Microbiota analysis and microbiological hazard assessment in poultry carcasses from conventional and antibiotic free farms

Alessandra De Cesare,[1] Antonio Parisi,[2] Alex Lucchi,[1] Loredana Capozzi,[2] Angela Bianco,[2] Frederique Pasquali,[1] Gerardo Manfreda[1]

[1]Department of Agricultural and Food Sciences, *Alma Mater Studiorum* – University of Bologna; [2]Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata, Sezione di Putignano, Bari, Italy

Abstract

The aim of this study was to assess microbiota and microbiological hazards in poultry carcasses from animals reared in conventional (n=15) and antibiotic free (n=15) farms. An aliquot of neck and breast skin was obtained from each individual carcass at the end of the refrigeration tunnel and submitted to DNA extraction. Total DNA was sequenced in the 16S rRNA and reads analysed with MG-RAST to classify the colonising bacteria up to the genus level and compare each taxonomic group in terms of mean relative frequency of abundance in conventional and antibiotic free carcasses. Firmicutes displayed abundances always higher than 38% but did not show statistically significative differences between conventional and antibiotic free carcasses. On the contrary, Bacteroidetes and Actinobacteria were significantly higher in antibiotic free then conventional carcasses (21.57 *vs* 10.95%; 19.29 *vs* 12.05%), whereas Proteobacteria were higher in the latter (33.19 vs 19.52%). The genera significantly higher in antibiotic free than conventional carcasses were *Chryseobacterium* (10.07 *vs* 1.94%), *Rothia* (3.08 *vs* 0.77%) and *Micrococcu*s (1.12 *vs* 0.16%), while *Shewanella* was significantly higher in conventional carcasses (1.38 *vs* 0.26%). Among Firmicutes, the genera significantly higher in conventional carcasses were *Ureibacillus* (1.45 *vs* 0.11%) and *Bacillus* (3.28 *vs* 0.56%). The higher abundance of Proteobacteria in conventional carcasses might suggest that hygienic conditions in conventional farms are worse than antibiotic free farms. 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 always lower than 0.4%.

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 animal 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.

Correspondence: Alessandra De Cesare, Department of Agricultural and Food Sciences, *Alma Mater Studiorum*-University of Bologna, via del Florio 2, 40064 Ozzano dell'Emilia (BO), Italy.
Tel.: +39.051.2097583 - Fax: +39.051.2097852.
E-mail: alessandra.decesare@unibo.it

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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 be not 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 conventional farms mgm4795632.3, mgm4795640.3, mgm4795636.3, mgm4795648.3, mgm4795626.3, mgm4795635.3, mgm4795613.3, mgm4795622.3, mgm4795646.3, mgm4795638.3, mgm4795620.3, mgm4795634.3, mgm4795649.3, mgm4795625.3, mgm4795619.3; carcasses from antibiotic free farms mgm4795633.3, mgm4795627.3, mgm4795616.3, mgm4795637.3, mgm4795647.3, mgm4795650.3, mgm4795642.3, mgm4795641.3, mgm4795631.3, mgm4795643.3, 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	19.295	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	2.408	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%), Campylobacteriales (1,503 vs 0,906%), Lactobacillales (20,109 *vs* 16,586%), Enterobacteriales (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 Bacillaceae 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 (1.237 *vs* 1.173%), Pseudomonadaceae (13.535 *vs* 6.204%), Lachnospiraceae (2.154 *vs* 2.461%), Lactobacillaceae (18.845 *vs* 15.127%), Bifidobacteriaceae (5.016 *vs* 4.247%), Staphylococcaceae (1.011 *vs* 1.929%), Bacteroidaceae (2.7 *vs* 3.464%), Ruminococcaceae (6.088 *vs* 6.63%), Enterobacteriaceae (3.957 *vs* 1.891%), Rikenellaceae (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%), *Parabacteroides* (0.902 *vs* 1.061%), *Alistipes* (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 significative difference. In terms of alpha diversity, the genera identified in the carcasses obtained from conventional and antibiotic free farms did not show significative 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 signifi-

Table 4. Families with mean relative frequency of abundance (mean) >1% and significantly different between conventional (AC) and antibiotic free (AF) carcasses.

