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

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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 1,600 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.

Highlights:

- Metagenetic analysis was combined with classical microbiology
- Halal and classical methods were investigated in the same slaughterhouse
- Two swabbing areas: legal zone (rump, flank, brisket and forelimb) and neck area
- No significant differences in bacterial counts were observed
- More marked differences with metagenetics: *Corynebacterium* and *Macrococcus*

Keywords:

contamination; metagenetics; cattle; carcasses; Halal; swabbing

1. Introduction

In several European countries, two cattle slaughtering protocols coexist 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 seconds 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 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 (1600 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 (Figure 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). Figure 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 24h. The cotton swabs were placed in a Tempo® bag with a mesh screen liner (80 µm pore size) (bioMérieux, Basingstoke, England, ref 80015) with 100 ml of sterile physiological water and homogenized for 2 minutes 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 72h ± 3h 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/1,600 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) N° 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 manufacturer's recommendations. 16S rDNA profiling, targeting V1-V3 hypervariable region (forward primer 5'-GAGAGTTTGATYMTGGCTCAG-3' and reverse primer 5'-GAGAGTTTGATYMTGGCTCAG -3') and sequenced on Roche GS Junior was performed as described previously (Rodriguez et al., 2015). Briefly, all libraries were run in the same titanium pyrosequencing reaction using Roche multiplex identifiers, and amplicons were sequenced using the Roche GS-Junior Genome Sequencer instrument (Roche).

Sequence reads processing was treated as previously described (Rodriguez et al., 2015) using respectively MOTHUR software package v1.35, Pyronoise algorithm and UCHIME algorithm for alignment and clustering, denoising and chimera detection (Edgar et al., 2011; Quince et al., 2009; Schloss et al., 2009). 16S rDNA Reference alignment and taxonomical assignation to the genus level in MOTHUR were based upon the SILVA database (v1.15) of full-length 16S rDNA sequences (Pruesse et al., 2007). Clustering distance of 0.03 was used for OTU generation. Subsample datasets were obtained and used to evaluate ecological indicators, Richness estimation (Chao1 estimator), microbial biodiversity (non parametric Shannon index), and the population evenness (derived from Shannon index) at the OTU level using

MOTHUR (Chao and Shen, 2003; Gotelli and Colwell, 2010; Mulder et al., 2004). Non parametric dimensional scaling (NMDS) based upon a Bray-Curtis dissimilarity matrix were performed using Vegan, Vegan3d and rgl packages in R (Oksanen et al., 2016). Permutational Multivariate Analysis of Variance (AMOVA) was performed to assess the diversity clustering of the four groups using MOTHUR, based on Bray-Curtis distance matrix with 999 permutations (Martin, 2002).

2.5. Statistical analysis

With the help of R software, Wilcoxon tests with non-paired samples were used to compare the microbiological results of samples between the two slaughtering methods.

Statistical differences in bacterial alpha-diversity, richness and evenness between groups were respectively assessed using one way-ANOVA and Mann-Whitney test using PRISM 6 (Graphpad Software). In order to highlight statistical differences in the bacterial population abundance between groups, two-way ANOVA with Tukey-Kramer posthoc test were performed using PRISM 6 (Graphpad Software). Differences were considered significant for a p-value of less than 0.05.

All the biosample raw reads have been deposited at the National Center for Biotechnology Information (NCBI) and are available under de Bioproject PRJNA300932.

3. Results

3.1. Microbial counts

Supplementary table 1 shows the individual results for classical microbiology and the medians for the different groups. Figure 2 depicts the distribution of results showing the median, Q1 and Q3 quartiles, outliers and maximal values (boxplots). For both microbiological parameters investigated, there is a higher contamination in the "legal" zone in comparison with the neck area (data expressed in log CFU/100 cm^2). For TVC results in the "legal" swabbing area, the median was 4.09 for classically slaughtered carcasses and 4.87 for Halal slaughtering (W value = 33.5, p-value = 0.21, NS). In the neck area, median values for TVC were 4.12 ("Classical" group) and 3.95 ("Halal" group) (W value = 59.5, p-value = 0.47, NS). For *Enterobacteriaceae* counts, in the "legal" swabbing area, the median was 0.49 for classically slaughtered carcasses and 0.95 for Halal slaughtering (W value = 41.5, p-value = 0.50, NS). In the neck area, median values for *Enterobacteriaceae* were 1.94 ("Classical" group) and 1.85 ("Halal" group) (W value = 65, p-value = 0.25, NS). All the differences were not significant with the Wilcoxon test.

3.2. Analysis of the aponeurosis contamination microbiota

The overall microbial alpha diversity and richness have been assessed and do not reveal any statistical differences between groups (Figure 3). However, Halal group indices harbored higher values than the Classical group. Microbiota population structure has been visualized by Non Parametric Dimensional Scaling and show a group clustering (Figure 3). Permutational Multivariate Variance analysis (MANOVA) underlines that both Neck groups failed to be clustered independently but that there's a statistical clustering between both slaughtering techniques in the "Legal" swabbing zones and between the Neck groups (Supplementary table 3).

3.3. Distribution of bacterial communities identified by 16S rDNA profiling

The number of reads ranged between a minimum of 3,713 and a maximum of 8,080 with a median of 4,634 (data not shown).

Taking into account the aim of the present study, the metagenetic results for both sampling days were merged in order to compare both slaughtering techniques and both sampling areas, while discarding the day effect. Figure 4 shows the relative population abundance at different levels: phylum, family and genus, while figure 5 depicts the distribution of 6 selected taxa throughout the 3 taxonomic levels considered (Phylum, Family and Genus). These figures and supplementary Table 2 highlight the statistical differences between the slaughtering procedures for the swabbing areas.

For the “legal” swabbing area, at the phylum level, *Actinobacteria* were more abundant in carcasses obtained from the “Halal” group compared to carcasses derived from animals classically slaughtered (“Classical” group). The situation was opposite for *Fusobacteria*, which are more abundant in the “Classical group”: the abundance of this phylum was less than 5% (Figure 5).

