Animal 15 (2021) 100113

Contents lists available at ScienceDirect

Animal
The international journal of animal biosciences

Application of RNA-sequencing to identify biomarkers in broiler chickens prophylactic administered with antimicrobial agents

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ARTICLE INFO

Article history:
Received 15 April 2020
Received in revised form 8 October 2020
Accepted 15 October 2020
Available online 27 December 2020

Keywords:
Antibiotics
Coccidiostat
Next-generation sequencing
Poultry
Transcriptome

ABSTRACT

Antimicrobial (**AM**) resistance is largely acknowledged as one of the biggest global health and food safety challenges and the overuse of AMs is known to generate resistance in bacteria that may affect both animals and humans. Poultry meat is the second most-produced meat in the European Union and in recent years consumers are becoming more concerned about food safety, traceability, and animal welfare in poultry rearing system, increasingly requiring meats from broilers reared without AMs. In the present study, we performed RNA sequencing to analyze 64 liver and 54 muscle transcriptomic profiles in broilers reared without treatment or treated with different classes of AMs. Moreover, we validated the most differentially expressed genes among the treated groups to detect putative novel biomarkers able to discriminate meats of broilers reared without AMs. The *PDK4*, *IGFBP1*, and *RHOB* genes were identified as putative novel hepatic biomarkers, discriminating broilers treated with AMs compared to broilers reared without treatments. The whole transcriptome changes revealed the liver as a valuable target organ for AM administration screening. In addition, our results suggest a leading effect of the coccidiostat when associated with AMs, influencing several biological processes. Our study showed that RNA sequencing is a powerful and valuable method to detect aberrant regulated genes and to identify biomarker candidates for AM misuse detection in farm animals. Further validation on larger sample size and a wider spectrum of AMs are needed to confirm the viability of the aforementioned biomarkers in poultry population.

Implications

In the present study, we used for the first time the brand new technique of whole transcriptome sequencing to analyze liver and muscle transcriptomic profiles in a quite large population of broilers reared without treatment or treated with different classes of antimicrobials. Moreover, we validated the most differentially expressed genes among the treated groups. The entire study was aimed to detect novel biomarker candidates, able to identify antimicrobial misuse in broilers, and to discriminate broilers treated with antimicrobials compared to broilers reared without treatments, enhancing the efficiency and success rate of food screening and safety programs established by national authorities.

Introduction

Over the last half-century, antimicrobials (**AMs**) have been primarily used in poultry industry for therapeutic and prophylactic purposes. In addition, the ability of these molecules to improve growth and feed efficiency was largely exploited in food animals, leading to a widespread administration of feed supplements (Van Boeckel et al., 2015). One of the undesirable consequences of AMs overuse is the presence of their residues in edible tissues, causing health risks for consumers, including AM resistance and hypersensitivity reactions (Ghorbani et al., 2016). Therefore, inappropriate AMs usage in animal production as growth promoters was banned in 2006 by the European Union (**EU**). Consequently, other extra-EU countries (United States, Canada, and Japan) established local guidelines and recommendations to reduce the use of AMs (Brown et al., 2017). So far, the prophylaxis and metaphylaxis treatments are still permitted in all large poultry-producing countries, even if discouraged, and coccidiostats are authorized as feed additives in poultry breeding.

To decrease AMs use in animals, a new legislation has been recently approved by the European Parliament to ban the prophylactic use of antibiotics in farming, which will come into force in 2022. In addition, consumers are now demanding specific guarantees for the consumption of meat products with low or absent AMs residues and are also ready to pay extra costs for this objective. This dramatic shift has created strong

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https://doi.org/10.1016/j.animal.2020.100113

market pressure and significant changes have been recorded in poultry industry. The major restaurant chains and large-scale organized distribution have recently committed to market only chicken meat raised without the use of AMs.

Even if the use of AMs for therapeutic purposes is not prohibited in Europe, the Regulation (EU) 37/2010 has established the maximum residues limits in foodstuffs derived from animals to limit AMs residues risk for the consumers. Moreover, a withdrawal period after AMs administration is required before broilers are processed for human consumption. It is therefore imperative that animal products are analytically screened to ensure that residues do not exceed maximum residues limits. In recent years, LC coupled with triple quadrupole MS (**LC–MS/MS)** was the most extensively applied techniques for the determination of AMs residues in various food and feed samples, due to its versatility, specificity, and selectivity (Chiesa et al., 2018; Wang et al., 2019). These methods are highly sensitive but time-consuming and expensive. Additionally, identification of indirect tissue biomarkers rather than drug residues would be more appropriate, when tissue alterations are more persistent than the AMs administered or when they are common to a whole drug family or when the parent drugs and direct metabolites or complex food matrices (i.e. high fat, oil, protein, or sugar content) cannot be easily analyzed with LC–MS/MS (Chen et al., 2019). Indirect methods are focused on the identification of the biological effects of drugs or illegal compounds in the target organs, independently from the substance and the administration route. This approach has been successfully applied for many years as an alternative to chemical analysis to detect illegal treatments with growth promoters in cattle (Kinkead et al., 2015; Starvaggi Cucuzza et al., 2017). In this context, the application of next-generation sequencing technologies to indirectly detect administration of AMs in target tissues could enhance the efficiency and success rate of food screening and safety programs established by national authorities. Decreasing costs of next-generation sequencing technology and the development of bioinformatics pipelines have rendered RNA-seq an affordable and user-friendly technique for whole gene expression profiling in all the species (Wang et al., 2009). In food control, the application of RNA-seq enables to quickly obtain information on the biological processes and pathways altered by the different classes of AMs and it facilitates the identification of tissue biomarkers revealing the treatment (Stark et al., 2019).

