barington and Jensen, 2013).

The spectrophotometric color assessment also showed that overall the lesions assessed in this study were darker (-ΔL* value; $P|t| < 0.001$), redder (+Δa* value; $P|t| < 0.001$) and yellower (+Δb* value; $P|t| < 0.001$) than non-lesioned skin (Table 2). However, differently for the visual assessment, the objective color evaluation could detect differences between lesion age categories. Based on the ΔL* and Δa* color values, T1 lesions (< 7 h old) were darker and redder than T3 lesions ($P = 0.01$ for both color values) and T4 lesions ($P < 0.001$ and $P = 0.003$ for ΔL* and Δa* values, respectively). The same trend was observed for ΔL* and Δa* color values between T1 and T2 (P=0.06 and P=0.08, respectively). No time-dependent difference was found for Δb*, indicator of the yellowness of the lesion ($P > 0.10$). This latter result is not surprising as the yellow component, which results from the degradation of hemoglobin into bilirubin during the late phases of healing (Hughes et al., 2004b; Mimasaka et al., 2010), is well known as an indicator of lesions older than those assessed in this study. Indeed, the yellow color was observed up to 72 h in human adult bruises (Langlois, 2007) or from 5 d to 3 wks after the injury in children bruises (Mimasaka et al., 2010).

Thus, the spectrophotometric color assessment appears to be a useful method to discriminate between fresh and older lesion (< 7 h vs. > 25 h old) as previously reported in a number of studies on humans (Hughes and Langlois, 2010; Mimasaka et al., 2010; Grossman et al., 2011).

In this study, the efficiency of the use of ΔL* or Δa* parameters for the determination of the age of lesions was validated through a diagnostic test. The best threshold value identified for the ΔL* was -15. When a ΔL* value higher than the threshold was considered as an indicator of lesions older than 7 h, the diagnostic test presented a sensitivity of 0.40, a specificity of 0.95, a PPV of 0.73 and a NPV of 0.83. The best threshold value identified for the Δa* was 15. When a Δa* value lower than the threshold was considered as an indicator of lesions older than 7 h, the diagnostic test presented a

sensitivity of 0.50, a specificity of 0.88, a PPV of 0.59 and a NPV of 0.84. Based on this test, it may be concluded that there was 73% of chance that a lesion was old if the ΔL* value was higher than -15 and 59% of chance that the lesion was old if the Δa* value was less than 15. However, for both color parameters, the sensitivity values were low, indicating that the test was not sensitive enough to discriminate between fresh and old lesions consistently. Differences in the depth of the lesion and its location on the carcass may limit the efficiency of the spectrophotometric assessment as the thickness of the subcutaneous fat tissue may alter the changes in the bruise color and the speed of the healing process (Mimasaka et al., 2010). The individual variation in the degradation of hemoglobin over time should be also taken into account in the interpretation of the results (Grossman et al., 2011).

4.3.3. Histology and Enzyme Histochemistry

No difference was found in the necrosis and hemostasis scores between lesion age categories ($P > 0.10$; Table 3). Whereas, a relationship was found between inflammation score and the age of the lesion ($P < 0.001$) in this study, with a higher proportion of lesions with the highest score (score 2) being found in T3 and T4 lesions (> 25 h old) compared with T1 lesions ($P = 0.001$ and $P < 0.001$, respectively). A similar inflammation rate has been also reported in 33-49 h old humans wounds (Takamiya et al., 2008), and in mice, calves and lambs skin bruises between 24 and 71 h after the injury (McCausland and Dougherty, 1978; Takamiya et al., 2005). Erosion/ulceration score also tended to be higher ($P = 0.07$) in T3 and T4 than in T1 lesions (Table 4.3).