At the family level, regarding OTUs with an abundance higher than 5%, there were significant differences for *Corynebacteriaceae* (respectively 13.4% vs 3.2% for the surface swabbing area, $p < 0.001$) and *Planococcaceae* (respectively 1.6% vs 5.7% for the surface swabbing area, $p < 0.01$), which were encountered more on both swabbing areas from the “Halal” group in comparison to carcasses from the “Classical” group. When considering OTUs with an abundance below 5%, this was also observed for *Aerococcaceae* and *Clostridiaceae* (data not shown). In addition, regarding OTUs with an abundance below 5%, a significant difference was observed for the surface swabbing area (“legal” zone), with a more abundant proportion of *Brevibacteriaceae*, *Dietziaceae*, *Intrasporangiaceae*, and *Peptostreptococcaceae* in the “Halal” group compared to the “Classical” group (Suppl. Table 2).

Finally, at the genus level, the most important differences were observed with *Brevibacterium*, *Corynebacterium* and *Macrococcus*. *Brevibacterium* and *Corynebacterium* were more abundant in the “Halal” group compared to the “Classical” group in both swabbing areas, while

264 *Macrococcus* was significantly more recovered in the legal swabbing zone in the “Classical”
265 group compared to the “Halal” group. We observed a lower abundance of *Butyrivibrio,*
266 *Caryophanon, Clostridium, Dietzia, Flacklamia, Guggenheimella* and *Syntrophococcus* in the
267 “Halal” group compared to the “Classical’” group (Suppl. Table 2).
268

4. Discussion

The aim of this study was to assess the use of two analytical methods (a classical microbiological method and metagenetics) to see if there were any differences in the superficial contamination of carcasses between animals slaughtered classically or following the Halal procedure. The metagenetic approach was used in order to complement the classical method with an exploration of the microbiota present on both swabbing areas. Indeed, molecular technologies can further elucidate the microbial community by including the identification and quantification of non-culturable organisms, and can perform at a higher resolution compared to what was previously possible using culture-based methods (Ahn et al., 2011).

The “legal” zone investigated in our study is the zone stated by royal Belgian decree to be investigated in order to monitor the hygienic quality of cattle carcasses. The other swabbing zone was chosen to examine the local contamination in the neck area. Our objective was to compare the two slaughtering techniques inside one swabbing area and not compare the results between the two slaughtering areas.

In this study, microbiological values were always below the minimal values required by the royal Belgian decree, which translates the European regulation dealing with the microbiological criteria applied to foodstuffs from animal origin (European Commission, 2005; Ministère des affaires sociales de la santé publique et de l'environnement, 1996). For TVC and *Enterobacteriaceae* counts in Belgium the microbiological criteria are 0.5 log CFU/cm^2 lower than the European limits, mainly due to the fact that the Belgian competent authority has accepted the cotton swab technique in order to monitor the hygienic quality of carcasses (in other areas of Europe the excision technique is generally practiced). So, in cattle in Belgium the inferior limit (“m” value) was set at 3.0 log CFU/cm^2 (instead of 3.5 in Europe) for TVC and 1.0 log CFU/cm^2 (instead of 1.5 in Europe) for *Enterobacteriaceae*.

In this experiment, we observed no statistically significant difference in superficial contamination between both slaughtering methods. Although very few studies have been conducted on the microbiological quality of Halal meat, this is consistent with the experiment

296 of Sabow et al. (2015) who observed only a slight difference of contamination with lactic acid
297 bacteria at day 1 post mortem when comparing goats slaughtered with the Halal technique
298 and goats exsanguinated after anesthesia with halothane. Otherwise, there were no
299 statistically significant differences for the other microbiological parameters such as total
300 aerobic count, *Enterobacteriaceae* and *Pseudomonas* spp. However, our results need to be
301 confirmed on a wider scale by taking more samples and by taking into account in our statistical
302 analysis the hygiene practices in place during the two slaughtering techniques.
303 In relation to the metagenetic results, at the family level, the main findings of the present study
304 were a higher abundance of *Corynebacteriaceae* and *Planococcaceae* in the legal zone for
305 the “Halal” group in comparison to the “Classical” group.
306 *Corynebacteriaceae,* members of the *Actinobacteria* phylum, are mainly Gram-positive
307 bacteria and are closely related to *Mycobacterium* species. In ruminants and humans,
308 *Corynebacterium pseudotuberculosis* cause a condition involving the lymphatic vessels and
309 nodes with the development of granulomas, sores and ulcers (D’Afonseca et al., 2010; Müller
310 et al., 2011).
311 Bacteria from the *Planococcaceae* family belong to the *Firmicutes* phylum. This phylum seems
312 to be represented at a level higher than 60% in the fecal microbiota of cattle, while members
313 of the *Planococcaceae* family make up to 18% of the fecal bacterial population (Mao et al.,
314 2012). Therefore, *Planococcaceae* are considered as normal inhabitants of the bovine gut and
315 may be recovered on carcasses after evisceration and de-hiding. The fact that this bacterial
316 family is encountered more on the legal swabbing zone of the “Halal” group supports the
317 hypothesis that different hygiene practices might be applied during both slaughtering
318 processes, although no difference in conventional microbial results was observed. The
319 hypothesis of a difference in contamination levels between both slaughtering methods needs
320 to be confirmed by more extensive research.
321 At the genus level, the same hypothesis may be advanced in order to explain the increase of
322 *Corynebacterium* on both swabbing zones. For *Macrococcus*, the situation was opposite.
323 Bacteria from the genus *Macrococcus* belong to the *Staphylococcaceae* family and may

originate from the abattoir workers or the hides of animals. It is believed to be an important contaminant in processed meats (Jay et al., 2005).

In relation with the presumption that the neck swabbing area may harbor more bacteria coming from the digestive tract in catlle slaughered with the Halal technique in comparison with cattle classically slaughtered, suprisingly, we have not confirmed this hypothesis. Metagenetic results did not reveal a higher contamination with taxa originated from the digestive tract for Halal slaughtering in comparison with classical slaughtering.