Therefore, the aims of this study were: first, we evaluated liver and *pectoralis major* (**PM**) muscle transcriptomic profiles by RNA-seq in broilers reared without any treatment or treated with different classes of AMs and sampled at the end of the specific withdrawal period. Second, we validated the most differentially expressed genes among the treatment groups, to identify possible novel biomarkers able to discriminate meat from broilers raised without AMs and meat from broilers administered with AMs undergone to the legal withdrawal period.

Material and methods

Study set-up and sample collection

A total of 240 male broiler chickens (Ross 308) reared in identical conditions in the chicken broiler farm facility of the Department of Veterinary Sciences of the University of Turin were included in the study. Birds were obtained from a local hatchery, already vaccinated *in ovo* against Gumboro and Marek diseases. Additionally, chicks were vaccinated against coccidiosis (coarse spray; Hypracox, Amer, Spain), Newcastle and infectious bronchitis (fine spray) at day 1 after hatching. Chicks were randomly allocated in seven pens. For each pen, a prophylactic program was applied during production cycles, as reported in Fig. 1: thiamphenicol (**THP**); thiamphenicol in association with diclazuril (THP + **DCZ**); amoxicillin (**AMX**); AMX in association with DCZ (AMX + DCZ); sulfadiazine + trimethoprim (**TRIM**); and DCZ. Untreated animals were used as a control group (K). Each pen was equipped with a bucket-type feeder and drinker with fresh wood shavings as litter. The chicks had *ad libitum* access to water and feed from placement (day 0) until the end of the study (day 58). The environmental conditions (lighting program, temperature, relative humidity, and ventilation rates) were controlled accordingly to the Ross broiler management guidelines. The birds received the following feeding program to meet the standard nutritional requirements: a commercial starter diet (230 g/kg of CP) from day 0 to 23 (starter period) and a commercial grower diet (185 g/kg of CP) from day 24 to 58 (finisher period).

Animals in DCZ, THP + DCZ, and AMX + DCZ groups received a diet supplemented with coccidiostat (i.e. DCZ). Supplementary Table S1 reports prophylactic protocols.

Animals were regularly slaughtered at the end of the rearing cycle and samples of PM muscle and liver of each broiler were immediately collected from 120 animals. Samples were frozen and stored at −80 °C for molecular analysis. A complete necropsy of the animals was performed after sampling.

Growth performances

The BW of each bird was individually recorded with a precision balance at day 3 and 23 (starter period), and at day 28 and 56 (finisher period). The mortality was daily recorded. The daily BW gain (**BWG**) was determined for each group.

Histology

Samples of liver were fixed in 10% buffered formalin (pH 7.0), paraffin-embedded, and sections were stained with hematoxylin and eosin. Stained sections were examined, and alterations were scored following the system reported in Supplementary Table S2.

RNA isolation, library preparation, and sequencing

A total of 56 PM muscle and 64 liver tissue samples, randomly selected from each group**,** were processed for RNA extraction and sequencing. Total RNA was isolated from the samples using the TRIzol reagent (Ambion, Life Technologies, Carlsbad, CA, USA) and RNase free DNase kit for DNA digestion (Qiagen KIT Cat No./ID: 79254) according to the manufacturer's instructions. Ribonucleic acid concentration and integrity were measured with a NanoDrop ND-1000 spectrophotometer and assessed through the Bioanalyzer 2 100 instrument (Agilent Technologies, Santa Clara, CA, USA). Only RNA samples with a RIN > 8 were used for sequencing analysis. A total of 120 non-normalized libraries for RNA sequencing experiments were prepared using NEBNext® Ultra™ II Directional RNA Library Prep with Sample Purification Beads and NEBNext® Poly(A) mRNA Magnetic Isolation Module (New England Biolabs) and a single-end sequencing (60 SE) was carried out on an Illumina HiSeq4000 (Illumina Inc., San Diego, CA, USA). Raw Illumina reads were deposited in the NCBI SRA repository (https://www.ncbi.nlm.nih.gov/sra/) under the accession number PRJNA592039.

RNA sequencing data processing

The quality of all raw Illumina reads was checked using FastQC v.0.11.5 software (https://www.bioinformatics.babraham.ac.uk/projects/download.html). Clean reads were obtained by removing low-quality reads and/or trimming the adaptor sequences. After quality assessment, RNA-seq processed reads were mapped to the GRCg6a genome assembly (Genome Reference Consortium; released April 2018; downloaded from the Ensembl Genome Browser site) using STAR (Dobin et al., 2013) software tool. Post-alignment quality parameters of RNA-seq (insert length, gene mapping bias, and RNA junctions) were evaluated using RSeQC (Wang et al., 2012) in standard mode. Next, the counts of aligned reads per gene were obtained using htseq-count from the HTSeq (Anders et al., 2015) software package in single-stranded mode, with chicken gene annotations from Ensembl