Within the lesion age categories, no difference in the epidermis ATP and epidermis and derma ALP scores was found between the lesioned and the control skin (ΔATPE, ΔALPE and ΔALPD, respectively) in this study ($P > 0.10$; Table 3), suggesting that the use of enzyme histochemical techniques may not be appropriate for the evaluation of the age of a skin lesion, which is likely due to the rather inconsistent appearance of ATP and ALP in the stains (Betz, 1994; Cecchi, 2010).

Table 4. 3 Variation of the histological scores and the delta values (difference between the skin lesion and the control) for the enzymatic activities according to lesion age category

	N	Age category[2]	Score[1] 0	1	2	*P*-value
Histology						
Necrosis	78	T1	25.00	60.00	15.00	0.16
		T2	10.00	50.00	40.00	
		T3	15.00	55.00	30.00	
		T4	11.11	44.44	44.44	
Erosion/ulceration	77	T1	50.00	35.00	15.00	0.07
		T2	15.00	50.00	35.00	
		T3	26.32	42.11	31.58	
		T4	16.67	44.44	38.89	
Inflammation	78	T1	45.00	55.00	0.00	<0.001
		T2	20.00	50.00	30.00	
		T3	10.00	55.00	35.00	
		T4	5.56	50.00	44.44	
Hemostasis	78	T1	15.00	75.00	10.00	0.50
		T2	25.00	65.00	10.00	
		T3	0.00	95.00	5.00	
		T4	11.11	72.22	16.67	
Enzyme histochemistry						
ΔALPE[3]	79	T1	50.00	50.00	-	0.16
		T2	84.21	15.79	-	
		T3	60.00	40.00	-	
		T4	60.00	40.00	-	
Δ ALPD[4]	79	T1	40.00	45.00	15.00	0.76
		T2	57.89	21.05	21.05	
		T3	40.00	55.00	5.00	
		T4	35.00	45.00	20.00	
Δ ATPE[5]		T1	85.00	15.00	-	0.73
		T2	84.21	15.79	-	
		T3	94.74	5.26	-	
		T4	85.00	15.00	-	

Values represent the percentage of lesions in each age categories according to the score system for the different analyses (100% in row). The *P*-value corresponds to the Cochran-Mantel-Haenszel test (row mean score).

[1] Scoring system: 0 = absent; 1 = minimal to mild; 2 = moderate to severe lesions for inflammation and hemostasis. For Necrosis and Erosion, the system was modified as: 0 = 0%; 1 = < 25% - <50%; 2 = 50-100% of the epidermis. For the enzyme histochemistry, 0 = delta value of 0; 1 = delta value of 1 or 2; 2 = delta value of 3 or 4.

[2] Age categories: T1 = < 7 h; T2 = 7-25 h; T3 = 25-30 h; T4 = 49-54 h

[3] ALPE= Epidermis Alkaline Phosphatase

[4] ALPD= Derma Alkaline Phosphatase

[5] ATPE= Epidermis Adenine Triphosphatase

4.3.4. Gene Expression

Of the 10 tested genes, 6 (CCL2, IL6, ITGA3, SERPINE1, MMP1 and T1MP1) presented a significant time-dependent expression pattern in this study, with the fold change attaining the highest level in T1 lesions and then decrease in older lesions in this study (Fig. 4.5). Whereas, the other 4 genes (TNFα, IL8, IL10 and COX2) were equally over-expressed, regardless of the lesion age category, resulting in no variation in their expression over time ($P > 0.10$).

When compared with T2 lesions, T1 lesions presented a higher fold change for CCL2 ($P = 0.008$), ITGA3 ($P = 0.01$) and SERPINE1 ($P = 0.01$) genes. Furthermore, when compared with T3 and T4 lesions, a higher fold change was found in the T1 lesions for the CCL2 ($P < 0.001$ for both), IL6 ($P < 0.001$ for both), ITGA3 ($P < 0.001$ for both), MMP1 ($P < 0.001$ for both) and SERPINE1 ($P < 0.001$ for both) genes. No difference was found in the fold change of these genes between T3 and T4 lesion categories ($P > 0.10$). A different expression pattern was found for TIMP1 gene when compared to the other genes, with its fold change being greater ($P = 0.01$) in T1 and T2 lesions compared with T4 lesions. No difference was found in this gene expression between T1 and T2 lesions ($P > 0.10$).