We performed a rough analysis based on the bacterial populations detected in the gastro-intestinal tract (GIT) of dairy cattle, that measure the total relative abundance of GIT related bacterial populations in samples from both groups. It revealed that both groups shared a similar amount GIT bacterial contamination (data not shown). This rough analysis (data not shown) was performed by taking into account the work of Mao *et. al* (2015) who characterized the intestinal microbiota of dairy cattle.

In relation to the bacterial diversity, richness and evenness, no statistical difference has been observed between carcass swabbing areas or slaughtering processes. However, systematically, a higher level of bacteria was observed in the “Halal” group in comparison to the “Classical” group. This is true for both swabbing areas, i.e. the neck area and the “legal” zone. The number of OTUs observed in the “Halal” group is higher than in the “Classical” group. This was also true regarding the diversity and the distribution that were more even in the “Halal” than in the “Classical” group. The hypothesis may be that hygiene practices were different between both slaughtering protocols or that bacteria originating from the respiratory tract or from the digestive tract enriched the microbiota on the swabbing areas in the “Halal” group. A complementary study is needed to clearly ascertain these assumptions.

In addition, even if it was not the main purpose of this study, it should be confirmed that the observed bacterial contaminations on carcasses could indeed further induce spoilage of beef. By comparing our results with a similar study on meat or fish products (Chaillou et al., 2015), it appears that environmental contamination through water seems to affect microbial spoilage population in food more than animal contamination.

In this study, several biases may be pinpointed. The swabbing areas were very different in size with a ratio of 1/8. This can lead to a difference in recovery of bacteria from one swabbing area to another, which may impact the distribution of taxa during the metagenetic analysis (and so, the relative abundances of OTU). Indeed, the relative distribution of OTU may be different and this aspect needs to be confirmed by further investigation. However, in this study, we compare only the relative distribution between the slaughtering techniques inside each swabbing area. Secondly, the homogeneity of contamination may differ between the two swabbing zones: this hypothesis has to be also confirmed. Finally, as already mentioned, we did not investigate the difference between the two slaughtering techniques regarding the following parameters: slaughtering sequence (i.e. succession of slaughtering techniques along the day), the change in personnel working practices (i.e. compliance to good manufacturing practices) and the cleanliness of cattle when arriving at the abattoir.

In conclusion, despite the limitations cited above and the fact that metagenetic lacks of gold standard (Chistoserdova, 2010), these analysis offers a new tool for identifying microorganisms present in different matrices and sampling protocols such as, for instance, cotton swabbing of meat carcasses. In comparison to culture-based methods on selective media and previous culture-independent techniques, metagenetic analysis combined with the enumeration of total flora provides more valuable information, and its use should be considered as a technique for quality control in slaughterhouses. In theory, metagenetic analyses may elucidate the origins of carcass contamination. Indeed, it may be useful to know the sources of contamination (soil, workers, hides, intestinal tracts etc.) in order to implement or validate good hygiene practices and good slaughtering practices.

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531
532

Figure 1. Schematic representation of swabbing zones on cattle carcass

A. Swabbing areas for the "Legal" zone (1600 cm^2 in total). (Source: Royal Belgian decree of 4 July 1996 in relation to general and special exploitation conditions for slaughterhouses and other establishments)
B. Swabbing areas for the "Neck" zone (200 cm^2 in total).

Legend: a) Rump (posterolateral face of the hindlimb): 400 cm^2 b) Flank: 400 cm^2 c) Brisket (thorax): 400 cm^2 d) Forelimb (posterior surface of the forelimb): 400 cm^2 e) Neck area (close to the sticking point): 200 cm^2

Figure 2. Distribution of classical counts results for the two swabbing areas ("legal" zone and the neck) and for the two slaughtering protocols

A. Boxplot of Total Viable Counts results (TVC in log CFU/100 cm^2)
B. Boxplot of *Enterobacteriaceae* counts results (in log CFU/100 cm^2)

Legend: the box represents the distance between the quartile 1 (Q1) and quartile 3 (Q3) ; the solid line is the median ; the two dotted lines is the difference of 25% below the Q1 or above the Q3 ; the circles represent the "outliers" (atypical values)

Figure 3. Spatial ordination and alpha diversity deduced by 16S profiling.

A. Bacterial diversity (non parametric Shannon Biodiversity Index), bacterial richness (Chao1 Richness Index) and bacterial evenness (deduced from Shannon Index). Diversity indexes are expressed as the mean from subsampled datasets ± standard error of the mean.
B. Non metric dimensional scaling (stress value 0.097) with samples connected to their weighted centroid of the four groups;

Figure 4. Cumulated histograms of the relative abundance of taxa identified by metagenetics at Phylum, Family and Genus levels in relation to the slaughtering technique and the swabbing areas.

