University of Turin

Department of Veterinary Sciences

Research Doctorate in Veterinary Sciences for Animal Health and Food Safety

Cycle: XXXV

Application of Next-Generation Sequencing (NGS) as a new approach to study antimicrobial effects in farm animals

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Academic years: 2019-2022

SSD: VET 03

Table of contents

Summary

(English version)

Antimicrobials (AMs) are widely use to prevent and treat infectious diseases. The new European regulatory scenario asks for a substantial reduction of AMs use in order to control antimicrobial resistance (AMR) spreading. Therefore, this dissertation aimed to investigate poultry and dairy cows farming systems applying a different Next-generation sequencing (NGS) approach. In poultry farming, ensuring gut health and efficient growth performance has become one of the biggest challenges. Investigating the AMs effects on gut microbiota and host intestinal barrier allows the identification of the mechanisms underlying the intricate relationship between host, microbiota and AMs. The introduction of new limitations in AMs use requires the implementation and the improvement of the diagnostic tools, available for AMs identification in food matrixes. In this context, this thesis firstly investigated the role of AMs at gut barrier level using 16S metabarcoding, associated with histopathology, IHC and gene expression techniques. The results of this research suggest an influence of AMs on the gut microbiota, characterised by dysbiosis. Moreover, the identification of intestinal microscopic lesions and their association with microbiota alterations revealed a negative effect of AMs on the gut barrier of treated broilers. On the other hand, in dairy cows farming there is an urgent need to support new studies with the aim of identifying new biomarkers for an early diagnosis of mastitis. An early identification of subclinical forms will allow the application of preventive pharmacological treatments and efficient biosecurity measures. In this study, the role of miRNAs vehiculated by EVs has been investigated in the modulation of mastitis and in their application as potential early predictors of subclinical mastitis. A panel of 4 main miRNAs has been proposed. According to the functional analysis, their function is mainly related to immune and inflammatory functions. In conclusion, it is possible to confirm that NGS technology has been a useful choice in the investigations of the relationship between host, microbiota and drugs. NGS application can be a powerful tool to find new solutions and support the new requirements derived from the veterinary scenario related to AMR and AMs use.

Summary

(Italian version)

Gli antimicrobici (AM) sono ampiamente utilizzati per prevenire e curare le malattie infettive. Il nuovo scenario normativo europeo richiede una sostanziale riduzione dell'uso di AM per controllare la diffusione della resistenza agli antimicrobici (AMR). Pertanto, questa tesi aveva come obiettivo lo studio i sistemi di allevamento del pollo da carne e vacche da latte applicando un diverso approccio di Next-generation sequencing (NGS). Nell'allevamento del pollo da carne, garantire la salute dell'intestino ed efficienti tassi di crescita è diventata una delle maggiori sfide. Lo studio degli effetti degli AM sul microbiota intestinale e sulla barriera intestinale dell'ospite può consentire l'identificazione dei meccanismi alla base dell'intricata relazione tra ospite, microbiota e AM. L'introduzione di limitazioni nell'uso degli AM richiede l'implementazione e il miglioramento degli strumenti diagnostici disponibili per l'identificazione degli AM nelle matrici alimentari. Con la presente tesi si sono indagati in primo luogo il ruolo degli AM a livello della barriera intestinale utilizzando il 16S metabarcoding, associato a tecniche di istopatologia, IHC e di espressione genica. I risultati suggeriscono un'influenza degli AM sul microbiota intestinale, caratterizzato da disbiosi. Inoltre, l'identificazione di lesioni microscopiche intestinali e la loro associazione con alterazioni del microbiota ha rivelato un effetto negativo degli AM sulla barriera intestinale dei broiler trattati. Dall'altro lato, nell'allevamento delle vacche da latte è urgente condurre nuovi studi con l'obiettivo di identificare nuovi biomarcatori per una diagnosi precoce di mastite. Una precoce identificazione delle forme subcliniche consentirà l'applicazione di trattamenti farmacologici preventivi e di efficaci misure di biosicurezza. In questo studio, il ruolo dei miRNA veicolati dalle vescicole extracellulari è stato studiato nella modulazione della mastite e nella loro applicazione come potenziali marcatori precoci di mastite subclinica. È stato proposto un pannello di 4 miRNA principali. Secondo l'analisi funzionale condotta, la loro attività è correlata ai processi immunitari e infiammatori. In conclusione, è possibile confermare che la tecnologia NGS è stata una scelta utile nelle indagini relative al rapporto tra ospite, microbiota e farmaci. L'applicazione della tecnologia NGS può essere un potente strumento per trovare nuove soluzioni e supportare i requisiti derivati dallo scenario veterinario relativo all'AMR e all'uso di AM.

CHAPTER 1: General introduction

1.1 Antimicrobials and antimicrobial resistance

In 1928, Alexander Fleming discovered the antibacterial activity of penicillin starting one of the biggest revolutions in science. Antimicrobials (AMs) have been largely used to treat and prevent infectious diseases in human and veterinary medicine. However, a few years later after Fleming's discovery the first resistance phenomenon to penicillin has been observed in *Escherichia coli* strains (Lobanovska & Pilla, 2017). During the 21st century, several AMs molecules have been discovered and commercialized, but the time elapsed between the release on the market of a new AM and its first resistance appearance has become even shorter (Iskandar et al., 2022). Nowadays, antimicrobial resistance (AMR) is defined by the World Health Organization (WHO) as the phenomenon occurring when microorganisms acquire the ability to survive and grow even with an administered antimicrobial (AM), whose presence is enough to inhibit or kill other microorganisms of the same species (Loo et al., 2020). As highlighted by the WHO, AMR is a global emergency reducing the possibility of efficient treatments for many infectious diseases and increasing the risk of death (WHO, 2018). In addition, the development of new antimicrobial molecules is not going as fast as AMR spreading (Mohr, 2016). For these reasons, AM use needs to be limited and new guidelines for a more responsible use must be adopted both in human and veterinary medicine. Several actions can be adopted for more prudent AM use, for instance avoiding AM abuse and misuse, applying preventive measures such as biosecurity and vaccines, or improving hygiene and management conditions (Guardabassi et al., 2018). In this context, food-producing animals have been some of the main players in AMR phenomenon (Schwarz et al., 2001). In the past, excessive AMs use in animals was considered one of the main causes of AMR spreading also in human medicine. However, since the advent of the new millennium new regulation and restrictions have gradually mitigated and reduced AM use in food-producing animals resizing their role in AMR spreading (Nicola et al., 2021). In this new scenario, AMR is more appropriately considered as a One Health issue affecting humans, animals, and the environment (Fig. 1). A causal relationship has been demonstrated between the increased use of antimicrobials in both human and veterinary medicine, the greater movement of people, as well as domestic and wild animals, the increasing industrialization and the increased prevalence of antimicrobial-resistant bacteria (Cantas et al., 2013).

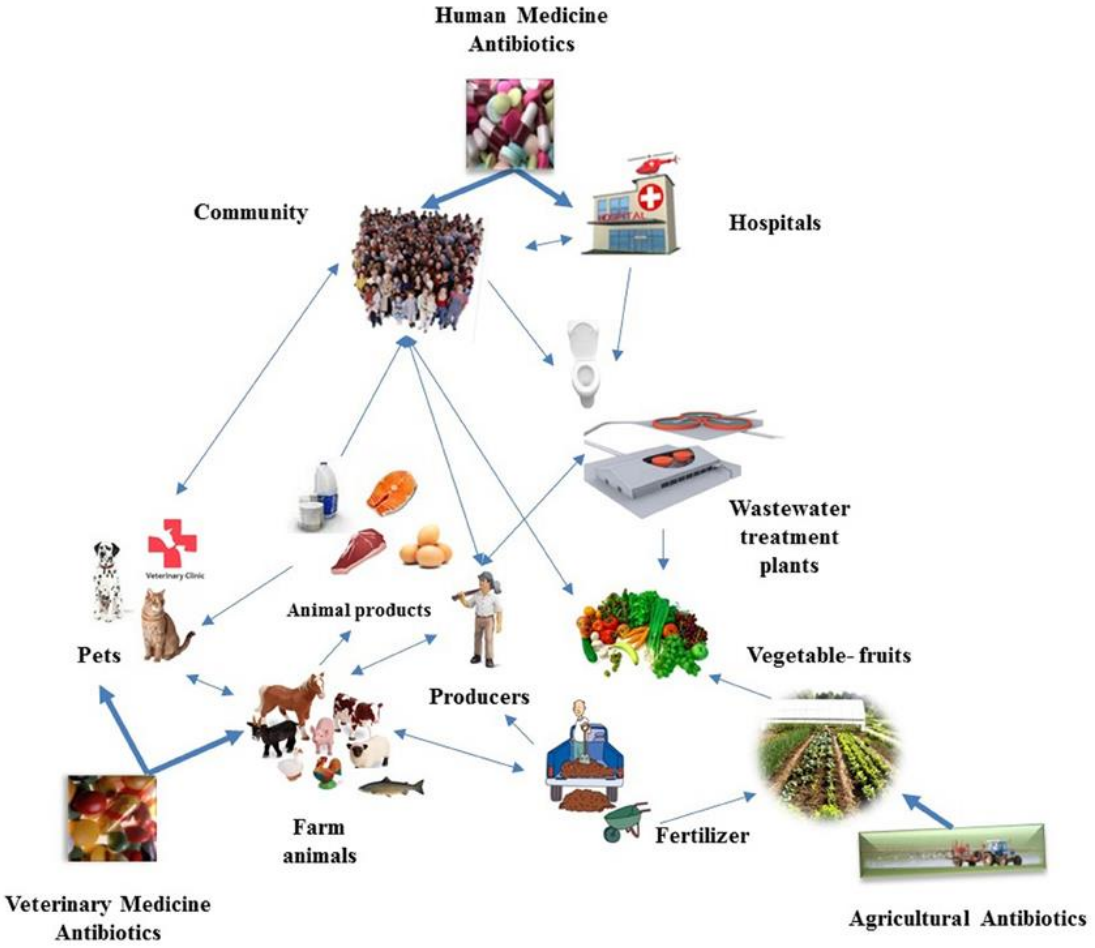

Figure 1. One Health approach for AMR spreading. Many different characters play a role in the arousal and dissemination of AMR. AMs use in human, veterinary medicine and agriculture can be identified as the main drivers of the phenomenon. Consequentely, soil, water and air are involved in AMR spreading and maintenance. Cited by (Cantas et al., 2013)

1.1.1 European legislation

In recent years, the European Union (EU) issued a series of directives and regulations:

- EU Regulation n. 1831/2003 has banned the use of AM as growth promoters in food-producing animals. In this way, it was anymore possible to treat animals with low doses of AM during the whole production cycle. This administration modality is still adopted in some Asian or American countries to improve growth performance.
- EU Regulation n. 6/2019 is the current regulation of veterinary medical products. It repealed the previous EU Directive n. 82/2001. One of the main differences with the past directive is the strict limitation of AM prophylaxes and metaphylaxes. Prophylactical use is defined as a preventive AM administration before the onset of any clinical signs to avoid

infectious diseases. Instead, metaphylaxis is the preventive AM administration in a group of animals, following the diagnosis of an infectious disease in a part of the group, to treat the sick animals and control the diffusion of the disease within the farm. At present, these types of AM administrations are strictly limited to exceptional occasions, when there is a huge risk for food animals contracting an infectious disease, that is demonstrated to be circulating in the farm or the area surrounding. In addition, the farm veterinarian must always prepare appropriate documentation.

1.1.2 Antimicrobial use control

This new legislative scenario requires an affordable and efficient system for AM control in farm animals. Although growth promoter and prophylaxis use have drastically decreased, it is still of primary importance to apply control measures to minimise AMs abuse and misuse. Moreover, public consumers are always more likely to purchase and consume animal food products with low or absent AMs amount (Mehdi et al., 2018). This change in food purchasing habits has required farmers, veterinarians, and other workers in the food-production industry to pay higher attention to AMs and to ensure the fulfilment of the new consumers requests. Therefore, the EU Regulation n. 37/2010 has already established the maximum residues limits (MRL) for most AMs and other drugs in food-animal products. In addition, each molecule is also defined with different withdrawal periods according to the food product typology. To apply European dispositions, every year the "Piano Nazionale Residui (PNR)" is arranged by the Italian ministry of health. The PNR program has the main aim of controlling authorized pharmacologically active drugs, banned drugs and envirromental contaminats on animal products. Control measures are defined as follows: animal species and productive type to control, specific molecules to investigate, methods to apply and frequency of the investigations. In this context, liquid chromatography coupled with triple quadrupole mass spectrometry (LC-MS) is mainly treated in order to control drug residues (Chiesa et al., 2018). This technique is a direct method targeting drug molecules ensuring specificity and can be applied to different food and feedstuff. Even though, it is still time-consuming and expensive. Moreover, not all LC-MS platforms can recognize metabolized molecules. Alternatively, others direct techniques can be applied to detect AMs residues, such as ELISA assay, gel electrophoresis or biosensors (Mungroo et al, 2014). However, the application of indirect measures to detect AMs residues can be more appropriate as control measures (Chen et al., 2019). Thus, indirect techniques can be more versatile than LC-MS allowing the identification of the biological effects of drugs and AMs on tissue or organs (Giannuzzi et al., 2021). Moreover, a long-lasting sign of AMs use is easily recognized, even when the AMs residue is barely detectable (Giannuzzi et al., 2021). Some different techniques can be applied as indirect

screening methods: ELISA assay, metabolomics, proteomics and other -omics technologies (Munteanu et al. 2018; Brown et al 2022). In particular, the application of next-generation sequencing technology can be an affordable method to implement the use of indirect control measures for AMs use and to further reduce AMs abuse (Stark et al., 2019).

1.2 Sequencing technology

1.2.1 Sanger sequencing

In 1977, Sanger and colleagues developed an innovative technology for sequencing DNA (Yin et al., 2021). Today, this method is known as Sanger sequencing and is based on chain termination and fragmentation techniques (McCombie et al., 2019). Concisely, Sanger reaction mimics the DNA replication processes using a DNA polymerase, oligo primers and dideoxynucleotide analogues (McCombie et al., 2019). Over time, radiolabelled nucleotides were substituted by safer fluorescent dyes easily detectable by gel electrophoresis (McCombie et al., 2019). Until 2004, Sanger was the sole sequencing technique and it allowed the completion of the first human genome sequence (Thermes, 2014; McCombie et al., 2019). The high cost and low throughput of Sanger sequencing were not enough to accomplish the requests in the new genomic era (Yin et al., 2021). However, this sequencing technique is well widespread and still adopted in diagnostic and research routine due to the decreased cost and increased accessibility gained nowadays (Crosslay et al., 2020).

1.2.2 Next generation sequencing

From 2005, sequencing technology underwent a revolutionary process with the advent of next-generation sequencing (NGS) (McCombie et al., 2019). Also known as massive parallel sequencing, NGS results in a faster generation of a huge amount of data in comparison with Sanger sequencing (McCombie et al., 2019). More precisely, achievable sequencing reactions passed from hundreds to many millions in parallel with NGS (Thermes, 2014). In addition, the sequencing output can be directly detected without the need for gel electrophoresis (Thermes, 2014). These improvements in sequencing technology allowed the sequencing of entire genomes in a relatively short time and at an extraordinary speed (Thermes, 2014). NGS produces relatively short reads requiring the development of new alignment bioinformatic tools (Thermes, 2014).

1.2.2.1 NGS application

In recent years, the fast development of NGS technology has constantly reduced the cost to access these applications. Moreover, the reduced cost has been associated with the important development of bioinformatic tools and pipelines (McCombie et al., 2019). In this way, the application of NGS has become always more accessible and affordable in several fields allowing a rapid expansion of scientific knowledge (van Dijk et al., 2014). The advantages of NGS technology have not only been observed in scientific research but also the medical field with many clinical applications.

One of the first use of NGS was to investigate genetic constitutional disorders in human medicine. In this field, several studies have been conducted to improve the efficiency of diagnostic tests and to characterize more in deep these diseases (Zhong et al., 2021). Moreover, another advantage of NGS application to the study of genetic diseases consists of the development of innovative therapies largely reducing the incidence of a large number of pathologies, such as inherited eye disorders (Consugar et al., 2015) atypical or fibrodysplasia ossificans progressive (Liu et al., 2015) and other diseases. The next field of NGS application is oncology. Here, similar improvements and advantages have been reached thanks to NGS, as for genetic diseases. Several studies have brought many new insights into cancer processes, from proliferation to diffusion and metastasis. NGS has made it possible to identify many genomic alterations and biomarkers, involved in the oncogenic process. This discovery has, therefore, led to the development of more specific and efficient therapies, and a large number of tests for prevention, diagnosis and monitoring as a consequence (Zhong et al., 2021), especially for lung and breast cancer (Luthra et al., 2017; Winters et al., 2017). Furthermore, also infectious diseases have been investigated through NGS. In particular, innovative diagnostic tests have been mostly developed for pathogen identification (Zhong et al., 2021). Finally, additional application fields for NGS are neurodegenerative, parasitic and metabolic diseases, plants and microbial communities (Alexander-Dann et al., 2018; Chen & Rechavi, 2021; Peng et al., 2022). Lastly, NGS has also allowed the identification of new biomarkers for early disease diagnosis and the development of new vaccines and therapies.

1.2.2.2 NGS platforms

Since 2005, the development of NGS has brought several sequencing platforms each based on a different technology (McCombie et al., 2019). The first discrovered method was pyrosequencing by 454 Life Sciences (now Roche) (Thermes, 2014). This sequencing reaction works with enzymes, primer, and unlabeled nucleotides, allowing the synthesis of the complementary DNA strand (Thermes, 2014). The incorporation of a nucleotide causes the release of pyrophosphate with subsequent light emission, which is monitored in real-time (Thermes, 2014). One year after, the second platform was commercialized by Solexa/Illumina (Thermes, 2014). In this case, DNA sequencing occurs by incorporation of the fluorescently labelled, reversibly terminating nucleotides, on a solid surface coated with adapter oligonucleotides (Thermes, 2014). Multiple cycles of this solid-phase amplification followed by denaturation allow the sequencing of DNA molecules (Thermes, 2014). At the end of each cycle, the different fluorescent emissions are recorded by a terminator, the fluorophore is then removed, and the cycle is repeated (Thermes, 2014). These sequencing technologies produce relatively short reads, but the cost has gradually decreased over time making sequencing technology always more affordable (Stark et al., 2019). Even if Illumina is still the main competitor in sequencing, a third-generation method was developed in 2010 by PacBio and subsequently by Oxford Nanopore allowing sequencing with longer reads (Thermes, 2014; Stark et al., 2019;). Its mechanism is based on the detection of natural DNA synthesis by a single DNA polymerase (Thermes, 2014). Incorporation of phosphate-labeled nucleotides leads to base-specific fluorescence, which is detected in real time (Thermes, 2014). Lastly, sequencing runs therefore last minutes or hours rather than days (Thermes, 2014).

1.3 Transcriptomics

The whole RNA content of a cell is defined as transcriptome and includes all complementary RNA transcribed from the cell genome (Klopfleisch & Gruber, 2012). Among the different RNA classes, the first being discovered and studied was messenger RNA (mRNA), which is directly involved in protein translation and is the only protein-coding class (Dalrymple & Joss, 2019). Different levels of mRNA depend on the gene expression of a cell and reflect the several cellular functions and the activated biological processes (Klopfleisch & Gruber, 2012). However, the whole gene expression machinery does not only depend on mRNA activity, but also on several other RNA classes, that have been more recently discovered (Dalrymple & Joss, 2019; Ala, 2020). After mRNA, transfer RNA (tRNA) and ribosomal RNA (rRNA) have been discovered. In particular, tRNAs are molecular adaptors involved in the translation from nucleic acid to peptide collecting amino acids and delivering them to the ribosome (Dalrymple & Joss, 2019; R. Peng et al., 2022). On the other hand, rRNA is the most abundant RNA class in a cell and is involved in mRNA translation (Dalrymple & Joss, 2019).

The technologies for the study of the transcriptome have been widely developed in the last 20 years. The main advantage of transcriptomics is providing a snapshot in time of the total transcripts present in a tissue or a cell (Lowe et al., 2017). In particular, transcriptomics has the main difference compared to genomics of describing a dynamic picture of how genes are expressed and regulated, also at post-transcriptional level (Dalrymple & Joss, 2019). Measuring the expression of an organism's genes in different tissues, conditions, or time points can give information on genes regulation and reveals details of an organism's biology (Lowe et al., 2017). It can also help to infer the functions of previously unannotated genes (Wang et al., 2019).

1.3.1 Small RNAs

Among more recently discovered RNA classes, a miscellaneous group is represented by small-non-coding RNAs (sncRNAs). As stated by their name, sncRNAs have a short length and their function is not directly related to protein-coding, but their activity supports indirectly the protein synthesis. More specifically, sncRNAs are further classified into micro RNAs (miRNAs), small interfering RNAs (siRNAs), piwi-associated RNAs (piRNAs), circular RNA (circRNAs), small nuclear and small nucleolar RNA (snRNAs and snoRNAs) (Chen & Rechavi, 2021). The main studied class is miRNAs and their main function is to regulate gene expression processes at the post-transcriptional level (Dalrymple & Joss, 2019). This activity is strictly related to their structure, being 18-26 nucleotides long and having a complementary sequence to mRNA strands

(Dalrymple & Joss, 2019; Grešová et al., 2022). The binding between miRNA and a complementary mRNA can either lead to mRNA silencing or its degradation (Dalrymple & Joss, 2019). miRNAs have a regulatory activity on several biological processes: physiological responses, immune response, inflammation, cell ageing, cancer, neurodegenerative and other diseases (Lindsay, 2008; DN et al., 2021). Synthesis of miRNAs involves specific coding genes for miRNAs. Briefly, this process involves a few immature forms of miRNAs and the involvement of different enzymes (Peng & Croce, 2016). The Biogenesis and main function of miRNAs are described in Fig. 2.

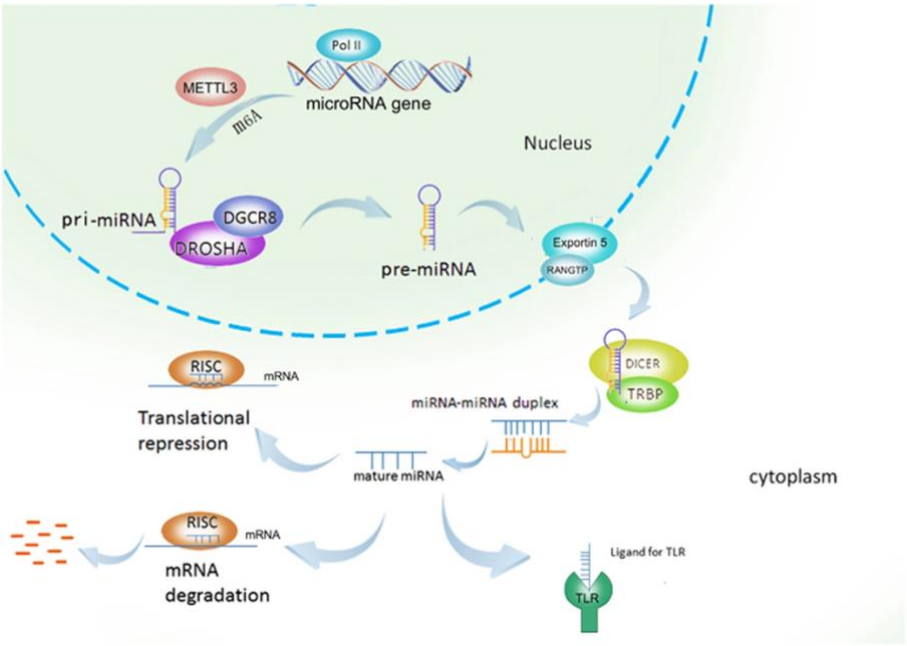

Figure 2. microRNA biogenesis and function. RNA polymerase II usually transcribes miRNA genes to produce to pri-mirRNAs. Following the cleavage process, the pre-miRNAs are transported from the nucleus to the cytoplasm, where are processed by an RNase III enzyme DICER to a 20-22-nucleotide miRNA/miRNA duplex. At this point, the mature miRNA is incorporated into a protein complex (RISC). Finally, miRNAs regulate the gene expression process at the post-transcriptional level by targeting their complementary mRNA strand. The transcript can be at this point either silenced repressing temporary its translation or degraded permanently. Cited by (Peng & Croce, 2016).

Afterwards, siRNAs are regulatory molecules, long between 20 and 27 nucleotides and their function and activity are similar to miRNAs. The difference between miRNAs and siRNAs is mainly ascribed to their different precursors (Chen & Rechavi, 2021). Conversely, piRNAs have more variable lengths (> 20 nucleotides) and they silence transposons genes and repetitive sequences (Dalrymple & Joss, 2019). Still not fully understood are circRNAs functions, they are known to have a role in gene regulation through interactions with specific spliceosome parts in the nucleus (Ala, 2020). The last group of sncRNAs are snRNAs and snoRNAs, which have the function of

regulating splicing processes (Dalrymple & Joss, 2019). Finally, the whole transcriptomic landscape is characterized by long non-coding RNAs (lncRNAs), which act as a target for miRNA silencing and cooperate with the abovementioned sncRNAs in the complex regulation of gene expression (Ala, 2020).

1.3.2 RNA-sequencing

Different techniques have been applied, individually or in combination, to study transcriptomic changes (Wang et al., 2009). Real-time quantitative polymerase chain reaction (RT-qPCR), microarray analysis and, more recently, RNA sequencing (RNA-Seq) techniques are mainly employed in the study mentioned above (Alexander-Dann et al., 2018). RT-qPCR is the most sensitive, but also the most time-consuming. It can be applied only for a limited number of genes, so it is used mostly for validation. DNA microarrays were first developed and employed in the 1990s. Shortly, microarrays use nucleotide sequences bound to a chip, called probes. These probes bind the fluorescently tagged reverse-transcribed sample cDNA. As the probes are designed with specific nucleotide sequences, microarrays are not able to detect unknown transcripts, which renders the technique often less suitable for more unknown parts of the transcriptome. RNA-seq was the least developed and it allows a deep and complete analysis of the transcriptome (Wang et al., 2009; Stark et al., 2019). Despite microarray, also unknown transcripts can be analyzed as well (Wang et al., 2009). Concisely, total RNA is retrotranscribed to cDNA with adaptors at both ends, then, libraries are sequenced with the chosen NGS platform (Wang et al., 2009).

1.3.3 Small-RNA sequencing

In recent years, innovative RNA-seq protocols have been developed to improve and select the sequencing of small-RNAs (Benesova et al., 2021). This technique allows a more reliable detection of shorter RNAs, which may not be identified through a canonical RNA-seq protocol (Giurato et al., 2013). In addition, in comparison with microarray small-RNA-seq allows the novel identification of small-RNAs (Benesova et al., 2021). Small-RNA-seq workflow is similar to RNA-seq, with the addition of a polyadenylation and ligation step (Benesova et al., 2021). Finally, the sequencing protocol varies according to the chosen platform (Stark et al., 2019).

CHAPTER 2: Aims of the PhD project

In recent years, the control of AMs use is one of the biggest concerns in veterinary medicine. The recently updated regulatory scenario about veterinary medical products has introduced a new strict limitation of AM prophylaxes and metaphylaxes. The increasingly growing concern about AMR is one of the main causes that has led to the new European regulation about veterinary medical products (EU Reg. n. 6/2019). Therefore, international and national authorities required a more prudent AMs use in both human and veterinary medicine. To that aim, a change in the farm management regarding AMs use is essential and new and alternative solutions may be necessary in veterinary medicine. Moreover, there is an urgent need to investigate the role of AM treatments on host tissue and organs and to implement the application of new potential biomarkers. On one hand, their application can allow the identification of AM treatments, reducing and discouraging AMs misuse. On the other hand, also the identification of new biomarkers for an early diagnosis of infectious diseases to reduce AMs use. Carrying on these studies will allow the application of new tools to control more efficiently the abuse and misuse of AMs.

In the last decade, the technological progress has brought many new, rapid, sensitive and specific tools for the scientific research. Among them, Next-Generation Sequencing (NGS) was applied to reach the main aim of this PhD project, as NGS is one the most appropriate and affordable tools in these contexts. For these reasons, this PhD project focused its attention on two different farming system: poultry and dairy cows farming, which are both blamed for an intense AMs misuse. In both these husbandry fields the use of AMs has always been a useful tool to control and prevent infectious diseases and to increase production performances (Mehdi et al., 2018; Loo et al., 2020). The application of NGS has two main advantages (van Dijk et al., 2014). First of all, in comparison with other techniques, NGS allows the achievement of a huge amount of data. Moreover, its application requires relative amount of time and results can be obtained in a relatively shorter time then with other techniques. Therefore, the main aim of this PhD thesis was to investigate the application of NGS technology as a useful tool in order to understand the role of AMs on host and to identify new putative biomarkers for AMs treatments and disease diagnosis.

Since this PhD project considers two farming systems, poultry and dairy cows, its development followed two separated research lines. The different approach was chosen considering the different conditions and the main problems affecting the studied farming system. However, the main aim was the application of NGS techniques in both cases in order to identify new putative biomarkers of AMs treatment or early diagnosis of the disease. The final consequence of this research will be the application of these biomarkers as tools to control and reduce AMs use.

Talking about poultry farming, one of the main pathologies, that always required AMs treatments, are gastrointestinal diseases (Awad et al., 2017). Ensuring gut health is a crucial challenge in broiler chickens. An efficient growth during the production cycle relies on the correct activity of the intestinal barrier (Chase, 2018). In addition, in the past AM growth promoters and prophylaxes were frequently adopted in this species to promote production performance (Mehdi et al., 2018). However, AM can have a negative effect on the gut microbiota, but also on the host influencing its metabolism and other biological processes (Broom, 2018; Xiong et al., 2018; Greene et al., 2022). For these reasons, this PhD project aims to investigate the role of AMs on the intestinal barrier and host through the application of 16S rRNA gene metabarcoding and histopathology. In addition, to further investigate the consequences of AMs on the intestinal barrier an evaluation of tight-junction proteins whether these proteins could be involved in the modulation of the intestinal barrier in AMs treated broilers. Finally, a panel of putative biomarkers for AMs treatment, previously identified by Giannuzzi and colleagues (Giannuzzi et al., 2021) applying an NGS approach, were evaluated in liver and muscle samples of broilers treated with different AM protocols.

As reported above, this PhD project considers a second research area, which is dairy cow farming. Considering this, one of the main challenging diseases is mastitis (Ruegg, 2017). AMs have always been useful tools to treat bacterial mastitis and to prevent the udder health during the dry-off period (Ashraf & Imran, 2020). Also, in this field the limitation of AMs use introduced with the new regulation, requires new approaches to control mastitis and a reduction in the use of AMs. In recent years, a growing body of evidence shows that miRNAs vehiculated by EVs have an important role in the regulation of mastitis and can be used as potential biomarkers for mastitis detection (Ryman et al., 2022; Saenz-de-Juano et al., 2022a). For these reasons, the specific aim of this PhD was to investigate the role of miRNAs vehiculated by EVs in the regulation of mastitis and to identify putative biomarkers for an early diagnosis of mastitis.