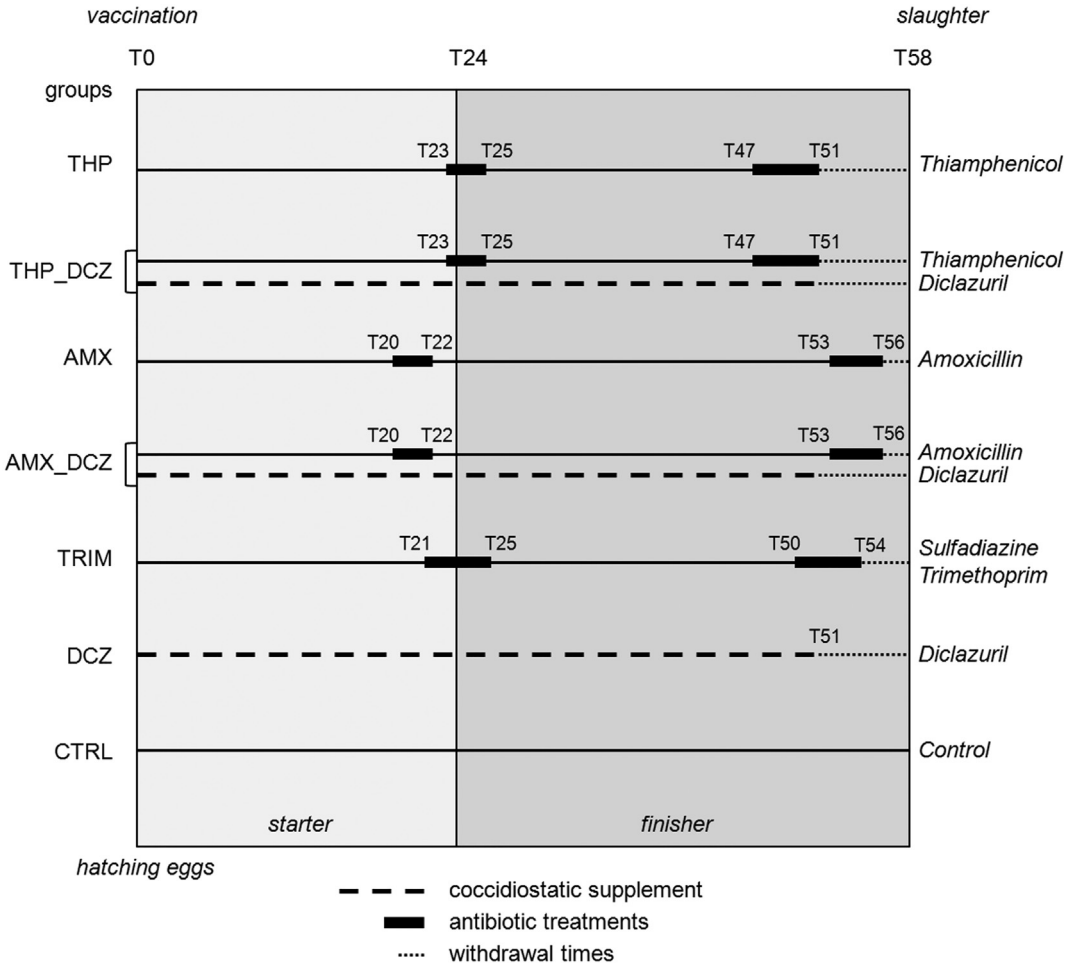


Fig. 1. Prophylactic treatment schedule. Chicks received a starter diet from 0 (T0) to 23 days (T23) and a grower diet from 24 to 58 days (T58). Animals in thiamphenicol + diclazuril (THP + DCZ), amoxicillin (AMX) + DCZ, and DCZ groups received a diet with a coccidiostatic supplement. Animals were vaccinated against coccidiosis, Newcastle, and infectious bronchitis at day 1 after hatching (T0) and they were slaughtered at T58. For each group, two antibiotic treatments were performed and the prescribed withdrawal times were respected.

Release 96. Only reads that were uniquely aligned were retained. The code is available in Supplementary Material S1.

Gene expression evaluation and differential expression analysis

Counts filtering, normalization, and differential expression analysis were performed using EDAseq (Risso et al., 2011) and EdgeR with GLM approach (Robinson et al., 2010) R package. To explore sample stratifications in the dataset according to the expression values of expression-filtered genes, principal component analysis, unsupervised clustering using k-means clustering, and supervised clustering using the 95th percentile highly variable expressed genes were applied. To evaluate how antibiotics, coccidostat, and their association affect gene expression, differential expression analysis was performed comparing each of the six groups of treatment (THP,THP + DCZ, AMX, AMX + DCZ, TRIM, and DCZ) to the control group (K) both in liver and muscle tissues. Deregulated expression of genes was considered as significant when $P < 0.05$ (false discovery rate (**FDR**) corrected) and log-2 fold change (logFC) > 1 or <−1. Modulated genes were annotated through BioMart (http://www.ensembl.org/biomart/martview/).