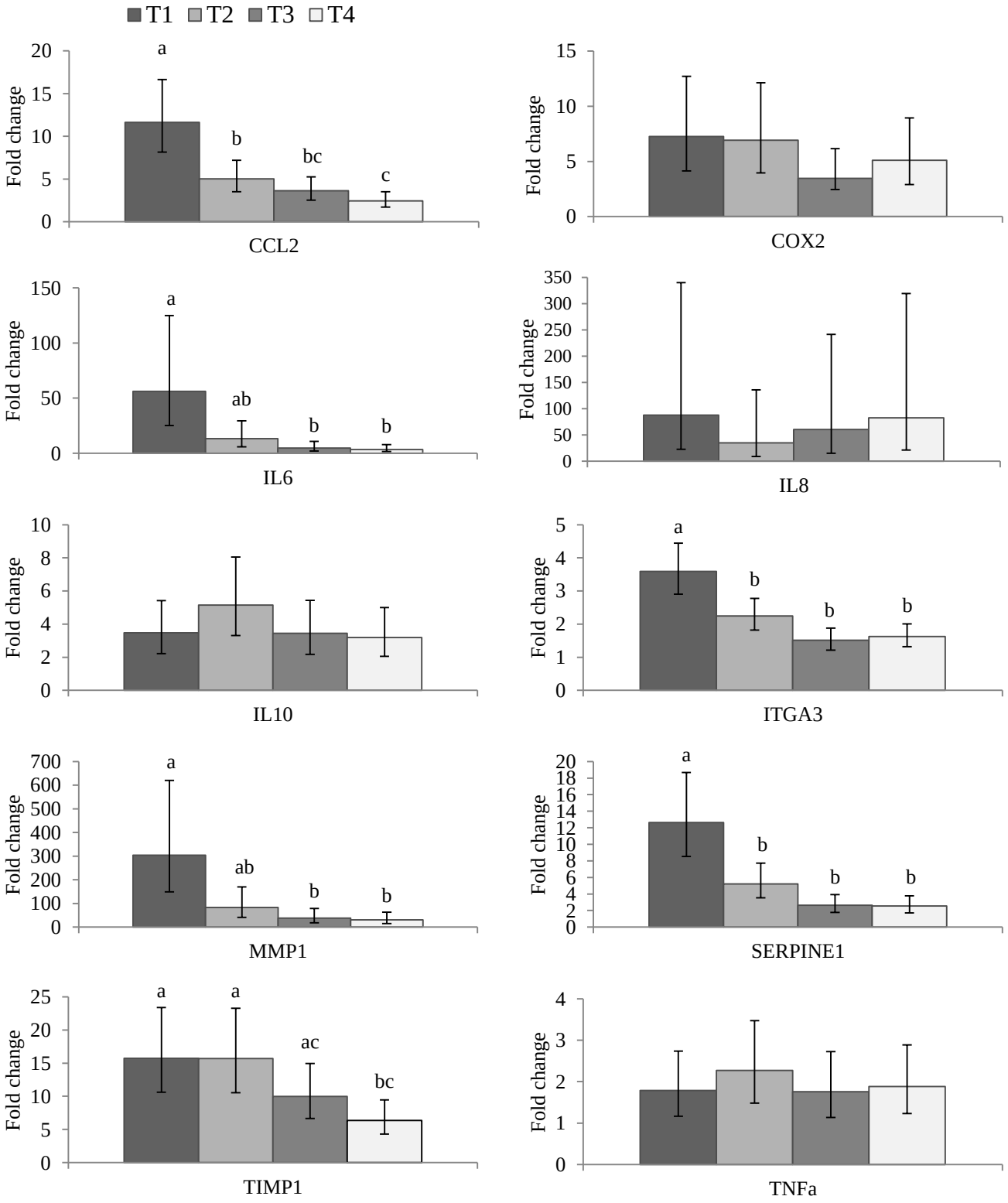
Except for CCL2, IL6 and MMP1, to our knowledge, the expression of the genes tested in this study was never assessed for the determination of lesion age in any species, including humans, before. CCL2 is a chemokine mainly involved in the inflammatory response, controlling the recruitment of monocytes/macrophages (Werner and Grose, 2003; Kondo and Ishida, 2010). The increased CCL2 gene expression found in fresh lesions (T1) in this study is in agreement with other studies assessing skin wound healing in mice (Kondo and Ishida, 2010; Wang et al., 2015). The expression observed in this study of IL6, which is proinflammatory cytokine playing an essential role to start the healing response (Lin et al., 2003; Werner and Grose, 2003), agrees with what reported by Sato and Ohshima (2000) in mice wound healing, but is in contrast with Wang et al. (2015) who reported a highest expression of this gene 1 d after wounding in humans and mice. MMP1, which is associated with inflammation, control of matrix degradation, cells migration