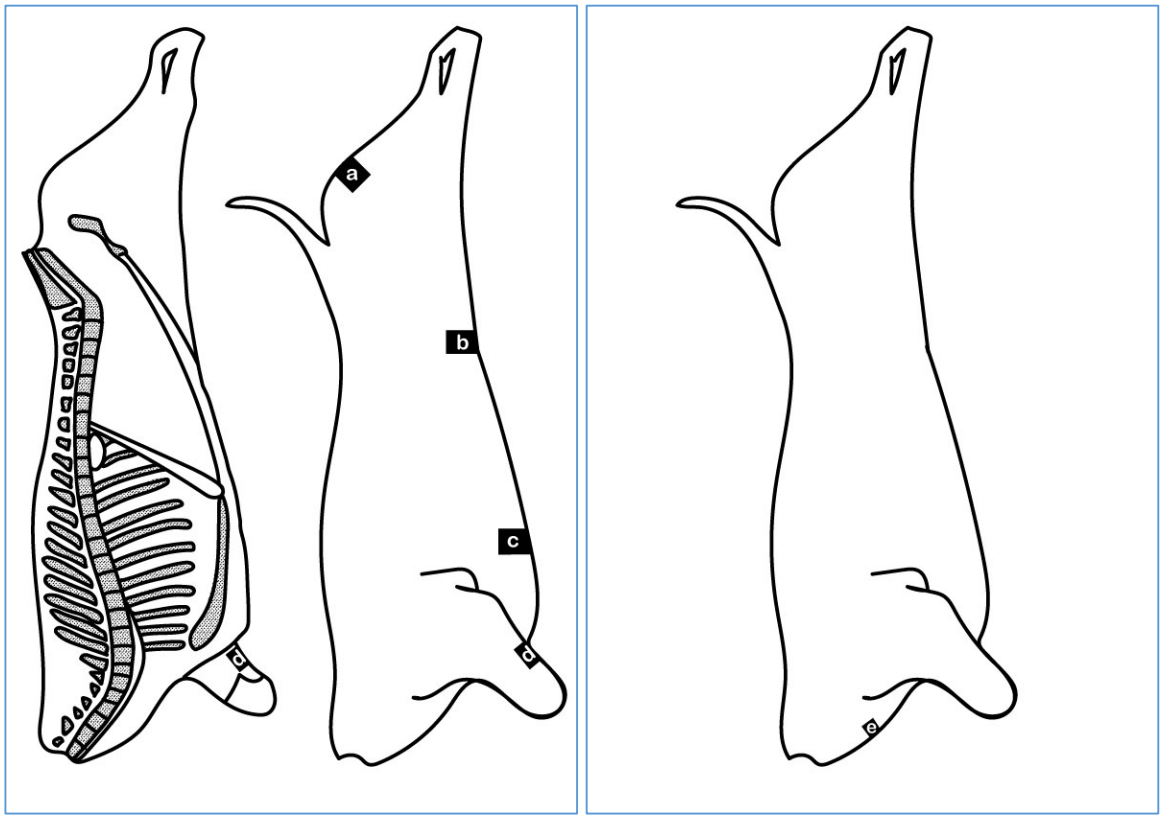
Legend: at Phylum, Family and Genus levels, the taxa representing less than 5% in relative abundance were merged in the category of "Others"

Figure 5. Histograms of the relative abundance of 6 selected taxa identified by metagenetics at Phylum, Family and Genus levels in relation to the slaughtering technique and the swabbing areas.

A. Relative population abundance in the neck swabbing area for selected taxa significantly different in proportion between the two slaughtering techniques (i.e. Halal vs Classical slaughtering technique).

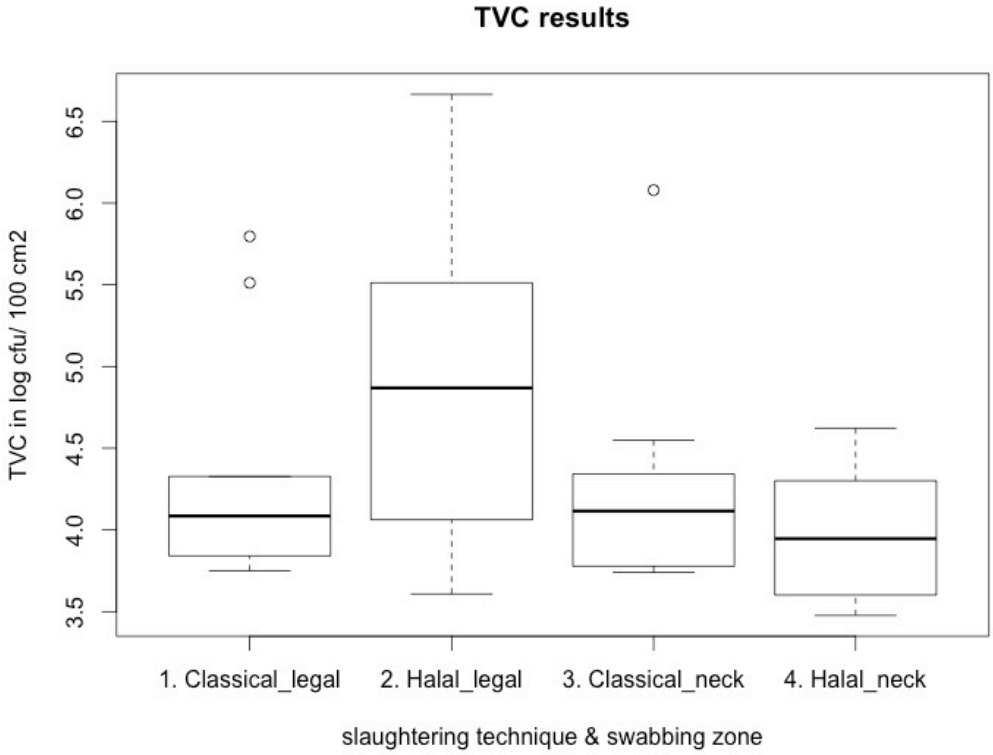
B. Relative population abundance in the legal swabbing area for selected taxa significantly different in proportion between the two slaughtering techniques (i.e. Halal vs Classical slaughtering technique);

Legend: superscript letters assignation for each bacterial taxa reflecting statistical difference ($p<0.05$) according to 2-way ANOVA tests followed by Tukey post hoc test._p: Phylum _f: Family _g: Genus