Functional analyses

The functional analyses were performed using the Kyoto Encyclopaedia of Genes and Genomes pathways and the gene ontology terms. To detect functional pathways with mean or variance significantly altered, and to evaluate which portions of the pathways (signal paths) are expected to be affected, we analyzed the expression values with Clipper (Martini et al., 2013; Ihnatova et al., 2018). To identify functional categories related to differentially expressed genes, a gene set overrepresentation and enrichment analysis was performed using PANTHER (Thomas et al., 2006) v.14.1 with a significant threshold of $P < 0.05$ (FDR corrected).

Selection and analysis of quantitative PCR reference genes

Genes from different functional classes were considered as reference genes (**RGs**) to minimize a potential co-regulation of genes. The list of RGs and information about each gene are listed in Supplementary Table S3. The expression stability (M) of the RGs and their optimal number (V) were determined using the geNorm algorithm (Vandesompele et al., 2002). Normalization by multiple RGs was applied for an accurate measure of expression levels (Vandesompele et al., 2002; Hellemans et al., 2007).

Validation of candidate genes

Inclusion criteria for candidate genes were logFC and FDR < 0.05, and quantitative real-time PCR (qPCR) was used for validation. For each treatment samples from three chickens previously analyzed with RNA-seq and three additional chickens reared in the same condition were randomly selected and analyzed. The QuantiTect Reverse

Transcription Kit (Qiagen) was used to synthesize the cDNA from 1 μg of total RNA. Primer sequences of the candidate genes were designed using Primer-BLAST (Ye et al., 2012) on the corresponding reference sequences. The expression levels of the candidate genes were analyzed on a Bio-Rad CFX Connect™ qPCR detection system using iTaq™ Universal SYBR®Green Supermix (Bio-Rad). The candidate genes selected in liver and PM muscle are listed in Supplementary Table S4.

Statistical analyses

All analyses were carried out with R v.3.5.0 software and GraphPad Prism v.6 (GraphPad Software, CA, USA). The Kolmogorov–Smirnov test was conducted to verify population normality. Grubb's test ($\alpha = 0.05\%$) was used to determine and exclude potential outliers. Body weight gain of each animal groups in starter and finisher period, were compared to control group using Kruskal–Wallis test. To compare lesion scores among the treatment groups and among inflammation sites (parenchymal, perivascular, and periportal) Kruskal–Wallis test or Friedman test were applied, followed by Dunn's post test. Association between different variables was ascertained by means of Chi-squared test. In the qPCR experiments, the results were stated as normalized expression, and one-way ANOVA or Kruskal–Wallis test were applied. Correlation analysis (Pearson/Spearman r) between the values of logFC obtained from RNA-seq and qPCR experiments was performed. Statistical significance was accepted at $P < 0.05$ level and P values for multiple comparisons were adjusted using the Benjamini–Hochberg correction.

Results

Growth performances

Body weight gain of each treated group compared to control group of animals during starter and finisher period are shown in Supplementary Table S5. In the starter period, the BWG of all treatments was significantly higher than the control's one; in the finisher period only the BWG of THP group was significantly higher ($P < 0.01$), while for TRIM group BWG was significantly lower ($P < 0.05$) compared to control group's one.

Histology

Histological examination of liver showed a variable degree (0–3) of lymphocytic or mixed inflammatory infiltration among groups, but no differences were identified regarding localizations.

Interestingly, lymphocytic aggregates composed of small lymphoid cells surrounded by a non-completed thin capsule were noted in 68 (56.2%) chickens. Localization was more frequent in the parenchyma (43.8%), compared to periportal (18.5%) and perivasal (7.4%) regions ($P < 0.0001$). Furthermore, the parenchymal lymphocytic aggregates were significantly associated ($P < 0.01$) to THP + DCZ and AMX + DCZ groups. Hydropic degeneration was also observed, but no significant differences among groups were recorded.

Bioinformatic analysis results

RNA-seq experiment in liver and PM muscle tissues produced an average of over 20 million reads per sample. Quality control and trimming procedures retained the vast majority of the sequences obtained (over 98% of the total, on average) and unique alignment was successful for 87% of the cleaned reads (Supplementary Table S6).

Differential expression and functional analysis in liver

The list of the differentially expressed genes (**DEGs**) for all the five comparisons is reported in Supplementary Table S7, and Fig. 2 shows the distribution of gene expression logFCs and *P*-values. When considering each comparison, a higher number of genes was differently regulated in liver compared to PM muscle, supporting the hypothesis that gene expression in the liver was more altered by treatments (Fig. 3).

The supervised clustering analysis with the 500 highly variable genes clearly separated treatment groups from controls in liver. When considering the three antibiotic treatments, gene signatures by gene set enrichment analysis were similar, conversely, the two AM associations showed a definite pattern of expression, enabling a clear clustering of two groups (Supplementary Figure S1). When compared to controls, the majority of DEGs were upregulated in THP and THP + DCZ groups (80 and 86%, respectively), whereas the majority of DEGs were downregulated (89%) in TRIM group. A total of 220 genes were shared by the three groups. These included *PDK4*, *IGFBP1*, *RHOB*, *PANX3,* and *ASB12,* involved in cell development, metabolism, and signaling, cellular response to molecule of bacterial origin, inflammatory response, and positive immune system regulation. Moreover, when taken together, gene expression profiles of the three antibiotic treatments revealed enrichment of apoptosis and immune system response (i.e. complement and coagulation cascade) pathways.