(Rohani and Parks, 2015), tissue remodelling and angiogenesis (Gallant-Behm and Hart, 2006; Wang et al., 2015), was the most expressed gene in this study. Madlener et al. (1998) also reported an increased expression of this gene in mice wounds, but only starting from 1 d of age. This study provides the first evidence that the expression of this gene increases in more recent lesions (< 7 h post-injury).

Before this study, there was only evidence of an effect of ITGA3 gene deficiency in delaying the wound healing process in mice (Reynolds et al., 2008), of a high quantity of SERPINE proteases in the exudates of human wounds (Eming et al., 2010) and of T1MP1 expression in cutaneous wounds in mice (Madlener et al., 1998). To our knowledge, the expression of these genes was never studied for the determination of the lesion age before. This study is worthy given the involvement of these genes in the biological process of wound healing (Li et al., 2003; Behm et al., 2012), with SERPINE1 stimulting MMPs and integrins, both mostly expressed in T1 lesions. Integrins, in turn, stimulate TIMPs which is an inhibitor of MMPs (Gill and Parks, 2008; Behm et al., 2012) and whose expression achieved the greatest increase in T3 lesions in this study.

The lack of variation in the expression of TNFα, IL8, IL10 and COX2 genes, all involved in the inflammatory response and often in the regulatory process of the other phases of wound healing (Werner and Grose, 2003; Takamiya et al., 2008; Gilroy et al., 1999; Cecchi, 2010), in the present study is in contrast with the results from other studies. Studies on pigs and mice (Wang et al., 2001; Wang et al., 2015; Wang et al., 2016) reported a great expression of TNFα in excisional wounds between 1 and 14 d of age. A similar variation in the expression was expected from IL10, whose expression is promoted by TNFα (Wanidworanun and Strober, 1993). However, its expression level is inconsistently reported in the literature with peaks observed at 60 min (Ohshima and Sato, 1998), or at 3 h and 3 d after the infliction of the lesion (Sato et al., 1999). The expression of IL8 and COX2 has been reported until 4 d after wounding in humans (Engelhardt et al., 1998) and from 1 h to 3 days in incised skin wound in rabbits (Bai et al., 2008), respectively.

Figure 4. 6 Fold change in gene expression for the four age categories of skin lesions. Means within a row with different superscripts differ ($P < 0.05$). The error bars represents the maximum and minimum values in fold change. T1= < 7h; T2= 7-25 h; T3=25-30 h; T4= 49-54 h.



Overall, the combined assessment of MMP1, SERPINE1, IL6 and CCL2 genes expression may be useful to discriminate between fresh and older lesions (< 7 h vs. 25 h), while the analysis of the TIMP1 expression can help distinguish between < 25 h and > 49 h old lesions on a pig carcass.