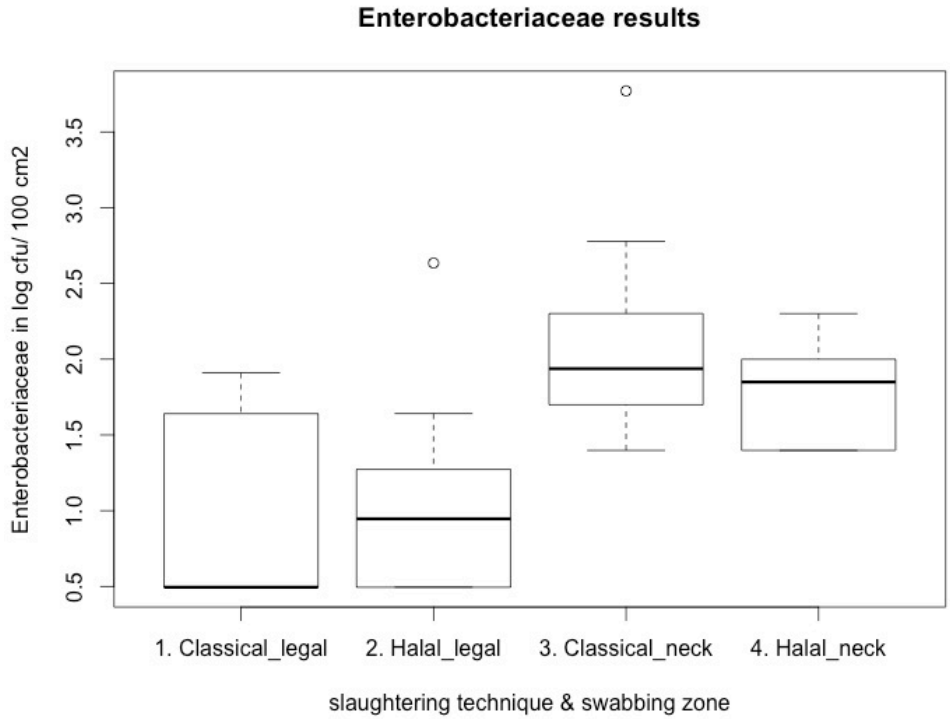


A B

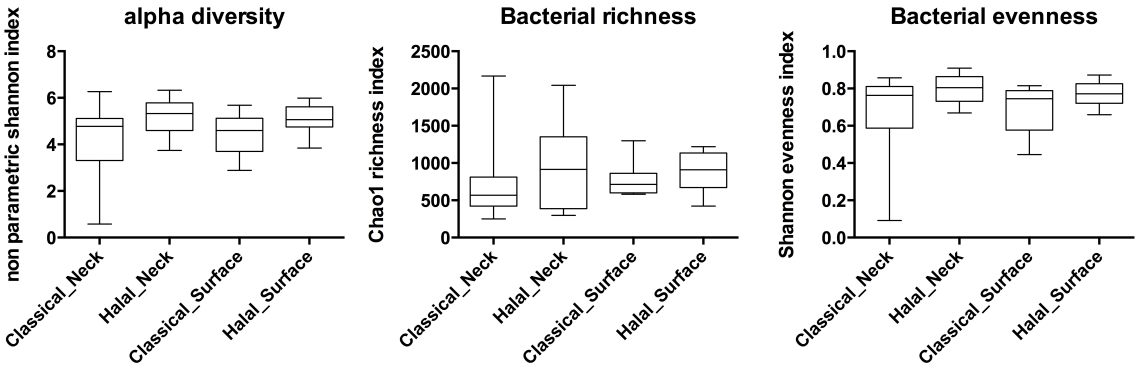
A


TVC results
TVC in log cfu/ 100 cm2
3.5
4.0
4.5
5.0
5.5
6.0
6.5
1. Classical_legal
2. Halal_legal
3. Classical_neck
4. Halal_neck
slaughtering technique & swabbing zone