The vast majority of DEGs were upregulated in THP + DCZ and AMX + DCZ groups (81 and 67%, respectively) compared to controls. Genes regulating lipid metabolic process were enriched in THP + DCZ group, including *FASN*, *FMC1*, *EEF1A2*, *ME1*, and *CPT1A*. Differently, DEGs in AMX + DCZ group showed involvement of chemokine-mediated signaling pathways, cellular protein metabolic processes, innate immune, and inflammatory response, including *CSF1R*, *CCR2*, *RAP2B*, and *IL2RG*. Taken the data together, broilers receiving the AM associations showed an altered expression of genes involved in the metabolism of glycine, serine, and threonine, vitamins and carbohydrate (i.e. porphyrin and propanoate), and insulin signaling pathway.

Differentially expressed genes analysis and functional analysis in muscle

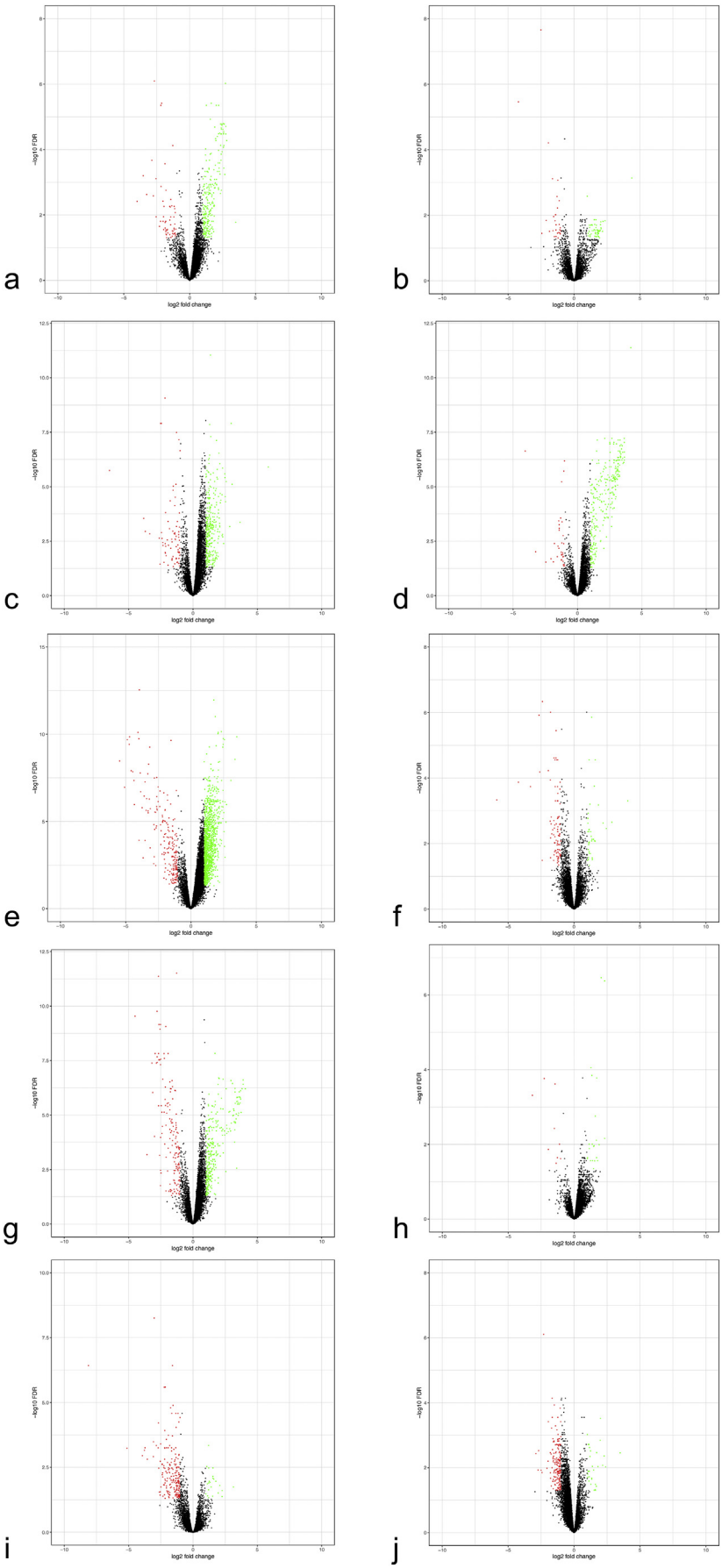
Similarly to liver, the supervised hierarchical cluster analysis using the 500 highly variable genes analysis in PM muscle showed a clear distinction between AM treatments and controls, but treatment groups were more homogenous. Differentially expressedgenes analysis highlighted a majority of upregulated genes (ranging from 67 to 86%), in all the treated animals, except for AMX and TRIM groups. Differentially expressed genes included genes related to lipid metabolism in the groups treated only with antibiotics (THP, AMX, and TRIM), and immune system in the groups treated with an antibiotic and coccidiostat association (THP + DCZ and AMX + DCZ). Functional analysis reflected these results, highlighting enrichment of lipid metabolism pathways (i.e. fatty acid elongation), in THP, AMX, and TRIM groups, whereas THP + DCZ and AMX + DCZ groups showed enrichment of pathways acting in glycine, serine, and threonine metabolism, sterols and carotenoids metabolism (i.e. terpenoid backbone synthesis), and regulation of immune system (i.e. RIG-I-like receptor signaling pathway).