Our results confirm the efficiency of the RT-PCR mRNA analysis for the assessment of the age of lesions reported in a previous study (Kondo and Ishida, 2010). However, this method may have some limitations under commercial conditions because it is laborious, time-consuming and expensive (Takamiya et al., 2008).

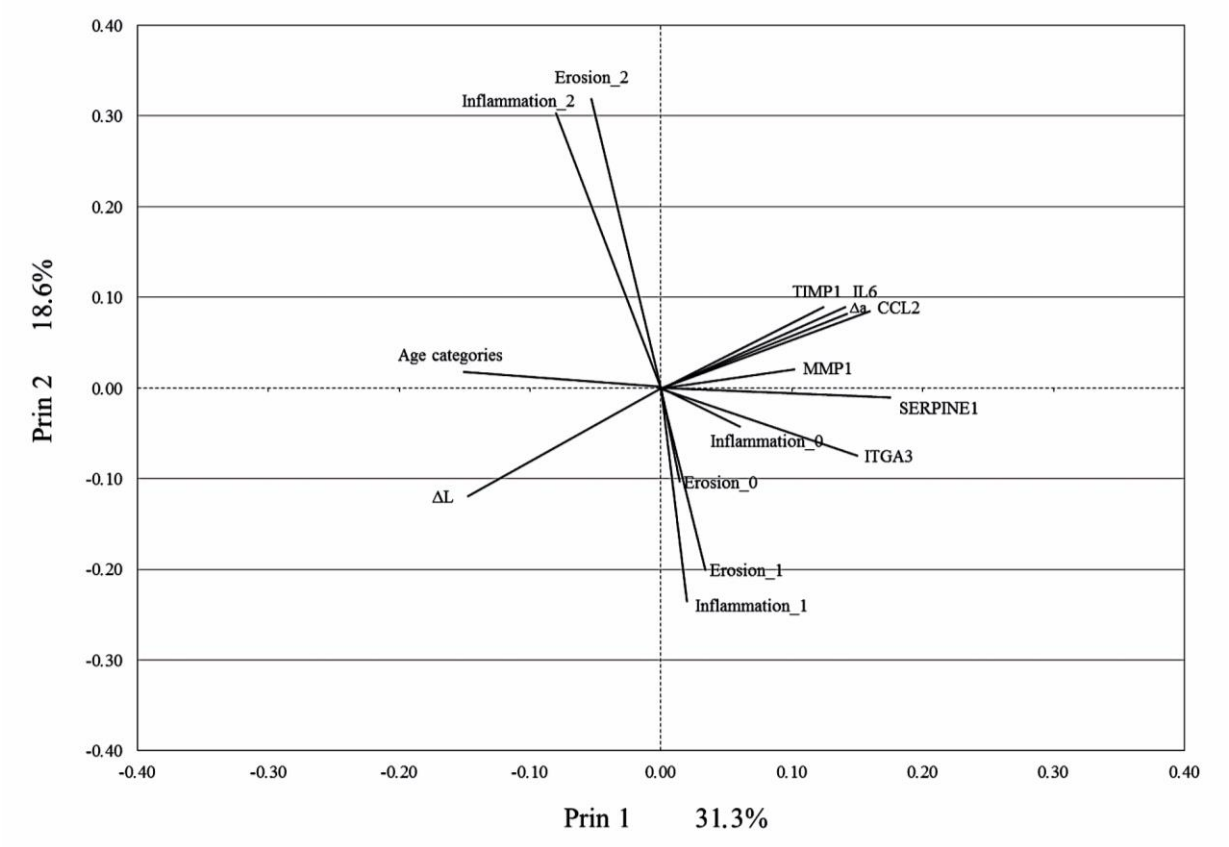
4.3.5. Multivariate Analysis

An approach based on the simultaneous analysis of many factors is considered as the best way to develop a reliable method on dating lesions (Cecchi, 2010). Factors used in the FAMD were selected from gene expression analysis (TIMP1, IL6, CCL2, SERPINE1, ITGA3 and MMP1), the spectrophotometric color analysis (Δa* and ΔL* color values) and from the histological parameters (i.e. inflammation and erosion), based on their relationship with lesion age categories. The multivariate analysis of the selected parameters shows that the variance in the lesion age can be explained by 49.9% by the combination of Principal 1 (Prin 1) and Principal 2 (Prin 2) components (Fig. 4.7), which may be attributed to the process of lesion healing. The other 50.1% of the variance may be related to other factors, such as the seriousness of the lesion (Barington and Jensen, 2016b), the individual physiological response, the animal body condition (i.e. body weight and fat thickness) and the anatomical region where the lesion is located (Cecchi, 2010; Mimasaka et al., 2010; Grossman et al., 2011). The position of variables on Prin 1 and 2 in the graphic (Fig. 4.7) showed that the lesion age categories are negatively associated with the gene expressions and the spectrophotometric Δa* color value, which indicates that the older is the lesion, the lower is the gene expression and the Δa* color value. In this study, fresh lesions were actually redder and presented a greater gene expression level. The opposite position of the ΔL* color vector in relation to the Δa* colour value and to all genes vectors may be explained by negative L* color value, which

increases as long as the lesion color gets dark. The 0 and 2 inflammation scores (absence and moderate to severe, respectively) were negatively and positively, respectively, associated with the age categories, indicating that the inflammation response in the skin tissue increased with the age of the lesions. No relationship was found between minimal inflammation rate (score 1) and the age of the lesions, which confirms the lack of variation in the percentage of lesions presenting a minimal or mild inflammation rate in the lesion age categories comparison (Table 4.3). This result suggest that this degree of inflammation may be more related to factors, such as the seriousness of the lesion or individual physiological response, as previously observed by Barington and Jensen (2016b) in pig bruises.