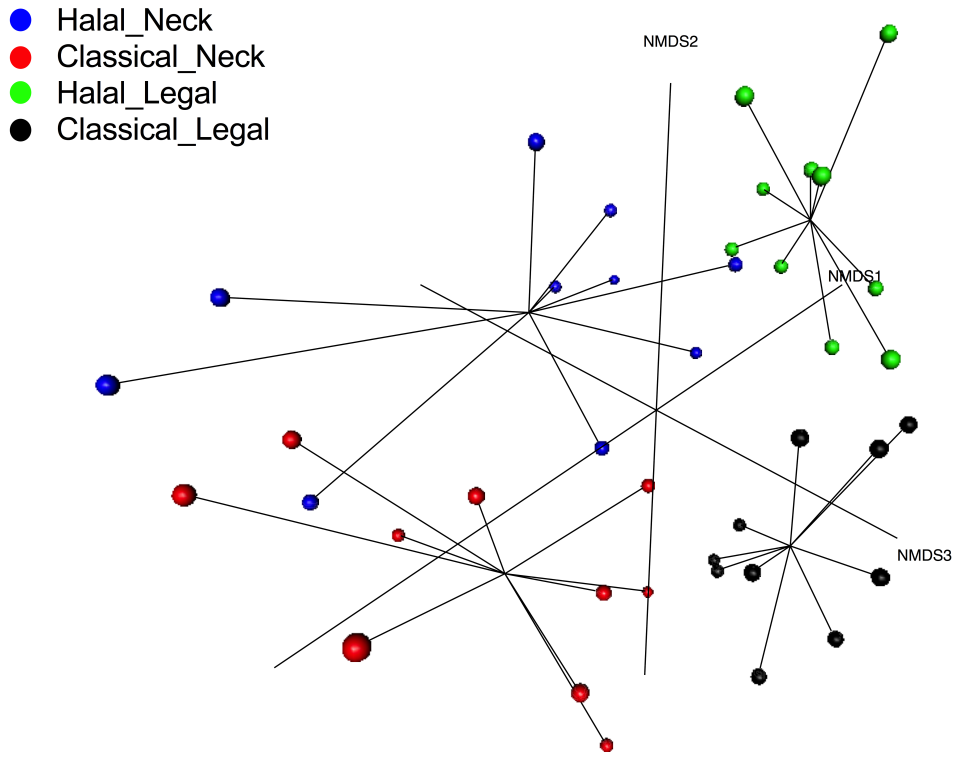
B

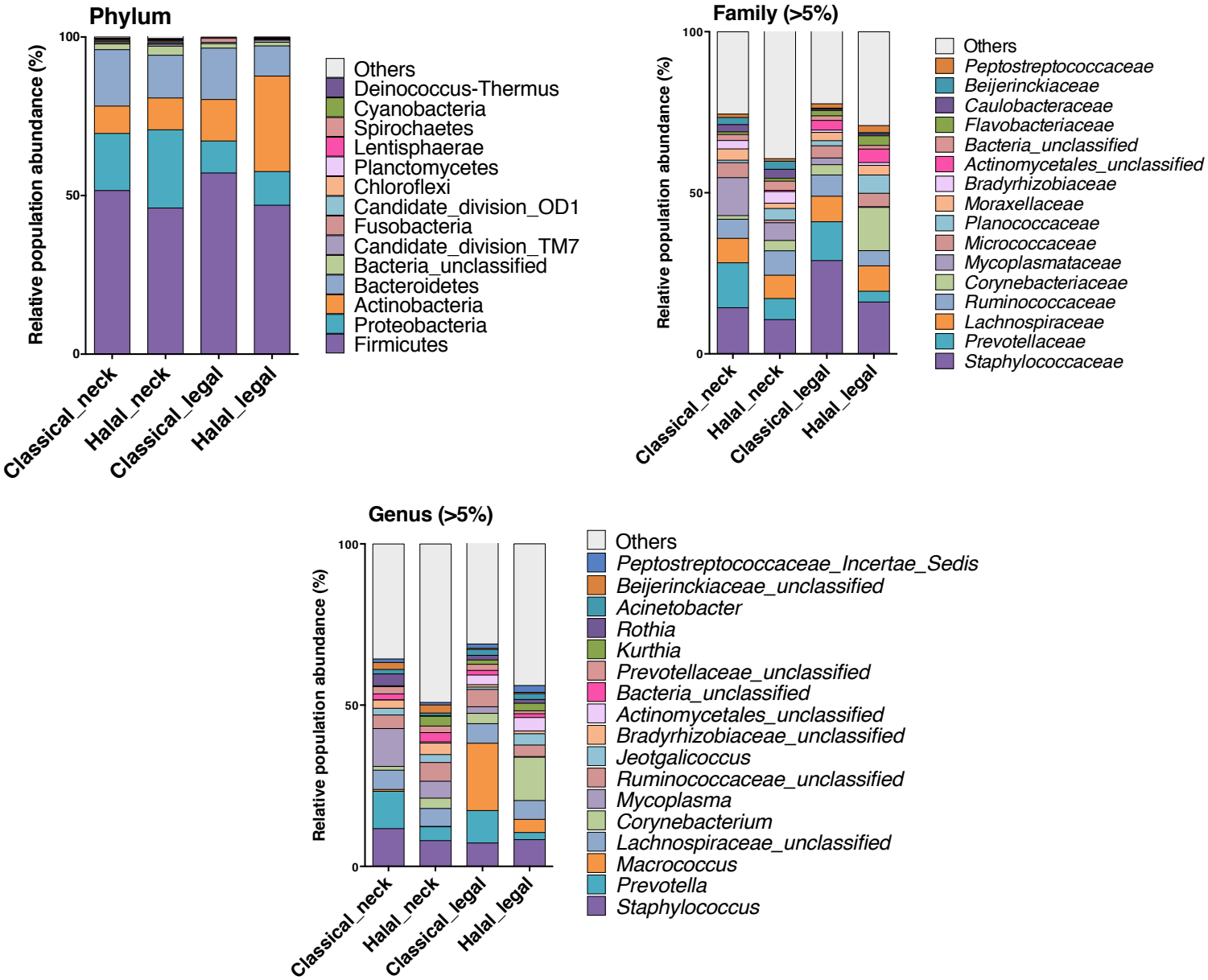

Enterobacteriaceae results
Enterobacteriaceae in log cfu/ 100 cm2
0.5
1.0
1.5
2.0
2.5
3.0
3.5
1. Classical_legal
2. Halal_legal
3. Classical_neck
4. Halal_neck
slaughtering technique & swabbing zone