CHAPTER 3: Poultry farming research line

3.1 Introduction

3.1.1 Chicken gut barrier

In the poultry industry, gut health is one of the main concerns for ensuring chickens health and growth performance (Dal Pont et al., 2020). Gastrointestinal disorders not only are animal health and welfare threats, but also they negatively affect production parameters at the slaughterhouse (Tabler et al., 2020). This complex function relies on the correct performance of the intestinal mucosal barrier (Fig. 3), consisting of gut microbiota (GM), intestinal epithelial cells (IECs) and mucosal immunity system (Pawłowska & Sobieszczańska, 2017; Iacob & Iacob, 2019).

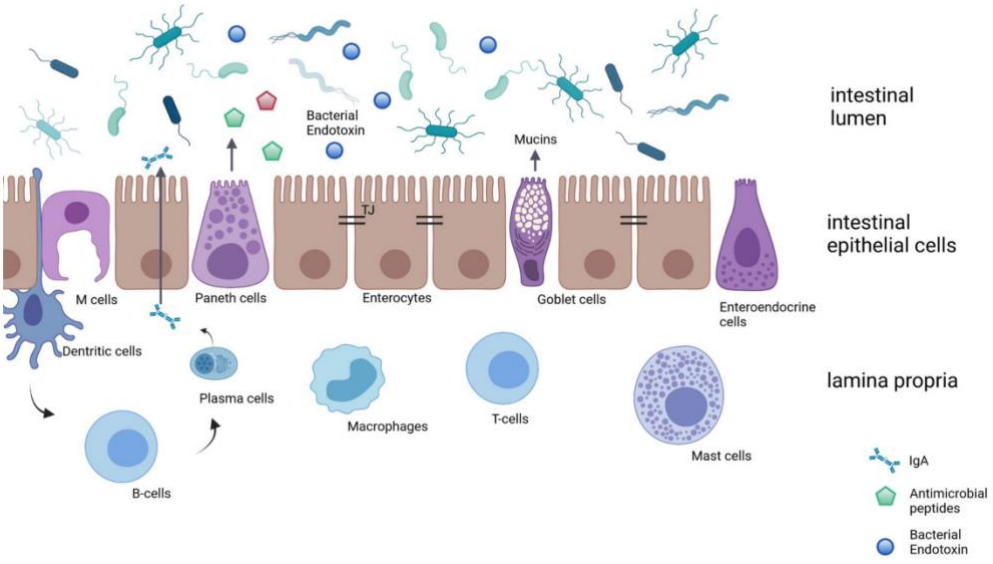

Figure 3. Graphic representation of the intestinal barrier. The gut barrier includes the GM, located in the intestinal lumen, a monolayer of specialized IECs and a complex immunity system underneath, located in the tissue lamina propria. The activity and function of the barrier are guaranteed by a complex system of cellular processes, which involve the production of secretory IgA, antimicrobial peptides and bacterial endotoxin. Cited by (Untersmayr et al., 2022).

The intestinal barrier not only forms a physical separation from the intestinal lumen and the bloodstream but also regulates water and nutrient absorption, protects from pathogen invasion and directly regulates intestinal inflammatory processes (Iacob & Iacob, 2019; Soderholm & Pedicord, 2019). In the poultry industry, the correct function is an essential requirement for the growth of animals and the fulfilment of the goals according to the specific hybrid line guidelines (Dal Pont et al., 2020). Considering broilers, the genetic selection applied in the improvement of these hybrid lines has led to animals with a very fast-growing rate in an always shorter time (Shah et al., 2019). This trend causes the selection of very efficient animals for meat production, but with huge and disproportionate breast muscle, weak legs and joints and consequent predisposition to metabolic, locomotor and welfare problems (Kong et al., 2017). In this farming scenario, the first and most difficult step in broiler farming is ensuring gut health (Dal Pont et al., 2020).

3.1.1.1 Gut microbiota

A community of different microorganisms residing in a specific environment is defined as microbiota (Marchesi & Ravel, 2015). The microbial composition varies between several species of bacteria, viruses, archaea, fungi and protozoa, in equilibrium with each other (Broom, 2018). When a microbial community resides in a specific environment or a particular anatomic region of an animal host, their relationship becomes a symbiosis with several benefits for each part (Kogut et al., 2020). On the other hand, the microbiome is the term for defining the whole habitat including not only the microorganisms but also their genomes and the surrounding environment (Marchesi & Ravel, 2015).

The GM is a complex microbial community of bacteria, viruses, fungi, protozoa and archaea, residing in the whole gastrointestinal tract and having a symbiotic relationship with the host (Le Roy et al., 2019). In the gut, GM is the first-line defence of the barrier and it prevents the arousal of intestinal infectious diseases (Kers et al., 2018; Iacob & Iacob, 2019). In poultry production, several host functions are strictly related to the GM, e.g. nutrients digestion, metabolism and immunity (Xiao et al., 2017a; Le Roy et al., 2019). The GM composition becomes stable at an early age and the equilibrium between the host and GM guarantees the prevention of pathogens colonization (Carrasco et al., 2019). Since the advent of NGS technologies, new information has been found about GM and its function, in particular about non-cultivable bacteria (Aruwa et al., 2021). The composition and characteristics of GM vary among the different tracts of the gastrointestinal system (Jurburg et al., 2019). In particular, the microbiota of ileum and caecum are the main investigated in poultry and their compositional differences reflect the main functions of the tract (Cuccato et al., 2021a; Yang et al., 2021; Di Marcantonio et al., 2022). GM differences are mainly related to composition and complexity, which increase considerably in distal tracts

(Rychlik, 2020). The ileum is the main site responsible for nutrient absorption and it is usually colonized by bacteria that stimulate nutrient availability and host metabolism (Stanley et al., 2014; Xiao et al., 2017). On the other hand, caecum is inhabited by bacteria involved in starch fermentation, water absorption and urea recycling (Stanley et al., 2014; Clavijo & Flórez, 2018).

Over time, different techniques have been developed in order to study both microbiota and microbiome. The first attempts were made with bacteriological tests on different culture media and varying growth conditions (Yeoman et al., 2012). However, achieved information during the culturomics era did not take into account those from uncultivable microorganisms (Du et al., 2022). With the advent of NGS, microbiota and microbiome data became accessible at an unprecedented speed (Jovel et al., 2016). One of the main NGS techniques employed in microbiota studies is 16S rRNA gene sequencing (or 16S rRNA gene metabarcoding). This method is based on the sequencing of 16S rRNA gene, which is a phylogenetic marker for bacteria and archaea (Yeoman et al., 2012). In detail, this gene has both well-conserved regions, which do not vary among species, and other variable regions, which allow a phylogenetic differentiation, even sometimes at the species level (Jovel et al., 2016). 16S rRNA gene metabarcoding is normally performed on Illumina platforms, with variable primers and protocols (Takahashi et al., 2014). Microbiota information, gained by this technique, is not only related to taxonomy data but to microbial community diversity as well (Takahashi et al., 2014; Jovel et al., 2016).

As stated before, 16S rRNA gene metabarcoding allows diversity analysis of microbial communities. Several diversity indexes can be calculated using sequencing data, divided into alpha and beta diversity (Durazzi et al., 2021). The first one, alpha, is defined as the complexity of a microbial community as the number of species composing it (Jovel et al., 2016). According to the similarity of each sequence, bacteria are grouped in Operational Taxonomic Unit (OTU) or Amplicon Variant Sequence (ASV) (Durazzi et al., 2021). Among alpha indexes, two aspects are mainly evaluated: the richness of a microbial community and the evenness (term used for the proportion between the different ASV) (Jovel et al., 2016; Durazzi et al., 2021). The most used alpha metrics are observed OTU, Shannon index, Pielou's and Faith phylogenetic diversity (Kowalska-Duplaga et al., 2019). In particular, observed OTU is a richness index and is represented by the absolute number of OTU identified in a microbial community (H. Lin & Peddada, 2020). This first index is highly dependent on the DNA extraction method and sequencing performance (Greathouse et al., 2019). Differently, the Shannon index considers both evenness and richness, giving more weight to the second (Lemos et al., 2011). Pielou's index is instead an evenness measure, which considers the different species composing a community and their proportion (Johnston & Roberts, 2009). Finally, faith phylogenetic diversity is calculated using the phylogenetic tree of the community (Armstrong et al., 2021). On the other hand, beta

metrics evaluated the difference in species composition between two or more microbial communities (Jovel et al., 2016). The most used beta indexes are Bray-Curtis, Jaccard and UniFrac, which are based on distance matrix calculations (Lemos et al., 2011; Jovel et al., 2016). Beta metrics usually consider two parameters: community membership, based on OTU presence or absence in a community, and community structure, which considers also the relative abundance of each OTU (Costa et al., 2017). In detail, Bray-Curtis index is based on abundance or read count data and its values can vary from 0 to 1, directly proportional to the different OTUs abundance (Willis & Martin, 2022). Jaccard distance, differently, is a community membership index and similar to Bray-Curtis index can vary from 0 to 1 (Lemos et al., 2011). Finally, UniFrac index is a sequence distance measure in phylogenetic terms, considering the fraction of branch length between two samples (Sarangi et al., 2019). UniFrac can be weighted or unweighted, if the relative abundances of OTUs are considered or not respectively (Aruwa et al., 2021).

Besides diversity data, 16S rRNA gene metabarcoding also provides taxonomy results. The taxonomical assignment is done by comparing the obtained sequences with those from an official database. The main databases used are Greengenes and Silva, the last more recently updated (McDonald et al., 2012; Callahan et al., 2016). Results depend on the percentage chosen for clustering OTU/ASV, normally 99% threshold is adopted (Johnson et al., 2019). However, the phylogenetic determination is accurate up to the family level and is less precise with genera and species, which is a well-known limit of this sequencing technique (Durazzi et al., 2021).

More recently developed, shotgun metagenomic sequencing has the main target to sequence the whole microbiome DNA content (Durazzi et al., 2021). Most studies aim to investigate the microbial resistome using shotgun metagenomic sequencing, correlating antimicrobial resistance (AMR) genes data to those from taxonomy (Rubiola et al., 2020; Rochegüe et al., 2021; Rubiola et al., 2022). In comparison with 16S rRNA gene metabarcoding, shotgun metagenomics is more accurate and reliable in taxonomical determination (Jovel et al., 2016; Johnson et al., 2019). However, diversity analysis cannot be conducted with shotgun metagenomics (Durazzi et al., 2021).

3.1.1.2 The host components of the intestinal barrier

Several cellular layers compose the intestinal barrier interacting on one side with the GM and on the other with the bloodstream. The main function lies in constituting a physical barrier between the lumen and the rest of the organism. Between the different types of cells, IECs represent the most diffused cells in the barrier. Furthermore, IECs are constituted by a heterogenous population with different functions. The most important difference is between crypts and villi. The first is

composed of less-differentiated enterocytes and also of pluripotent stem cells (Ramis et al., 2022). Differently, villi are formed by a multitude of specialized cells (Nii et al., 2020). Among them, enterocytes are the most frequent and involved in nutrient absorption. Another cellular type is enteroendocrine cells, goblet cells involved in mucus production, Paneth cells involved in the secretion of antimicrobial substances, and M cells involved in the immunity tolerance mechanism of GM (Chase, 2018; Shira & Friedman, 2018). In addition, the intestinal barrier is completed by a complex mucosal immunity system. Here, Paneth cells and M cells can be included, but other cells are included, such as dendritic cells, resident memory T-cells and B-cells involved in the production of secretory IgA (Chase, 2018). This immune population represents the first-line defence of the immune system protecting from pathogens (Kogut et al., 2018).

3.1.1.3 Tight-junction proteins

Furthermore, the proper activity of the intestinal barrier relies also on a monolayer of IEC, which includes different types of epithelial cells and are bound together by tight junction (TJ) protein complexes (Jin & Blikslager, 2020; Tabler et al., 2020). Both transmembrane and cytoplasmic proteins form the TJ complexes, schematically shown in Fig. 4.

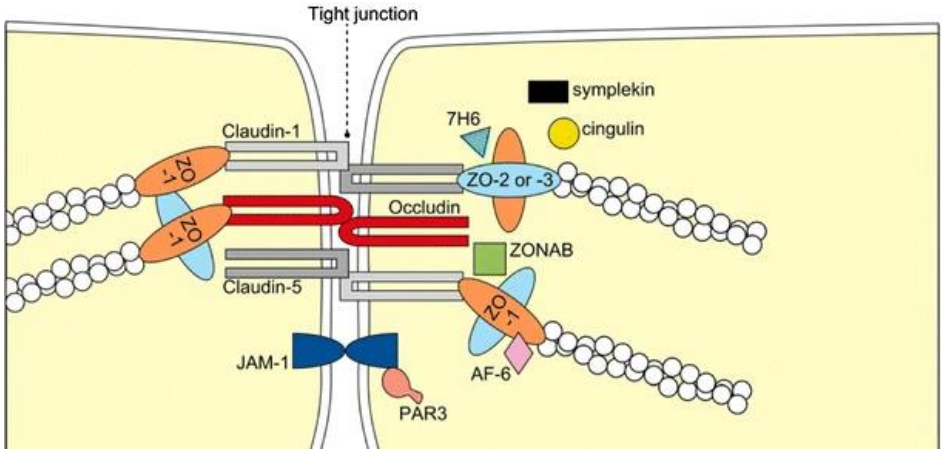

Figure 4. Proteins composing TJ complexes. This scheme shows the several proteins composing TJ complexes and their location in the cell. Claudin family is the most abundant group. Along with occludin, claudins form a transmembrane ring regulating the paracellular space. ZO proteins are located in the cytoplasm and form a link between the transmembrane TJ proteins and the cytoskeleton. Modified from (Förster, 2008).

Among the first ones there are claudins, occludin, tricellulin, and junctional adhesion molecules (Awad et al., 2017; Suzuki, 2020); on the other hand, the cytoplasmic zonula occludens (ZO) proteins form an intracellular plaque, linked with the cytoskeleton actin filaments (Fasano & Nataro, 2004; Suzuki, 2020). Claudins are the most abundant family composing TJ complexes and several isoforms have been described. These proteins have transmembrane localization and form a three-dimensional seal for the passage of ions and small molecules (Awad et al., 2017). Particularly, claudins can be divided into pore-forming and pore-sealing TJ. The first group is mainly composed of claudin-2 and -15 can form paracellular ion pores and water channels, enabling them to decrease epithelial tightness and increase solute permeability by allowing the passage of sodium ions. Differently, claudin-1, -3, -4, -5, -7, and -19 are pore-sealing claudins and their increased expression can lead to very tight epithelia and reduced permeability (Awad et al., 2017). Occludin is a TJ protein consisting of four transmembrane domains with the ability to shift to various paracellular locations and, therefore, modify epithelial permeability. Displacement of occludin from the tight junction into cytoplasmic vesicles occurs frequently during barrier function loss and can be induced by multiple stimuli, such as oxidative stress and inflammation (Awad et al., 2017). Finally, ZO is a group of cytosolic proteins connecting the TJ complexes to the cytoskeleton. ZO-1 isoform is the most abundant, but also ZO-2 and -3 have been described. The main functions of this group are related to the stability of the TJ complexes, interaction with the perijunctional actomyosin ring via their C-terminus and also to adherens-junction proteins in the cell basement. Among the different TJ isoforms, claudin-1, claudin-3, claudin- 5, claudin-16, ZO-1, and ZO-2 have been investigated to be expressed in the chicken intestinal epithelium (Ozden et al., 2010; Awad et al., 2017). TJ complexes serve as a selective barrier for the passage of ions and molecules regulating the paracellular pathway (Awad et al., 2017). The barrier permeability depends on the expression and interaction of the different TJ proteins (Fasano & Nataro, 2004; Awad et al., 2017). Specifically, the composition and expression regulation of TJ complexes can be affected by different internal and external factors, such as inflammatory regulators (i.e., cytokines, chemokines), dietary components, microorganisms or enterotoxins (Ulluwishewa et al., 2011; Awad et al., 2017; Suzuki, 2020). TJ dysregulation may lead to the onset of leaky gut syndrome, characterized by increased gut permeability, and loss of nutrients, water and ions (Awad et al., 2017; Lin & Lee, 2021). Furthermore, TJ proteins can be targeted during the pathogenesis of specific enteric pathogens, disrupting or relocating these proteins (Fasano & Nataro, 2004; Assimakopoulos et al., 2011; Awad et al., 2015; Awad et al., 2017;). However, also intestinal inflammation can lead to the modulation of TJ expression by myosin light chain kinase or Rho-associated GTPase pathway activation (Awad et al., 2017).

3.2 Materials and methods

3.2.1 Zootechnical trials

The samples analysed during this PhD project were collected at end of three zootechnical trials previously described (Cuccato et al., 2021; Giannuzzi et al., 2021; Cuccato et al., 2022; Laconi et al., 2022). The trials were conducted in the chicken broiler farm facility of the Department of Veterinary Sciences of the University of Turin and regularly approved by the Ethics and Animal Welfare Committee of the Department of Veterinary Sciences (protocol n. 2796/2020; protocol n. 275/22), University of Turin (Italy). During each trial, broilers were vaccinated against Marek's disease and Infectious Bursal disease at the hatchery. Additionally, one-day old chicks were vaccinated against coccidiosis (coarse spray), Newcastle disease and Infectious Bronchitis disease (fine spray). Each pen was equipped with a bucket-type feeder and a drinker with wood shavings as litter. Water and feed were provided "ad libitum" during the whole cycle. The environmental conditions (lighting, temperature, relative humidity, and ventilation) were controlled according to the Ross broiler management guidelines. During the 3 zootechnical trials, different prophylactic protocols were adopted. AMs were administered via drinking water. In Trial 1, prophylactic treatments were done twice during the production cycle and the second treatment was stopped before slaughtering, always respecting the withdrawal periods of AMs. In the other trials, prophylactic protocols were administered with longer withdrawal periods with the aim of understanding whether findings observed in trial 1 could be confirmed. The experimental design of this research line is shown in ANNEX A – Figure 1.

3.2.1.1 Zootechnical trial 1

During 2018, a total of 240 male broilers (Ross 308) were reared under the same conditions in the chicken broiler farm facility of the Department of Veterinary Sciences of the University of Turin. Chicks were randomly allocated into 7 groups. During production cycle, six groups received a different antimicrobial prophylactic program: thiamphenicol (Tirsan O.S. 200 mg/g) (THP), amoxicillin (Amoxid Polv 1430 g) (AMX), sulfadiazine + trimethoprim (Trimethosulfa orale) (TRIM), thiamphenicol (Tirsan O.S. 200 mg/g) + diclazuril (Coxiril) (THP + DCZ), amoxicillin (Amoxid Polv 1430 g) + diclazuril (Coxiril) (AMX + DCZ), and diclazuril (Coxiril) (DCZ). Untreated animals were used as a control group (K). Chickens received a starter diet from the first day to the 25th day and a grower diet from 26th to 58th day of rearing and AMs were administered during both periods. Detailed prophylactic protocols are available in Tab. 1. The withdrawal periods were respected before slaughtering. At the end of the rearing cycle (58 days), all broilers were regularly

slaughtered and samples were collected from a total of 120 broilers (15 animals per each treated group and 30 animals of the untreated group).

Group	AMs	Prophylactic protocols	Period of treatment (Rearing days)	Withdrawal (days)
AMX	Amoxicillin	30 mg/kg BW (twice/day)	20-22/ 53-56	1
AMX + DCZ	Amoxicillin + Diclazuril	30 mg/kg BW (twice/day) + 1 mg/kg	20-22/ 53-56 0-52	1
THP	Thiamphenicol	65 mg/kg BW/day	23-25/ 47-51	5
THP + DCZ	Thiamphenicol + Diclazuril	65 mg/kg BW/day + 1 mg/kg	23-25/ 47-51 0-52	6
TRIM	Sulfadiazine + Trimethoprim	20 mg/kg BW/day + 4 mg/kg BW/day	21-25/ 50-54	6
DCZ	Diclazuril	1 mg/kg	0-52	5
K	-	-	-	-

Table 1. Detailed AM protocols for the seven groups of Trial 1. AMs were administered via drinking water and withdrawn before slaughtering.

3.2.1.2 Zootechnical trial 2

During 2019, a total of 210 male broilers (Ross 308) were reared under the same conditions in the chicken broiler farm facility of the Department of Veterinary Sciences of the University of Turin. Chicks were randomly allocated into 7 groups. During the production cycle, six groups received an antimicrobial prophylactic program with the same molecules of Trial 1, but with different

protocols (Tab. 2). Untreated animals were used as a control group (K). Chickens received a starter diet from the first day to the 28th day and a grower diet from 29th to 42nd day of rearing and AMs were administered in both these periods. The withdrawal periods were respected before slaughtering and they were longer than trial 1. At the end of the rearing cycle (42 days), all broilers were regularly slaughtered and, particularly, samples were collected from 15 animals per each group.

Group	AMs	Prophylactic protocols	Period of treatment (Rearing days)	Withdrawal (days)
AMX	Amoxicillin	30 mg/kg BW (twice/day)	14-16	25
AMX + DCZ	Amoxicillin + Diclazuril	30 mg/kg BW (twice/day) + 1 mg/kg	14-16 0-36	25
THP	Thiamphenicol	65 mg/kg BW/day	14-16	25
THP + DCZ	Thiamphenicol + Diclazuril	65 mg/kg BW/day + 1 mg/kg	14-16 0-36	25
TRIM	Sulfadiazine + Trimethoprim	20 mg/kg BW/day + 4 mg/kg BW/day	14-18	23
DCZ	Diclazuril	1 mg/kg	0-36	5
K	-	-	-	-

Table 2. Detailed AM protocols for the seven groups of Trial 2. AMs were administered via drinking water and withdrawn before slaughtering.

3.2.1.3 Zootechnical trial 3

During 2020, 120 one-day-old healthy Ross 308 chicks from a single hatchery were randomly allocated in 6 different pens in the animal facility of the Department of Veterinary Science,

University of Turin, and reared until 46 days of age as part of a zootechnical trial. Birds received the following feeding program to meet the standard nutritional requirements: a commercial starter diet (230 g/kg of CP) from the first day to the 12th (starter period) and a commercial grower diet (185 g/kg of CP) from the 13th day to the 46th (finisher period). During production cycle, five groups received an antimicrobial prophylactic program, with the same molecules of the previous trials, but with different protocols (Tab. 3). No treatments were administered to control group (K). On the 30th day of age, birds of all groups started showing respiratory and intestinal symptoms; therefore, the farm veterinarian prescribed and administered 20mg of doxycycline per kg of live weight a day for five consecutive days from day 33rd onwards. All treatments were administered via drinking water. At the end of the rearing cycle (42 days), all broilers were regularly slaughtered and particularly, samples were collected from 36 animals, randomly selected from each group.

Group	**AMs**	**Prophylactic protocols**	**Period of treatment (Rearing days)**	**Withdrawal (days)**
AMX1	Amoxicillin	20 mg/kg BW (twice/day)	4-7	38
AMX2	Amoxicillin	20 mg/kg BW (twice/day)	21-23	22
THP1	Thiamphenicol	65 mg/kg BW/day	5-7	38
THP2	Thiamphenicol	65 mg/kg BW/day	21-23	22
TRIM	Sulfadiazine + Trimethoprim	20 mg/kg BW/day + 4 mg/kg BW/day	21-25	24
K	-	-	-	-

Table 3. Detailed AM protocols for the seven groups of Trial 3. AMs were administered via drinking water and withdrawn before slaughtering.

3.2.2 Sample collection

At the end of all trials, broilers were regularly slaughtered, and samples were appropriately collected. In particular, tissue and organs for molecular biology investigation were collected and snap frozen on liquid nitrogen. Intestinal contents of ileum and caecum were instead collected in an aseptic way and snap frozen on liquid nitrogen. Finally, tissue and organs were also fixed into 10% buffered formalin (pH 7.0) and paraffin-embedded according to routine histological procedures. A detailed list of samples collected at the end of each trial is reported in Tab. 4.

Zootechnical trial	Formalin-fixed samples	Snap-frozen samples
1	Liver and intestine	Liver, pectoralis major muscle, ileum, caecum and relative intestinal contents
2	Liver and intestine	Liver, pectoralis major muscle, ileum and caecum
3	Liver and intestine	Liver, pectoralis major muscle, ileum, caecum and relative intestinal contents

Table 4. List of samples collected at the end of each zootechnical trials.

3.2.3 Intestinal barrier study

In order to clarify the AMs effects on the intestinal barrier, the GM was investigated through the application of 16S rRNA gene metabarcoding. Furthermore, an histopathological approach was applied to evaluate the presence and the characteristics of intestinal lesions. Finally, TJ proteins were evaluated in intestinal samples trough IHC and qPCR to verify their possible role in the modulation of the intestinal barrier.

3.2.3.1 DNA extraction

Genomic DNA was isolated from 12 samples of ileal content (n= 6) and caecal content (n = 6), randomly selected in each group. In samples of Trial 1, DNAzol (Thermo Fisher Scientific) and DNeasy PowerClean Pro Cleanup Kit (QIAGEN) were respectively used for DNA extraction and

purification according to the manufacturer's procedures. Differently, DNA was extracted using Maxwell RSC Fecal Microbiome DNA Kit (Promega). In both trials, DNA was quantified in parallel using a Nanodrop spectrophotometer (Thermo Fisher Scientific) and QuantiFluor dsDNA System (Promega).

3.2.3.2 16S rRNA gene metabarcoding

Library preparation and 16S rRNA gene sequencing were performed by an external laboratory. The variable V3 and V4 regions were amplified with universal prokaryote primers Pro341F and Pro805R (Takahashi et al., 2014). A Nextera XT Index kit (Illumina) was used for libraries preparation. Finally, amplicons were sequenced with the Illumina MiSeq platform using a 2×300 bp paired-end protocol.

3.2.3.3 Bioinformatic analysis

Raw reads were divided according to intestinal origin (ileum and caecum) and analysed separately with the same bioinformatic pipeline. The quality of raw reads was checked with FastQC v. 0.11.9 software. Then, raw reads were processed following Qiime2 v. 2019.10 pipeline. Primers were trimmed using Cutadapt. Low-quality reads with a q-score < 20 were removed. After trimming and filtering steps, clean reads were processed with DADA2. The amplicon sequence variant (ASV) table was used for generating the rooted and unrooted phylogenetic trees. Afterwards, alpha (Pielou's even- ness and Faith's phylogenetic Diversity) and beta diversity indexes (unweighted UniFrac, weighted UniFrac, Jaccard, and Bray-Curtis distances) were determined. The PCoA plots were generated using Emperor tool in Qiime2. Statistical analyses were conducted using Qiime2 plugins. A pairwise Kruskall-Wallis test and nonparametric Permutational Multivariate Analysis of Variance (PERMANOVA) tests were conducted, respectively, for alpha and beta metrics. The alpha-rarefaction curve was generated to understand if the sequencing depth was enough to describe all microbial communities. Finally, taxonomy was assigned to the obtained ASV table using the Greengenes database clustered at 99% identity. The Greengenes database was previously trained to the region targeted by sequencing primers. Box plots were generated for taxonomy results visualization.

3.2.3.4 Histopathology of gut barrier

Representative sections of each intestinal sample were stained with haematoxylin-eosin (HE). Observation was performed by a Nikon Eclipse E600 light microscope (Nikon Corporation, Tokyo, Japan). A multiple grading scoring system, previously adopted in the histopathological evaluation

of the swine intestine (Ruggeri et al., 2014), was implemented for the evaluation of the intestinal tissues of broilers. The status of the villi/epithelia, inflammatory infiltrates in the mucosa and submucosa considering the main cellular components (i.e., lymphocytes, eosinophils) and haemorrhages were the main features evaluated. Severity and distribution scores for each of the analysed parameters were defined as mild, moderate, and severe. To describe distribution, lesions were defined as focal, disseminated and diffuse. Moreover, for intestinal epithelia and villi, a further score, reflecting lesions severity, was assigned: normal aspect, presence of epithelial detachment, fusion, and necrosis. Finally, hyperaemia and caecal tonsils reactivity were recorded. Caecal tonsils were considered reactive when an evident hypertrophy of the secondary lymphoid follicles was present and/or septa of connective tissue dividing tonsillar units were not evident, due to the proliferation of the lymphoid cells. A detailed scheme of the scoring system used with all parameters evaluated and their corresponding numerical values are presented in Tab. 5. A total score was obtained for each of the analysed parameter: villi/epithelial score, mucosal inflammatory score and submucosal inflammatory score.

Parameter evaluated	**Lesion**	**Severity**	**Distribution**
Villi and epithelium	0.5 = epithelial detachment 1 = villi fusion 2 = necrosis	1 = mild 2 = moderate 3 = severe	1 = focal 2 = disseminated 3 = diffuse
Mucosal and submucosal infiltrate		1 = mild 2 = moderate 3 = severe	1 = focal 2 = disseminated 3 = diffuse
Hyperemia	+ = present - = absent		
Hemorrhages		1 = mild 2 = moderate 3 = severe	1 = focal 2 = disseminated 3 = diffuse
Cecal tonsils	+ = activated - = silent		

Table 5. Histopathological scoring system used for the evaluation of intestinal tissues in broilers. Each parameter was evaluated for all chickens in ileum, cecum and colon.

3.2.3.5 Immunohistochemistry of tight-junction proteins

Immunohistochemistry (IHC) was performed on 6 randomly selected animals of each treatment group for ileum, caecum and colon of Trial 1. After deparaffinization, endogenous peroxidases were blocked using a 0.3% H_2O_2 solution for 30 minutes. Antigen retrieval was achieved by incubation in Tris-EDTA buffer (pH 9.0) at 98◦ C for 30 minutes. After a 5min washing step in phosphate-buffered saline (pH 7.4), slides were incubated with Normal Horse Serum 2.5% for 10 minutes in a humidifier chamber. To evaluate TJs proteins, two primary antibodies were selected: anti-claudin 3 (rabbit polyclonal antibody specific to Claudin 3, ab15102, Abcam) and anti- ZO-1 tight junction protein antibody (rabbit monoclonal [EPR19945-224] antibody specific for ZO-1 tight junction protein, ab221546, Abcam). The tissue slides were incubated overnight at 4◦C with primary antibodies in a humidifier chamber. Antibodies detection was performed by avidin-biotin-peroxidase complexes, using the Vectastain Universal Quick HRP Kit (Vector Laboratories, Burlingame, VT). A diaminobenzidine-hydrogen peroxide solution (DAB substrate kit, Vector Laboratories, Burlingame, VT) was used for 5 minutes as a chromogen. Tissue slides were washed in water, hematoxylin counterstained, dehydrated and mounted with a coverslip. Positive controls were performed to ensure the reactivity of primary antibodies;negative controls without primary antibodies were also conducted to avoid the presence of non-specific signals in chicken intestinal samples. Chicken ileal tissue was used as positive control for claudin-3 antibody and murine renal tissue for ZO-1 antibody, as suggested by the manufacturer. A quantitative scoring system was used to evaluate ZO-1 and claudin-3 expression levels (0– 100%). The examination was carried out by a Nikon Eclipse E600 microscope (Nikon Corporation, Tokyo, Japan). Score assignment was performed for each sample using a 40x lens and five different areas were randomly evaluated in ileum and colon, as 10 different areas in cecum, equally divided between the two ceca.