In summary, the results of the multivariate analysis show the positive association between TIMP1, IL6 and CCL2 gene expression and the Δa* color value and confirm the potential of spectrophotometric color method for the reliable assessment of the age of a lesion.

Figure 4. 7 Factorial Analysis of Mixed Data for the selected parameters. The scores 0, 1 and 2 in the Erosion and Inflammation parameters represent each category of the healing condition of the lesion. The age categories parameter consists of the numerical categories (1= < 7h; 2 = 7-25 h; 3=25-30 h; 4 = 49-54 h). Delta (Δ) values indicate the differences between the lesions and controls for the L* (lightness) and a* (redness) color parameters.



4.4. Conclusions

The need to monitor handling and critical areas preslaughter in order to improve animal welfare and pork quality is resulting in increasing interest of the pork sector towards the development of a practical and accurate technique for the determination of the age of lesions on the carcass at slaughter.

Based on the assessment of preslaughter carcass lesions in this study, the spectrophotometric color assessment of the lesion on the carcass appears to be a suitable and rapid technique to discriminate between fresh lesions (< 7 h) occurring between loading and slaughter and old lesions (> 25 h old) originating from the farm. However, the high variability in color results, likely due to pig individual variation in body composition (i.e. subcutaneous fat thickness), prevented from obtaining a color threshold allowing an accurate discrimination between lesion age categories in this study. Further investigations are needed to assess the influence of this factor on the spectrophotometric lesion color measurement.

The analysis of MMP1, SERPINE1, IL6, ITGA3, TIMP1 and CCL2 gene expression, and the inflammation response in the skin lesion at slaughter supported the spectrophotometric assessment of the lesion color in this study. This relationship either may indicate the potential of these techniques for the validation of on-line objective determination of the lesion age or their usefulness for this type of assessment using the pig as a model for scientific purposes in human studies and for forensic investigation.