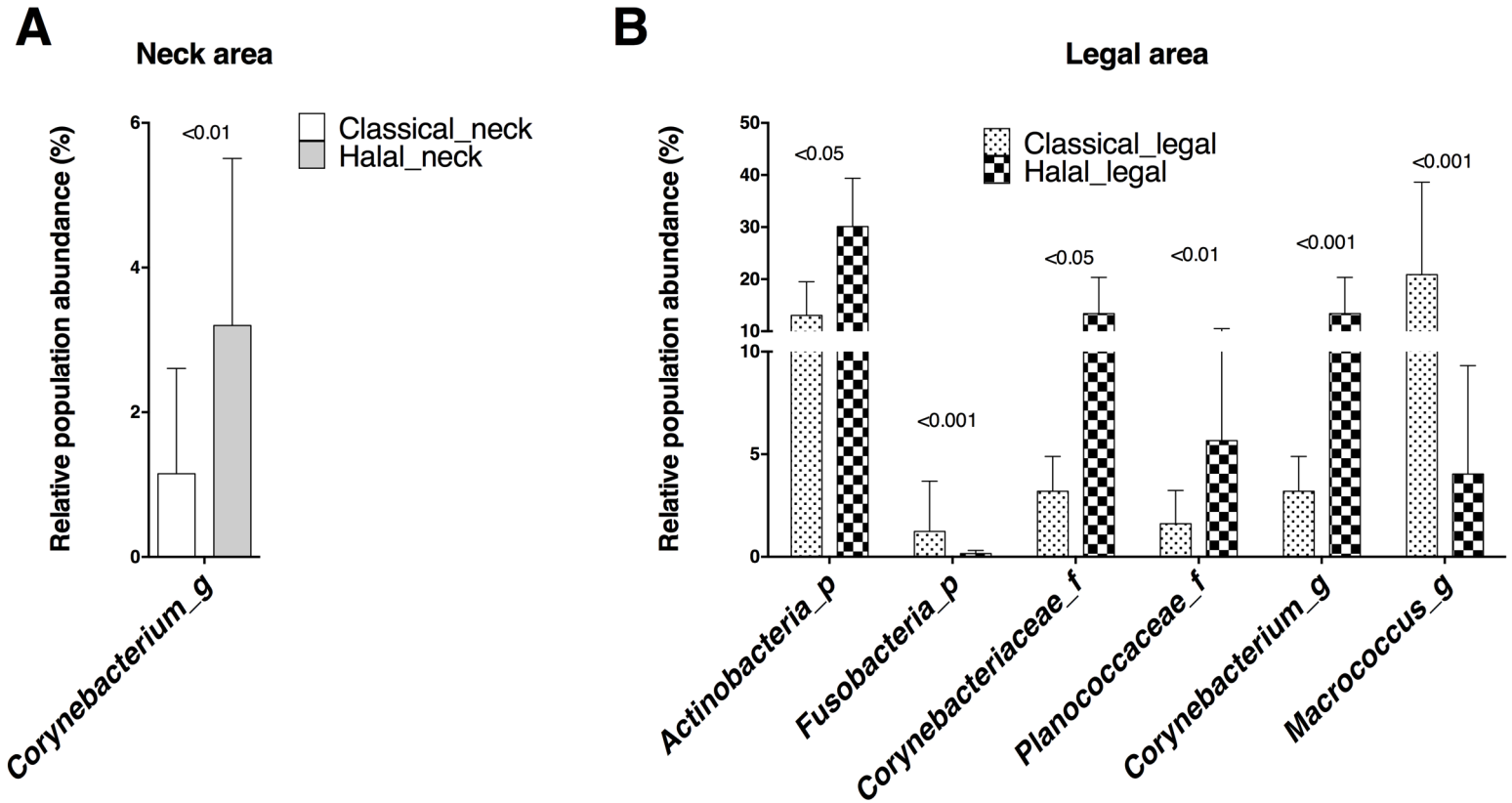
A


alpha diversity
non parametric shannon index
0
2
4
6
8
Classical_Neck
Halal_Neck
Classical_Surface
Halal_Surface
Bacterial richness
Chao1 richness index
0
500
1000
1500
2000
2500
Classical_Neck
Halal_Neck
Classical_Surface
Halal_Surface
Bacterial evenness
Shannon evenness index
0.0
0.2
0.4
0.6
0.8
1.0
Classical_Neck
Halal_Neck
Classical_Surface
Halal_Surface

B


Halal_Neck
Classical_Neck
Halal_Legal
Classical_Legal
NMDS2
NMDS1
NMDS3


Phylum
Relative population abundance (%)
100
50
0
Classical_neck
Halal_neck
Classical_legal
Halal_legal
Others
Deinococcus-Thermus
Cyanobacteria
Spirochaetes
Lentisphaerae
Planctomycetes
Chloroflexi
Candidate_division_OD1
Fusobacteria
Candidate_division_TM7
Bacteria_unclassified
Bacteroidetes
Actinobacteria
Proteobacteria
Firmicutes
Family (>5%)
Relative population abundance (%)
100
50
0
Classical_neck
Halal_neck
Classical_legal
Halal_legal
Others
Peptostreptococcaceae
Beijerinckiaceae
Caulobacteraceae
Flavobacteriaceae
Bacteria_unclassified
Actinomycetales_unclassified
Bradyrhizobiaceae
Moraxellaceae
Planococcaceae
Micrococcaceae
Mycoplasmataceae
Corynebacteriaceae
Ruminococcaceae
Lachnospiraceae
Prevotellaceae
Staphylococcaceae
Genus (>5%)
Relative population abundance (%)
100
50
0
Classical_neck
Halal_neck
Classical_legal
Halal_legal
Others
Peptostreptococcaceae_Incertae_Sedis
Beijerinckiaceae_unclassified
Acinetobacter
Rothia
Kurthia
Prevotellaceae_unclassified
Bacteria_unclassified
Actinomycetales_unclassified
Bradyrhizobiaceae_unclassified
Jeotgalicoccus
Ruminococcaceae_unclassified
Mycoplasma
Corynebacterium
Lachnospiraceae_unclassified
Macrococcus
Prevotella
Staphylococcus


A
Neck area
Relative population abundance (%)
Classical_neck
Halal_neck
<0.01
6
4
2
0
Corynebacterium_g
B
Legal area
Relative population abundance (%)
Classical_legal
Halal_legal
<0.05
<0.001
<0.05
<0.01
<0.001
<0.001
50
40
30
20
10
10
5
0
Actinobacteria_p
Fusobacteria_p
Corynebacteriaceae_f
Planococcaceae_f
Corynebacterium_g
Micrococcus_g

Suppl. Table1. Microbiological Results (log CFU/100 cm^2) obtained by carcass swabbing (performed 2 to 4 hours after slaughter)

n° bovine (sex)	Sampling day	Slaughter method	Total viable counts		*Enterobacteriaceae* counts	
			"legal" zone	Neck zone	"legal" zone [a]	Neck zone [b]
1 (M)	19/8/2013	Classical	3.84	4.04	0,49	2,30
2 (M)	19/8/2013	Classical	4.11	3.74	1,64	2,78
3 (M)	19/8/2013	Classical	3.75	4.19	0,49	2,18
4 (M)	19/8/2013	Classical	4.33	4.34	1,80	3,77
5 (M)	19/8/2013	Classical	4.06	4.28	1,10	1,70
6 (M)	19/8/2013	Classical	4.24	3.90	0,49	1,40
7 (M)	19/8/2013	Halal	4.06	4.33	1,64	2,00
8 (M)	19/8/2013	Halal	5.51	3.96	1,10	2,30
9 (M)	19/8/2013	Halal	4.40	4.28	0,80	2,18
10 (M)	19/8/2013	Halal	4.07	3.48	0,49	1,40
11 (M)	19/8/2013	Halal	3.99	4.62	0,49	1,40
12 (M)	19/8/2013	Halal	5.94	3.60	1,27	1,40
13 (M)	26/8/2013	Classical	5.51	6.08	1,91	2,30
14 (M)	26/8/2013	Classical	5.80	3.74	0,49	1,70
15 (M)	26/8/2013	Classical	3.96	3.78	0,49	1,70
16 (M)	26/8/2013	Classical	3.78	4.55	0,49	1,40
17 (M)	26/8/2013	Halal	3.61	3.48	0,80	2,00
18 (F)	26/8/2013	Halal	5.42	4.30	1,10	2,00
19 (M)	26/8/2013	Halal	5.34	3.80	0,49	1,70
20 (M)	26/8/2013	Halal	6.67	3.93	2,63	1,40
	Median day 1 (19/8/2013)		4.09	4.12	0.95	2.09
	Median day 2 (26/8/2013)		5.38	3.86	0.65	1.70
		Median "Classical"	4.09	4.12	0.49	1.94
		Median "Halal"	4.87	3.95	0.95	1.85

Legend: see text for the description of the swabbing area [a] if absence in the zone, the value assigned was 0.49 log CFU/100 cm^2 (corresponding to 50 CFU/1600 cm^2, half value of the detection limit of 100 CFU/1600 cm^2) [b] if absence in the zone, the value assigned was 1.40 log CFU/100 cm^2 (corresponding to 50 CFU/200 cm^2, half value of the detection limit of 100 CFU/200 cm^2)

Suppl. Table2. Supporting Information Table SX (1) Abundance of bacteria taxa, expressed in percentage, that are impacted by the slaughtering method and/or the swabbing area, as determined by 16sRNA profiling.

