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Assessment of bacterial superficial contamination in classical or ritually slaughtered cattle using metagenetics and microbiological analysis

N. Korsak [a,*], B. Taminiau [a], C. Hupperts [a], L. Delhalle [b], C. Nezer [b], V. Delcenserie [a], G. Daube [a]

[a] University of Liège, FARAH, Faculty of Veterinary Medicine, Department of Food Science, Quartier Vallée 2, Avenue de Cureghem 10, 4000, Liège, Belgium
[b] Quality Partner, 62 Rue Hayneux, 4040, Herstal, Belgium

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ABSTRACT

The aim of this study was to investigate the influence of the slaughter technique (Halal vs Classical slaughter) on the superficial contamination of cattle carcasses, by using traditional microbiological procedures and 16S rDNA metagenetics. The purpose was also to investigate the neck area to identify bacteria originating from the digestive or the respiratory tract. Twenty bovine carcasses (10 from each group) were swabbed at the slaughterhouse, where both slaughtering methods are practiced. Two swabbing areas were chosen: one "legal" zone of 1600 cm^2 (composed of zones from rump, flank, brisket and forelimb) and locally on the neck area (200 cm^2). Samples were submitted to classical microbiology for aerobic Total Viable Counts (TVC) at 30 °C and *Enterobacteriaceae* counts, while metagenetic analysis was performed on the same samples. The classical microbiological results revealed no significant differences between both slaughtering practices; with values between 3.95 and 4.87 log CFU/100 cm^2 and 0.49 and 1.94 log CFU/100 cm^2, for TVC and *Enterobacteriaceae* respectively. Analysis of pyrosequencing data showed that differences in the bacterial population abundance between slaughtering methods were mainly observed in the "legal" swabbing zone compared to the neck area. Bacterial genera belonging to the *Actinobacteria* phylum were more abundant in the "legal" swabbing zone in "Halal" samples, while *Brevibacterium* and *Corynebacterium* were encountered more in "Halal" samples, in all swabbing areas. This was also the case for *Firmicutes* bacterial populations (families of *Aerococcaceae*, *Planococcaceae*). Except for *Planococcoceae*, the analysis of Operational Taxonomic Unit (OTU) abundances of bacteria from the digestive or respiratory tract revealed no differences between groups. In conclusion, the slaughtering method does not influence the superficial microbiological pattern in terms of specific microbiological markers of the digestive or respiratory tract. However, precise analysis of taxonomy at the genus level taxonomy highlights differences between swabbing areas. Although not clearly proven in this study, differences in hygiene practices used during both slaughtering protocols could explain the differences in contamination between carcasses from both slaughtering groups.

1. Introduction

In several European countries, two cattle slaughtering protocols co-exist and are permitted under regulation: the "Classical" method, that encompasses a stunning step (where the animal is rendered unconscious) before the sticking procedure (where major blood vessels supplying the brain are severed, resulting in rapid blood loss and death), and the Halal method, that combines the stunning and the sticking in one step (Council of the European Union, 2009; Dunoyer, 2008). The main difference is that, in the Halal protocol, a single cut with a sharp knife is practiced directly on live cattle, instead of two cutting steps with two different knives for the sticking of unconscious cattle in the classical slaughtering technique. The single cut used in the Halal technique generally results in the cross section of the trachea and esophagus of cattle at the same time as blood vessels are cut, which may lead to contamination of carcasses with bacteria originating from the respiratory or digestive tract (Dunoyer, 2008). Other authors have reviewed the Halal slaughtering procedures to be applied in slaughterhouses in order to minimize the suffering of animals during bleeding and reduce the time to unconsciousness (Anil, 2012; Farouk et al., 2014).

