Introduction

Over several decades, antibiotics have been used as feed additives to mitigate early chick mortality due to bacterial infections, as well as to ensure bacteria-free and safe products to consumers (Diarra and Malouin, 2014; Gaucher et al., 2015). However, there is growing concern about indiscriminate use of antibiotics in animal production and emergence of antibiotic-resistant strains of bacteria that may eventually adversely affect animals and human health (Diarra and Malouin, 2014; Gaucher et al., 2015; Laxminarayan et al., 2013). Indeed, evidence from many studies suggest that bacteria carrying antibiotic resistance genes can be transmitted from animals to humans (Folster et al., 2012; Luangtongkum et al., 2006; Sahin et al., 2012; Tremblay et al., 2011; White et al., 2002). Based on these results and in line with the precautionary principle, the European Union Commission banned the use of antibiotic growth promoters in animal feed in 2006 (Castanon, 2007), although anticoccidial ionophore inclusion in broiler feed is still permitted (Gaucher et al., 2015).

In 2014, the World Health Organization concluded that the use of antimicrobial agents as feed additives in agricultural animals is a public health issue and that an urgent global coordinated action plan is needed to reduce the use of these compounds in animal husbandry, as many antimicrobial agents used in farm animal production are also used to treat important human infections (WHO, 2014). Strategies to reduce the use of antibiotics in poultry include improved biosecurity, vaccination, genetic selection and competitive exclusion. However, to date there are no universal standards for antibiotic free productions and poultry supply chains have their own protocols in place (Poultry World, 2018). Those range from not using antibiotics important to human health, to no antibiotics at all for the broiler or the parent bird (Poultry World, 2018).

As a matter of fact, all meat that ends up in the supermarket should be “antibiotic-free”, as all farmers have to comply with the compulsory withdrawal periods (certain amount of days) after animals are treated with antibiotics, to make sure no traces or residues of the drug are left behind. Therefore, the term “antibiotic-free” should be replaced with “antibiotic-free production” (Poultry World, 2018). The “no antibiotic” claim has recently become a selling point for many supermarket and restaurant brands. A recent US representative Consumer Reports survey of over 1,000 people, found that 43% say they always or often buy meat raised without antibiotics and nearly 6 in 10 people would be more likely to eat at a restaurant if the meat and poultry was raised without antibiotics, and would pay more for a “no-antibiotic” burger (Consumer report, 2018).

Despite the lack of details concerning antibiotic free productions, in this study the microbiota of conventional and antibiotic free broiler carcasses has been compared in terms of mean relative frequency of abundance of colonising bacteria and foodborne pathogens.

Materials and Methods

A total of 30 poultry carcasses, belonging to the first two batches slaughtered at the beginning of the day, were collected at the same slaughterhouse at the end of the refrigeration tunnel and transported to the laboratory at 0-4°C within 8 hours. A total of 15 carcasses were obtained from animals reared in conventional farms, meaning intensive farms where the administration of antibiotics for therapeutic treatments is allowed, whereas 15 carcasses were reared...
in antibiotic free farms, where the use of antibiotics should not be permitted for any reason during the rearing period. A total of 10 g of neck and breast skin were aseptically collected from each individual carcass and diluted in 90 ml of sterile physiological solution (0.90% NaCl) before homogenization in the Pulsifier® at normal speed for 1 minute. The solution was then centrifuged at 6800 rpm at 4°C for 20 min and the pellet re-suspended in further 5 ml of sterile physiological solution before centrifugation as previously described. The DNA was extracted from the pellet by using the PowerFood® Microbial DNA Isolation kit (MoBio). The libraries were prepared following the Illumina 16S Library preparation protocol, amplifying the variable V3 and V4 regions of the 16S rRNA in order to obtain a single amplicon of approximately 460 bp. Sequencing was performed in paired-end in the Illumina MiSeq. At the end of sequencing run the samples were demultiplexed using Illumina Basespace (https://basespace.illumina.com) and uploaded as fastq files in MG-RAST (Keegan et al., 2016). After applying the quality control procedure, following the instructions of the MG-RAST manual, the taxonomic classification of the sequencing data was performed by applying the Best Hit Classification method and using the M5RNA database. The following parameters were set: maximum e-value 1e-5, minimum identity 60%, and minimum alignment length 15 bp. The mean values of the relative frequency of abundance of each taxonomic level within each carcass group were obtained by the normalized read counts and compared by using the t test of Turkey-Kramer in the software Statistical Analysis of Metagenomic Profile (STAMP) v2.0.9. The p values < 0.05 were considered statistically significant. Furthermore, the Shannon index was used to represent the alpha diversity, meaning the level of biodiversity within each group of carcasses, while the principal coordinate analysis (PCoA) with Bray-Curtis dissimilarity was applied to estimate the beta diversity, meaning the level of biodiversity between conventional and antibiotic free carcasses. The metagenomic sequences are public available in MG-RAST (http://metagenomics.anl.gov/) under project label as “first trial antibiotic free” with the following IDs: carcasses from antibiotic free farms mgm4795633.3, mgm4795651.3, mgm4795639.3, mgm4795629.3, mgm4795645.3, mgm4795618.3.