3.2.3.6 Gene expression assay of tight-junction proteins

Total RNA was extracted from 82 intestinal samples, collected from trial 3, equally divided in caecum and ileum. Trizol was used to perform RNA extraction following manufacturer instructions. Quantity and quality of RNA samples were respectively checked using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the Bioanalyzer 2100 instrument (Agilent Technologies, Santa Clara, CA, USA). The QuantiTect Reverse Transcription Kit (Qiagen) was used to synthesize the cDNA from 1µg of total RNA. A multi-reference gene-study was firstly performed to identify the more stable housekeeping genes in intestinal samples. A total of 10 different references genes (RGs) were selected from literature, as the most used in gene expression studies in poultry. The expression stability (M) of the RGs and their optimal

number (V) were determined using the geNorm algorithm. Normalization by multiple RGs was applied for an accurate measure of expression levels. The candidate genes for tight-junction proteins selected in both intestinal tracts were selected among tight-junction proteins: claudin-1 (CLDN-1), claudin-2 (CLDN-2), claudin-3 (CLDN-3), claudin-5 (CLDN-5), occludin (OCLN), zonula-occludens-1 (ZO-1), zonula-occludens-2 (ZO-2) and zonula-occludens-3 (ZO-3). Primer sequences of the candidate genes were designed using Primer-BLAST on the corresponding reference sequences. The expression levels of the candidate genes were analysed on a Bio-Rad CFX Connect™ qPCR detection system using iTaq™ Universal SYBR®Green Supermix (Bio-Rad).

3.2.4 Biomarkers evaluation

A previous study, conducted at the animal pathology section of the University of Turin, proposed a panel of putative biomarkers for the identification of broilers treated with AMs using liver and pectoralis major (PM) muscle samples (Giannuzzi et al., 2021). In order to validate the results of above cited study, the application of these potential biomarkers were examined in samples of the other trials. In particular, liver samples from trial 1, 2 and 3 and PM muscle from trial 1 and 3, were analysed using qPCR and droplet digital PCR (ddPCR) technology respectively.

Total RNA was extracted from liver and pectoralis major muscle samples. Trizol was used to perform RNA extraction following manufacturer instructions. Quantity and quality of RNA samples were respectively checked using a Nanodrop spectrophotometer (Thermo Fisher Scientific) and the Bioanalyzer 2100 instrument (Agilent Technologies). The QuantiTect Reverse Transcription Kit (Qiagen) was used to synthesise the cDNA from 1µg of total RNA. RGs were selected from Gianuzzi et al.: HMBS and TBP were selected for liver samples in qPCR and only HMBS for PM muscle samples in ddPCR. The analysed genes selected in liver and PM muscle samples were respectively: DIO2, IGBP1, GPR27, PDK4 and RHOB in liver samples and ELOVL1 and CERS2 in PM muscle samples. Primer sequences of the candidate genes were either chosen from Giannuzzi's study or designed using Primer-BLAST on the corresponding reference sequences for ddPCR expression studies. The expression levels of the candidate genes were analysed on a Bio-Rad CFX Connect™ qPCR detection system using iTaq™ Universal SYBR®Green Supermix (Bio-Rad). Differently, ddPCR experiments were performed using QX200 ddPCR EvaGreen Supermix (BioRad) and BioRad's QX200 ddPCR system were used according to the manufacturer's protocol. In order to establish the best conditions to perform the HMBS gene expression analysis in ddPCR, the optimal annealing temperature for both target gene and the selected RG primers was assessed throughout a gradient from 55–65℃. After optimization, the PCR protocol was kept at 95℃ for 5 minutes, 40 cycles at 95℃ for 30 seconds and at 58,5℃ for

1 minute with an overall ramp rate of 2 ℃/s and three final steps at 4 ℃ for 5 minutes, 95 ℃ for 5 minutes, and 4 ℃ indefinite hold for dye stabilization.

3.2.5 Statistical analysis

Data from the taxonomic assignment of OTUs were tested for differentially abundant taxa for both phyla and families. Statistical analyses were performed by two-way ANOVA using GraphPad Prism v.8 (GraphPad Software, San Diego, CA, USA). The two effects evaluated consisted of the AM treatment group and the top five phyla and families represented in ileal and caecal samples. We tested multiple comparison corrections with Tukey's test when two-way ANOVA showed a p-value < 0.05 or a significant interaction was revealed. Histology results were analysed using GraphPad Prism v.8 (GraphPad Software, California, USA). Data were checked for population normality with the Kolmogorov-Smirnov test. Scores were compared among the different groups from the three gut tracts using the Kruskal-Wallis test, followed by Dunn's post- tests. Regarding IHC, data obtained from different groups from the three intestinal tracts were analysed by nested one-way ANOVA. P-value <0.05 was considered significant. Gene expression data were analysed using GraphPad Prism v.8 (GraphPad Software, California, USA). Data were checked for population normality with the Kolmogorov-Smirnov test. Grubb's test (α = 0.05%) was used to determine and exclude potential outliers. One-way ANOVA or Kruskal-Wallis test was applied, followed by post hoc tests to identify significantly differences within groups. Statistical significance was accepted at P <0.05 level. In addition, in order to understand the effects and the differences of the different AMs protocols applied during the 3 zootechnical trials, a statistical analysis was conducted using alpha diversity metrics data, histological scores and gene expression data about biomarkers evaluation using GraphPad Prism v.8 (GraphPad Software, California, USA). To allow a comparison between the groups of the three trials, groups were paired according to the AM molecule administered:

- AMX vs AMX1,
- AMX + DCZ vs AMX2,
- THP vs THP1,
- THP + DCZ vs THP2,
- TRIM vs TRIM.

Alpha diversity metrics data were analysed using Mann Whitney tests and groups were compared according to abovementioned pairs. The remaining data were analysed by two-way ANOVA. Sidak's post-test was conducted when a significant p-value (< 0.05) was observed for interaction or trial effects. In this case, the groups treated with DCZ were separated from those without DCZ in order to conduct the abovementioned analyses.

3.3 Results

3.3.1 Microbiota analysis – Zootechnical trial 1

A total of 84 samples were analysed both for ileal (n = 42) and caecal (n = 42) microbiota by 16S rRNA sequencing. A total of 4,407,187 raw reads (2 × 300 bp) were obtained in caecum after sequencing, with an average value of 104,933 reads/sample. In ileum instead, a total of 3,618,011 raw reads (2 × 300 bp) were obtained after sequencing, with an average value of 86,143 reads/sample. After joint and quality filtering, a total of 3,331,206 and 2,637,370 reads, respectively for caecum and ileum, passed the filters applied through the DADA2 plugin in the QIIME2 software package, with an average value of 79,314 reads/sample in caecum and 62,795 in ileum. Following primer trimming and raw read quality filtering, ileal and caecal samples were analysed separately. In order to avoid biases due to different sequencing depths, ileal samples and caecal samples were rarefied at 31,100 reads and 5600 reads, respectively; one sample from ileum was excluded from further analyses due to a low read count. After assigning taxonomies, a total of 3421 unique operational taxonomic units (OTUs) at 99% nucleotide sequence identity were identified in caecum samples, while 1153 unique OTUs were identified in ileum samples.

Alpha diversity analysis has shown that AM treatments have different consequences on ileum and caecum microbiota. Considering caecal samples, Pielou's evenness ($H = 5.38$; $p = 0.5$) and Faith's phylogenetic diversity ($H = 7.69$; $p = 0.26$) indicated no significant differences between samples. Instead, ileum samples were characterised by alpha diversity metrics statistically different with Pielou's evenness ($H = 13.53$; $p = 0.03$) and Faith's phylogenetic diversity ($H = 13.12$; $p = 0.04$). In addition, the Permutational Multivariate Analysis of Variance (PERMANOVA) test has shown that beta diversity metrics were always statistically different between groups ($p = 0.001$ for caecum and $p = 0.002$ for ileum).

For caecal samples, the dominant phyla were in order: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, an unassigned bacterial phylum, and *Cyanobacteria*. At the family level, OTUs were assigned to an unassigned family of *Clostridiales* order, *Ruminococcaceae*, *Bacteroidaceae*, *Rikinellaceae*, and *Barnesiellaceae*. Relative abundances of the aforementioned taxa are shown in bar plots of Fig. 5 for caecum.

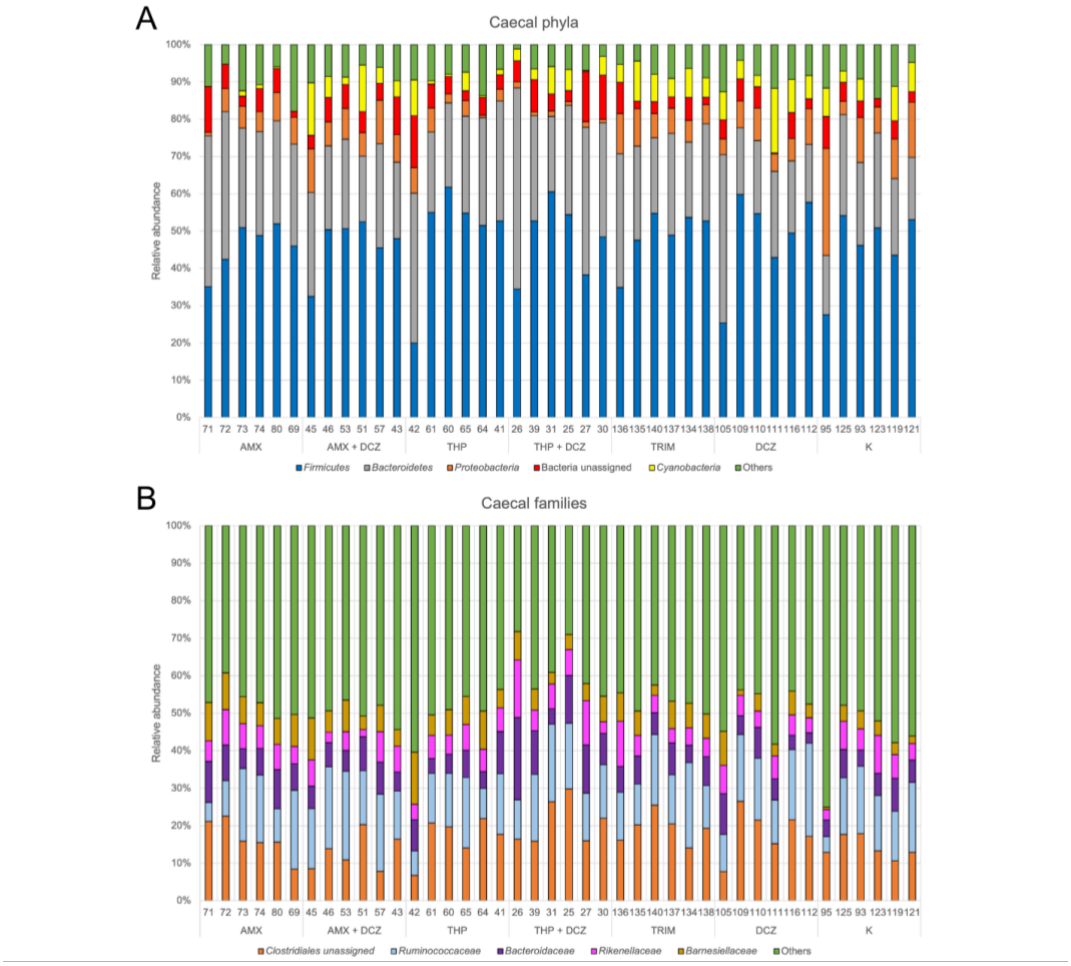

Figure 5. Relative abundance at phylum (A) and family level (B) in caecum of broilers from Trial 1. Specific legends are reported for each bar plot.

Among ileal samples, the main five phyla were in order: *Firmicutes*, *Proteocbacteria*, *Bacteroidetes*, *Cyanobacteria* and an unassigned bacterial phylum. At the family level, the main taxa identified were, respectively: *Turibacteriaceae*, an unassigned family of *Clostridiales* order, *Enterococcaceae*, *Clostridiaceae*, and *Peptostreptococcaceae*. Relative abundances of the aforementioned taxa are shown in bar plots of Fig. 6 for ileum.

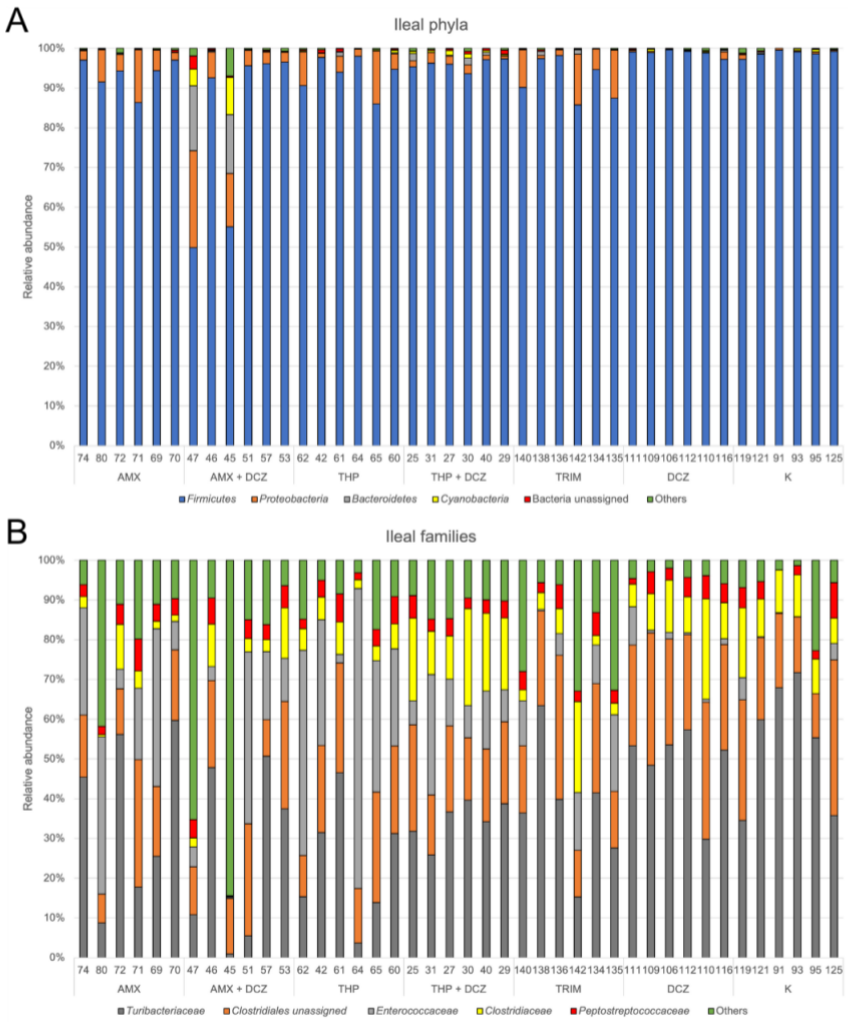

Figure 6. Relative abundance at phylum (A) and family level (B) in ileum of broilers from Trial 1. Specific legends are reported for each bar plot.

Two-way ANOVA was performed on the abundance of OTU values belonging to the aforementioned phyla and families. Related p values are presented in Tab. 6.

Source of variation	p-values			
	Phyla		Families	
	Caecum	Ileum	Caecum	Ileum
Interaction	0.92	0.31	0.32	< 0.0001
Groups	0.64	0.32	0.81	0.17
Taxa	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Table 6. p-values summary from two-way ANOVA analysis of the top five phyla and families in caecal and ileal samples of trial 1.

The results show that an effect of the studied taxa was always significant on the total variance of the samples. Box plots are shown in Fig. 7 (caecum) and Fig. 8 (ileum). Most intriguingly, in Dunn's post-test the ileal families *Enterococcaceae* have been shown to be significantly overrepresented in our samples in amoxicillin group (p-value=0.004) and in thiamphenicol group (THP) (p-value < 0.0001) groups in comparison to control group K.

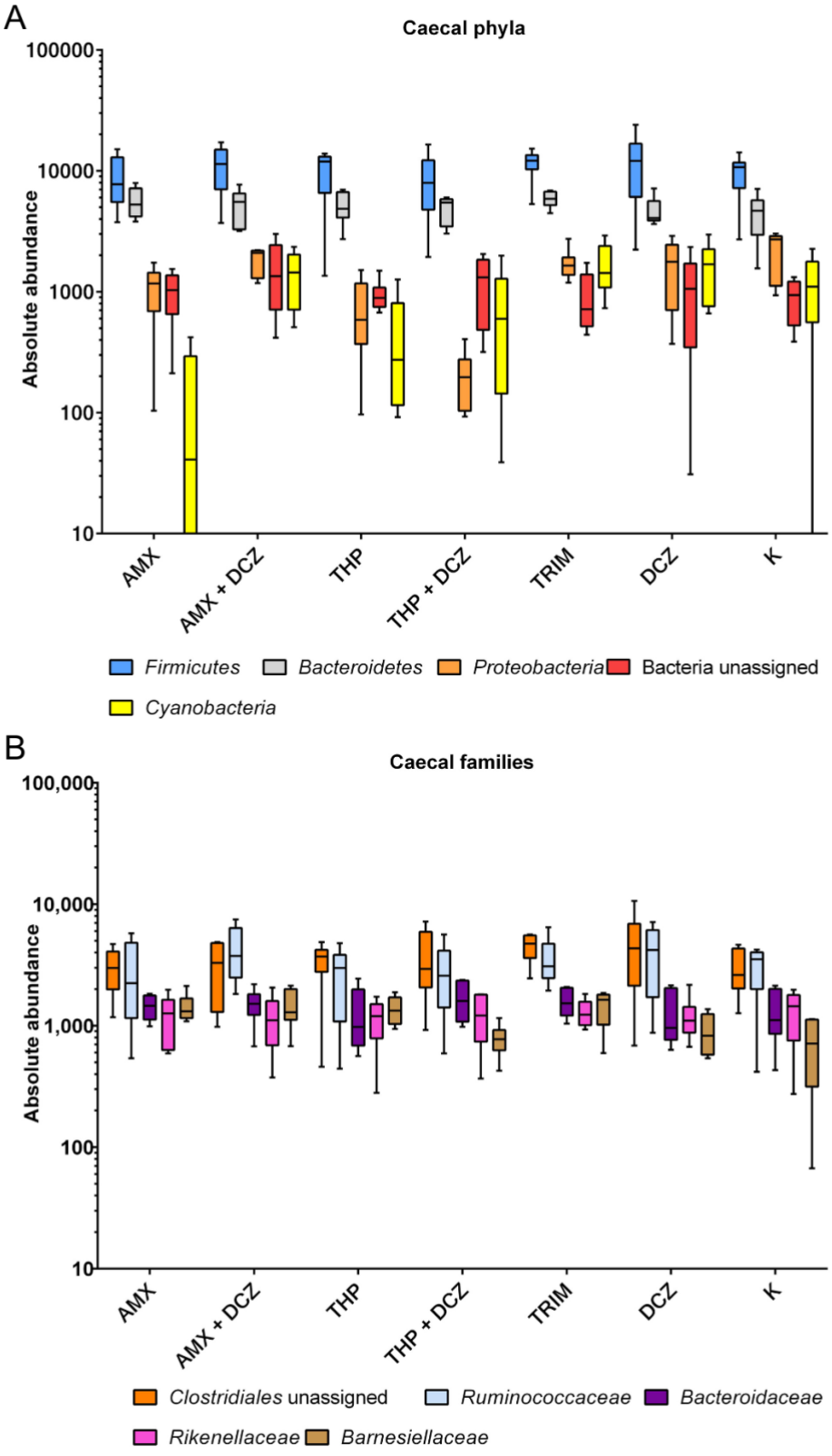

Figure 7. Box plots of two-way ANOVA analysis for caecal phyla (A) and families (B) of Trial 1.

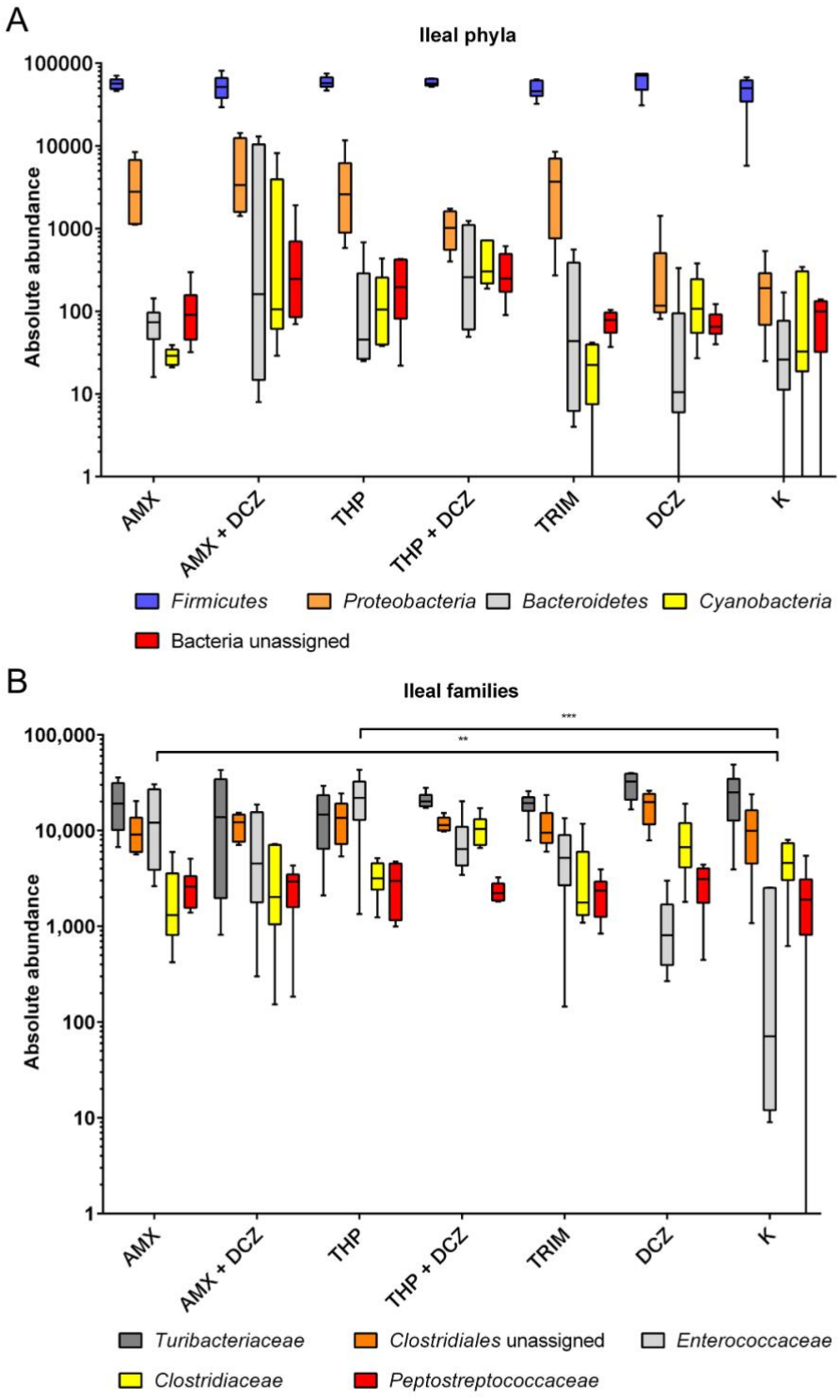

Figure 8. Box plots of two-way ANOVA analysis for ileal phyla (A) and families (B) of Trial 1. (** p-value < 0.01; *** p-value < 0.001).

3.3.2 Microbiota analysis – Zootechnical trial 3

The same protocol analysis was performed on ileal and caecal samples of Trial 3. A total of 72 samples were analysed both for ileal (n = 36) and caecal (n = 36) microbiota by 16S rRNA sequencing. A total of 4,536,635 raw reads (2 × 300 bp) for caecum and 2,771,519 for ileum were obtained after sequencing, with an average value of 126,018 reads/sample in caecum and 76,209 in ileum. After joint and quality filtering, a total of 2,743,953 for caecum and 1,706,441 for ileum reads passed the filters applied through the DADA2 plugin in the QIIME2 software package, with an average value of 76,209 reads/sample in caecum and 47,401 in ileum. Following primer trimming and raw read quality filtering, ileal and caecal samples were analysed separately. In order to avoid biases due to different sequencing depths, ileal samples and caecal samples were rarefied at 34,371 reads and 27,650 reads, respectively; one sample from ileum was excluded from further analyses due to a low read count. After assigning taxonomies, a total of 2,593 unique operational taxonomic units (OTUs) at 99% nucleotide sequence identity were identified in caecum samples, while 3,028 unique OTUs were identified in ileum samples.

Alpha diversity analysis has shown that AM treatments have different consequences on ileum and caecum microbiota. Considering caecal samples, Pielou's evenness ($H = 16.41$; $p = 0.006$) and Faith's phylogenetic diversity ($H = 4.52$; $p = 0.48$) highlighted no significant differences between samples. Instead, ileum samples were characterised by alpha diversity metrics statistically different with Pielou's evenness ($H = 14.58$; $p = 0.01$) and Faith's phylogenetic diversity ($H = 8.39$; $p = 0.14$). In addition, the Permutational Multivariate Analysis of Variance (PERMANOVA) test has shown that beta diversity metrics were always statistically different between groups ($p = 0.001$ for caecum and $p = 0.056$ for ileum).

For caecal samples, the dominant phyla were in order: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Synergistetes*, and *TM7*. At the family level, OTUs were assigned to *Ruminococcaceae*, an unassigned family of *Bacteroidales* order, *Bacteroidales S24-7, Lachnospiraceae*, and an unassigned family of *Clostridiales* order. Relative abundances of the aforementioned taxa are shown in bar plots of Fig. 9 for caecum.

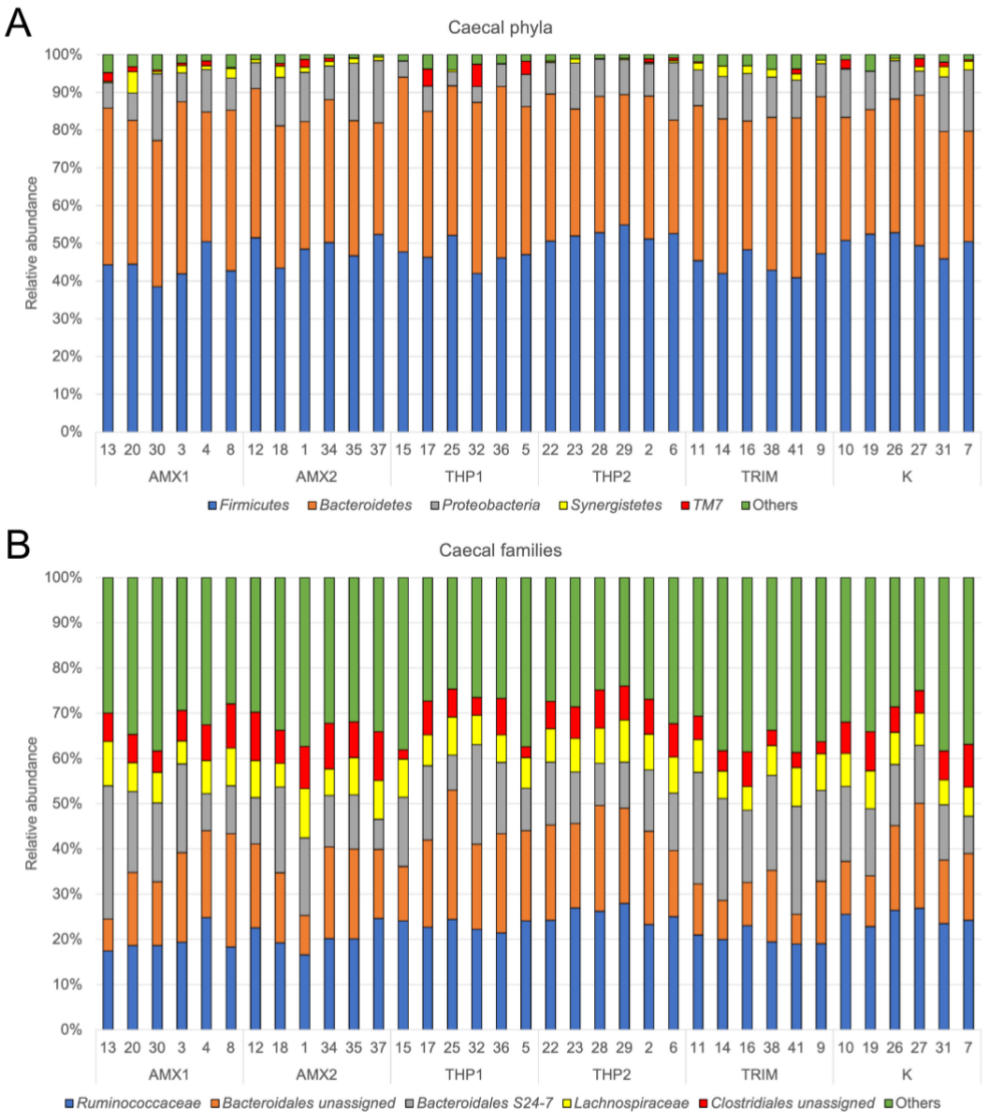

Figure 9. Relative abundance at phylum (A) and family level (B) in caecum of broilers from Trial 3. Specific legends are reported for each bar plot.

Among ileal samples, the main five phyla were in order: *Firmicutes*, *Proteocbacteria*, *Bacteroidetes*, an unassigned bacterial phylum and *Actinobacteria*. At the family level, the main taxa identified were, respectively: *Peptostreptococcaceae, Campylobacteraceae,* an unassigned family of *Bacillales* order, *Clostridiaceae*, and a second unassigned family of *Bacillales* order. Relative abundances of the aforementioned taxa are shown in bar plots of Fig. 10 for ileum.