Finally, comparing broilers receiving only coccidiostat with controls, no separation was achieved by hierarchical cluster analysis, and only a few genes were differentially expressed in liver and muscle tissue (Supplementary Table S8).

Validation of differentially expressed genes by quantitative PCR

A total of 54 and 26 DEGs were in common in broilers treated with antibiotics in liver and in PM muscle, respectively. Broilers treated with antibiotics and coccidiostat association (THP + DCZ and AMX + DCZ) shared 24 and 2 DEGs in liver and in PM muscle, respectively.

To validate RNA-seq results, six genes in the liver (*GPR27*, *DIO2*, *RHOB*, *ACTG2*, *PDK4*, and IGFBP1) and six genes in PM muscle (*PDK4*, *SLC25A30*, *RRAD*, *SLC43A2*, *PDZRN3*, and *ELOVL1*) were selected for qPCR analysis (Supplementary Figures S2 and S3). According to geNorm validation, the optimal number of RGs was two, both in liver (V2/3 =


a
b
c
d
e
f
g
h
i
j
−log10 FDR
log2 fold change

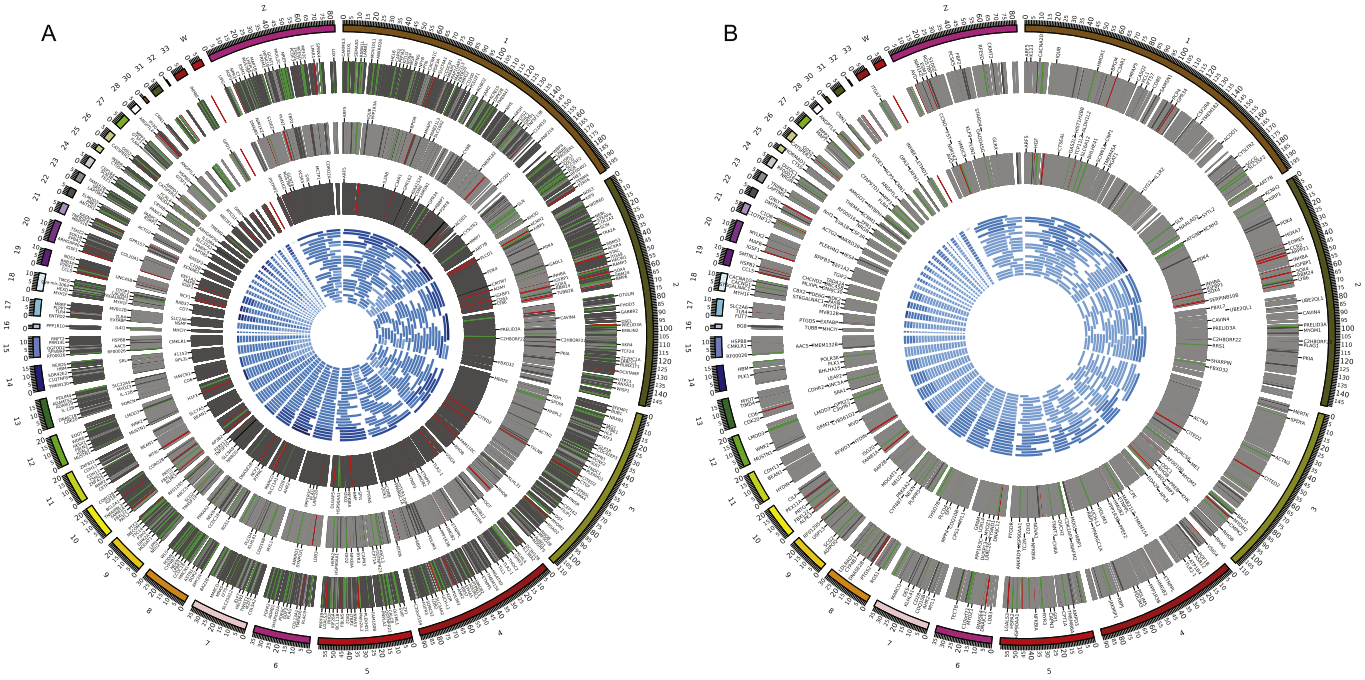


Fig. 3. Circos plots depicting significant differentially expressed genes in groups treated only with antibiotics (A) and with antibiotics with cocciodiostat (B) versus controls in the liver of broiler chickens. Chromosome numbers are identified in the outer-most ring. The inner-most ring shows as blue tiles the genome-wide distribution of differential expressed genes (DEGs). A: from outer to inner layer, third, fifth, and seventh circles represent as heatmap the genome-wide distribution of DEGs in thiamphenicol group (THP) versus controls, amoxicillin group (AMX) versus controls and sulfadiazine + trimethoprim group (TRIM) versus controls, respectively. Differential expressed genes are labeled in the second, fourth, and sixth circles, respectively. B: from outer to inner layer, third, and fifth circles represent as heatmap the genome-wide distribution of differentially expressed genes in THP + Diclazuril group (THP + DCZ) versus controls and AMX + DCZ group versus controls, respectively. Differential expressed genes are labeled in the second and fourth circles, respectively. Green lines represent up-regulated genes and red lines represent the downregulated ones. Gray lines represent genes with no differential expression.