1a. At the phylum level

Phylum	Corrected p-value	Classical_neck mean	Classical_neck SD	Halal_neck mean	Halal_neck SD	Classical_legal mean	Classical_legal SD	Halal_legal mean	Halal_legal SD
Actinobacteria	9.03E-06	8.656	11.447	10.003	5.015	13.065	6.448	30.120	9.262
Fusobacteria	0.014	0.463	1.041	0.208	0.257	1.242	2.442	0.163	0.154

2a. At the phylum level

Phylum	p-value cut-off	Classical_neck mean	Halal_neck mean	Classical_legal mean	Halal_legal mean
Actinobacteria	0.05	a	a	a	b
Fusobacteria	0.001	a	a	b	a

1b. At the family level

Family	Corrected p-value	Classical_neck mean	Classical_neck SD	Halal_neck mean	Halal_neck SD	Classical_legal mean	Classical_legal SD	Halal_legal mean	Halal_legal SD
Aerococcaceae	0.033	0.327	0.351	0.857	0.705	0.869	0.737	1.585	1.338
Brevibacteriaceae	8.51E-05	0.061	0.111	0.485	0.364	0.529	0.585	1.573	1.031
Clostridiaceae	0.003	0.080	0.138	0.626	0.736	0.658	0.556	1.223	0.717
Corynebacteriaceae	1.62E-07	1.149	1.458	3.200	2.309	3.196	1.694	13.413	6.941
Dietziaceae	1.46E-06	0.096	0.125	0.313	0.206	0.125	0.074	0.748	0.388
Intrasporangiaceae	0.005	0.088	0.138	0.410	0.319	0.189	0.154	1.142	1.221

Peptostreptococcaceae	0.031	1.062	1.164	0.782	0.704	1.349	1.178	2.172	0.820
Planococcaceae	0.047	0.797	1.025	3.600	5.595	1.614	1.621	5.667	4.829

2b. At the family level

Family	p-value cut-off	Classical_neck mean	Halal_neck mean	Classical_legal mean	Halal_legal mean
Aerococcaceae	< 0.0001	a	a	a	b
Brevibacteriaceae	< 0.0001	a	a	a	b
Clostridiaceae	0.01	a	b	b	c
Corynebacteriaceae	< 0.0001	a	a	a	b
Dietziaceae	0.02	a	ab	a	b
Intrasporangiaceae	< 0.0001	a	a	a	b
Peptostreptococcaceae	0.001	a	a	a	b
Planococcaceae	0.001	a	ab	a	b

1c. At the genus level

Genus	Corrected p-value	Classical_neck mean	Classical_neck SD	Halal_neck mean	Halal_neck SD	Classical_legal mean	Classical_legal SD	Halal_legal mean	Halal_legal SD
Brevibacterium	8.51E-05	0.061	0.111	0.485	0.364	0.529	0.585	1.573	1.031
Butyrivibrio	0.038	0.162	0.195	0.369	0.401	0.335	0.244	0.684	0.538
Caryophanon	0.025	0.135	0.196	0.218	0.254	0.185	0.268	2.500	3.702
Clostridium	0.010	0.080	0.138	0.354	0.341	0.621	0.572	0.684	0.404
Corynebacterium	1.62E-07	1.149	1.458	3.200	2.309	3.196	1.694	13.413	6.941
Dietzia	1.46E-06	0.096	0.125	0.313	0.206	0.125	0.074	0.748	0.388
Flacklamia	0.002	0.088	0.099	0.216	0.258	0.052	0.082	0.425	0.282
Guggenheimella	0.003	0.000	0.000	0.252	0.406	0.036	0.065	0.539	0.481

Macrococcus	5.76E-05	0.558	1.004	0.128	0.152	20.892	17.741	4.039	5.283
Syntrophococcus	0.007	0.403	0.340	0.122	0.180	0.388	0.251	0.080	0.099

2c. At the genus level

Genus	p-value cut-off	Classical_neck mean	Halal_neck mean	Classical_legal mean	Halal_legal mean
Brevibacterium	< 0.0001	a	b	b	c
Butyrivibrio	0.001	a	a	a	b
Caryophanon	0.005	a	a	a	b
Costridium	0.05	a	b	c	c
Corynebacterium	< 0.0001	a	b	b	c
Dietzia	< 0.0001	a	a	a	b
Flacklamia	0.005	a	ab	a	b
Guggenheimella	0.05	a	b	ab	c
Macrococcus	< 0.0001	a	a	b	c
Syntrophococcus	0.05	a	b	a	b

Legend: Superscript letters assignation for each bacterial taxa reflecting statistical difference ($p<0.05$) according to 2-way ANOVA followed by Tukey post hoc test. SD: standard deviation.

Suppl. Table3. Permutational multivariate variance analysis (AMOVA) of the samples groups based on the bacterial species distribution (999 permutations). Centroid of each group show a statistical separation from the others if the F statistic value is >1. Pair-wise p value cut off is 0.001.

Global analysis

Group	F statistic	P value
Classical_Legal-Classical_Neck-Halal_Legal-Halal_Neck	2.91715	<0.001

Pairwise analysis

Group	F statistic	P value
Classical_Legal-Classical_Neck	2.8825	<0.001
Halal_Legal-Halal_Neck	2.61168	<0.001
Classical_Legal-Halal_Legal	3.16922	<0.001
Classical_Neck-Halal_Neck	1.43304	0.041

Legend: Centroid of each group show a statistical separation from the others if the F statistic value is >1. Pair-wise p value cut off is 0.001.