In Europe, great attention is paid to animal welfare in general and especially during the slaughter of animals. Indeed, some experiments proved that the time between blood artery sectioning and complete loss of consciousness (collapse) was 20 s on average in cattle slaughtered with the Halal technique (Gregory et al., 2010). Conversely, a good mechanical stun causes the animal to collapse instantaneously, with a complete disappearance of the corneal reflex (Food and

* Corresponding author.
E-mail addresses: nkorsak@ulg.ac.be (N. Korsak), bernard.taminiau@ulg.ac.be (B. Taminiau), carohup@hotmail.com (C. Hupperts), laurent.delhalle@keyfood.be (L. Delhalle), carine.nezer@quality-partner.be (C. Nezer), Veronique.delcenserie@ulg.ac.be (V. Delcenserie), georges.daube@ulg.ac.be (G. Daube).

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Agriculture Organization of the United Nations, 2004). In this respect, the Council Regulation (EC) No 1099/2009 has been promulgated in order to protect farmed animals during killing (Council of the European Union, 2009). It established stunning standards, while allowing each EU member state to regulate the Halal ritual method. Important debates have been taking place in some member states to adapt or ban the Halal method, or to specifically label meat originating from animals not conventionally slaughtered.

Beside the potential adverse effect in relation to animal welfare, another issue with Halal slaughtering might be the difference in bleeding efficiency between the classical stunning method and the Halal ritual slaughtering practice. However, the latter technique did not modify the total blood yield as attested by experiments conducted in England on sheep and on cattle (Anil et al., 2004; Anil et al., 2006).

Carcass contamination can lead to an increase in food microbiological contamination with bacteria such as *Pseudomonas* sp., *Brochothrix thermosphacta*, *Acinetobacter* or *Psychrobacter*, leading to a decreased shelf life of some products such as steaks (De Filippis et al., 2013). However, food and storage conditions can also have a selective effect on some microbial population (Pothakos et al., 2015; Stellato et al., 2015).

The methods of carcass swabbing used here were based on research performed during the last two decades (McEvoy et al., 2004). Although in comparison to the excision technique there is a slight underestimation of the microflora present on carcass surfaces, the swabbing method for carcass monitoring has proven its efficiency, requires little equipment and is non-destructive (Ghafir et al., 2008; Korsak et al., 1998).

The main purpose of this study was to compare the two slaughter techniques regarding the superficial contamination of cattle carcasses, by using classical microbiological and metagenetic analyses. In this context, 16S rRNA metagenetics (also called metagenomic analysis targeting 16S ribosomal DNA) has emerged as a powerful tool for exploring the bacterial composition of various ecosystems (Esposito and Kirschberg, 2014). During the last decade, many applications in the field of microbiology have been developed to elucidate the microbiota of different foods such as fermented food, marinated poultry, sausages, cheese, tea, and bottled water (Benson et al., 2014; Delcenserie et al., 2014; Hansen et al., 2013; Jung et al., 2011; Lyu et al., 2013; Nam et al., 2012; Nieminen et al., 2012). With the help of these techniques, researchers can clarify the microfloral distribution of various ecosystems at a higher resolution than had been observed previously (Hanning and Ricke, 2011).

2. Material and methods

2.1. Cattle slaughterhouse

During August 2013, 20 samples were collected in a cattle slaughterhouse located in eastern Belgium, which is approved by the competent authority. This abattoir practices both slaughtering techniques: classical slaughter and following the Halal ritual. In the same stunning area, two different containment boxes are used for the two different slaughtering techniques, before a common slaughtering line for the remaining parts of the slaughter (shackling, dressing, evisceration, marking…). For conventional slaughter, workers use a non-penetrative captive bolt to stun animals, while for Halal slaughtering, a separate containment box is used to restrain the animal in order to practice direct sticking on animals. Both stunning methods are practiced on an upright animal in the containment box. For the Halal ritual method, the cattle are supported by a metallic device at the level of the brisket in order to prevent them from falling. Muslim slaughterers are certified by the Belgian Muslim Council. For both slaughtering techniques, ligation of the esophagus is performed. On a daily basis, the slaughter line is cleaned with a chlorinated foaming agent and sanitized with a quaternary ammonium combined with glutaraldehyde. The frequency is lowered to a weekly basis for the chilling rooms and the holding pens.