Family	P-value	AC mean (%)	AC std. dev. (%)	AF mean (%)	AF std. dev. (%)
Flavobacteriaceae	0.000	3.753	2.458	13.784	13.244
Planococcaceae	0.000	1.451	1.853	0.117	0.114
Microbacteriaceae	0.001	0.165	0.108	1.189	1.803
Sphingobacteriaceae	0.002	0.487	1.257	1.009	1.238
Shewanellaceae	0.002	1.369	2.469	0.255	0.341
Bacillaceae	0.006	4.162	4.785	1.177	2.127
Micrococcaceae	0.012	6.425	10.262	11.623	12.499

Table 5. Genera with mean relative frequency of abundance (mean) >1% and significantly different between conventional (AC) and antibiotic free (AF) carcasses.

Genus	P-value	AC mean (%)	AC std. dev. (%)	AF mean (%)	AF std. dev. (%)
Chryseobacterium	0.000	1.945	1.179	10.071	11.851
Ureibacillus	0.000	1.451	1.855	0.108	0.111
Rothia	0.002	0.775	0.559	3.080	2.245
Shewanella	0.002	1.380	2.481	0.258	0.342
Bacillus	0.003	3.287	3.848	0.563	0.331
Micrococcus	0.010	0.162	0.280	1.121	1.296

cant. 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

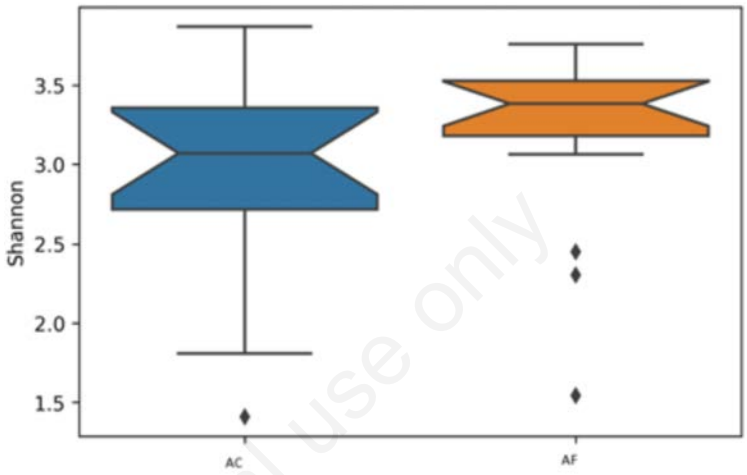

Figure 1. Shannon indexes associated to the genera detected in the carcasses obtained in conventional (AC) and antibiotic free (AF) farms.

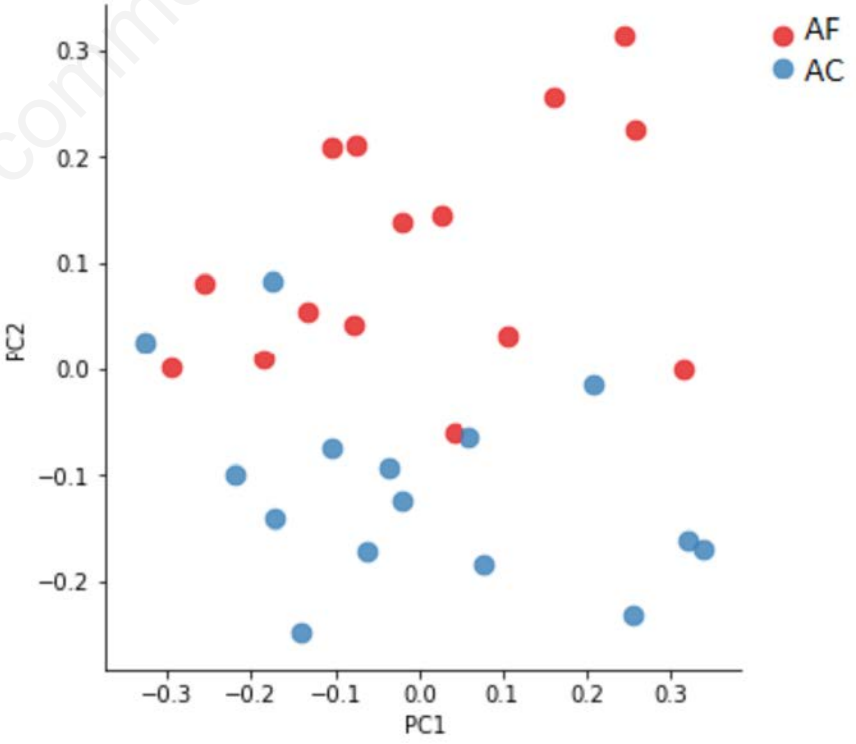

Figure 2. Principal Coordinate Analysis (PCoA) with Bray-Curtis dissimilarity plots showing the genera detected in in the carcasses obtained in conventional (AC) and antibiotic free (AF) farms.