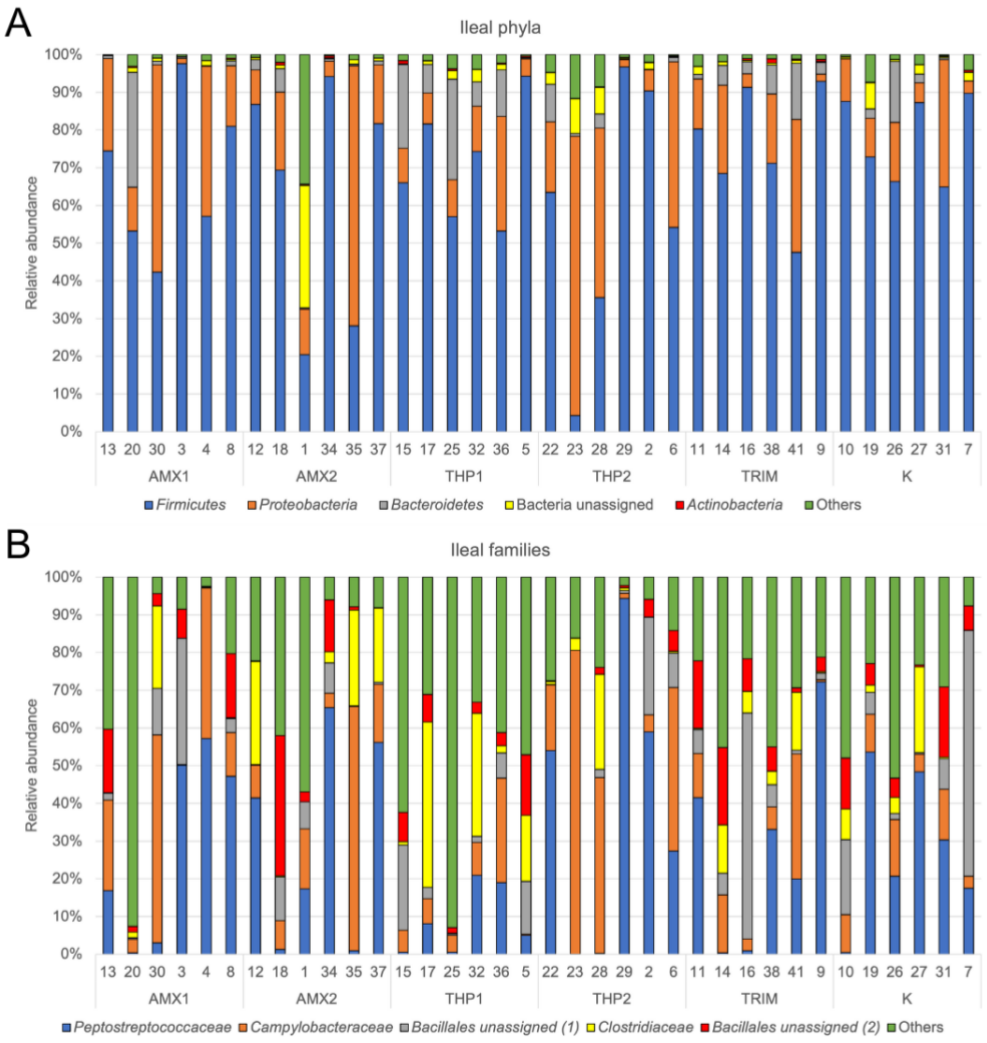

Figure 10. Relative abundance at phylum (A) and family level (B) in ileum of broilers from Trial 3. Specific legends are reported for each bar plot.

Two-way ANOVA was performed on the abundance of OTU values belonging to the aforementioned phyla and families. Related p values are presented in Tab. 7.

Source of variation	p-values			
	Phyla		Families	
	Caecum	Ileum	Caecum	Ileum
Interaction	< 0.0001	0.0653	< 0.0001	0.5547
Groups	0.0024	0.0003	0.0067	0.0164
Taxa	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Table 7. p-values summary from two-way ANOVA analysis of the top five phyla and families in caecal and ileal samples of trial 3.

From broilers of Trial 3, many significant differences in microbiota population were observed. In particular, most significant results were from caecum (Fig. 11):

- *Firmicutes* were overrepresented in AMX2 (p-value < 0.0001), THP1 (p-value = 0.0018), THP2 (p-value < 0.0001) and TRIM (p-value = 0.0034) in comparison to control group K;
- *Bacteroidetes* were overrepresented in AMX1 (p-value = 0.0001), AMX2 (p-value < 0.0001), THP1 (p-value < 0.0001), THP2 (p-value = 0.0002) and TRIM (p-value < 0.0001) in comparison to control group K;
- *Ruminococcaceae* were overrepresented in AMX2 (p-value = 0.0365) and THP2 (p-value = 0.0001) in comparison to control group K;
- An unassigned family of *Bacteriodales* order was overrepresented in AMX2 (p-value = 0.0093), THP1 (p-value = 0.0016) and THP2 (p-value = 0.0002) in comparison to control group K;
- *Bacteroidales S24-7* were overrepresented in AMX1 (p-value = 0.0166) and TRIM (p-value < 0.0001) in comparison to control group K;
- An unassigned family of *Clostridiales* order was overrepresented in AMX2 (p-value = 0.0482) in comparison to control group K.

In ileum of broilers from trial 3 (Fig. 12), only *Firmicutes* were significantly overrepresented in group TRIM (p-value < 0.0001) in comparison to control group K.

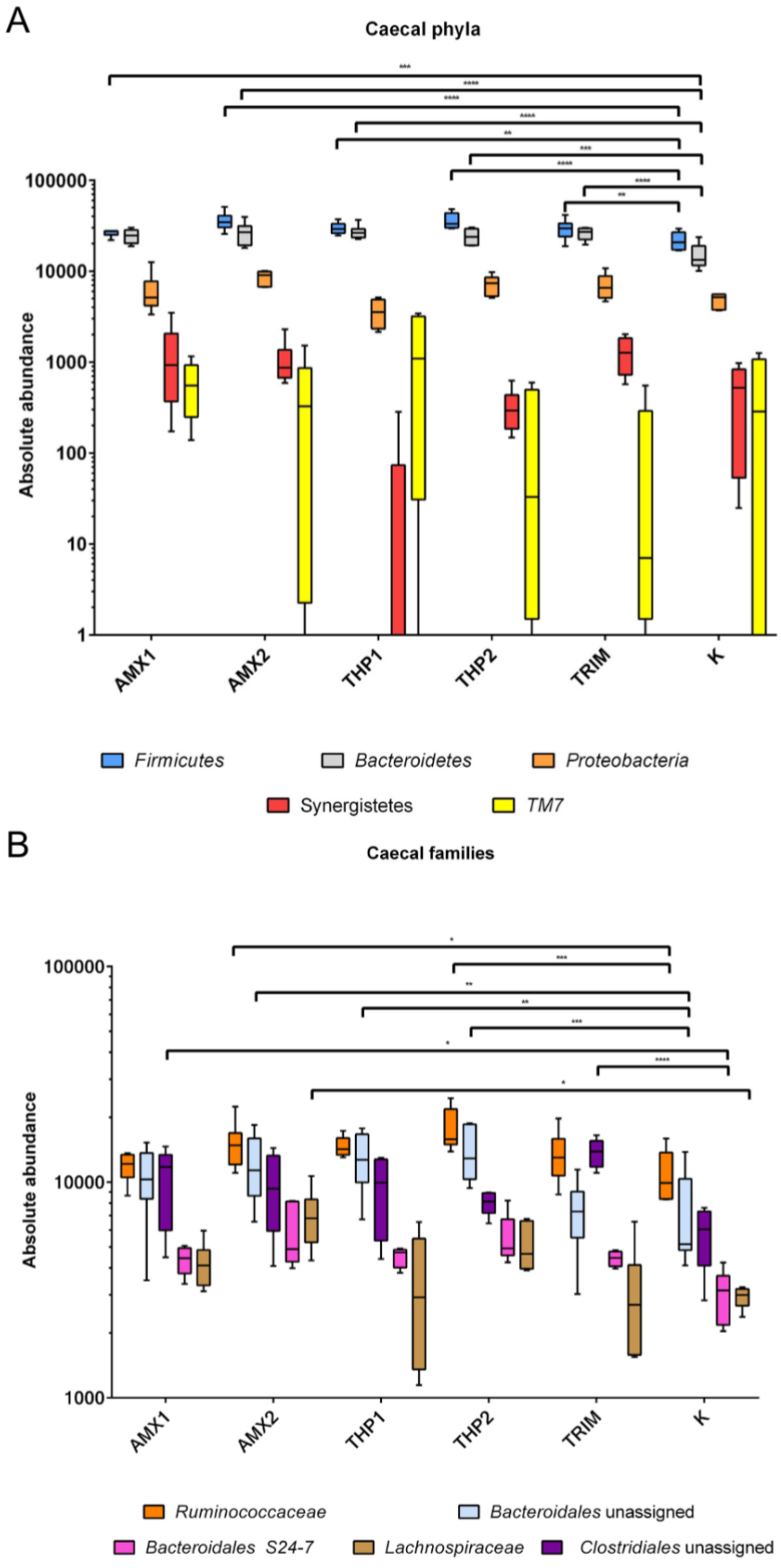

Figure 11. Box plots of two-way ANOVA analysis for caecal phyla (A) and families (B). (* p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001; **** p-value < 0.0001).

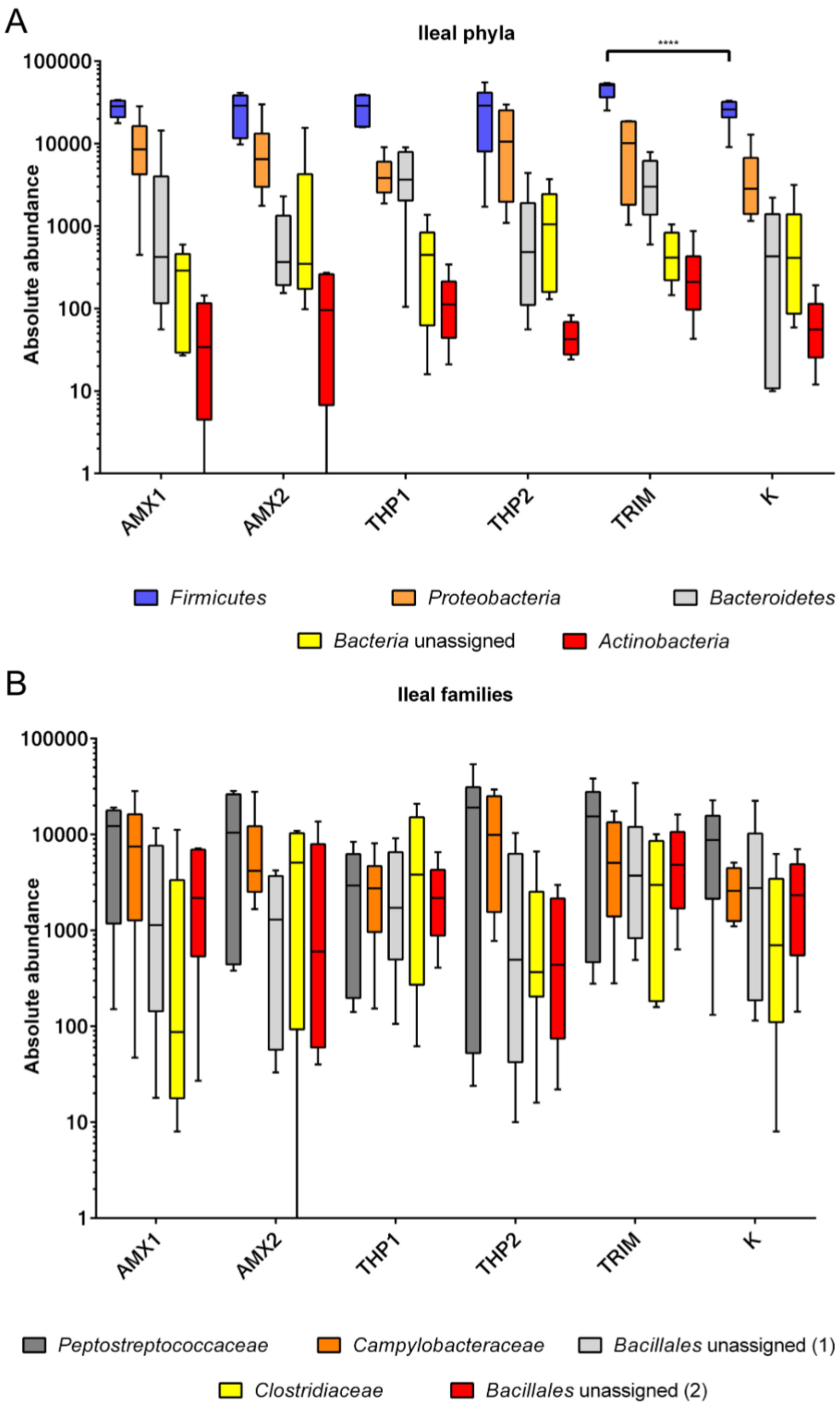

Figure 12. Box plots of two-way ANOVA analysis for ileal phyla (A) and families (B). (**** p-value < 0.0001).

3.3.3 Histopathology of gut barrier – Zootechnical trial 1

In this study, the effects of different AM prophylaxis protocols on the gut health of treated broilers were evaluated. Histopathological evaluation revealed that intestinal villi of the AM-treated groups were characterised by epithelial detachment and fusion often associated with lymphocytic infiltration (Fig. 13). Moreover, several samples were characterised by hyperaemia and reactivity of caecal tonsils. Histological features of the intestinal tracts analysed are showed in Fig. 14. A list of p-values generated by Kruskal Wallis analyses is presented in Tab. 8.

Intestinal tract	Epithelial lesions	Mucosa infiltrate	Submucosa infiltrate
Ileum	< 0.0001	0.0141	0.0063
Caecum	0.0002	0.0139	0.0031
Colon	0.0007	0.043	0.0064

Table 8. Summary results (*p*-value) of **HE** histology analysed by Kruskal Wallis test.

Results of total scores analyses of histology evaluation (epithelial lesions and mucosa/submucosa infiltration) are presented in the box plots of Fig. 15. Bars and asterisks point out significant differences revealed by post-tests. Intriguingly, THP and AMX groups most frequently showed statistically significant increased score for the epithelial lesions and severe and diffuse lymphocytic infiltration in the mucosa and submucosa of all intestinal tracts. In addition, a significantly increased lymphocytic infiltrate was also found in the mucosa and submucosa of animals belonging to DCZ group in all the examined tracts.

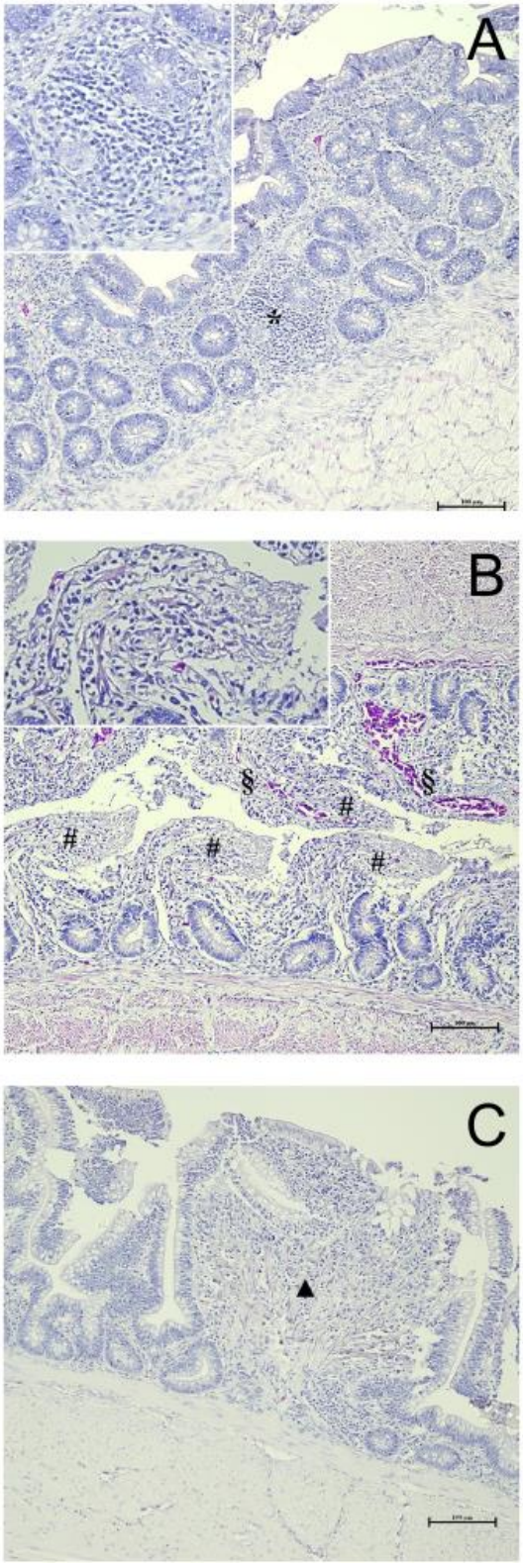

Figure13. Main histological gut findings in AM-treated groups. (A) Focal lymphocytic infiltration (*) of the mucosa (HE, 200x – insert 400x). (B) Diffuse and severe epithelial detachment (#) associated with hyperemia (§) (HE, 200x – insert 400x). (C) Focal epithelial fusion of the villi (▲) (HE, 200x – insert 400x).

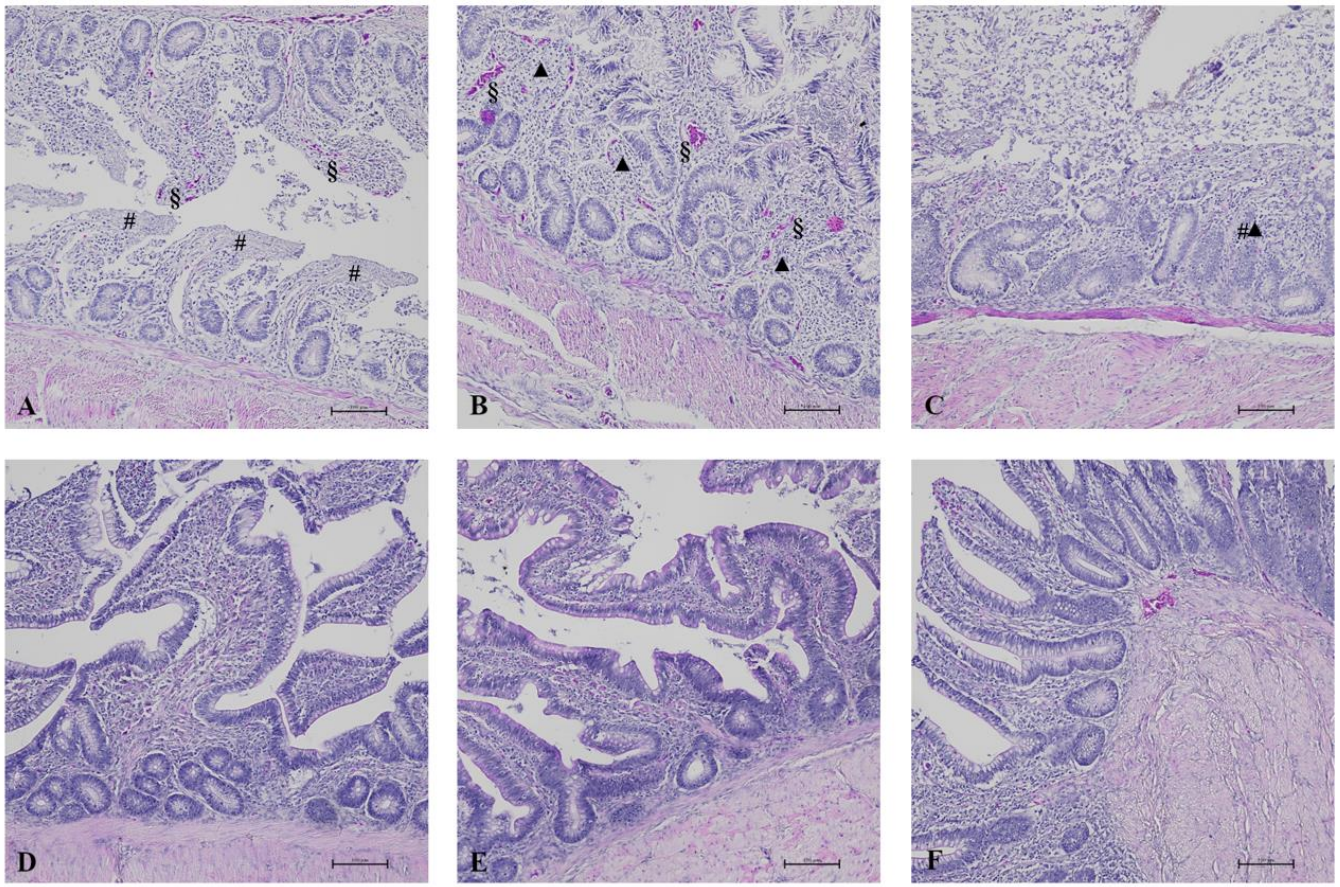

Figure 14. HE results in broilers of zootechnical trial. In AM-treated groups, ileum (A) showed diffuse and severe epithelial detachment (#) associated with hyperaemia (§) (HE, 100x) and caecum (B) showed severe and diffuse epithelial fusion (▲) of the villi associated with focal epithelial detachment of the villi (#) and hyperaemia (§) (HE,100x). Colon (C) was characterised by severe and diffuse epithelial fusion of the villi (▲) associated with severe and diffuse epithelial detachment of the villi (#) (HE, 100x). These HE results were compared to the normal aspect of intestinal villi and epithelium of the respective intestinal tract observed in K group (D–F

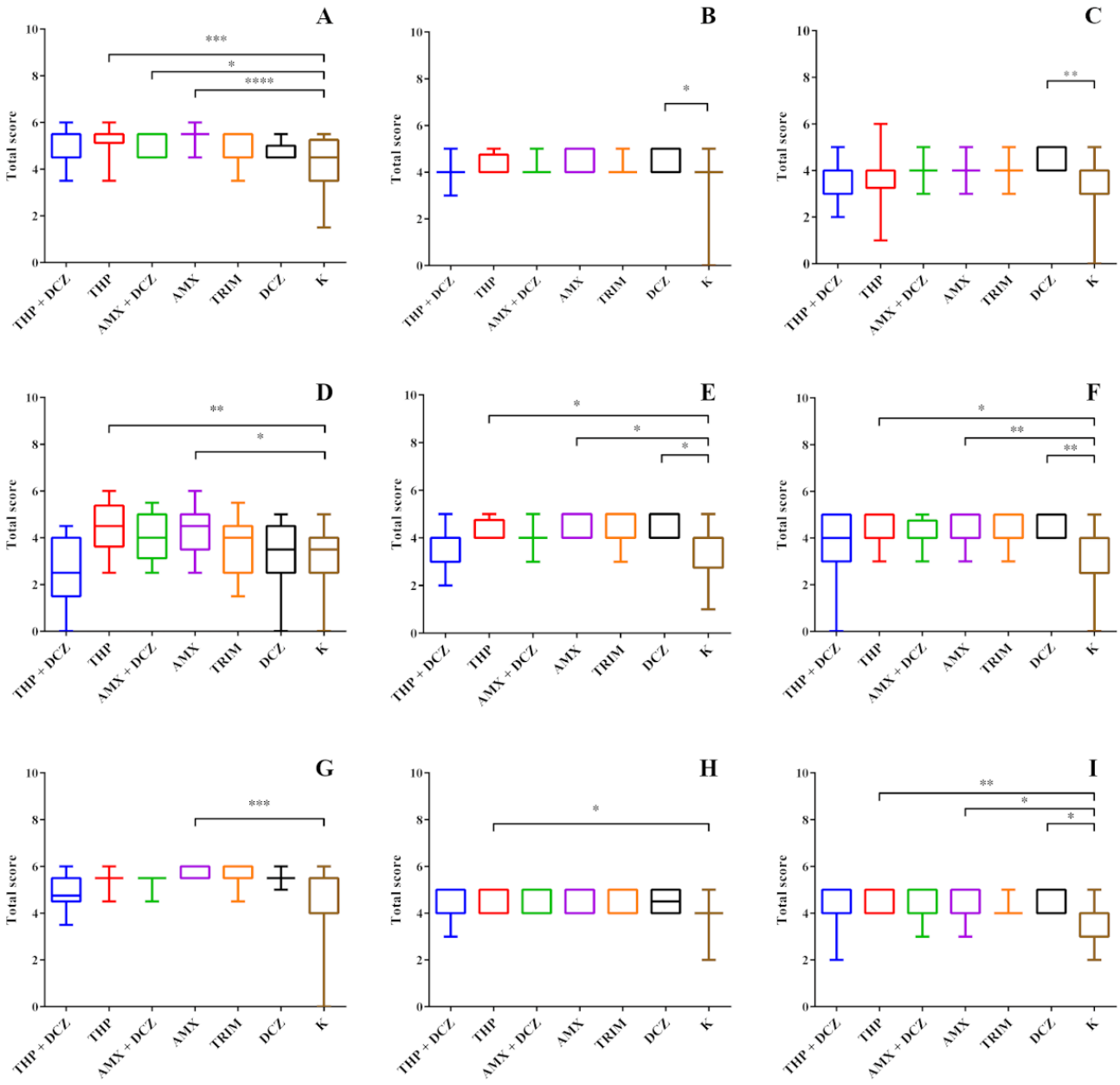

Figure 15. Statistical analysis of HE scores. Box plot graphs were generated for ileum (A, D, G), caecum (B, E, H) and colon (C, F, I). The data for the villi total scores (A–C), the lymphocyte score in the mucosa (B, E, H) and the lymphocyte score in the submucosa (C, F, I) are, respectively, shown. Results were considered significant with p-value < 0.05 (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).

3.3.4 Histopathology of gut barrier – Zootechnical trial 3

Similar lesions to the previous trial were observed in broilers of trial 3 (Fig. 16). The intestinal villi of the AM-treated groups were characterised by epithelial detachment and fusion. However, necrosis was also observed more frequently than broilers in trial 1. In addition, the intestinal mucosa and submucosa were in this case characterised by inflammatory infiltrate diffused in all the three tracts analysed too. Differently from trial 1, the inflammatory population was mixed with lymphocytes and eosinophils. Moreover, several samples were characterised by hyperaemia and reactivity of caecal tonsils. Another lesion recorded was the presence of apical haemorrhages in the mucosa. A list of p-values generated by Kruskal Wallis analyses is presented in Tab. 9.

Intestinal tract	Epithelial lesions	Mucosa infiltrate	Submucosa infiltrate
Ileum	0.8634	0.1547	0.4519
Caecum	0.4978	0.0243	0.1083
Colon	0.9409	0.0383	0.2998

Table 9. Summary results (*p*-value) of **HE** histology analysed by Kruskal Wallis test.

The results of total scores analysed of histology evaluation (epithelial lesions and mucosa/submucosa infiltration) are presented in the box plots of Fig. 17. In particular, the total score for the lymphocyte inflammatory infiltrate in the colon mucosa was significantly higher in group AMX1 (Dunn's post-test p-value = 0,018) in comparison with group K.

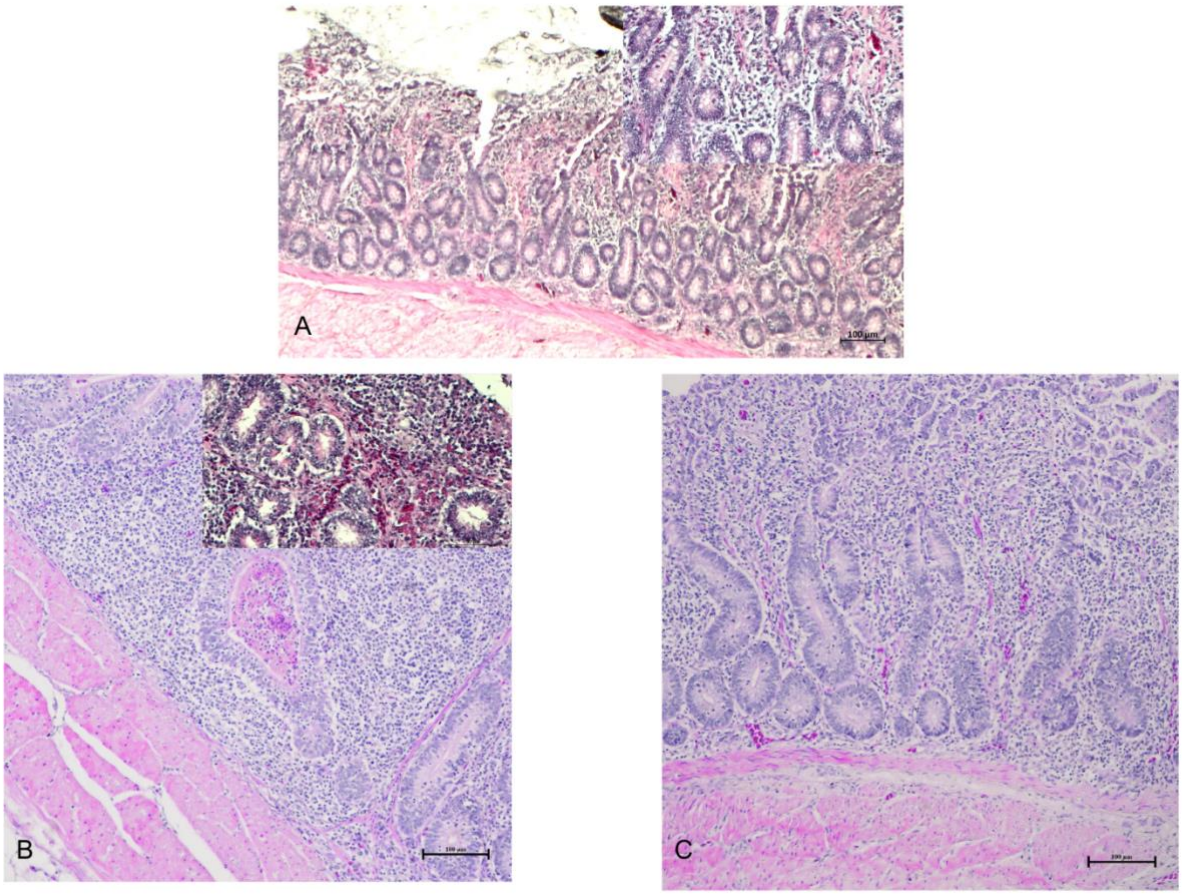

Figure 16. Main histological gut findings in AM-treated groups of trial 3. (A) Diffuse and severe villi fusion with lymphocytic infiltration of the ileal mucosa (HE, 200x – insert 400x). (B) Diffuse and severe villi fusion associated with hyperaemia and mixed (lymphocytes and eosinophils) inflammatory population in the caecum (HE, 200x – insert 400x). (C) Focal epithelial fusion of the villi in the colon (HE, 200x – insert 400x).