Acknowledgements

This research project was funded by Swine Innovation Porc within the Swine Cluster 2: Driving Results Through Innovation research program. Funding was provided by Agriculture and Agri-Food Canada through the Agri Innovation Program, provincial producer organizations and industry partners. The authors thank Janaina Braga, Davide Carbonari, Ariane de Castro, Taciana Diesel and

Fidèle Z. Kabemba for their valuable contribution in the data collection at the farm and abattoir. Many thanks go to Catherine Thibault for her contribution to the gene analysis, Steve Méthot for his assistance in the statistical analysis and to the staff of the AAFC swine unit for the care of animals with expertise and professionalism. We are grateful to the COOP Fédérée for supplying pigs and feed, and to the Rousseau abattoir staff for their collaboration during the slaughter trials. Authors declare no conflict of interest.

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5.

GENERAL CONCLUSIONS

The presented results allow to underline the importance of lesions as indicators of animal welfare in growing-finishing pigs.

In the study presented in chapter 2, the assessment of lesions was paramount to enlighten the efficacy of different environmental enrichment devices for Italian Heavy Pigs. In particular, the presence of tail biting was linked to a lack of effectiveness in one device, affecting also carcass traits (backfat thickness, F-o-M) and water-holding capacity of the meat. Moreover, the results suggest to be careful in choosing edible enrichment devices, because the vantages may be effective in keeping interest during all the growing-finishing stage; but the occurrence of adverse effects related to their consumption (e.g. the prevalence of gastric lesion at slaughter) should also be considered.

In chapter 3, results demonstrated that, through the use of a spectrophotometer, it is possible to see differences in lesion color according to the age of the lesion, and so to discriminating between young lesions occurred pre-slaughter at lairage or older lesion occurred at farm level. Results were confirmed also from histological and molecular indicators. Given the actual findings, to develop a reliable diagnostic test, further research is required in order to better understand the role played by the region where the lesion is located, the thickness of subcutaneous fat tissue, the force of the trauma and the individual response in the lesion healing response. Overall, the method was found suitable at abattoir, and should be considered for being applied to automated devices, thus becoming part of the meat inspection at abattoir.

AKNOWLEDGEMENTS

These three years were deeply formatives and challenging, both professionally and personally. It would never have happened if I was not in an invaluable guidance and company.

First, I would like to thanks my tutor Prof. Luca Sardi, and Prof. Giovanna Martelli. They were insuperable for humanity and support and was a great privilege to work with them.

Many thanks to Dr. Luigi Faucitano for his welcoming in his team. Thank you for the trust they reposed in me, and to push me in new situations, I have learned a lot about my limits and capacities.

I am very grateful to Dr. Eleonora Nannoni and Dr. Sabine Conte. They encourage me and teach me with incredible patience, professionality and competence during the everyday working life.

Many thanks to Nicole Vitali and Marco Petrelli for the graphical and the linguistic revisions of the manuscript, for their kindness and availability all the time I needed.

Thank you to my family, which through its imperfection gave me skills and braveness to the everyday challenges, and to support my decisions also if sometimes they do not completely understand.

Many thanks to Enrico, to be always on my side. For his unconditional support also through distance.

I am glad to my colleagues at University of Bologna and Agriculture and Agri-food Canada. Your friendship is a precious gift and I will keep the memory of this time forever with me.

Thank you to all my friends from everywhere, who have shared the road with me, really thank you for make me smile.

Especially Federica and Elisa deserve a special mention for their support, patience, grace during this manuscript writing and for have been always present. Thank you to Agata, which always remember me that almost everything you want you can reach if you are ready to work hard for that.

LIST OF SCIENTIFIC PRODUCTION DURING THE PHD

AND NOT INCLUDED IN THE MANUSCRIPT

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Nannoni E., Liuzzo G., Serraino A., Giacometti F., Martelli G., Sardi L., **Vitali M**., Romagnoli L., Moscardini E., Ostanello F. (2016). Evaluation of pre-slaughter losses of Italian heavy pigs. Animal Producton Science. p.1-10.

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