### Results

Firmicutes was the most abundant phylum, although its mean relative frequency of abundance was not significantly different between conventional and antibiotic free carcasses (i.e., 43.364 vs 39.042%). On the contrary, among the other phyla with abundances >1%, Bacteroidetes and Actinobacteria were significantly higher in antibiotic free carcasses, while Proteobacteria in conventional carcasses (Table 1). At class level, Flavobacteria and Actinobacteria were significantly higher in antibiotic free carcasses, whereas Gammaproteobacteria in conventional carcasses (Table 2). The other classes with mean relative frequency of abundance >1%, but not significantly different in conventional and antibiotic free carcasses, were Bacilli (26.913 vs 19.515%), Clostridia (15.560 vs 17.322%), Epsilonbacteria (1.494 vs 0.923%), Bacteroidia (7.144 vs 7.560%) and Negativicutes (1.679 vs 1.739%). At order level, Flavobacteriales

### Table 1. Phyla with mean relative frequency of abundance (mean) >1% and significantly different between conventional (AC) and antibiotic free (AF) carcasses.

| Phylum          | P-value | AC mean (%) | AC std. dev. (%) | AF mean (%) | AF std. dev. (%) |
|-----------------|---------|-------------|------------------|-------------|------------------|
| Proteobacteria  | 0.010   | 33.188      | 15.013           | 19.523      | 10.387           |
| Bacteroidetes   | 0.011   | 10.949      | 6.014            | 21.573      | 12.916           |
| Actinobacteria  | 0.026   | 12.046      | 8.515            | 19.285      | 12.631           |

### Table 2. Classes with mean relative frequency of abundance (mean) >1% and significantly different between conventional (AC) and antibiotic free (AF) carcasses.

| Class            | P-value | AC mean (%) | AC std. dev. (%) | AF mean (%) | AF std. dev. (%) |
|------------------|---------|-------------|------------------|-------------|------------------|
| Flavobacteria    | 0.000   | 3.576       | 2.271            | 13.057      | 12.380           |
| Actinobacteria   | 0.029   | 12.256      | 8.453            | 19.343      | 12.658           |
| Gammaproteobacteria | 0.033 | 28.888      | 16.517           | 16.857      | 10.458           |

### Table 3. Orders with mean relative frequency of abundance (mean) >1% and significantly different between conventional (AC) and antibiotic free (AF) carcasses.

| Order             | P-value | AC mean (%) | AC std. dev. (%) | AF mean (%) | AF std. dev. (%) |
|-------------------|---------|-------------|------------------|-------------|------------------|
| Flavobacteriales  | 0.000   | 3.663       | 2.426            | 13.449      | 13.187           |
| Alteromonadales   | 0.002   | 1.355       | 2.048            | 0.252       | 0.338            |
| Actinomycetales   | 0.004   | 7.589       | 10.347           | 15.567      | 13.368           |
| Bacillales        | 0.049   | 7.208       | 7.241            | 3.181       | 2.371            |
and Actinomycetales were significantly higher in antibiotic free carcasses, whereas Alteromonadales and Bacillales in conventional carcasses (Table 3). The other orders with mean relative frequency of abundance >1% but not significantly different in conventional and antibiotic free carcasses were Pseudomonadales (21.423 vs 12.341%), Clostridiales (15.699 vs 17.621%), Bifidobacteriales (4.859 vs 4.068%), Campylobacterales (1.503 vs 0.906%), Lactobacillales (20.109 vs 16.586%), Enterobacterales (3.843 vs 1.827%), Bacteroidales (7.235 vs 7.716) and Selenomonadales (1.700 vs 1.773).

At family level, Flavobacteriaceae, Microbacteriaceae, Sphingobacteriaceae and Micrococcaceae were significantly higher in antibiotic free carcasses, whereas Planococcaceae, Shewanellaceae and Bacteroidaceae in conventional carcasses (Table 4). The other families with mean relative frequency of abundance >1%, but not significantly different in conventional and antibiotic free carcasses, were Enterococcaceae (2.549 vs 2.235%), Helicobacteraceae (1.216 vs 0.825%), Moraxellaceae (8.315 vs 6.545%), Clostridiaceae (3.573 vs 3.881%), Porphyromonadaceae (2.153 vs 2.341%) and Veillonellaceae (1.178 vs 1.277%).