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.

References

de Beer H, Hugo CJ, Jooste PJ, Willems A, Vancanneyt M, Coenye T, Vandamme PA, 2005. Chryseobacterium vrystaatense sp. nov., isolated from raw chicken in a chicken-processing plant. Int J Syst Evol Microbiol 55:2149-53.

Castanon J I, 2007. History of the use of antibiotic as growth promoters in European poultry feeds. Poult Sci 86:2466-71.

Consumer report, 2018. Available from: https://www.consumerreports.org/overuse-of-antibiotics/what-no-antibiotic-claims-really-mean/

Diarra MS, Malouin F, 2014. Antibiotics in Canadian poultry productions and anticipated alternatives. Front Microbiol 5:282.

Folster JP, Pecic G, Singh A, Duval B, Rickert R, Ayers S, Abbott J, McGlinchey B, Bauer-Turpin J, Haro J, Hise K, Zhao S, Fedorka-Cray PJ, Whichard J, McDermott PF, 2012. Characterization of extended-spectrum cephalosporin-resistant Salmonella enterica serovar Heidelberg isolated from food animals, retail meat, and humans in the United States 2009. Foodborne Pathog Dis 9:638-45.

Gaucher ML, Quessy S, Letellier A, Arsenault J, Boulianne M, 2015. Impact of a drug free program on broiler chicken growth performances, gut health, Clostridium perfringens and Campylobacter jejuni occurrences at the farm level. Poult Sci 94:1791-801.

Hang'ombe BM, Sharma NR, Skjerve E, Tuchili LM, 1999. Isolation of bacteria during processing of chicken carcasses for the market in Lusaka, Zambia. Veterinarski arhiv 69:191-7.

Keegan KP, Glass EM, Meyer F, 2016. MG-RAST, a Metagenomics Service for Analysis of Microbial Community Structure and Function. In: Martin F., Uroz S. (eds) Microbial Environmental Genomics (MEG). Methods in Molecular Biology, vol 1399. Humana Press, New York, NY.

Laxminarayan R, Duse A, Wattal C, Zaidi AK, Wertheim HF, Sumpradit N, Vlieghe E, Hara GL, Gould IM, Goossens H, Greko C, So AD, Bigdeli M, Tomson G, Woodhouse W, Ombaka E, Peralta AQ, Qamar FN, Mir F, Kariuki S, Bhutta ZA, Coates A, Bergstrom R, Wright GD, Brown ED, Cars O, 2013. Antibiotic resistance-the need for global solutions. Lancet Infect Dis 13:1057-98.

Luangtongkum T, Morishita TY, Ison AJ, Huang S, McDermott PF, Zhang Q, 2006. Effect of conventional and organic production practices on the prevalence and antimicrobial resistance of Campylobacter spp. in poultry. Appl Environ Microbiol 72:3600-7.

Mai T, Conner D, 2001. Identification of bacteria found in broiler deboning operations. J Dairy Sci 84, 297.

Poultry World, 2018. Available from: https://www.poultryworld.net/Health/Articles/2018/3/Transition-to-antibiotic-free-production-264348E/

Sahin O, Fitzgerald C, Stroika S, Zhao S, Sippy J, Kwan P, Plummer PJ, Han J, Yaeger MJ, Zhang Q, 2012. Molecular evidence for zoonotic transmission of an emergent, highly pathogenic Campylobacter jejuni clone in the United States. J Clin Microbiol 50:680-7.

Tremblay CL, Letellier A, Quessy S, Boulianne M, Daignault D, Archambault M, 2011. Multiple-antibiotic resistance of Enterococcus faecalis and Enterococcus faecium from cecal contents in broiler chicken and turkey flocks slaughtered in Canada and plasmid co localization of tetO and ermB genes. J Food Prot 74:1639-48.

White DG, Zhao S, Simjee S, Wagner DD, Mc-Dermott PF, 2002. Antimicrobial resistance of foodborne pathogens. Microb Infect 4:405-12.

WHO, 2014. Antimicrobial Resistance Global Report on Surveillance 2014. World Health Organization, Geneva, CH.