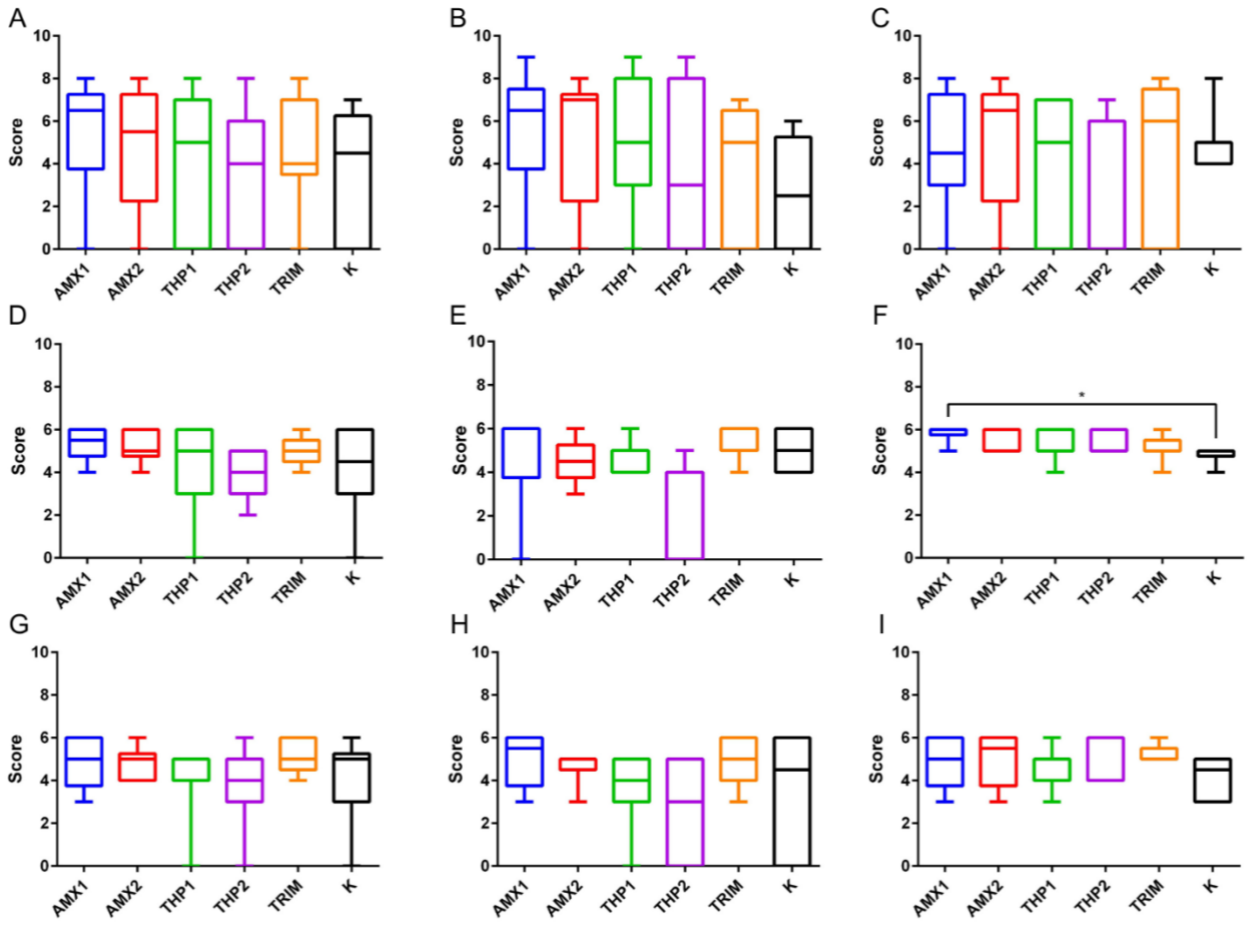

Figure 17. Statistical analysis of HE scores. Box plot graphs were generated for ileum (A, D, G), caecum (B, E, H) and colon (C, F, I). The data for the villi total scores (A–C), the lymphocyte score in the mucosa (B, E, H) and the lymphocyte score in the submucosa (C, F, I) are, respectively, shown. Results were considered significant with p-value < 0.05 (*p < 0.05)

3.3.5 Immunohistochemistry of tight-junction proteins

IHC evaluation of TJ proteins was performed only in intestinal samples of broiler from trial 1. Results for the expression of claudin-3 and ZO-1 proteins evaluated by IHC in intestinal samples are summarised in Fig. 18. No significant differences between treated groups and controls were revealed for both proteins.

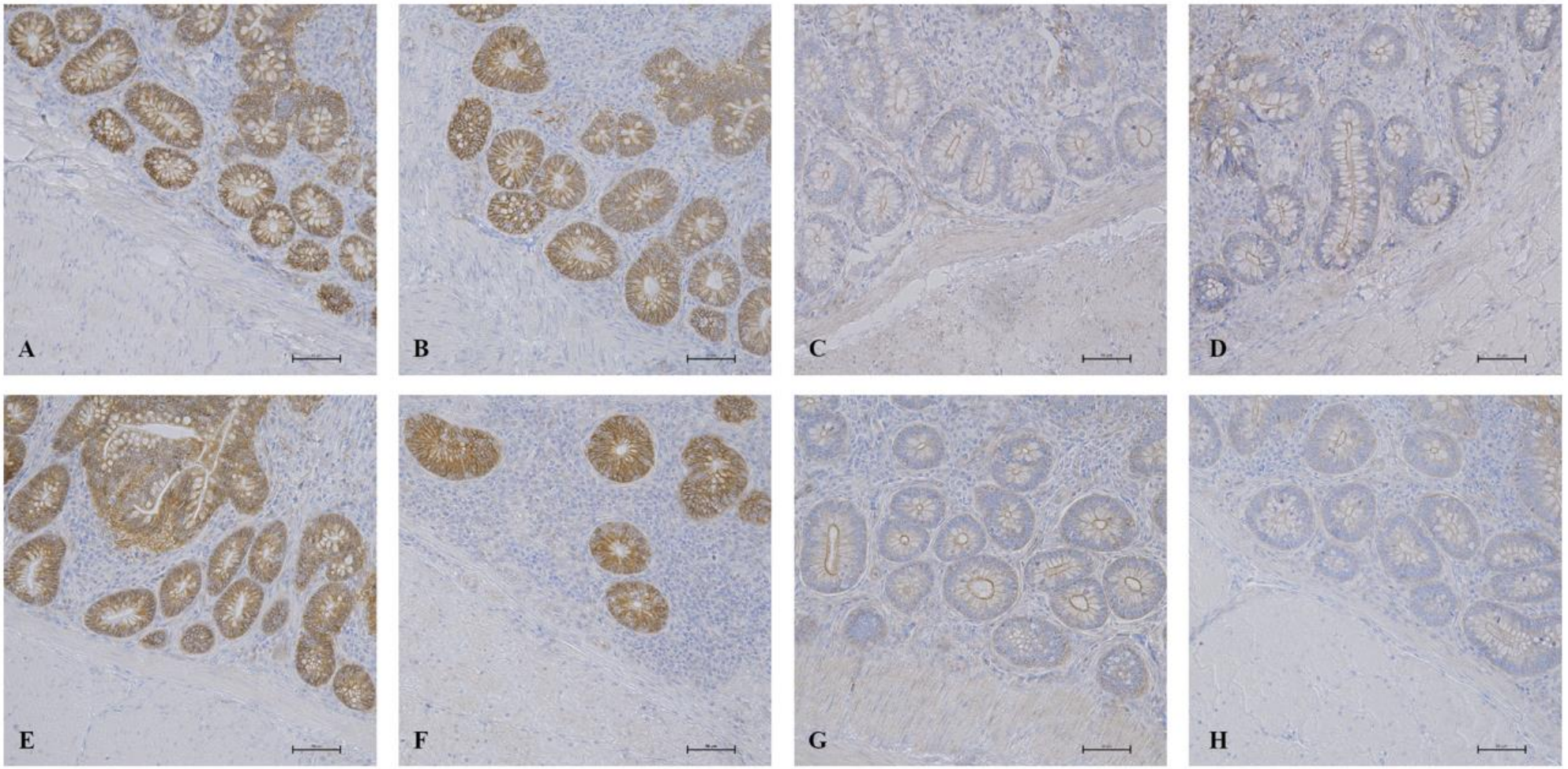

Figure 18. IHC results in the intestinal mucosa. Histological pictures of claudin-3 in ileum (A) and cecum (B) of AM-treated groups and in ileum (E) and caecum (F) of K group. ZO-1 expression in ileum (C) and caecum (D) of AM-treated groups and in ileum (G) and caecum (H) of K group. IHC, 200x.

3.3.6 Gene expression assay of tight-junction proteins

In ileum and caecum of broilers from trial 3, several targets from tight-junction proteins were evaluated in qPCR. According to geNorm validation, the optimal number of RGs was two, both in ileum (V2/3 = 0.1) and caecum (V2/3 = 0.079). Among claudins, only claudin-1 was significantly downregulated in caecum samples according to the Kruskal-Wallis test (p-value = 0.0494). In particular, the Dunn's post-test revealed that the downregulation of claudin-1 was significant in broilers belonging to THP2 group in comparison with control group K (p-value = 0.0326). Detailed results of the statistical analysis for TJ gene expression data are presented for the caecum (Fig. 19) and ileum (Fig. 20).

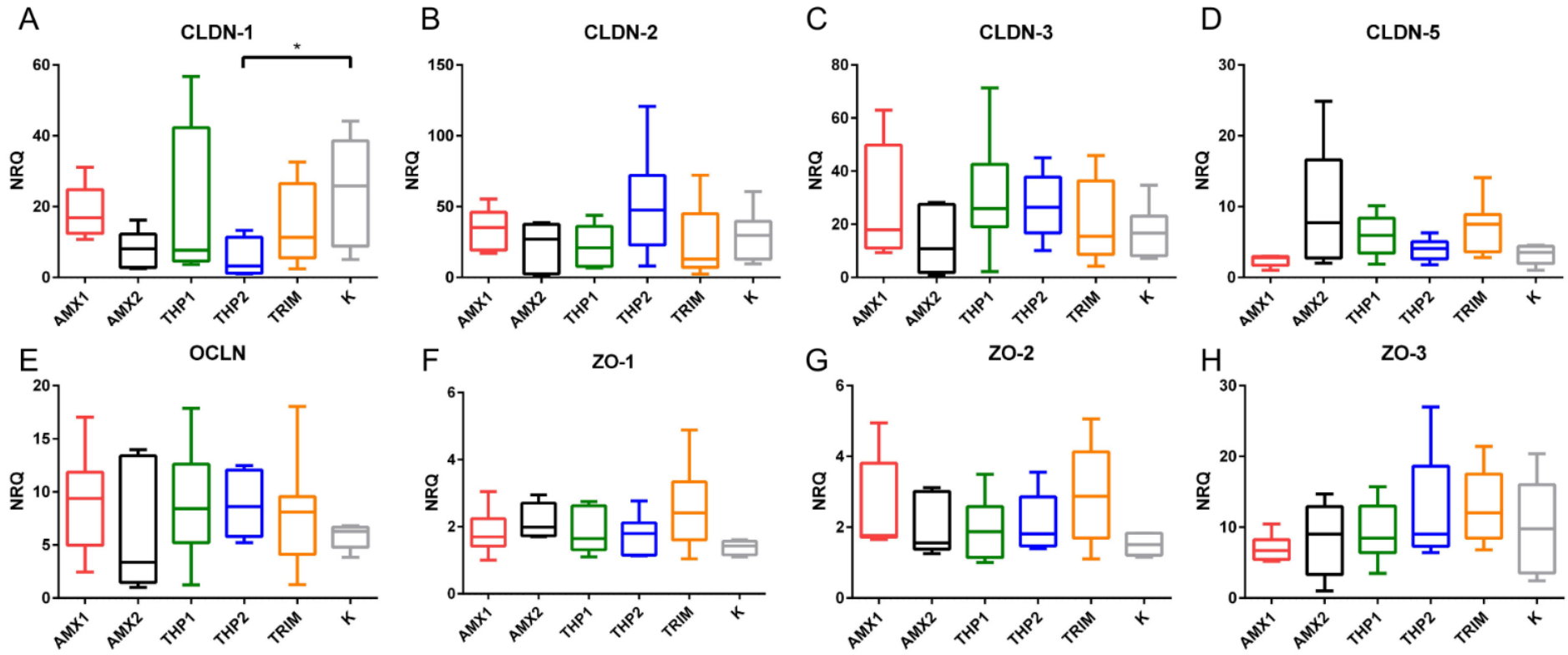

Figure 19. Statistical analysis of TJ gene expression in the caecum of broiler from trial 3. Values are expressed as normalised relative quantity (NRQ) using qPCR. Box plot graphs were generated for claudin-1 (CLDN-1), claudin-2 (CLDN-2), claudin-3 (CLDN-3), claudin-5 (CLDN-5), occludin (OCLN), zonula occludens-1 (ZO-1), zonula occludens-2 (ZO-2) and zonula occludens-3 (ZO-3). The results were considered significant with p-value < 0.05 (*p < 0.05).

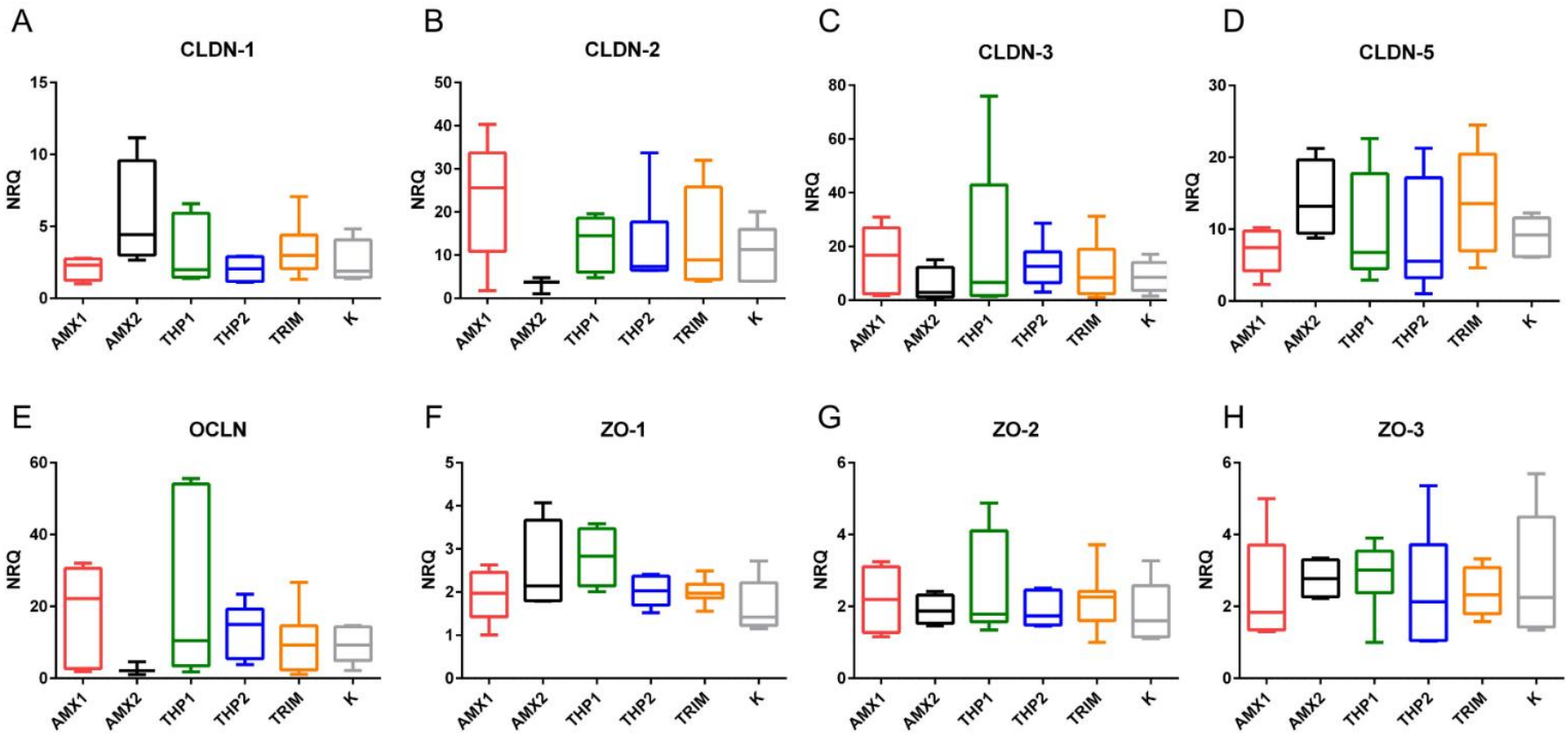

Figure 20. Statistical analysis of TJ gene expression in the ileum of broiler from trial 3. Values are expressed as normalised relative quantity (NRQ) using qPCR. Box plot graphs were generated for claudin-1 (CLDN-1), claudin-2 (CLDN-2), claudin-3 (CLDN-3), claudin-5 (CLDN-5), occludin (OCLN), zonula occludens-1 (ZO-1), zonula occludens-2 (ZO-2) and zonula occludens-3 (ZO-3).

3.3.7 Biomarkers evaluation

To confirm RNA-seq results obtained in a previous study (Giannuzzi et al., 2021), five genes in the liver (GPR27, DIO2, RHOB, PDK4, and IGFBP1) and two genes in PM muscle (ELOVL1 and CERS2) were selected for qPCR or ddPCR analysis in samples collected in trial 2 and trial 3. According to the previous results, HMBS and TBP were selected as RGs in qPCR for liver targets. Differently, only HMBS was selected in ddPCR for PM muscle targets. Prior to test PM samples in ddPCR, the protocol was set up on samples from trial 1. The results were in line with qPCR ones, already published (Giannuzzi et al., 2021). Gene expression results were analysed with Kruskal-Wallis test (p-value = 0,0362). As shown in Fig. 21, Dunn's post test revealed that broilers from AMX + DCZ, THP + DCZ and TRIM have a significant downregulation of ELOVL1 (p-value < 0.05). Differently, ELOVL1 downregulation was not confirmed in trial 3. Considering PM muscle target CERS2, the expression was not significantly influenced by AMs treatments in trial 1 and 3. In liver of trial 2 and 3, all genes analysed in qPCR did not show significant difference in the expression comparing treated groups with control group K (Fig. 22).

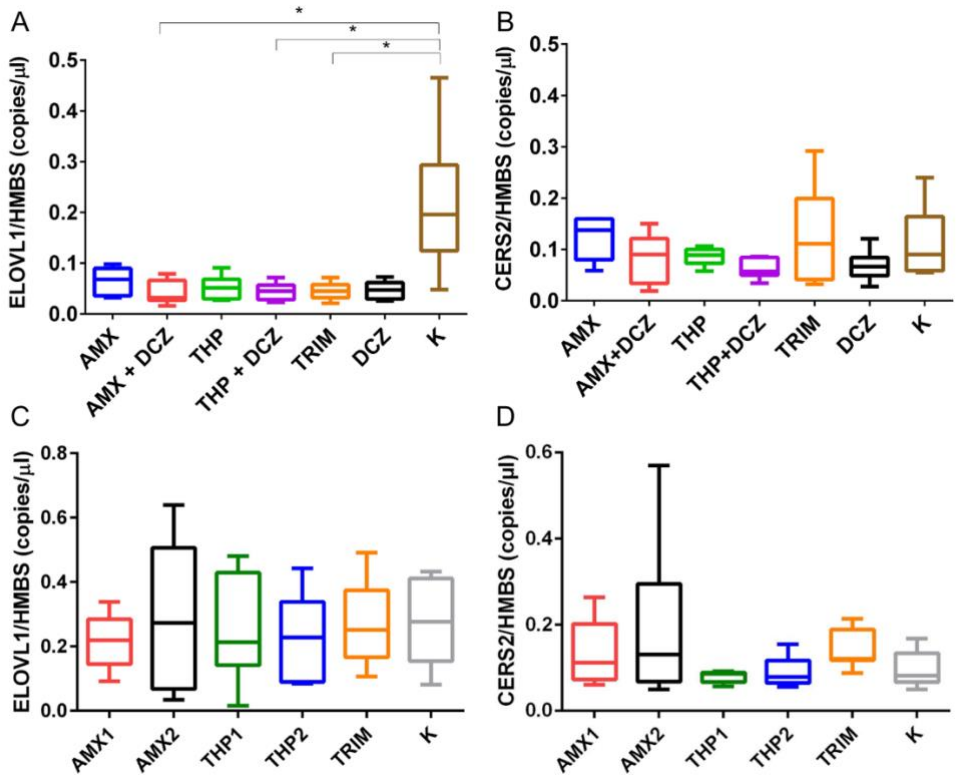

Figure 21. Statistical analysis of ELOVL1 and CERS2 gene expression in the PM muscle of broiler from trial 1 (A and B) and 3 (C and D). Values are expressed as copies/µl of target/reference gene using ddPCR. Results were considered significant with p-value < 0.05 (*p < 0.05).

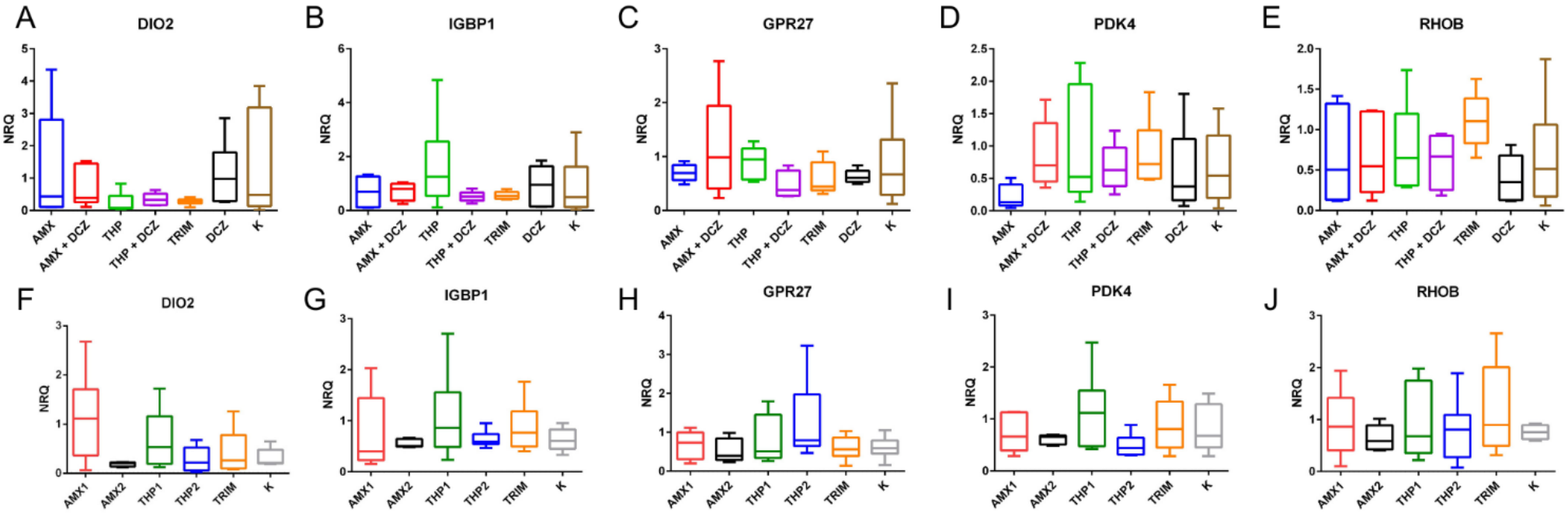

Figure 22. Statistical analysis of liver gene expression of broiler from trial 2 (A, B, C, D and E) trial 3 (F, G, H, I and J). Values are expressed as normalized relative quantity (NRQ) using qPCR. Box plot graphs were generated for DIO2, IGBP1, GPR27, PDK4 and RHOB.

3.3.8 Zootechnical trials comparison

Alpha diversity data, both for Pielou's evenness and Faith phylogenetic diversity, were analysed separately for each comparison pairs. Results are summarised in Fig. 23 for caecum and Fig. 24 for ileum. Considering Pielou's evenness in ileal samples, a significantly reduced index was observed in trial 3 in comparison with trial 1:

- in group AMX1 from trial 3 in comparison with AMX group from trial 1,
- in group THP1 from trial 3 in comparison with THP group from trial 1,
- in group THP2 from trial 3 in comparison with THP + DCZ group from trial 1.

An opposite situation was observed considering Faith PD in ileal samples with a significantly increased index in trial 3 in comparison with trial 1:

- in group THP1 from trial 3 in comparison with THP group from trial 1,
- in group THP2 from trial 3 in comparison with THP + DCZ group from trial 1.

In caecum instead, Pielou's evenness index was significantly reduced index in trial 3 in comparison with trial 1:

- in group AMX2 from trial 3 in comparison with AMX + DCZ group from trial 1,
- in group THP2 from trial 3 in comparison with THP + DCZ group from trial 1.

Finally, a different situation was observed considering Faith PD in caecal samples in trial 3 in comparison with trial 1:

- an increased index in group THP2 from trial 3 in comparison with THP + DCZ group from trial 1.
- a reduced index in group TRIM3 from trial 3 in comparison with TRIM1 group from trial 1.

Histological scores and gene expression data were, instead, analysed using two-way ANOVA test. Results are showed as box plot graphs for ileum (Fig. 25), caecum (Fig. 26) and colon (Fig. 27). Intriguingly, a significantly higher score in histological lesions was observed in trial 3 in comparison with trial 1:

- in caecum of TRIM3 group from trial 3 in comparison with TRIM1 group from trial 1,
- in caecum of DCZ/K group from trial 3 in comparison with K group from trial 1,
- in colon of AMX1 group from trial 3 in comparison with AMX group from trial 1,
- in colon of THP1 group from trial 3 in comparison with THP group from trial 1,
- in colon of TRIM3 group from trial 3 in comparison with TRIM1 group from trial 1,
- in colon of K group from trial 3 in comparison with K group from trial 1,

- in colon of AMX2 group from trial 3 in comparison with AMX + DCZ group from trial 1,
- in colon of THP2 group from trial 3 in comparison with THP + DCZ group from trial 1.

Finally, gene expression data for liver biomarkers are presented in Fig. 28 and for PM muscle in Fig. 29. No statistically significant differences were observed in liver gene expression data. In PM muscle samples, a statistically significant higher expression for ELOVL1 was observed:

- in THP1 group from trial 3 in comparison with THP group from trial 1,
- in TRIM3 group from trial 3 in comparison with TRIM1 group from trial 1,
- in AMX2 group from trial 3 in comparison with AMX + DCZ group from trial 1,
- in THP2 group from trial 3 in comparison with THP + DCZ group from trial 1.
- in K group from trial 3 in comparison with DCZ group from trial 1.

A summary of the main results related this research line is shown in ANNEX B – Figure 1.

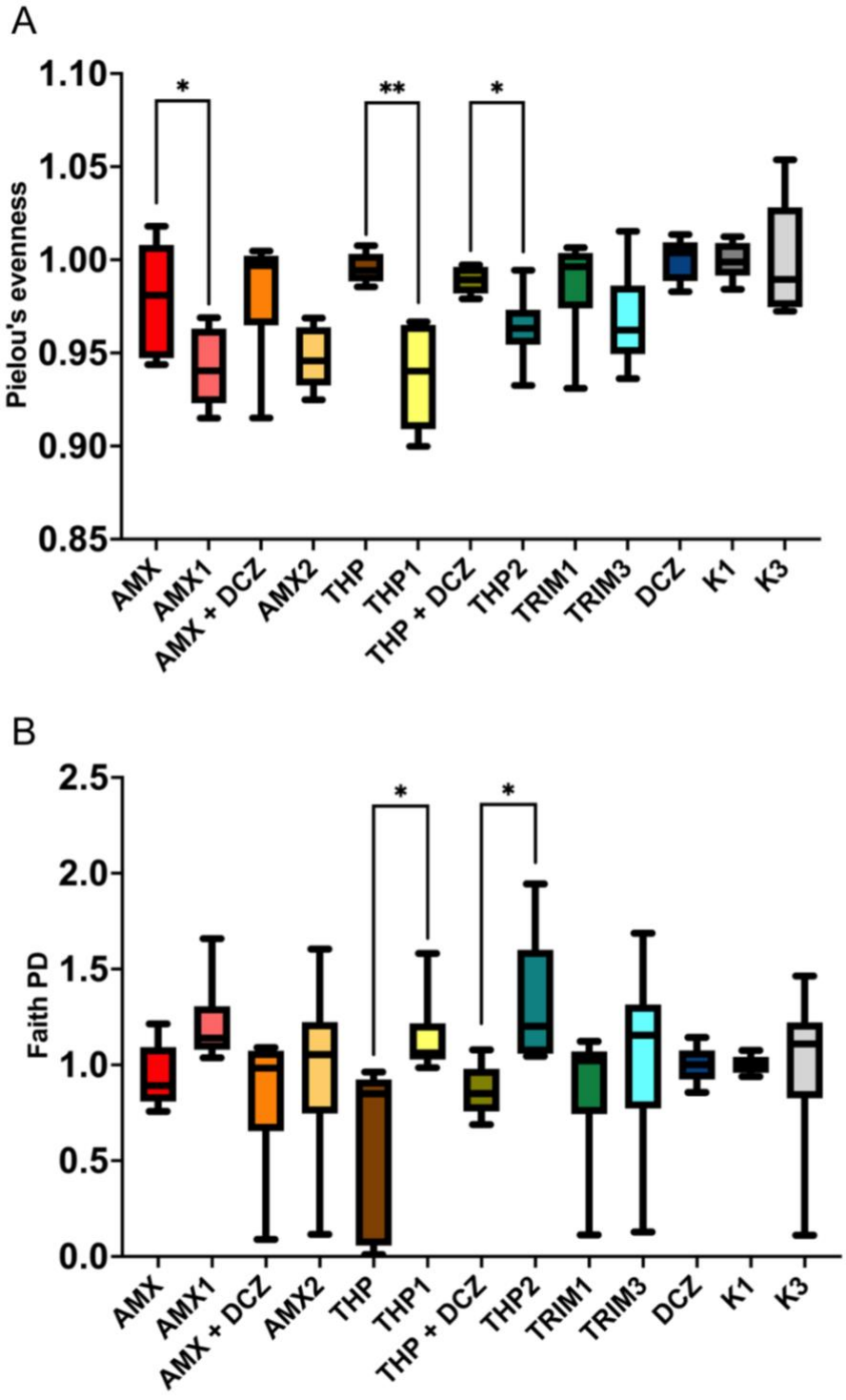

Figure 23. Alpha diversity in caecal microbiota. Comparison between trial 1 and 3. Statistical analysis was performed using Mann Whitney tests. Results were considered significant with p-value < 0.05 (*p < 0.05).