At genus level, Chryseobacterium, Rothia and Micrococcus were significantly higher in antibiotic free carcasses, whereas Ureibacillus, Shewanella and Bacillus in conventional carcasses (Table 5). The other genera with mean relative frequency of abundance >1%, but not significantly different in conventional and antibiotic free carcasses, were Psychrobacter (6.711 vs 3.772%), Ruminococcus (1.433 vs 1.833%), Enterococcus (1.230 vs 1.159%), Pseudomonas (13.598 vs 6.289%), Escherichia (2.682 vs 0.162%), Acinetobacter (1.533 vs 2.644%), Lactobacillus (18.281 vs 15.158%), Bifidobacterium (5.074 vs 4.312%), Bacteroides (2.758 vs 3.548%), Myroides (0.717 vs 1.231%), Arthrobacter (5.493 vs 7.478%) and Parabacteroides (0.902 vs 1.061%). Alstistes (2.273 vs 1.665%), Faecalibacterium (3.509 vs 3.249%), Helicobacter (1.248 vs 0.845%) and Clostridium (2.517 vs 2.513%). The mean relative frequency of abundance of Campylobacter was always lower than 0.4% and higher in conventional carcasses than antibiotic free carcasses (0.348 vs 0.124%) but without any statistical significant difference. In terms of alpha diversity, the genera identified in the carcasses obtained from conventional and antibiotic free farms did not show significant difference (P=0.433) (Figure 1). However, in the principal coordinate analysis (PCoA) with Bray-Curtis dissimilarity plot the genera colonising the two groups of carcasses clustered one from the other with few exceptions (Figure 2).

**Discussion**

The antibiotic free claim is increasing in many food products, including egg and meat. In this study the microbiota of chicken carcasses obtained from broilers reared in conventional and antibiotic free farms were compared to investigate the impact of each kind of farming on the microbiological composition of the meat consumers eat. Overall, the microbiota associated to conventional and antibiotic free carcasses were significantly different, with a higher mean relative frequency of abundance of Bacteroidetes and Actinobacteria in antibiotic free carcasses and Proteobacteria in conventional carcasses. At genus level, the main differences regarded degradative bacteria, while concerning the genera listed in EU Regulation 2073/2005, *Salmonella* was not detected, while *Campylobacter* showed abundances always lower than 0.4% and higher in conventional carcasses, although this difference was not statistically significant.
Firmicutes was the most abundant phylum in both kinds of carcasses and genera Bacillus and Ureibacillus were significantly higher in conventional carcasses. The same was for Lactobacillus, Enterococcus, Faecalibacterium and Clostridium, while Ruminococcus was higher in antibiotic free carcasses but without any statistical significance. Bacteroidetes and Actinobacteria were significantly higher in antibiotic free carcasses. The higher abundance of Bacteroidetes was mainly due to the order Flavobacteriales, family Flavobacteriaceae, genus Chryseobacterium. Flavobacteria are well known degradative bacteria in foods, including meat (de Beer et al., 2005). They might originate from both animals and slaughterhouse environment (Hang’ombe et al., 1999) and show a prevalence in poultry meat around 16% (Mai and Conner, 2001). The higher abundance of Actinobacteria in antibiotic free carcasses was mainly due to the order Actinomycetales, families Micrococcaceae and Microbacteriaceae, genera Rothia, Microbacterium and Micrococcus. Proteobacteria was the only phylum with an abundance significantly higher in conventional carcasses and this difference was mainly due to the classes Gammaproteobacteria and Epsilonbacteria. Shewanellaceae was the only family belonging to the Gammaproteobacteria significantly higher in conventional carcasses. The same was for Pseudomonadaceae, Enterobacteriaceae, Campylobacteraceae and Helicobacteriaceae but without a statistical significance. At genus level, Shewanella was significantly higher in conventional carcasses, while Stenotrophomonas and Pantoea in antibiotic free carcasses. Finally, Pseudomonas, Escherichia, Helicobacter and Campylobacter were higher in conventional carcasses without a statistical significance. As a matter of fact, both Campylobacter and Escherichia were much higher in conventional carcasses than antibiotic free carcasses but those differences were not statistically significant due to the high standard deviation associated to the mean relative frequency of abundance of those genera. Such high standard deviation is linked to the high variability of the value of relative frequency of abundance associated to each carcass, which is intrinsic to the nature of the sample and might be possibly attenuated increasing further the number of carcasses included in each tested group.

Conclusions

The higher abundance of Proteobacteria in conventional carcasses might suggest that hygienic conditions in conventional farms are worse than antibiotic free farms, where most effective biosecurity measures should be generally applied. However, from a food safety point of view, Salmonella was not detected in both kinds of carcasses and the Campylobacter mean relative frequency of abundance was lower than 0.4% in both conventional and antibiotic free carcasses. The results of this preliminary study obtained by target sequencing of neck and breast skin provided an overview of the carcass microbiota as well as abundances of genera relevant from a food safety point of view, although further data are need to confirm the present results which might be further exploited at species level characterised by lower abundances. Despite precise

Figure 1. Shannon indexes associated to the genera detected in the carcasses obtained in conventional (AC) and antibiotic free (AF) farms.

Figure 2. Principal Coordinate Analysis (PCoA) with Bray-Curtis dissimilarity plots showing the genera detected in the carcasses obtained in conventional (AC) and antibiotic free (AF) farms.
results on the correspondence between relative frequency of abundance and colony forming units are still lacking, trials with mock communities containing known concentrations of different foodborne bacteria are currently under development as part of this research.

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