2.2. Sampling protocol

Twenty samples were gathered in August 2013, by swabbing carcasses in two visits separated by one week. As recommended by the Belgian law, the swabbing was performed between 2 and 4 h after the killing step on carcasses stored in the chilling room (Agence fédérale pour la sécurité de la chaîne alimentaire, 2015). After dressing and marking, the carcasses are directly moved to a chilling room at a temperature of 4 °C. Contacts with other carcasses may occur after the post-mortem examination step. From the twenty samples, ten were from bovines classically slaughtered ("Classical" group) and ten came from bovines slaughtered following the Halal procedure ("Halal" group), twelve were swabbed on the first day of visit (six for each group), and eight on the second day of visit (four for each group) (Suppl. Table 1). The population of the cattle was very heterogeneous owing to the fact they were mainly purchased in different Belgian farms. Nineteen samples originated from male carcasses and one from a heifer. For the swabbing protocol, two swabbing areas were investigated with the wet-cotton swabbing method: one zone specified by law for monitoring contamination (1,600 cm^2), the "legal" zone, and one rectangular area of 200 cm^2 close to the sticking point in the neck area. The "legal" zone is composed of 4 different sub-zones of 400 cm^2 each: rump, flank, brisket and forelimb (Fig. 1). Samples were collected aseptically by a trained food specialist without delimiter and by changing gloves between two carcasses. Four sterile cotton pads were used for the "legal" zone and pooled in the same plastic bag while one other sterile cotton pad was used for the neck zone. The swabbing techniques have already been described in the scientific literature (Ghafir et al., 2008; Korsak et al., 1998). Fig. 1 presents the swabbing areas on the carcasses. In Belgium, the "legal" zone has to be swabbed on a weekly basis in sampled animals in order to monitor the hygiene quality of cattle carcasses (Ministère des affaires sociales de la santé publique et de l'environnement, 1996).

2.3. Microbial counts

Microbiological analyses were performed by a laboratory licensed by the Belgian Ministry of Public Health and accredited in accordance with the requirements of "ISO standard 17025" (ISO, 2005). In the laboratory, samples were stored at 4 °C and analyzed within 24 h. The cotton swabs were placed in a Tempo® bag with a mesh screen liner (80 μm pore size) (bioMérieux, Basingstoke, England, ref. 80,015) with 100 ml of sterile physiological water and homogenized for 2 min using a Mix 2 Stomacher apparatus (AES Chemunex, Bruz Cedex, France). Total viable counts (TVCs) were performed following the ISO 4833 method using plate count agar (PCA) medium with an aerobic incubation period of 72 h ± 3 h at 30 °C (Bio-Rad, Marnes La Coquette, France, ref. 356-3989) (ISO, 2003). Counting of *Enterobacteriaceae* was performed after aerobic incubation for 25 h at 30 °C on Violet Red Bile Glucose (VRBG) agar plates in accordance with the international norm ISO 21528–2:2004 (ISO, 2004). In accordance with other studies, when no colony of *Enterobacteriaceae* was observed in the samples, the value of half the detection limit was assigned: 0.49 log CFU/100 cm^2 for the "legal" zone corresponding to 50 CFU/1600 cm^2 and 1.40 log CFU/100 cm^2 for the neck zone corresponding to 50 CFU/200 cm^2 (Ghafir and Daube, 2008; Hutchison et al., 2005). The evaluation of hygienic conditions of carcasses was evaluated in accordance with the Commission Regulation (EC) No 2073/2005 by enumerating aerobic colony count and *Enterobacteriaceae*. These two bacterial parameters (TVC and *Enterobacteriaceae*) are classified, along with *Salmonella* spp., as process hygiene criteria (European Commission, 2005).

2.4. 16S rDNA pyrosequencing and data analysis

Total bacterial DNA was extracted from the samples with the Blood and Tissue DNA extract kit (QIAGEN, Venlo, The Netherlands), following the

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