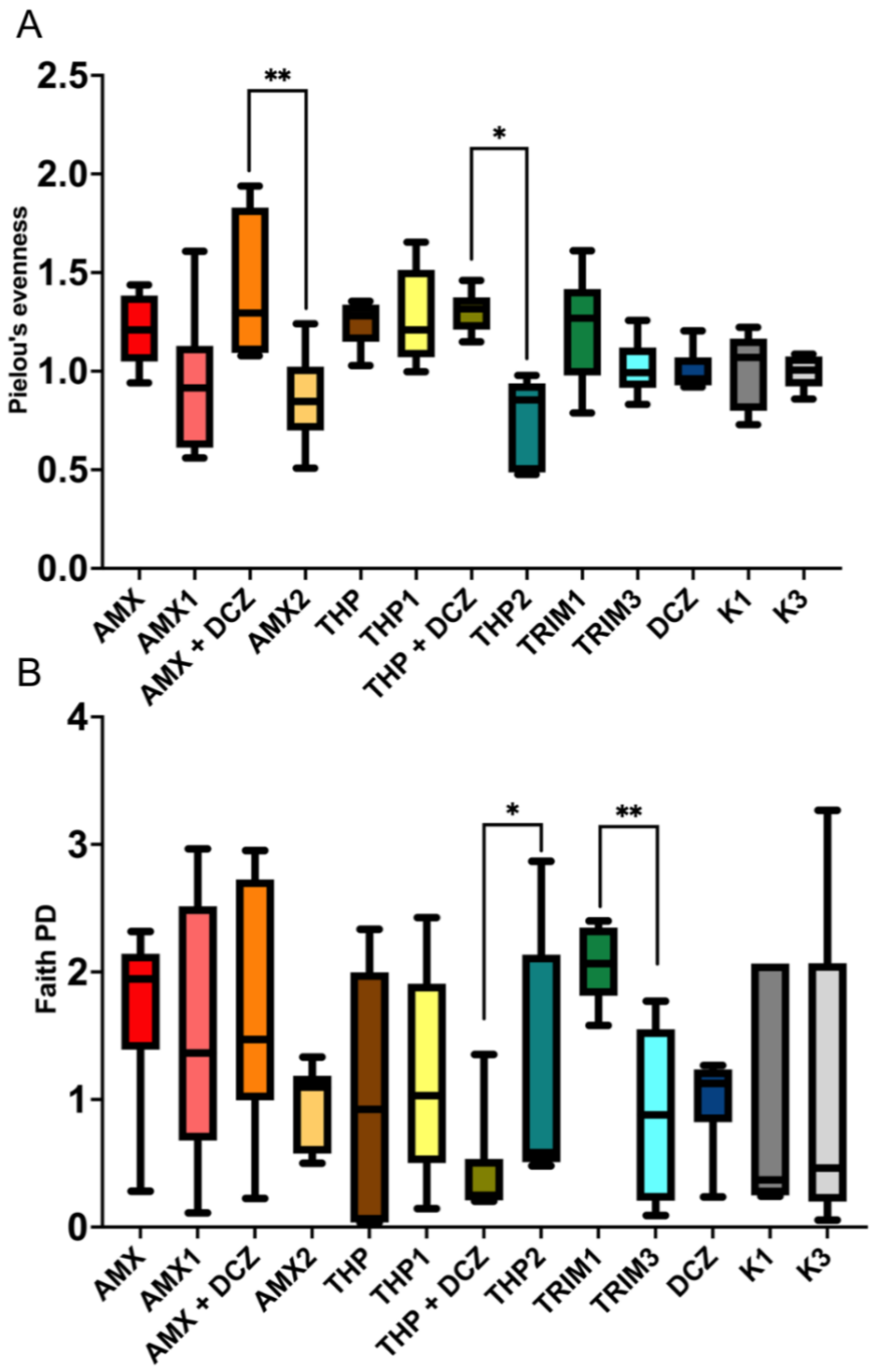

Figure 24. Alpha diversity in ileal microbiota. Comparison between trial 1 and 3. Statistical analysis was performed using Mann Whitney tests. The results were considered significant with p-value < 0.05 (*p < 0.05, **p < 0.01).

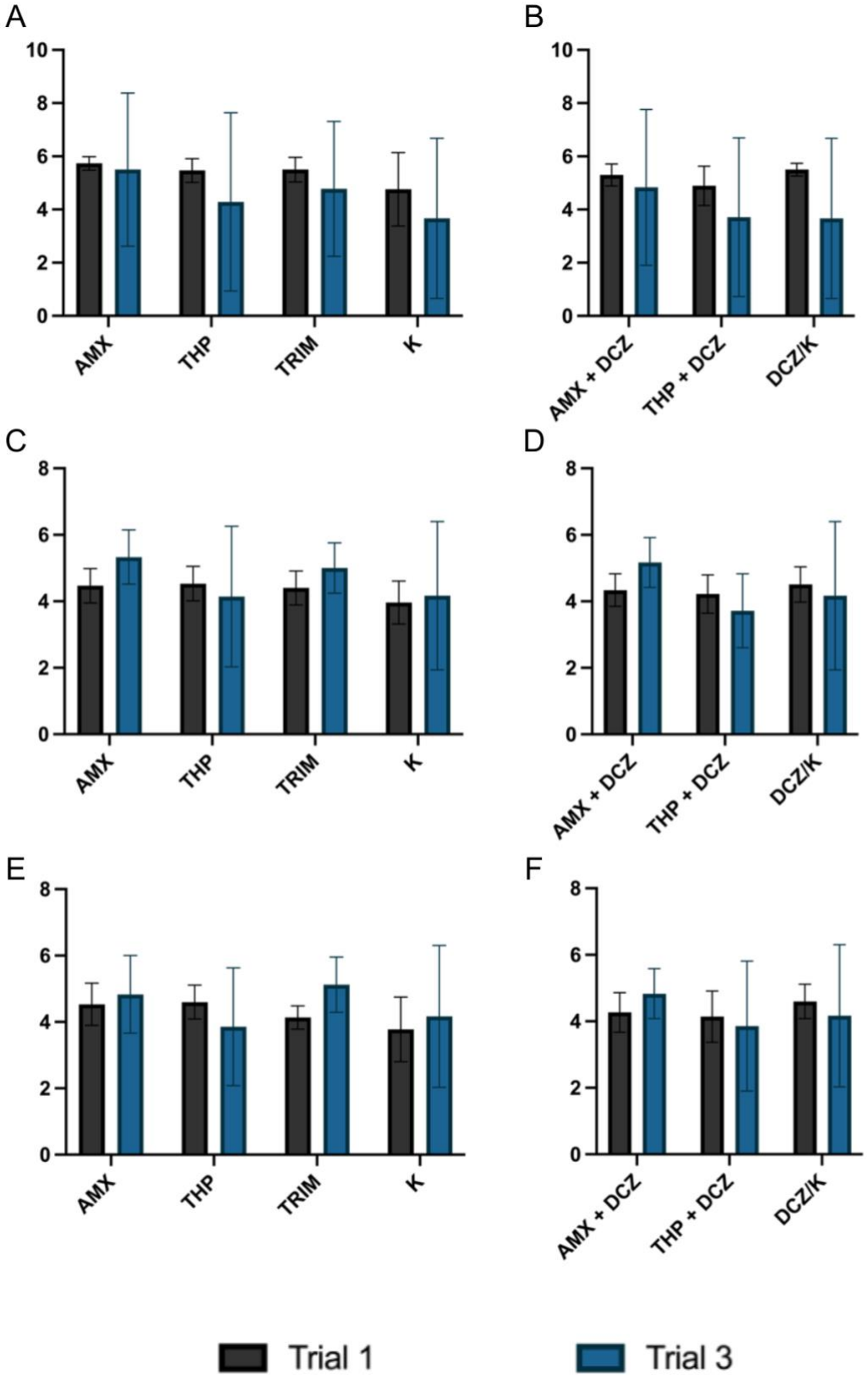

Figure 25. Histological scores comparison in ileum. Comparison between trial 1 and 3. Statistical analysis was performed using two-way ANOVA test. The results were considered significant with p-value < 0.05.

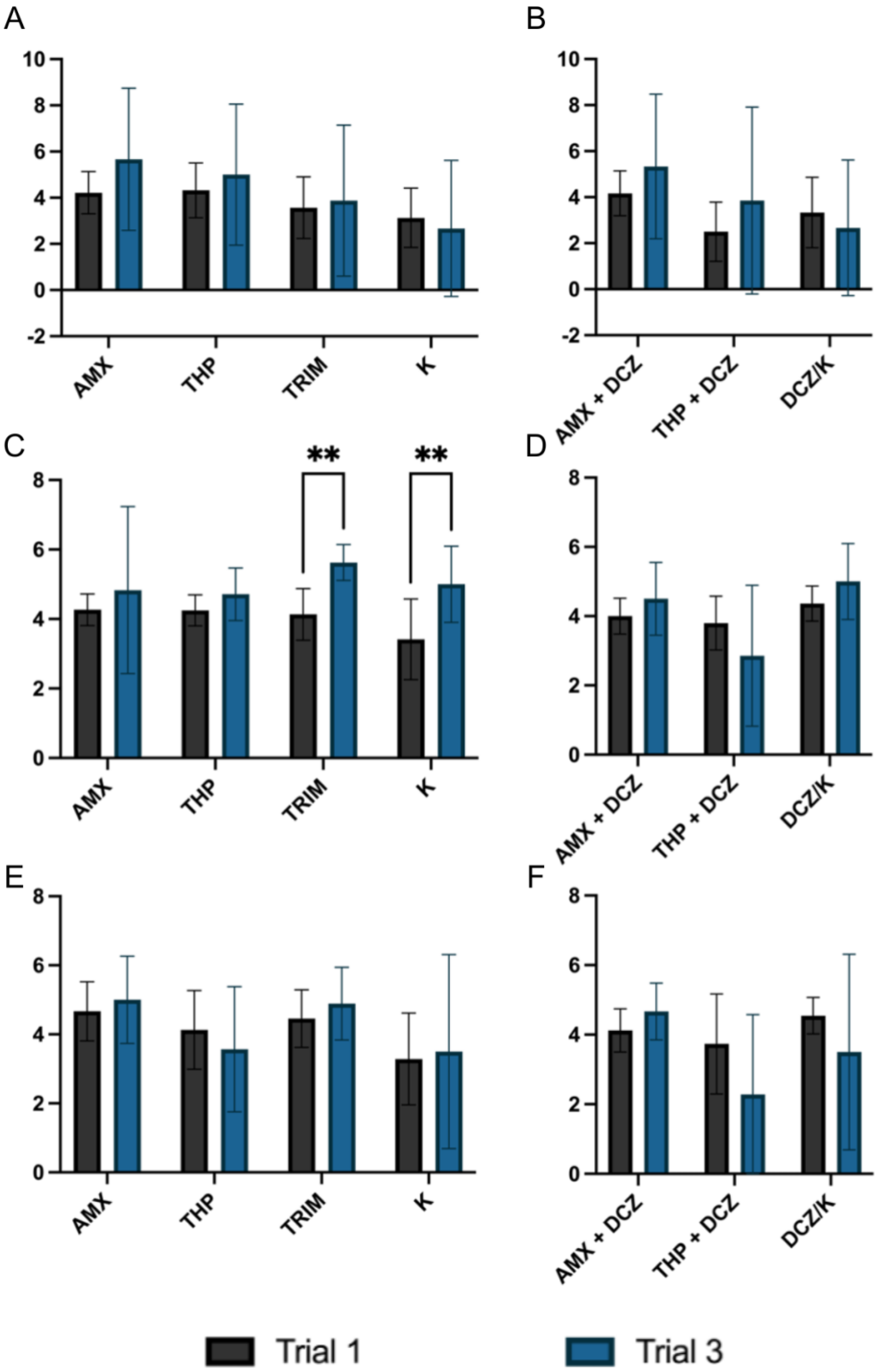

Figure 26. Histological scores comparison in caecum between trial 1 and 3. The statistical analysis was performed using two-way ANOVA test. The results were considered significant with p-value < 0.05 (**p < 0.01).

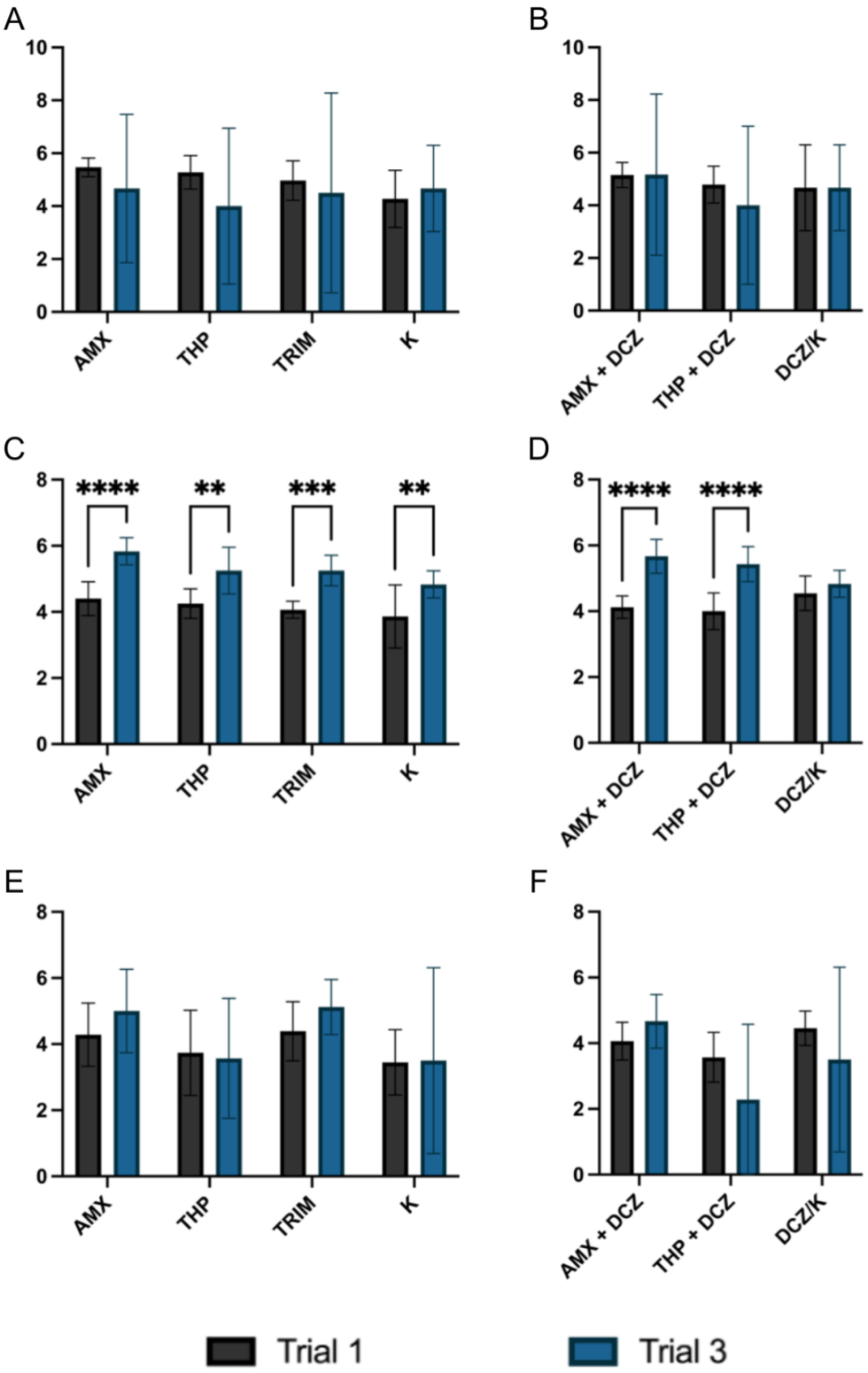

Figure 27. Histological scores comparison in colon between trial 1 and 3. The statistical analysis was performed using two-way ANOVA test. The results were considered significant with p-value < 0.05 (**p < 0.01, ***p < 0.001, ****p < 0.0001).

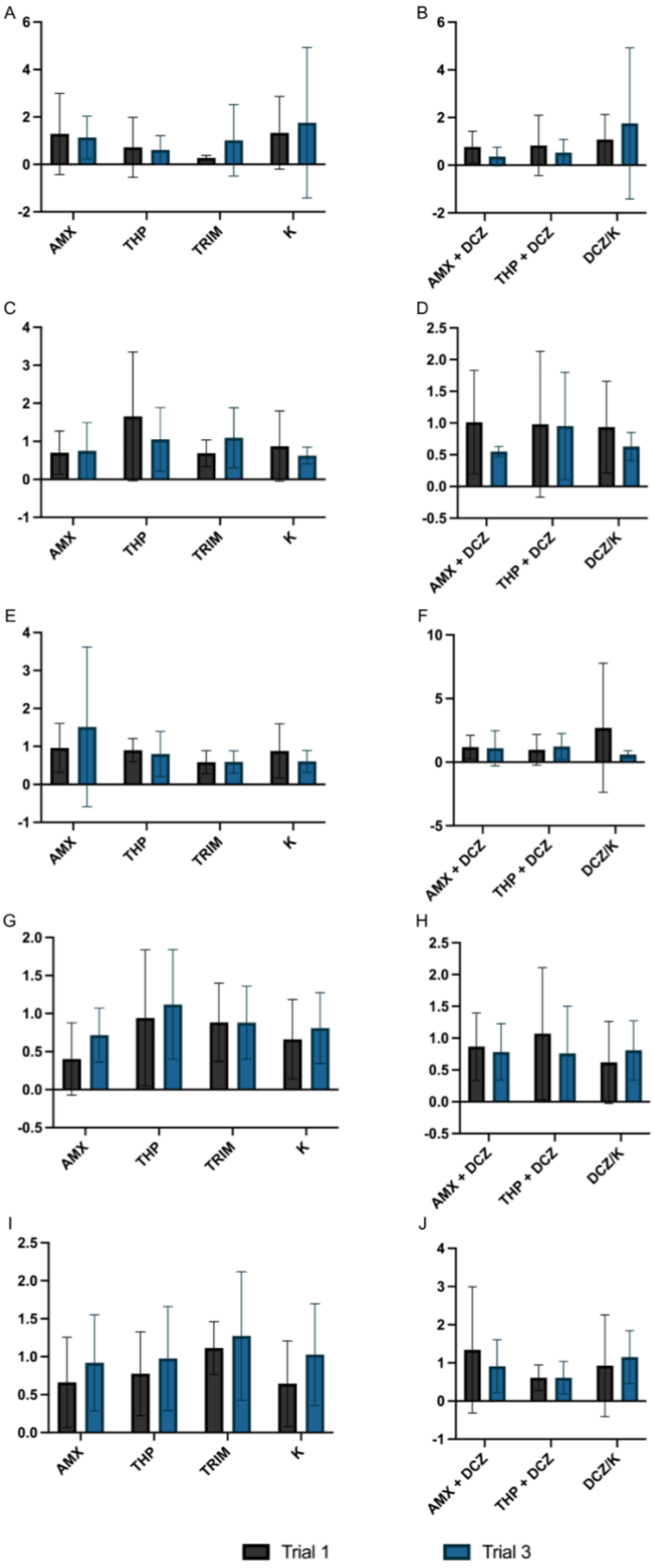

Figure 28. Liver gene expression. Comparison between trial 2 and 3. The statistical analysis was performed using two-way ANOVA test. The results were considered significant with p-value < 0.05.

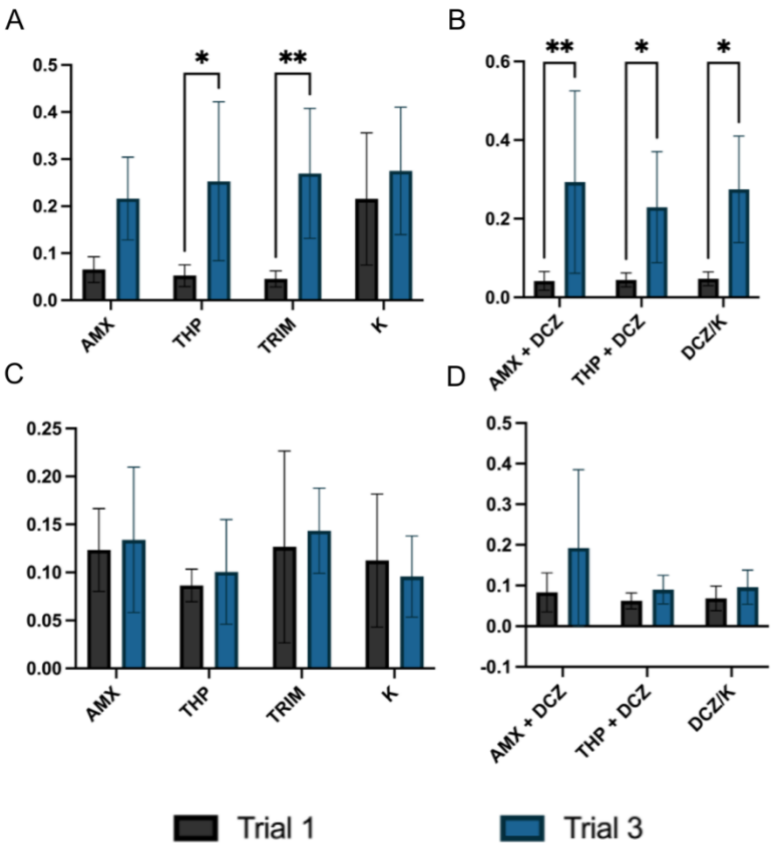

Figure 29. PM muscle gene expression. Comparison between trial 1 and 3. The statistical analysis was performed using two-way ANOVA test. The results were considered significant with p-value < 0.05 (*p < 0.05, **p < 0.01).

3.4 Discussion

In the last two decades, AMs use in veterinary medicine has gone through several changes to control the spreading of AMR. According to the current European regulation n. 6/2019, the therapeutic use of AMs is still permitted, but many limitations have been applied to AM prophylaxes and metaphylaxes. Poultry production is particularly affected by these changes, since AMs are still a very useful option against bacterial infectious diseases. Moreover, gut health has assumed a pivotal role in the farm management of the modern poultry industry. It relies on the maintenance of the feeble balance between the host, the intestinal microbiota, the intestinal environment and dietary compounds, affecting also other organs and the whole organism. This balance can be significantly affected by factors such as bird management, feed quality and the environment. In this context, the usage of AMs can lead to different negative consequences primarily for the gut microbiota (Xiong et al., 2018; Zhu et al., 2020), but even more interestingly, AMs can modulate the host transcriptome in different intestinal tracts and other organs, e.g., liver and skeletal muscle (Yu et al., 2017; Zhang et al., 2017; Giannuzzi et al., 2021). It is well known that broiler gut microbiota (GM) is a favourable environment for the dissemination of AMR genes between commensal and pathogenic bacteria (Y. Wang et al., 2020). Moreover, the selective pressure of AM treatments can induce the selection of resistant bacteria exacerbating the AMR issue in poultry productions (Guardabassi et al., 2018). On the other hand, any modifications of GM, also known as dysbiosis, could alter broiler physiology and increase susceptibility to infectious diseases, or also metabolic and inflammatory dysfunctions (Iacob & Iacob, 2019). However, few studies have focused their attention on the role of prophylactic and therapeutic doses of AMs in broiler gut health.

In this context, one of the main aims of this PhD project was to investigate the role of AMs in the modulation of the intestinal barrier morphology and function in broilers. First of all, it was investigated whether different AM treatments could determine different effects on broiler GM in ileum and caecum. Several studies have described the microbial communities that colonized the two different intestinal tracts (Xiao et al., 2017; Kers et al., 2019). The microbial populations differ between gastrointestinal compartments and reflect their different functions and their relationship. In particular, the ileum is the main site of nutrient absorption, and it is generally colonised by bacteria that affect nutrients availability and host metabolism (Stanley et al., 2014; Xiao et al., 2017). For example, *Lactobacillus* spp. is one of the main represented genera and its functions have been related to the production of essential vitamins, but also the recycling of intestinal bile acids (Xiao et al., 2017). On the other hand, caecum is an important site of starch fermentation, water absorption and urea recycling and these activities are strictly dependent on the metabolic capacity of caecum microbiota (Stanley et al., 2014; Clavijo & Flórez, 2018). Caecal

GM has always been reported as the one with the highest richness and abundance in bacterial composition while *Bacteroides* spp., *Ruminococcus* spp., and *Faecalibacterium* spp. are the main genera that have been found (Xiao et al., 2017; Kers et al., 2019; M. P. L. Lemos et al., 2020).

The results of these studies highlighted that different AM treatments have a distinct impact on ileal and caecal communities. Considering alpha diversity metrics in trial 1, our results show significantly different Pielou's evenness ($p = 0.03$ for ileum; $p = 0.5$ for caecum) and Faith's phylogenetic diversity ($p = 0.04$ for ileum; $p = 0.26$ for caecum) only in ileum.

A different scenario, instead, describes alpha diversity metrics in trial 3. The results show significantly different Pielou's evenness ($p < 0.001$ for ileum; $p < 0.006$ for caecum) and Faith's phylogenetic diversity ($p = 0.13$ for ileum; $p = 0.48$ for caecum). The two alpha diversity metrics are not in line, suggesting that the AMs prophylaxes may cause a partial affection of the abundance and richness of the microbial communities. Moreover, another explanation for this result may depend on the different characteristics of the two alpha diversity metrics. In fact, there is a substantial difference between the two alpha diversity metrics. Pielou's eveness focuses on species abundances, referring more about the number of species composing the microbial community. Differently, Faith's phylogenetic diversity is calculated as the sum of branch length of all species in a community using the phylogenetic tree, and therefore it is more related to the different number of species composing the microbial community. The more different bacterial species and more distant they are according to the phylogenetic tree, the higher and higher will be the index. Therefore, the effect of AMs on microbial communities from trial 3 may be more related to species diversification than richness reduction.

Generally, according to these results the withdrawal periods may be not enough for the restoral of the abundance of microbial communities in the ileum and partially also in the caecum for trial 3. For sure, "*a restitutio ad integrum*" of the GM, with similar characteristics to control group K seems not to be possible. Moreover, broilers are normally raised in a very short time thanks to their optimal growth performance and this short time probably is not enough for the restoral a eubiotic gut microbiota. It is well known that broiler caecum harbours the richest and most diverse microbiota in comparison with other intestinal tracts (Shang et al., 2018; Glendinning et al., 2019; Ngunjiri et al., 2019). Moreover, Mohd Shaufi et colleagues have shown that caecum microbiota presented a significant difference in the expression of the bacterial colonisation pathway (e.g., motility proteins, two-component system, and bacterial system) (Mohd Shaufi et al., 2015). All these considerations can contribute to partially explain the higher tolerance of caecal microbiota in comparison with ileum.

On the other hand, beta diversity metrics are significantly affected by AM treatment ($p = 0.001$ in the caecum and $p = 0.002$ in the ileum) in trial 1 and ($p = 0.001$ for caecum and $p = 0.056$ for

ileum) for trial 3. Beta indexes describe compositional changes between different microbial communities (Wagner et al., 2018). As expected, the different AM protocols caused different changes in GM of ileum and caecum. However, no significant p-value of ileal microbiota was observed in trial 3.

Firstly, an explanation of the significant results can be related to the different broad-spectrum action of amoxicillin, thiamphenicol, and trimethoprim-sulfadiazine. In addition, the association with diclazuril can modulate the AM activity against gut bacteria. In our case, sulfadiazine-trimethoprim is not active against bacteria belonging to *Enterococcus* and *Pseudomonas* genera (Kalkut, 1998). On the contrary, thiamphenicol and amoxicillin have a wider antibacterial activity than sulfadiazine-trimethoprim. Amoxicillin is often associated with clavulanic acid to improve antibacterial activity against lactamases producers (Bush & Bradford, 2016). In Italy, amoxicillin is only permitted as monotherapy in poultry production, and several pieces of evidence support that broiler GM is a reservoir of different-lactamases producing bacteria, e.g., Extended-Spectrum-Beta-Lactamases producing *Enterobacteriaceae* (Saliu et al., 2017; Apostolakos et al., 2019). Finally, the non-significant result in ileum of trial 3 most likely is related to the additional treatment with doxycycline to all animals. Having a broad-spectrum antibacterial activity, doxycycline may have modulated the microbial communities flattening the differences caused by the previous prophylactic protocols. In addition, as already seen before, the microbial communities of ileum and caecum are known to be different and thus they may have reacted differently to doxycycline.

Furthermore, the status of the gut barrier was investigated through the application of a multiple scoring system in HE stained samples. In treated broilers of trial 1, the observed intestinal lesions were characterised by detached or fused epithelia. Moreover, a severe and diffuse lymphocytic infiltrate was found in the mucosa and submucosa of ileum, cecum and colon of AM-treated broilers. These findings suggest that AM use in broilers has a direct impact on the gut barrier.

On broilers from trial 3, similar lesions to the first trial were observed. In particular, intestinal samples were characterised by villi fusion and epithelial detachment similarly to trial 1. The inflammatory population in the mucosa and submucosa was mainly composed by lymphocytes, but also by eosinophils. However, the statistical analysis did not reveal significant differences between the AMs-treated group and the control, excepting the more severe lymphocytic infiltrate in the colon mucosa of THP2 group.

It is widely known that specific enteric pathogens can induce an inflammatory response in the broiler intestine (Awad et al., 2017; Lauridsen, 2019). However, gut dysbiosis can induce the production of pro-inflammatory cytokines and initiate the inflammatory cascade (Awad et al., 2017; Iacob & Iacob, 2019). All administered AMs have a broad-spectrum activity and the gut

microbiota in ileum and cecum were negatively affected in alpha and beta metrics by AM treatments, as discussed before. It is well-known that a healthy gut microbiota is a crucial part of the intestinal barrier and thus for the defence of the host. Normally, microbe-associated molecular patterns (MAMPs) of the gut microbiota do not induce any activation of the pattern recognition receptors and consequently do not initiate a pro-inflammatory response, keeping a controlled homeostasis of the intestine (Kogut et al., 2018; Iacob & Iacob, 2019).

The AM treatments induced a dysbiosis status in the ileal and caecal microbiota, that was not recovered at the end of the rearing cycle. The presence of an altered microbiota in treated broilers may have possibly led to break the balanced homeostasis between gut microbiota and host, inducing the activation of the pro-inflammatory cascade. Moreover, these results support the hypothesis that AM effects are not only related to the gut microbiota, but directly involve the animal host and its tissues (Morgun et al., 2015). Intriguingly, AMX and THP groups from trial 1 showed the most significant differences in all the scores analysed. In those groups, broilers were fed without adding DCZ to the diet, but they received a coccidiosis vaccine (coarse spray; Hypracox, Amer, Spain) to ensure protection against these protozoa. The difference between groups with and without DCZ suggests a possible role of this molecule in the regulation of gut health. Moreover, statistical analysis revealed a significant increase of inflammatory infiltrate in mucosa and submucosa of DCZ treated broilers. In reality, these results were unexpected since DCZ is supposed to be active only against coccidia and other protozoa, even if the mechanism of action of DCZ is still not clearly understood (Noack et al., 2019). A recent study demonstrated that DCZ has a dose-dependent antibacterial activity against *Staphylococcus aureus*, *Enterococcus faecalis*, and *Streptococcus agalactiae* (Zheng et al., 2021). This latest discovery suggests that probably DCZ has a broader spectrum of action than the sole anticoccidial activity. Regarding trial 3, it may not be possible to understand if the observed lesions are dependent on the AMs prophylactic protocols or on the gastrointestinal syndrome. However, the statistically significant and more severe score attributed to the colonic lymphocytic infiltrate in comparison with the control group suggested a possible co-participation of AM prophylaxes in the pathogenesis of the intestinal lesions.