0.059) and PM muscle (V2/3 = 0.058). Therefore, candidate RGs with a lower geNorm M value (ranking of candidate genes stability) were used, namely *HMBS* and *TBP* in liver and *HPRT1* and *HMBS* in PM muscle. They were employed for the multireference gene normalization.

In liver, a statistically significant overexpression of *GPR27* was observed in THP + DCZ (2-fold; $P < 0.01$) and AMX + DCZ (2.9-fold; $P < 0.0001$) groups compared to controls (1.8-fold). The *PDK4* expression in liver was significantly reduced by 12.5-fold ($P < 0.01$) in AMX + DCZ group, compared to control group. Furthermore, *PDK4* gene downregulation was also detected in liver of THP (12.5-fold; $P < 0.01$) and TRIM (11-fold; $P < 0.05$) groups. In PM muscle, *ELOVL1* was altered by the antibiotic treatments and in particular it was downregulated by about 6.8, 6.7, 9.1, and 8.3-fold in DCZ ($P < 0.05$), THP + DCZ ($P < 0.05$), AMX + DCZ ($P < 0.01$), and TRIM ($P < 0.01$) groups, respectively.

Finally, correlation analysis demonstrated that the values of logFC obtained from RNA-seq and qPCR were significantly correlated. Spearman's r was 0.6627 ($P < 0.0001$) in liver and Pearson's r was 0.8965 ($P < 0.0001$) in PM muscle.

Discussion

Antimicrobial resistance is one of the major emergencies of the century and the most recent studies have revealed that over 33 000 people in the EU die every year due to infections related to antibiotic-resistant bacteria. The economic impact is constantly increasing, and AM resistance costs are estimated around 1.5 billion euros per year in healthcare expenses and productivity losses. Efforts to reduce AM resistance have steadily grown over the last two decades, and European poultry producers are aiming for a sustainable reduction of antibiotic use. Preventing AM resistance requires new diagnostic tools and the use of sequencing methods in the fields of food quality and food safety is strongly encouraged by the scientific community.

During the breeding starter period, antibiotics supplement, alone or in association with coccidiostat, increased the weight of treated chickens, compared to the untreated ones. In this phase, our data showed a powerful effect on performances in animals treated with the association of antibiotics and coccidiostat. Differently, during the finisher period, only THP group showed a significant increase in BWG compared to the control group, whereas TRIM group exhibited a significant decrease in BWG. It is known that the taxonomic composition of the microbiota is affected by different factors, such as age, diet, and the use of AMs, especially during the first 2 weeks of life. Even if the mechanism remains unclear, antibiotics are likely to act by remodeling microbial diversity and relative abundance in the intestine, providing an optimal microbiota for growth (Dibner and Richards, 2005).

We applied whole transcriptomic analysis to investigate genes and pathways affected by AM treatments and to detect potential novel biomarkers. Our findings highlight that liver is a valid target organ for the detection of indirect biomarkers of AM treatments. This was quite expected, since liver catabolizes most biochemical burdens directly or indirectly

Fig. 2. Volcano plots of distribution trends for differentially expressed genes in antimicrobials-treated groups versus controls in liver (a, c, e, g, and i) and pectoralis major muscle (b, d, f, h, and j) of broiler chickens. The log-2 fold-change (logFC) indicates the mean expression level for each gene and is represented on the x-axis. The y-axis shows the -log10 of the *P*-value with a false discovery rate (FDR) correction. Each dot represents one gene. Green dots represent up-regulated genes (FDR < 0.05 and logFC > 1) and red dots represent downregulated genes (FDR < 0.05 and logFC < −1). Black dots represent genes with no differential expression. a and b: thiamphenicol group (THP) versus controls; c and d: THP + diclazuril group (THP + DCZ) versus controls; e and f: amoxicillin group (AMX) versus controls; g and h: AMX + DCZ group versus controls; i and j: sulfadiazine + trimethoprim group (TRIM) versus controls.

produced by environmental chemicals, diets, and drugs, including AMs. Furthermore, liver represents one of the most important immune relevant organs in mammals and avian species (Zaefarian et al., 2019).

In animals treated only with antibiotics, a total of 220 differently regulated genes, including *PDK4*, *IGFBP1*, *RHOB*, *PANX3*, and *ASB12,* were common in liver. Functional analysis revealed an enrichment of signatures involved in glucose and lipid metabolism, cellular response to molecule of bacterial origin, inflammatory response, and positive immune system regulation. This is in line with the antibiotics ability to influence the microbiota composition and the expression of microbial genes, including their derived metabolites, such as bile acid products, short-chain fatty acids, methylamines, and indoles. Moreover, the modification of gut microbiota composition alters liver metabolism and rearranges immune defenses, leading, in some cases, to detrimental effects on health. Our results strengthen the hypothesis that liver disorders in glucose and lipid metabolism regulation and alterations of immune response might be related to antibiotic treatments and gut dysbiosis (Iacob and Iacob, 2019). Indeed, liver is essential for the regulation of immune defense during systemic infections, through mechanisms such as bacterial clearance, acute-phase protein or cytokine production, and metabolic adaptation to inflammation (Zaefarian et al., 2019).