Finally, the maintenance of intestinal homeostasis depends on the integrity of the intestinal epithelium supported by TJ proteins. In this study the effects of AM prophylaxis on the expression of TJ proteins was evaluated. Also, it was investigated if these proteins may be a link between the altered gut microbiota and the observed intestinal lesions. According to recent studies, enteric pathogens, e.g., *Escherichia coli*, *Salmonella enterica*, *Campylobacter jejuni* and *Clostridium perfringens*, have the ability to target TJ components and to exploit the so-increased paracellular pathway reaching other extra-intestinal organs (Awad et al., 2017). Moreover, dysbiosis has been suggested to play a role in the regulation of TJ proteins (Suzuki, 2020; Zhu et al., 2020). In our

case, no statistically significant differences were shown in the expression of claudin-3 and ZO-1 proteins using IHC. Maybe, these not significant results could be due to the semi-quantitative scoring employed by pathologists on the sections stained using diaminobenzidine-hydrogen peroxide solution. It is demonstrated that in some cases TJ proteins can be regulated also by changing their location in the cells (Awad et al., 2017). Using immunofluorescence, Roxas et al. demonstrated that enterohemorrhagic *E. coli* infected mice showed a redistribution of claudin-3 and occludin in the enterocytes (Roxas et al., 2010). Although our results do not confirm the abovementioned studies, a possible role in the regulation of TJ by AM-induced dysbiosis may not be excluded. Further studies are needed to better clarify the role of dysbiosis and inflammation of TJ expression, probably by immunofluorescence and the application of a High-Content Screening System in substitution of the scoring system. For a better comprehension of TJ dynamics, a gene expression study was conducted on ileum and caecum of trial 3. This different approach allowed firstly to evaluate a broader group of TJ including different claudins, occludin and all ZO proteins. Unfortunately, the same broad evaluation of TJ proteins on IHC is still not possible for the absence of specific antibodies targeting chicken proteins. In veterinary science, this fact is a remarkable issue limiting the possibilities in research projects. Moreover, the cost required for developing specific antibodies is most of the time not affordable for a single study. Considering the gene expression results, a significant downregulation of claudin-1 was observed in the caecum of broilers treated with thiamphenicol in group THP2 in comparison with control group K. The consequences of claudin-1 downregulation may be related to a reduction of enterocytes binding capacity leading to an increased tissue permeability. Moreover, claudin-1 is one of the major components of TJ protein complexes contributing for the most at cellular binding (Suzuki, 2020). Similar results were not observed for other TJ targets; thus, this study can only suggest an implication of claudin-1 in the modulation of intestinal barrier after AMs exposure. The absence of other TJ significant down- or upregulations is most likely caused by the additional treatment with doxycycline administered to all broilers including the control animals.

Another aim of this PhD project related to poultry farming was the evaluation of putative biomarkers in order to identify AMs-treated broilers, previously published (Giannuzzi et al., 2021). In particular, 5 liver targets were evaluated in broilers from trial 2 and 3 liver samples. The previous data obatained were not confirmed. In trial 2 the reasons probably are due at the adopted prophylaxes protocols characterized by long withdrawal periods. Most likely, the expression differences imputable to AMs prophylaxes have been attenuated during the cycle after the AMs administration and the downregulation of the putative biomarkers may be restored in comparison with the control group. A different situation was observed with PM muscle biomarkers, ELOLV1 was identified by Giannuzzi and colleagues as possible biomarkers in PM muscle for identifying AMs treated broilers. Moreover, the strong downregulation of ELOVL1 suggested a promising

ability to distinguish treated broilers from un-treated. The strong downregulation was also the reason bringing to the decision of evaluating ELOVL1 gene expression on ddPCR, which is much more sensitive and precise in the quantification of very low expressed targets than qPCR (Divari et al., 2022). Indeed, the results in ddPCR of this study confirmed the feasibility of ddPCR application in gene expression studies. Considering trial 1, ELOVL1 results in ddPCR were in line with qPCR results, already published (Giannuzzi et al., 2021). The same evaluation was performed for trial 3, but no significant differences were observed among groups. Also in this case, the most probable reason, explaining the no significant result, is the additional treatment with doxycycline. Finally, CERS2 gene expression was investigated in PM muscle since this enzyme is strongly related to ELOVL1 activity. Both enzymes regulate the metabolic pathway of very-long-chain fatty acids and ceramides production (Ohno et al., 2010). In particular, recent studies suggested a possible interaction between the two genes that could bring to a negative regulation of ELOVL1 (Edagawa et al., 2018). Considering the results in ddPCR obtained in this study, CERS2 was not affected by the AMs treatments and no interaction between the two targets can be demonstrated. Nevertheless, other mechanisms for gene expression regulation, such as miRNA or epigenetic modifications, may be acting in PM muscle explaining ELOVL1 downregulation.

For a better and complete comprehension of these results, a statistical comparison was performed between the trials. Only when the same analysis was conducted in at least two trials, the statistical evaluation was conducted. First, alpha diversity metrics were evaluated between trial 1 and 3 using Mann Whitney tests. No significant differences were observed in both alpha diversity metrics between the two trials. However, the different kit used for DNA extraction is not a marginal bias. It is well known that the extraction methods can influence the sequencing results (Fiedorová et al., 2019), but the absence of statistically significant differences between the two trials suggests that the results may not be influenced by the extraction methods. A different scenario is presented by the comparison of histopathological results, which were compared by two-way ANOVA test between trial 1 and 3. Here, a significant effect of the trial was observed for caecal and colon lymphocytic infiltrate in several groups. Above all, the inflammatory infiltrate was more severe in trial 3 than 1. However, these differences are easily explainable with the occurrence of the gastrointestinal syndrome during trial 3. Indeed, the comparison between control groups of the two trials is also significant both in caecum and colon. Finally, biomarkers results were compared by two-way ANOVA test. The only significant difference was observed for ELOVL1 comparing trial 1 and 3. In this case, the main bias in the comparison of gene expression data between the trials is the additional treatment with doxycycline happened in trial 3.

In this study, the effects of AMs prophylaxes were investigated in treated broilers and compared to control animals. First, the consequences of AMs were investigated at gut barrier level through

histopathology, IHC and gene expression of TJ proteins. Second, some putative biomarkers for AMs treatment were evaluated in different trials. Both gut health and AMs control have assumed a prominent role in poultry farming and therefore in veterinary research. This study demonstrates that AM prophylactic protocols, as they are normally adopted in poultry production, are responsible for GM compositional alterations. Moreover, the respected withdrawal periods were not enough for the restoral of a GM similar to the control group. This conclusion could seem to be in accordance with Elokil and colleagueas, who negative consequences on GM after enrofloxacin administration in adult broilers (Elokil et al., 2020). However, Elokil's study is not comparable with this PhD study, indeed the age of broilers involved and the AMs treatments applied are different. Nevertheless, to my best knowledge of the scientific literature few studies can be compared with this thesis. GM results can still be partially compared with literature. However, most studies applied growth promoter protocols to broilers, which differ from prophylaxes protocol in the AM molecule, dose and administration periods. Therefore, a useful and complete comparison may not be possible. Another bias in the literature comparison happens with the histopathology results. Most published studies tried to evaluate the effects of drugs or feed additives applying a morphometric approach (Dal Pont et al., 2020). This method is suitable when evaluating the functionality of the gut tracts and the effects of the molecules on the gut physiological functions of digestion and absorption. Differently, the aim of this study was to describe the indirect consequences of AMs on the intestinal tissue and the possible negative effects on tissue caused by a dysbiotic microbiota. Therefore, a scoring system evaluating the observed lesions and their severity and diffusion seems to be more appropriate than a morphometric evaluation. Regarding the AMs treatment biomarkers, more studies are needed to validate and confirm the proposed biomarkers and new zootechnical trials with similar and different AMs protocols may be conducted. In addition, considering the investigated organs, results related to PM muscles are less consistent than liver (Giannuzzi et al., 2021). Although PM muscle is an easy organ to collect at slaughterhouses for conducting laboratory analysis, the results obtained with this organ suggested its inadequacy in this kind of studies. A different and more promising situation seems to be with the liver and the intestine, which can be more directly affected by the AMs and are also connected through the entero-hepatic circulation. The GM changes can lead to negative consequences for broilers, and, in particular, future studies should focus on how GM alterations affect host intestinal physiology. Moreover, further investigations focusing on the selective pressure applied by AM treatments on the expression of AMR genes in GM need to be carried out. TJ proteins expression would seem unaffected by the use of AM. However, qPCR evaluation of TJ suggested a transcriptional regulation of these targets.

In poultry farming, gut health is considered of great importance for production performance, understanding the mechanisms of gut barrier dysfunction may allow future prevention of the serious sequelae of bacterial translocation and sepsis in veterinary medicine.

CHAPTER 4: Dairy cows research line

4.1 Introduction

4.1.1 Mastitis

Mastitis is the most frequent and detrimental disease in dairy cows. The inflammation of the udder during lactation is the principal cause of economic losses in the dairy industry, due to the reduction in milk production and quality, the increased costs for treatments, and the grown herd turnover (Ruegg, 2017; El-Sayed & Kamel, 2021). It is a complex multi-etiological disease caused by a variety of microorganisms, mainly bacteria (*Staphylococcus* spp., *Streptococcus* spp., and *Enterobacteriaceae*). Bovine mastitis is classified into clinical and subclinical forms according to the presence or absence of symptoms and signs, such as visibly abnormal milk, swelling, heat, pain and redness of the udder (Ashraf & Imran, 2020). Subclinical mastitis is very challenging and difficult to diagnose because of the normal appearance of both the mammary gland and milk; the only indicators of infection are the increased somatic cell count (SCC) and the bacterial population in milk (Ashraf & Imran, 2018). According to a recent systematic review and meta-analysis of the prevalence of bovine mastitis worldwide, the subclinical forms of the disease are the most prevalent and cause the major loss of milk production (Krömker & Leimbach, 2017; Krishnamoorthy et al., 2021). Therefore, the early detection of subclinical mastitis is crucial for effective treatment and successful dairy herd management. More to the point, in European Union the recent implementation of Reg. EU No. 6/2019 about veterinary medicinal products has strictly limited the use of antimicrobials for prophylaxis and metaphylaxis purposes to prevent the insurgence and spread of resistance phenomena. In the last decades, the use of antibiotics for the preventive control of possible outbreaks of mastitis has been regularly adopted, especially during the dry period (National Mastitis Council, 2001; El-Sayed & Kamel, 2021;). In the new regulatory scenario, the veterinary practitioners acting in the dairy industry need alternative solutions for the control of mastitis, including novel diagnostic and therapeutic tools. The routinely used methods to diagnose the subclinical forms of mastitis are the measurement of SCC and the microbiological test (Ashraf & Imran, 2020). The SCC level is also one of the parameters to assess milk suitability for human consumption, therefore influencing milk pricing (National Mastitis Council, 2001). It is widely accepted that mastitic milk has an SCC value higher than 400,000 cells/ml, regardless of the presence of clinical symptoms (Ruegg, 2017; Zecconi et al., 2021). On the contrary, the milk collected from a healthy mammary gland is characterized by an SCC value lower than 100,000 cells/ml (National Mastitis Council, 2001; Ruegg, 2017). An SCC measurement comprised between those boundaries should be interpreted: it could be suggestive

of subclinical mastitis or the result of the recovering phase of udder infection, when inflammation and microbial pathogens could still be detected (Adkins & Middleton, 2018; Zecconi et al., 2021). Currently, an SCC measurement equal to 200,000 cells/mL has been set as a threshold to classify subclinical mastitis (Dufour & Dohoo, 2013). However, inflammation of the mammary gland has been also observed at values around 100,000 cells/mL, especially in primiparous cows (Kandeel et al., 2018).

4.1.2 Extracellular vesicles

In recent years, extracellular vesicles (EVs) have been widely investigated in human and veterinary medicine. EVs are membrane-limited nanoparticles involved in intercellular communication and their presence has been proven in several biological fluids, such as blood, urine, milk and saliva (Blans et al., 2017; Benmoussa et al., 2020; Yu et al., 2022). Classification of EVs is continuously being revised since the development of new technologies provides always more new insights in this field. Among EVs, three main classes are included: exosomes, ectosomes and apoptotic bodies (Dang et al., 2020). Their classification is based according to the different routes of biogenesis, as shown in Fig. 30.

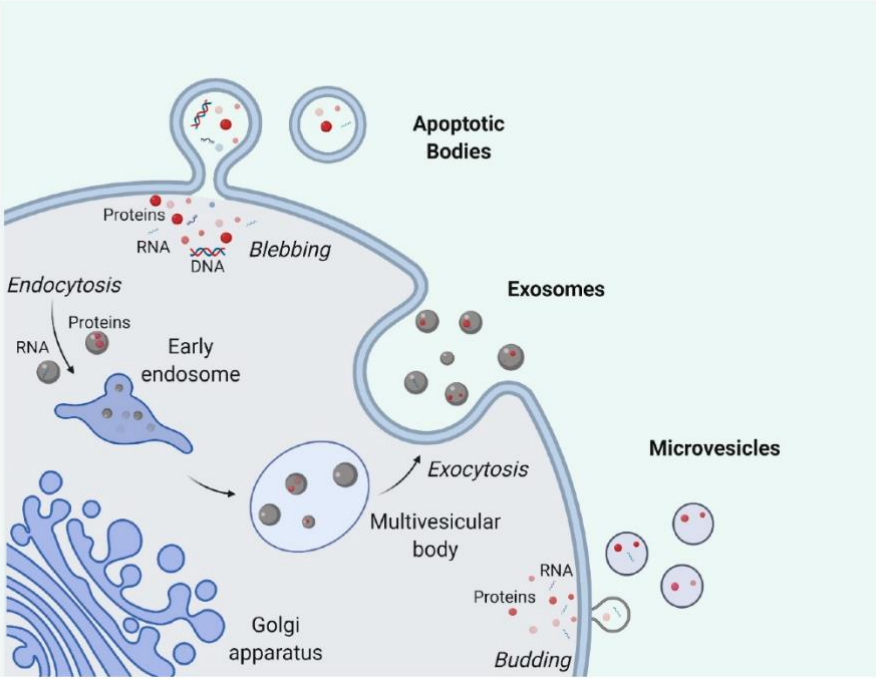

Fig. 30. Extracellular vesicles biogenesis and classification. Exosomes are intraluminal vesicles which are released thanks to the fusion of a multivesicular body with the cell membrane. Microvesicles, or ectosomes, are formed by the outward shedding of the membrane into the extracellular space. Apoptotic bodies are generated when cells undergo apoptosis. Cited by (Dang et al., 2020).

However, an unanimity about EVs classes and their definition is still missing. Thanks to the "Minimal Information for Studies of Extracellular Vesicles" (MISEV) guidelines of the International Society for Extracellular Vesicles (ISEV) a general agreement about the main characteristics has been reached (Théry et al., 2018). First of all, EVs are delimited by a double lipid layer and are anucleate. Classes, instead, are defined according to the range size of EVs and divided into small-size EVs (< 200 nm) and medium/large EVs (> 200 nm). Another important mechanism for understanding EVs nomenclature is their biogenesis. Three main different ways have been described as originating EVs. The first views involved the endosome and the multivesicular bodies, which originate EVs usually called exosomes (Abels & Breakefield, 2016). In this context, exosome biogenesis can occur through the endosomal complex required for transport (ESCRT) or a ceramide-dependent pathway. Exosome size is normally lower than 100 nm (Zempleni et al., 2017). A second mechanism involved in EVs biogenesis is the exocytosis of the cellular membrane. In particular, this EVs class originates through an outer budding of the membrane into the extracellular space and ectosomes is the general term used for this class. In this case, dimensions are bigger than exosomes and their size can be up to 1000 nm (Raposo & Stoorvogel, 2013). Finally, one last class of EVs are apoptotic bodies and their classification into EVs is still under debate. However, they originate during the apoptotic process and their size can be highly variable, even higher than 1000 nm (Raposo & Stoorvogel, 2013).

The ability of EVs in regulating cellular and organ processes is due to the presence of different types of cargo inside EVs, such as non-coding RNA, mRNA, DNA, proteins and lipids, which can be delivered to the targeted recipient cell (Zempleni et al., 2017). However, their ability to regulate the cellular communication relies also on the presence of several protein and glycoprotein membrane markers (Admyre et al., 2007). These transmembrane proteins exploit the antigen-receptor binding mechanism in the regulated cells (Zempleni et al., 2017). Among the different membrane markers identified, the main ones are tetraspanins (CD65, CD81, CD9 and other cluster of differentiation proteins), integrins, LAMP1 and LAMP2 (Théry et al., 2018). Moreover, these transmembrane markers are used for EVs characterization and their presence demonstrates the availability of a membrane-limited structure. On the other hand, another group of protein markers used for EVs characterization are cytosolic proteins. Several markers can be used, such as TSG101 (involved in the ESCRT mechanism), ALIX, flotillin1 and 2, annexins and others (Théry et al., 2018). Anyway, cytosolic proteins are more promiscuous than transmembrane markers and depend on the EVs originating mechanism (Abels & Breakefield, 2016).

4.1.2.1 Extracellular vesicles in medicine

Several biological functions are attributed to EVs, both physiological and pathological. Most information derives from cancer studies, which demonstrated the involvement of EVs in several oncogenic mechanisms. EVs can regulate the initiation, proliferation and progression of tumours (Cintio et al., 2020). According to the originating type of cells and their cargos, EVs involved in cancer can induce or suppress oncogenic processes (Moccia et al., 2022). Among the several processes regulated, EVs can contribute to tumour angiogenesis, immune response silencing and also cell migration during metastasis (Groot Kormelink et al., 2018; Brennan et al., 2020). Additionally, also EVs act in the regulation of immune response (Admyre et al., 2007). For example, EVs can activate lymphocytes and other inflammatory cells inducing also their differentiation (Driedonks et al., 2018). For this reason, EVs are involved in most infectious disease pathogenesis. Moreover, EVs can regulate nervous system activities and, thus, are involved in neurodegenerative disease (de la Torre Gomez et al., 2018). Finally, another important function of EVs is the regulation of mother-infant communication. Not only some EVs can pass the placenta barrier and affect embryonic development during gestation, but also can be vehiculated by colostrum and milk (Cintio et al., 2020). During its early life, the infant can assume them during alimentation. In this context, most EVs resist the stomach environment and can be absorbed by the intestinal mucosa. In that way, this regulation of the infant development can continue also after birth (Van Hese et al., 2020).

Since EVs are involved in all the above-mentioned processes, studying EVs can bring many new insights into disease mechanisms. Clarifying EVs role can lead to a better knowledge of the disease and the development of new therapies and diagnostic tools. Moreover, EVs can also be energized in the laboratory and used for the development of new vaccines and therapies (Heinemann et al., 2014; Moccia et al., 2022). However, the most interesting application field for EVs is biomarker discovery. In the last ten years, EVs have been largely studied and evaluated as innovative biomarkers in human medicine, particularly for cancer and neurodegenerative diseases. Both EVs and their cargo can be evaluated in these studies to validate new diagnostic tools.

4.1.3 microRNAs in mastitis

In veterinary medicine, microRNAs (miRNAs) role has been particularly investigated in mastitis pathogenesis among domesticated ruminant species (i.e. cow, sheep and goat). Among non-coding RNAs, miRNAs are an extremely important group of small non-coding RNAs, long from 20 to 22 nucleotides, that regulate gene expression through mRNA silencing at the post-transcriptional level. Several studies have been conducted in experimentally infected cows and several putative miRNAs differently regulated mainly under *Staphylococcus aureus* or *Escherichia coli* infections have been observed (Jin et al., 2014; Cai et al., 2021; Chen et al., 2021). Similarly to other infectious diseases, during mastitis miRNAs are involved in the regulation of several pathways, such as pathogen response, arousal and regulation of the inflammatory processes and regulation of the immune response (Lawless et al., 2014). However, the experimental conditions and the limited sampled cows didn't allow for validation of these miRNAs as mastitis biomarkers yet. The identification of early indicators for rapid and accurate detection of mastitis could lead to earlier and more effective treatment allowing the animal to recover faster, and therefore reducing the associated economic losses. A better understanding of the molecular regulation of the mammary response to inflammation would allow the identification of such indicators. Another bias of mastitis studies is the different matrix choice for investigating miRNAs role. Milk represents the most accessible sample, first avoiding dairy cows welfare disturbances during sampling and second allowing an easier collection than blood or mammary biopsies (Leroux et al., 2022). However, also using milk as an investigation sample, it is possible to work with whole milk, or its components like cells, fat globules or milk whey (Pawlowski et al., 2020).

4.2 Materials and methods

4.2.1 Study design and sample collection

Primiparous Holstein Friesian cows belonging to ten dairy farms located in the provinces of Cuneo and Turin (Piedmont Region, Northern Italy) were selected, as previously described (Cuccato et al., 2022). Animals were managed according to the local farm production practices. All manipulations were performed kindly to avoid animal distress. Before milk collection, teats were disinfected, and the first squirts were collected in a specific container and discarded. Individual samples were collected in sterile polypropylene tubes (2 aliquot of 50 mL) from all quarters of each cow and immediately refrigerated. Milk sampling has been conducted during winter (December 2020 – February 2021) and summer (June 2021 – September 2021). During the first period of sampling (winter), a total of 98 milk samples were collected. Additionally, during summer other 76 milk samples were collected. During each sampling date, information about parity, days in milk (DIM) and milk yield were collected for all sampled dairy cows. The experimental design of this research line is shown in ANNEX A – Figure 2.

4.2.2 Microbiology and SCC

The routine follow up of milk quality through functional feature analyses (i.e., SCC measurement and bacteriological examination) was conducted in the A.R.A.P. laboratory (Associazione Regionale Allevatori Piemontesi, Cuneo, Italy). The SCC measurement was performed with the Fossomatic 7DC (Foss Italia, Padua, Italy) according to the certified protocol (ISO 13366-2 / IDF 148- 2:2006). Bacterial culture through non-selective conditions on blood agar medium and subsequent confirmation tests were performed according to the A.R.A.P. laboratory routine methods.

4.2.3 Statistical analysis

The dataset related to the collected milk samples, including parity, DIM, season, farms, milk yield, microbiology and SCC, was fitted to a linear mixed model using the lmer() function from lme4 package in RStudio (R v.4.1.2, RStudio v. 1.4.1103). Parity and DIM data were set as fixed parameters, instead time of the sampling (season), farms, milk yield, microbiology and SCC results were set as random effects and the model was then tested using anova() function.

4.2.4 EVs isolation

Milk aliquots for the EVs isolation were submitted to a differential centrifugation protocol, whose steps were as follows:

- 3,000 g for 10 min at + 4° C to remove milk fat and somatic cells,
- 3,000 g for 10 min at + 4° C to remove additional fat and cells residuals,
- 5,000 g for 30 min at + 4° C to remove larger cell debris,
- 10,000 g for 30 min at + 4° C to remove smaller cell debris.

After these centrifugation steps, the skimmed milk was stored at −80◦C until further analysis.

At a later time, skimmed milk samples were thawed gradually in ice. EVs isolation was performed using a size exclusion chromatography (SEC) system, in detail qEV original 70mm columns (Izon Science). Before loading milk samples on the SEC column, the volume was reduced using a centrifugation filter tube (AMICON ULTRA-4 50 kDa, Merck Millipore) in order to reach a volume of 500μL, the maximal loadable capacity of the SEC column. Centrifugations were performed at 4,000 × g at + 4° C with a variable time from 40’ to 60’ depending on sample viscosity. To avoid protein precipitation at filter level, during centrifugation samples were gently mixed by slow pipetting on ice. Once reached the 500 μL volume size, milk samples were loaded on the SEC columns, which was previously mounted on its automatic fraction collector (qEV AFC system, Izon Science). Following manufacturer's instructions, the first 5 aliquot (50 μL each) were separately collected on 2 mL tubes corresponding to the most rich and pure EVs fractions. Subsequently, the 5 EVs fractions (250 μL) were mixed and the volume was reduced with a centrifugation filter tube (AMICON ULTRA-4 50 kDa, Merck Millipore) in order to reach a volume of 50 μL, the minimal useful volume for downstream applications. The protocol was similar to the previously described, with a reduced time of centrifugation variable from 15’ to 30’ due to a lower viscosity than milk samples. Finally, concentrated EVs were stored at -80 until further analysis.

4.2.5 Western blot

To validate the EVs isolation method and prove the presence of EVs in our samples, a western blot analysis was conducted. Particularly, two EVs markers were selected as suggested by the MISEV guidelines: TSG101 and CD9 (Théry et al., 2018). Total proteins were extracted from EVs lysate using RIPA buffer supplemented with a protease inhibitor cocktail (Sigma). Twenty-five μL of EVs lysate were added with 25 μL of Laemmli buffer (with a 2-mercaptoethanol supplementation just for TSG101) and resolved by 10% SDS–PAGE for TSG101 and CD9 assessment respectively. Proteins were blotted to PVDF membranes (Bio-rad) using the Mini Trans-Blot cell (Bio-Rad). The blotted membranes were blocked with a 10% BSA solution (Millipore KGaA, Darmstadt, D) for 1 hour at room temperature, followed by an overnight 4°C

incubation with the mouse monoclonal anti-TSG101 primary antibody (1:200; sc-136111, Santa Cruz Biotechnology) and with the rabbit monoclonal anti-CD9 primary antibody (1:1000; ab92726, Abcam). The membranes were subsequently incubated with a secondary horseradish peroxidase (HRP)-conjugated anti-goat antibody (1:10000), developed using Clarity Western ECL Substrate (Bio-rad) and recorded on CL-XPosure X-ray film (Thermo Fisher Scientific). During WB analysis, a protein extract from TE-1 cells lysate was used as positive control.

4.2.6 miRNAs extraction

Total smallRNAs were extracted from EVs samples using Maxwell RSC miRNA Blood kit, following manufacturer's instructions. Then, miRNAs were quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific) and the Qubit™ microRNA Assay kit following an adapted protocol for the Quantus fluorometer (Promega). Finally, smallRNAs were analysed using the Agilent small RNA kit (Agilent Technologies) for the Bioanalyzer 2100 instrument (Agilent Technologies).

4.2.7 smallRNA-sequencing

Library preparation and smallRNA-seq were performed by an external laboratory. Library preparation for next-generation sequencing was performed using SMARTer® smRNA-seq kit for Illumina (Cat. no. #635031, Clontech Laboratories Inc.) according to the manufacturer's protocol. Following PCR amplification, purification, and validation, sequencing libraries required size selection performed using SPRIselect beads (Cat. no. #B23318, Beckman Coulter Life Science). Quality controls were performed on Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and Qubit V4 (Invitrogen Thermo Fisher Scientific, Waltham, MA, USA). Next-generation sequencing was performed on NextSeq500 (Illumina, San Diego, CA, USA) with the reagents kit V2 (75 cycles; Illumina). Samples were processed starting from single-ended 75bp-long sequencing reads.

4.2.8 Bioinformatic analysis

Fastq files were trimmed using cutadapt (cutadapt 3.5 with Python 3.7.7) following the kit manufacturer instructions (parameters: -m 15 -u 3 -a AAAAAAAAAA). Trimmed fastq files were processed using the miRNAseq workflow implemented in docker4seq (Beccuti et al., 2018; Ferrero et al., 2018; Kulkarni et al., 2018). Briefly, quality control of trimmed reads was performed using FastQC software v. 0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Quality of trimmed reads was checked to evaluate the overall distribution of sequenced fragments length. Then, trimmed reads were mapped on bovine miRNA precursors (miRbase 22) using BWA aligner (v. 0.7.12) (H. Li & Durbin, 2009), and mature 5p and 3p miRNAs were counted with a R script embedded in the docker4seq workflow.

Counts filtering, data normalisation, and differential expression analysis were performed in RStudio (R v.4.1.2, RStudio v. 1.4.1103). We first normalised the miRNAs count matrix with the sequencing depth for each sample by calculating counts per million (CPM). Then, we filtered out genes expressed in less than 10 samples with CPM < 0.5 using the cpm() function from edgeR package (v. 3.36.0) (Robinson et al., 2010). miRNAs failing these criteria were removed from the count matrix before the exploration, and differential expression steps.

Once achieved a filtered miRNAs matrix, exploratory analysis of the expressed miRNAs was performed using unsupervised principal component analysis (PCA) and the non-parametric multidimensional analysis (NMDS) with ggplot2 (v 3.3.5) R package. Differentially expressed (DE) miRNAs analysis was performed pairwise using edgeR (v. 3.36.0, Robinson et al., 2010) packages. The differential analysis was performed by comparing samples according to their SCC level. In particular, samples were clustered using a SCC threshold level of 200.000 cells/mL, normally considered as an SCC level of subclinical mastitis, in two groups high SCC (group H) and low SCC (group L). Counts from expressed genes were first normalised with thecalcNormFactors() function. Then, limma's voom() function (v.3.50.0) was used to fit a generalised linear regression model to correct the data with the group as a fixed effect. The p-values were adjusted for multiple testing using the Benjamini and Hochberg (BH) procedure. Only DE miRNAs with an adjusted P-value < 0.05 were used for the downstream pathway analysis.

Using only DE miRNAs, a predictive functional analysis was performed to evaluate the effects of DE miRNAs regulation of biological processes and pathway. *In silico* analyses were performed using human orthologs (*hsa*-), instead of bovine orthologs (*bta*-), since information about miRNAs activity is more detailed and extensive in human than bovine. In this particular step, we used miRWalk 2.0 (http://mirwalk.umm.uni-heidelberg.de), OmicsNet 2.0 (https://www.omicsnet.ca) and Cytoscape v.3.9.1 with ClueGO plugin v.2.5.9 (https://cytoscape.org) softwares. First, the most deposited mature form (-3p or -5p) of DE miRNAs were checked in miRBase database and modified accordingly in the DE miRNAs list with the aim of retrieving the most relevant results with the functional analysis. Subsequently, the partially modified DE miRNAs list was used in miRWalk to retrieve all the miRNA-gene interactions. In particular, only validated interactions deposited in miRTarBase database and with biding position data (related to 3UTR, 5UTR and CDS) were selected. In parallel, to identify functional biological categories related to differentially expressed miRNAs, Panther Biological Processes analysis was performed using OmicsNet. The categorization of biological processes was manually performed. In addition, target genes involved in the regulation of immunity processes were obtained with ClueGO plugin in Cytoscape using the function GO-ImmuneSystemProcess. These gene lists were filtered by selecting only validated miRNA-gene interactions, previously obtained using miRWalk. The miRNAs-genes network was visualised using Cytoscape, which was used to identify potential networks.

4.3 Results

4.3.1 Microbiology and SCC

According to SCC results, samples were grouped in two different groups using a threshold value of 200,00 cells/ml. From now on, animals with SCC level < 200,00 cells/mL will be defined as group L, and animals with SCC > 200,00 cells/mL as group H. In particular, during winter a total of 93 samples (95% of winter samples) belonged to group L and only 5 (5%) to group H. Differently, during the summer sampling 64 samples (84% of summer samples) belonged to group L and 12 (16%) to group L.