Animals treated with antibiotics in association with coccidiostat (THP + DCZ and AMX + DCZ) revealed an altered expression of genes involved in the metabolism of serine and threonine, carbohydrate, and insulin signaling pathway. Interestingly, these pathways have been shown to be differentially expressed in recent transcriptomic analyses on liver of chickens under stressing conditions (e.g. temperature) (Coble et al., 2014; Lan et al., 2016). Previous studies showed that the insulin signaling pathway in the liver is altered by antibiotic treatments in healthy mice (Rodrigues et al., 2017), but the effects of DCZ are unknown. Indeed, the mechanism of action of DCZ is not yet fully known (Noack et al., 2019). Recently, DCZ was shown to downregulate mRNA expression of the serine/threonine protein phosphatase type 5 (**PP**5) in *Eimeria tenella* (Zhou et al., 2013). In eukaryotic organisms, protein phosphatase type 5 has complex regulatory functions and its major role is to regulate phosphorylation of signaling molecules, especially nuclear receptors, involved in carbohydrate and lipid metabolism and cellular response to stress. Moreover, since many pathways affected by PP5 are comparable to our results, we hypothesize here a synergic effect of the coccidiostats when administered with AMs compared to the antibiotic alone.

A subset of candidate genes affected by AM treatments in liver was identified. Among the genes with an altered expression, we focused on those closely related to lipid and glucose metabolism. To validate RNA-seq results, *GPR27*, *DIO2*, *RHOB*, *ACTG2*, *PDK4*, and *IGFBP1* were selected for qPCR analysis. Three of them deserve particular attention for future investigations. In our study, *PDK4* was downregulated in all the treated animals compared to the control group. The *PDK4* deficiency has been described to dramatically reduce the expression of genes related to fatty acid uptake, synthesis, and gluconeogenesis in mice (Zhang et al., 2018). Likewise, *IGFBP1* gene resulted downregulated in all the treated animals compared to controls. The liver is the major source of IGFBP-1 protein and insulin is the central regulator of its synthesis (Brismar et al., 1994). The IGFBP-1 is an important determinant of IGF activity, but it also enhances glucose uptake in peripheral tissues and it reduces glucose output in liver. Moreover, it plays a role in lipid metabolism. Finally, *RHOB* was downregulated in all the treated animals. As a member of the Rho family, *RHOB* participates in the modulation of numerous essential cellular processes, including actin organization, gene transcription, cell adhesion, proliferation, apoptosis, and vesicle traffic.

Results obtained in the muscle are less consistent. The number of DEGs was lower and the affected biological processes were less specific compared to liver, suggesting that AM effects did not remarkably influence the muscular transcriptome. Indeed, DEGs were enriched of genes related to main metabolic processes of the organism, such as biosynthesis of amino acids, gluconeogenesis and glycolysis, fatty acid metabolism, and immune system. These pathways have been commonly observed in transcriptome analyses of non-treated broilers (Zhang et al., 2017; Liu et al., 2020). Anyhow, six candidate genes were selected in the muscle for validation manually reviewing genes with activities directly related to AMs. Among them, *ELOVL1* was the most intriguing. This gene is involved in the production of C20 to C28 very long-chain fatty acids, whose regulation is directly implicated in meat quality (Lopez-Ferrer et al., 2001). Indeed, poultry has a favorable balance between polyunsaturated and saturated fatty acids, enabling chicken meat to be optimally incorporated into almost all consumer diets (Marangoni et al., 2015). The qPCR showed significant downregulation in all treated animals, imposing a focus on potential detrimental effects of AM treatments in chicken meat. This study demonstrates that RNA-seq is a powerful method to screen aberrantly regulated genes in liver and optimal to identify potential biomarkers, discriminating AMs misuse in farm animals. Nevertheless, diverse factors (e.g. feed changes and stress conditions) might alter the same biological pathways resulting in similar differential expressed genes. Therefore, the promising new biomarkers need to be validated in a larger sample size and in individuals treated with other AMs frequently used in poultry farm.

Supplementary materials

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2020.100113.

Ethics approval

All applicable international, national, and/or institutional guidelines for care and use of animals were followed.

Data and model availability statement

Raw Illumina reads of RNA-seq were deposited in the NCBI SRA repository (www.ncbi.nlm.nih.gov/sra) under the accession number PRJNA592039.

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Diana Giannuzzi: Data creation, methodology, software, formal analysis, writing – original draft, visualization. Bartolomeo Biolatti: Conceptualization, resources, writing – review & editing, supervision. Erica Longato: Investigation, writing – original draft. Sara Divari: Data curation, validation, formal analysis, investigation, writing – original draft, visualization. Laura Starvaggi Cucuzza: Validation, investigation. Paola Pregel: Validation, formal analysis, writing – original draft. Frine Eleonora Scaglione: Investigation, writing – original draft. Andrea Rinaldi: Investigation. Luca Maria Chiesa: Investigation. Francesca Tiziana Cannizzo: Conceptualization, resources, writing – review & editing, supervision, project administration, funding acquisition.

Declaration of interest

None.

Acknowledgements

None.

Financial support statement

This study was supported by Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR) under the program "Dipartimenti di Eccellenza ex L.232/2016" to the Department of Veterinary Science, University of Turin. The funders had no role in study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

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