According to microbiology results, in winter, 17 out of 98 winter samples microbiologically tested (17%) were positive for *Staphylococcus* spp, and 1 out of 98 samples (1%) was positive for *Streptococcus uberis*. The rest of winter samples (n= 80 – 82%) showed no positivity to any of the tested microorganisms. Differently, in summer, 13 out of 76 winter samples microbiologically tested (17%) were positive for *Staphylococcus* spp, 2 (2.5%) for *Streptococcus uberis* and 2 (2.5%) for *Serratia* spp. The rest of summer samples (n= 59 – 78%) showed no positivity to any of the tested microorganisms. The summary results of SCC and microbiology may be observed in Fig. 31.

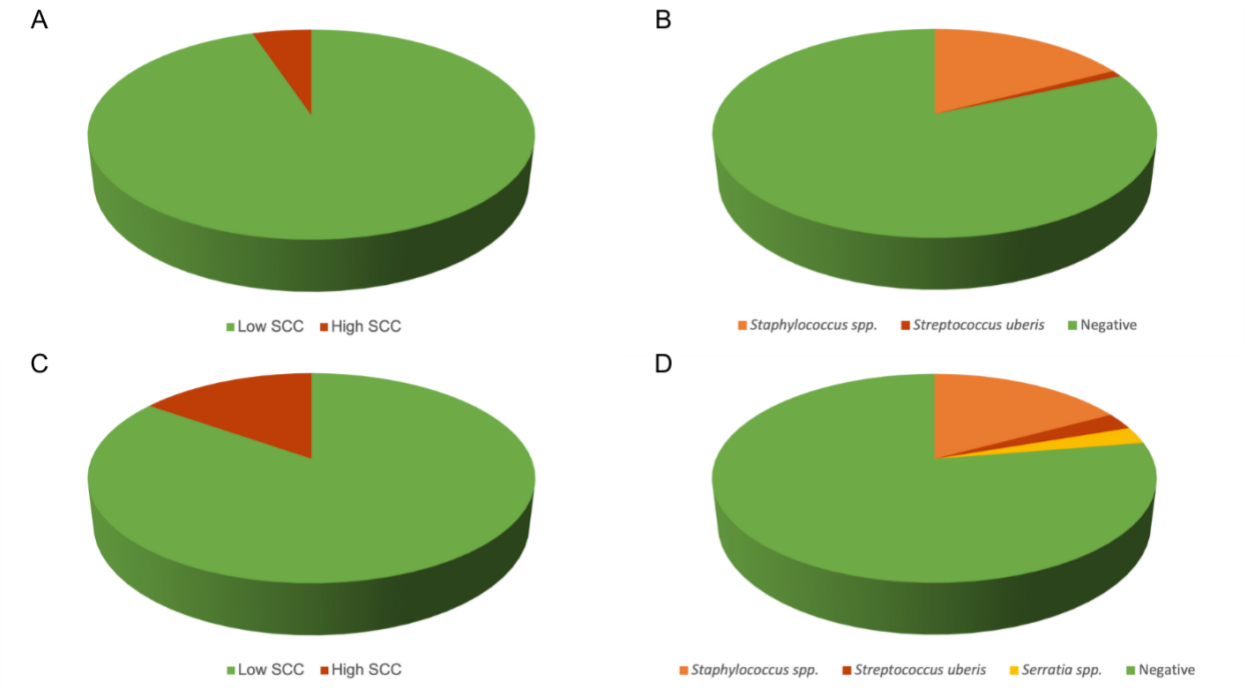

Figure 31. Summary results of SCC (A and C) and microbiology (B and D). Samples were collected in winter (A and B) and summer sampling (C and D). Milk samples were classified according to a threshold SCC value of 200,000 cells/mL.

4.3.2 EVs isolation and Western blot

To validate the presence of EVs in our samples, a western blot analysis was performed on representative samples, randomly selected from the collected samples. Both EVs markers, TSG101 and CD9, were positive in our samples. Specific bands of the two targets are reported in Fig. 32.

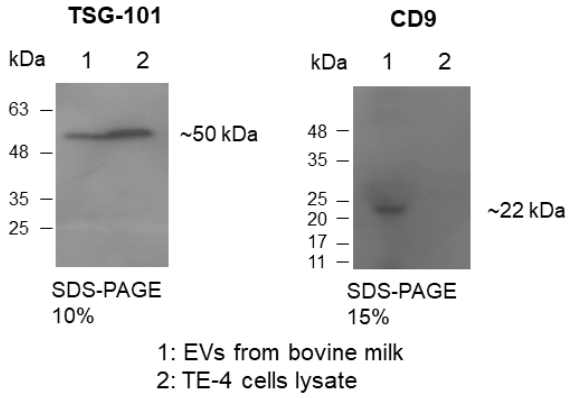

Figure 32. Western blot analysis of EVs samples. Analysed EVs markers were TSG-101 (~ 50 kDa) and CD9 (~ 22 kDa).

4.3.3 smallRNA-sequencing and bioinformatic analysis

The differential analysis was performed using a cut-off value of 200,000 cells/ml. A total of 1997 miRNAs were differentially expressed (DE) between group L and group H. Among them, 1267 were upregulated and 730 downregulated. Further investigations were performed filtering DE miRNAs using a false discovery rate (FDR) < 0.05. In this way, a total of 68 miRNAs were obtained, which were mostly downregulated. Intriguingly, only 1 miRNA, *bta-miR*-361-3p, was upregulated with 2.7 fold change (FC) value. Among the downregulated miRNAs, a total of 15 had a FC < -2 and are listed in Tab. 10.

miRNA	FC	p-value
bta-miR-223-5p	-2.7	1.31E-09
bta-miR-4286-2-3p	-2.2	1.31E-09
bta-miR-3432b-5p	-2.1	1.08E-08
bta-miR-2340-5p	-2.3	3.94E-08
bta-miR-11986c-3p	-2.7	5.37E-08
bta-miR-10161-3p	-2.2	1.24E-07
bta-miR-124a-1-3p	-2.5	2.85E-07
bta-miR-12026-1-5p	-2.6	5.07E-07
bta-miR-2285k-1-3p	-2.4	3.30E-06
bta-miR-11986-5p	-2.3	5.12E-06
bta-miR-12002b-5p	-2.3	7.06E-06
bta-miR-2285u-5p	-2.6	9.77E-06
bta-miR-568-5p	-2.1	1.24E-05
bta-miR-11998-5p	-2.1	5.63E-05
bta-miR-12010-5p	-2.1	8.28E-05

Table 10. Most significantly downregulated miRNAs in group H in comparison with group L. The results were considered significant with p-value < 0.05.

4.3.4 Functional analysis

The functional analysis was conducted using human (*bta*-) orthologs. The targets for 17 known miRNAs were obtained from OmicsNet with Panther database. Biological processes were clustered in two main macro-categories: cell life processes (including cell cycle, regulation of cell cycle, cell proliferation and apoptotic process) and gene expression machinery processes (including regulation of transcription by RNA polymerase II, Transcription DNA-templated, mRNA processing, mRNA splicing via splicesome, regulation of translation and protein phosphorilation). In addition, the regulation of immune system processes was investigated using ClueGO plugin in Cytoscape. The main immune processes significantly influenced by the DE miRNAs are listed below:

- somatic recombination of immunoglobulin gene segments,
- activation of innate immune response,
- immunoglobulin production involved in immunoglobulin-mediated immune response,
- somatic diversification of immunoglobulins,
- positive regulation of isotype switching,
- antigen processing and presentation of peptide antigen via MHC class I,
- somatic diversification of immunoglobulins involved in immune response,
- somatic recombination of immunoglobulin genes involved in immune response,
- isotype switching,
- positive regulation of B cell mediated immunity,
- positive regulation of immunoglobulin mediated immune response,
- negative regulation of hematopoietic progenitor cell differentiation,
- stimulatory C-type lectin receptor signalling pathway,
- alpha-beta T cell lineage commitment,
- positive regulation of lymphocyte mediated immunity,
- CD4-positive or CD8-positive, alpha-beta T cell lineage commitment,
- establishment of T cell polarity,
- monocyte differentiation,
- establishment of lymphocyte polarity.

These immune processes were mainly related with mediated immunity including immunoglobulins productions and lymphocytes regulation. Therefore, the miRNA-gene networks were displayed using Cytoscape and three separated reactomes were prepared considering cell life (Fig. 33), gene expression (Fig. 34) and immunity processes (Fig. 35). Two large nodes of regulation of miRNA-gene interactions involved *miR*-503-5p and *miR*-455-3p both cell life and gene expression

processes. Considering the immunity reactome, here the network is less complex than the previous. However, it is possible to highlight 4 main miRNAs involved in the regulation of this processes: *miR*-455-3p, *miR*-503-3p, *miR*-1301-3p and *miR*-361-5p. A summary of the main results related this research line is shown in ANNEX B – Figure 2.

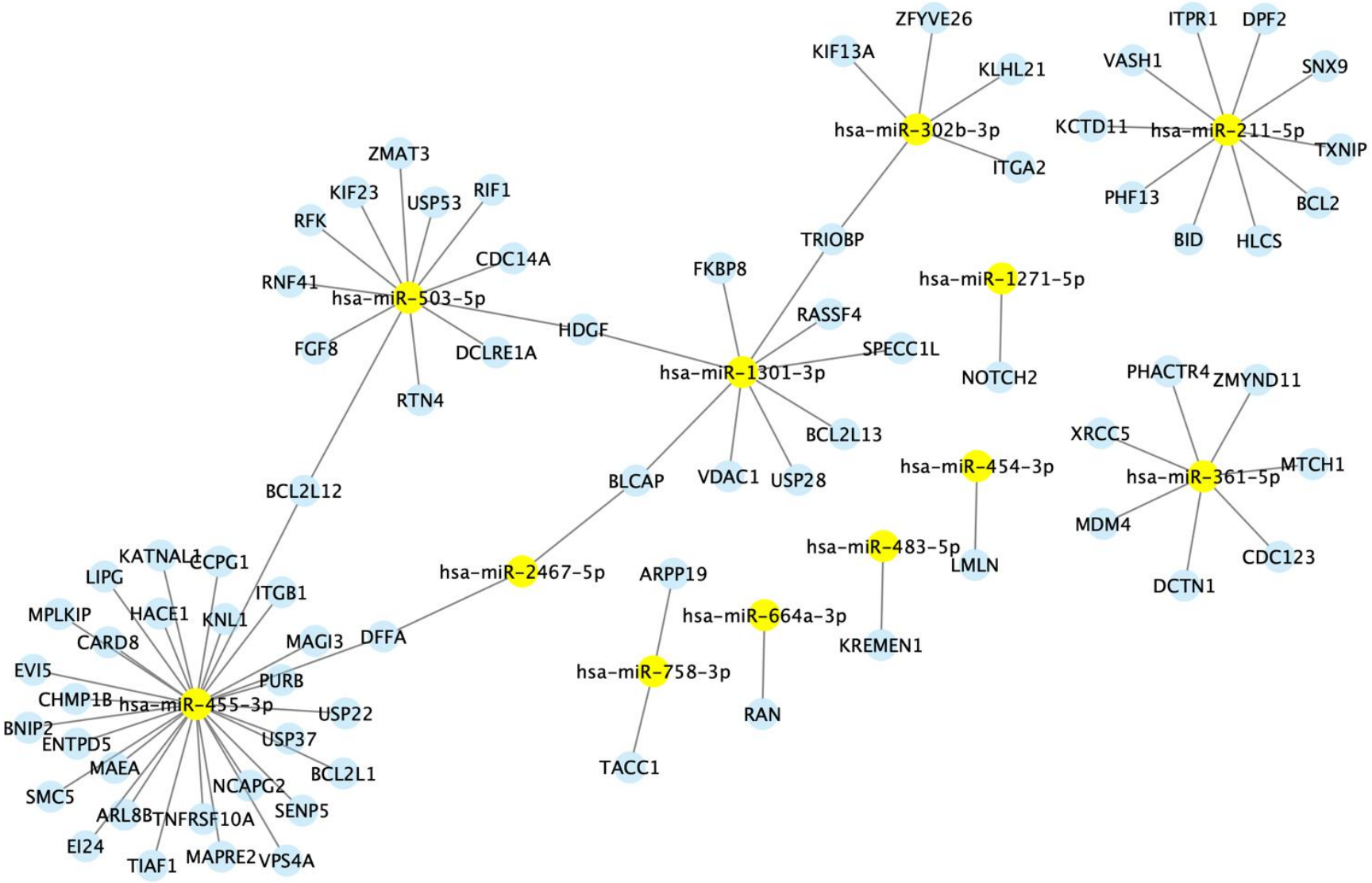

Figure 33. Networks of the differentially abundant miRNAs identified using Cytoscape. The results are related to miRNA-gene interactions involved in cell life processes.

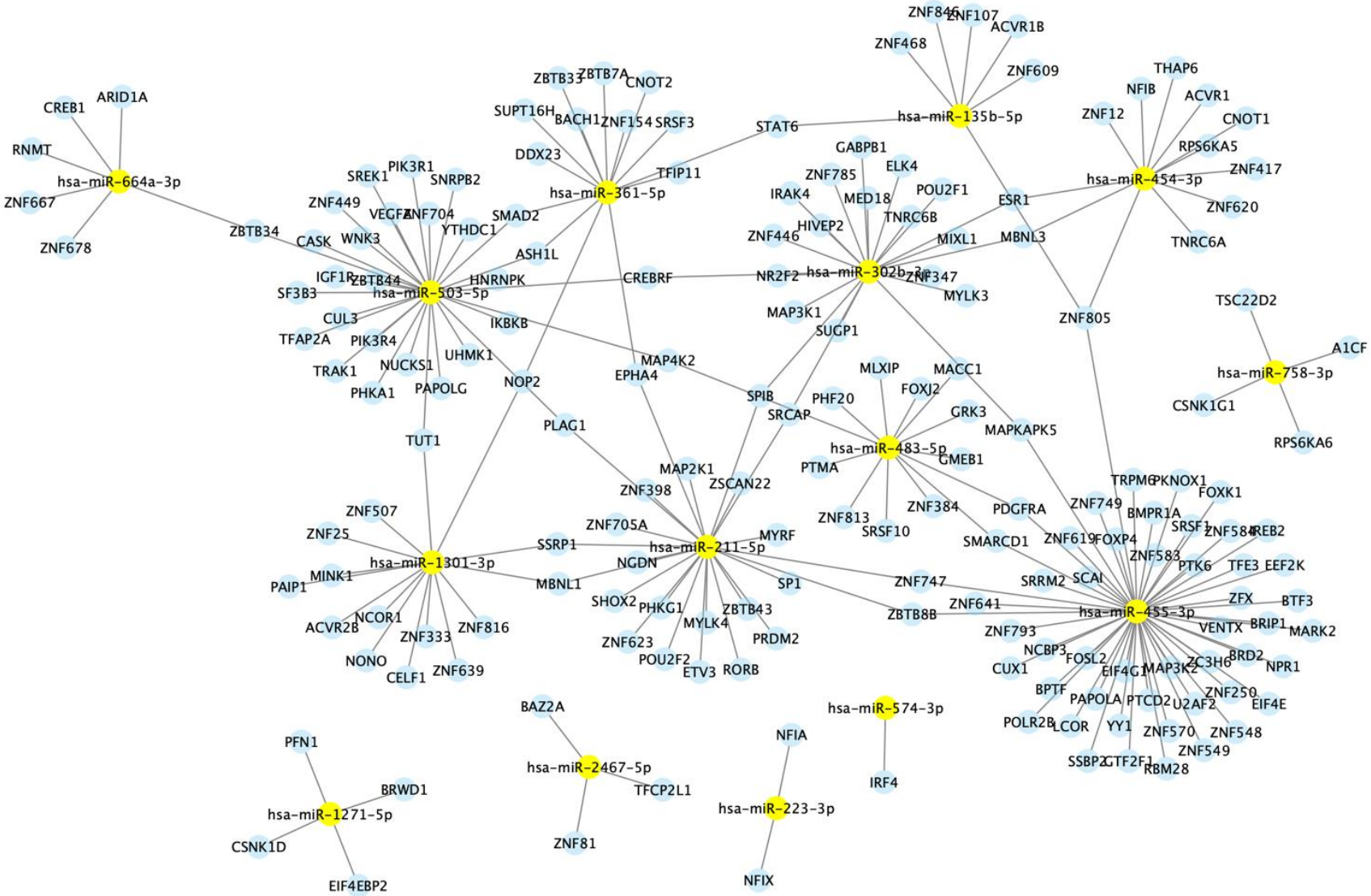

Figure 34. Networks of the differentially abundant miRNAs identified using Cytoscape. The results are related to miRNA-gene interactions involved in gene expression machinery.

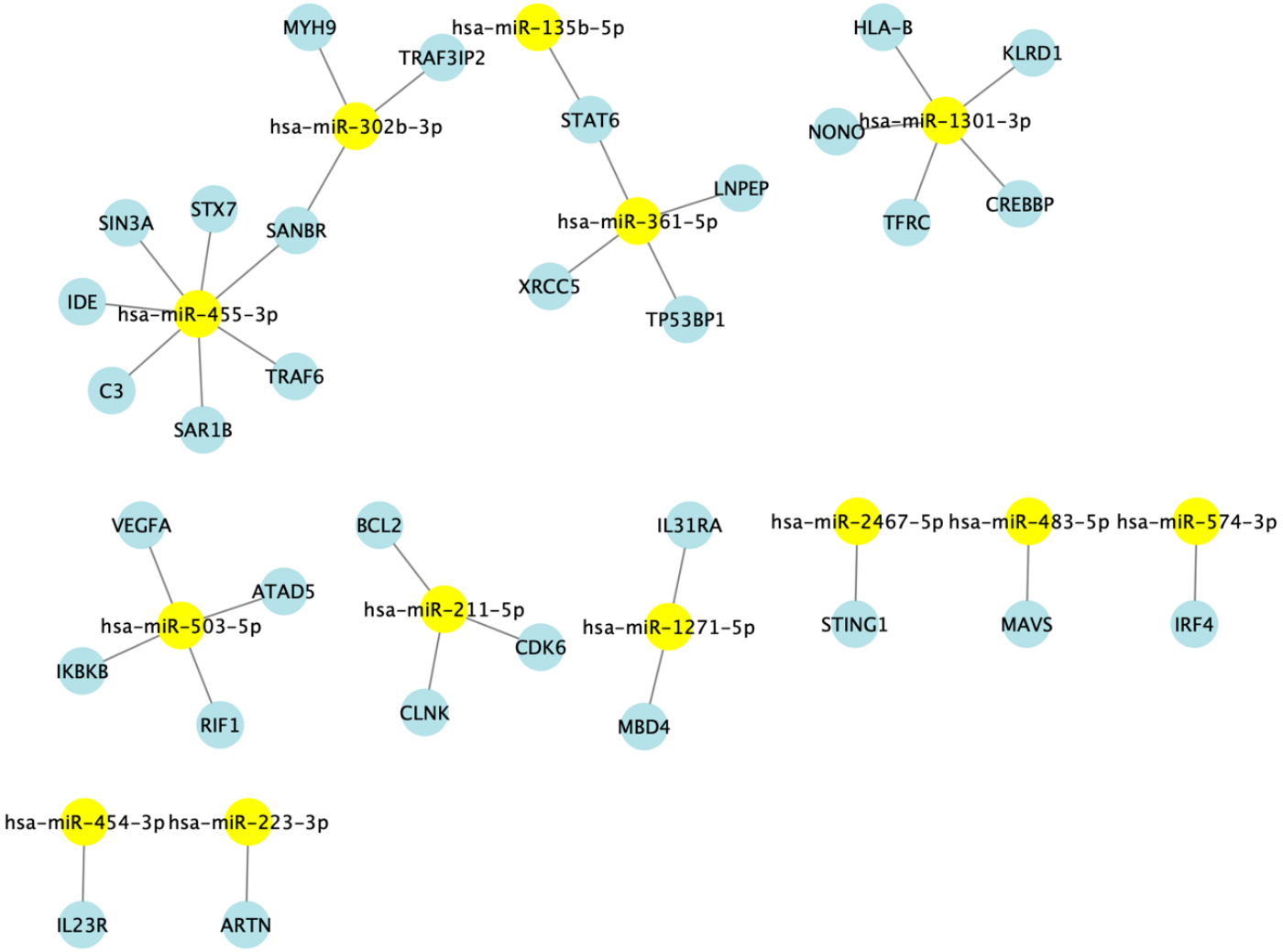

Figure 35. Networks of the differentially abundant miRNAs identified using Cytoscape. The results are related to miRNA-gene interactions involved in immune system processes.

4.4 Discussion

Bovine mastitis is one of the main challenges that farmers and veterinarians routinely must face in dairy farming. Moreover, the spread of antimicrobial resistance and the recent entering into force of the new European regulation about the use of veterinary medicinal products amplified the need for the implementation of diagnostic tools to early detect subclinical mastitis, to reduce/avoid antimicrobial treatments. In this study, individual milk samples were collected from a total of 174 Holstein Friesian cows during routinely mastitis screening tests and subjected to smallRNA-seq analysis to detect miRNome profiles suggestive of the subclinical form of the disease. Indeed, bovine mastitis is able to modify the milk miRNome and several miRNAs have been reported to be differentially expressed during mammary gland inflammation (Do et al., 2021). Among the different parameters of mammary gland infection, the increase in the somatic cell count (SCC) in milk is considered a prognostic value. According to the International Dairy Federation guidelines, the optimal SCC value to differentiate between affected and healthy cows is around 200,000 cells/ml. Therefore, to compare healthy subjects with cows potentially affected by subclinical mastitis, the milk samples were classified according to the SCC measurement, setting the 200,000 cell/ml value as a threshold.

In our study, the functional features analyses of milk showed that a total of 28 (16 in winter and 12 in summer) samples with SCC lower than 200,000 cells/ml were positive for *Staphylococcus* spp. Such result is in line with other studies reporting low SCC levels in primiparous cows and/or positive for non-aureus staphylococci (Sampimon et al., 2009; Narayana et al., 2021). However, recent studies have also described less pathogenic Staphylococcus strains, whose virulence factors could induce less severe mastitis or a delayed SCC increase (Wellnitz & Bruckmaier, 2012; Rossi et al., 2021). Indeed, the host-pathogen interaction varies according to the stage of mastitis and the arousal of the inflammatory response depends on the responsiveness of the host immune system (Wellnitz & Bruckmaier, 2012; Abdelmegid et al., 2018; Maity et al., 2020). In conclusion, there is still a large debate in the veterinary and scientific communities regarding the SCC level characterising mastitis. First of all, the Regulation (EC) N. 853/ 2004 of the European Parliament and of the Council of 29 April 2004 has established 400,000 SCC as the cut-off value to consider the milk suitable for human consumption. However, many studies reported mastitis forms with SCC lower than 200,000, mainly caused by environmental pathogens (Cobirka et al., 2020). In addition, the SCC can vary according to the stage of the disease and be also influenced by any pharmacological treatments, such as antimicrobial and/or anti-inflammatory molecules (Laevens et al., 1997). Although numerous factors can influence the SCC at individual cow- and udder quarter level, such as parity, lactation stage, incorrect milking machine settings, stress and other factors including genetics, the most important cause remains the infection status of the mammary gland (Schepers et al., 1997).

On the other hand, 10 (3 in winter and 7 in summer) milk samples presented negative results to the microbiological analysis, but SCC higher than 200,000 cells/ml. Such discrepancy can be explained by the well-known limitations of the microbiological assays (Ashraf & Imran, 2020; Cobirka et al., 2020; Saenz-de-Juano et al., 2022b). In addition, *Mycoplasma* spp. should be considered, due to difficulties in its cultivation and isolation procedures (Ashraf & Imran, 2020). Also, viruses should be considered as potential mastitis pathogens and in this study their presence was not checked, mainly because their research is not well established in the Italian diagnostic routine.

The results of the milk miRNome profiling showed statistically significant differences between samples with high SCC level (> 200,000 cell/ml, group H) and samples with low SCC level (< 200,000 cell/ml, group L). In particular, 68 miRNAs are differentially expressed (DE), mostly downregulated in group H compared to group L, considered as healthy. Among these 68 DE miRNAs, 15 miRNAs were downregulated with FC value < -2. Many of these DE miRNAs were specifically related to bovine species and no relevant information can be retrieved from scientific literature. However, 3 miRNAs can be highlighted: miR-223, miR-124 and miR-568.

First, miR-223 is largely described in literature as regulating the inflammatory response in bovine mastitis (Naeem et al., 2012; Chen et al., 2014; Fang et al., 2016; Pu et al., 2017; Cai et al., 2018). All these studies reported an upregulation of miR-223 in response to *S. aureus*, *S. uberis*. or *S. agalactiae* experimental infections. Therefore, miR-223 downregulation in the present study is not in line with literature. However, miR-223 is either expressed almost exclusively or highly enriched in several subsets of white blood cells including T- and B- lymphocytes, neutrophils, mast cells and monocytes (Tzelos et al., 2022). Considering this, miR-223 downregulation observed in this PhD study may be due to the removal of SCC from milk samples by centrifugation.

On the other hand, miR-124 and miR-568 were significantly downregulated in this study and for the first time reported as influencing bovine mastitis. According to literature, miR-124 was already described as regulating *Schistosoma japonicum* and *Fasciola gigantica* parasitic pathogenesis in bovine and buffalo (Guo & Guo, 2019; Yu et al., 2019; Zhou et al., 2022). Intriguingly, miR-568 inhibitory activity on CD4+ T cells was previously demonstrated suggesting a role in the modulation of lymphocytes activity (Lie et al., 2013).

According to the functional analysis, four miRNAs were mainly involved in the regulation of the biological processes investigated: miR-455, miR-361, miR-1301 and miR-503. The functional analysis was conducted using human orthologs (*hsa*-) and not bovine (*bta*-), since human databases are more complete and information about miRNAs, their function and interaction with genes are constantly updated. This study was conducted on bovine and the functional results

obtained may just be considered as predictive and further studies must be conducted to validate these results.

However, results can be compared with the scientific literature to contextualise and explain the role of these 4 miRNAs:

- miR-455 has already been reported in dairy cows subjected to dietary conditioning (Webb et al., 2020). Webb and colleagues observed a downregulation of miR-455 in the period after calving in cows fed with a high balanced diet. Our results are in line with this study, since both reported miR-455 downregulation and its involvement in the regulation of the inflammatory response. Considering human literature, miR-455 downregulation is associated with the activation of inflammatory pathways in multiple sclerosis (Torabi et al., 2019). Moreover, the anti-inflammatory activity of miR-455 was also verified and confirmed by recent studies reporting an efficient therapeutic role of this miRNA in acute liver injury and cerebral ischemia/reperfusion injury (Shao et al., 2020; Zhang et al., 2022).
- Another interesting result is miR-361 upregulation, which seems also to be involved in the regulation of the biological processes investigated. In bovine, miR-361 was already reported as downregulated in serum of grazing cows in comparison with housed cattle (Muroya et al., 2015). This study may suggest a role of miR-361 in the regulation of metabolism in cows. In fact, it is well-known that during mastitis metabolism and milk composition can vary according to its protein and fat composition (Addis et al., 2020; Giagu et al., 2022). Therefore, the upregulation of miR-361 in our samples may depend on metabolic factors indirectly modified by mastitis. In humans, miR-361 is reported as a promising biomarker for tuberculosis diagnosis and it is most likely involved in the regulation of host-pathogen interaction (Qi et al., 2012; Ndzi et al., 2019;).
- Furthermore, also miR-1301 downregulation was firstly reported in mastitis by this study. Luoreng and colleagues reported an upregulation of miR-1301 in dairy cows experimentally infected by a *Staphylococcus aureus* strain (Luoreng et al., 2018). This controversial result may be partially explained by the different biological fluid analysed, milk in the present study and blood in Luoreng study. However, *S. aureus* was never identified in the milk samples of this study. It is well-known that etiological agents can differently influence mastitis pathogenesis (Jin et al., 2014; Luoreng et al., 2018), and differences in the milk miRNome between Escherichia coli and S. aureus mastitis have already been reported (Mansor et al., 2013). Therefore, a different regulation of miR-1301 depending on the specific pathogens could not be excluded.
- Finally, miR-503 represents another important node of regulation according to the functional analysis. Most information regarding miR-503 are related to human medicine. This miRNA seems to be involved in the pathogenesis of diabetes and LPS injury (De

Silva et al., 2018; Zapała et al., 2023), but more intriguingly its downregulation seems to be involved in the activation of NF-kB signalling and PPARγ pathway (Zhou et al., 2013; Lee et al., 2017). Finally, the downregulation of miR-503 was also reported in dog blood mononuclear cells infected by *Leishmania infantum* suggesting a role in the modulation of host-pathogen response (Soares et al., 2021).

In this study, the miRNome profile of dairy cows affected by subclinical mastitis was investigated and compared to healthy control samples. EVs were obtained from milk samples to better understand the role of miRNAs in the regulation of mastitis and to identify new potential biomarkers of the disease. EVs and miRNAs have been largely studied in human and veterinary medicine for their evaluation as potential biomarkers of different diseases such as cancer, neurodegenerative and infectious diseases. In scientific literature, some studies have been already published regarding bovine mastitis.

First, one innovative aspect of this study is the selection of dairy cows naturally affected by mastitis. Therefore, studies with an in-field scenario give the opportunity to evaluate more precise and truthful pathological conditions in comparison with experimental studies. Moreover, some studies, even if conducted on naturally infected cows, considered a very low sample size (Ju et al., 2018; Saenz-De-juano et al., 2022b) in comparison with our large sample size (174 Holstein Friesian cows). Considering the method applied another important consideration can be done. Omitting economic reasons, using qPCR instead of smallRNA-seq represents a less precise approach considering the wide and complete panorama of miRNA expression and activity. In fact, studies applying qPCR or microarray did not allow the identification of novel bovine miRNAs (Lai et al., 2017; Srikok et al., 2020; Tzelos et al., 2022). Finally, other two studies, from Bagnicka and colleagues and Ozdemir, represent the most comparable with the present study (Özdemir, 2020; Bagnicka et al., 2021). Similar methods for smallRNA-seq and a smaller (but still well representative) sample size have been applied. However, the main DE miRNAs reported are different from this study. The main reason is probably related to the animal selection modality, which analysed only dairy cows positive for coagulase +/- *Staphylococci* or *M. bovis*. Instead, the present study focused the selection of cows on SCC and not on bacteriology. Furthermore, the different biological matrices could influence the results. In fact, it is well known that according to the chosen sample miRNAs can vary. For example, whole milk has a different miRNA profile from skim milk, or somatic cells, or milk fat globules, or mammary gland biopsies (Li et al., 2020; Pawlowski et al., 2020; Leroux et al., 2022).

Based on these results and their discussion, the results obtained from this study could be a promising starting point in the investigation of miRNAs and EVs regulation of bovine subclinical mastitis. Moreover, the applied methods and the sample size represent for sure an advantage

considering the literature review conducted. The functional analysis conducted in this study revealed a panel of 4 miRNAs, promising putative biomarkers of subclinical mastitis: miR-455, miR-361, miR-1301 and miR-503. It should be reminded that these results were obtained through a predictive analysis, using human orthologs and their role may not be the same on bovine. To clarify the DE miRNAs results, a future validation using qPCR or ddPCR may be performed on other dairy cows. In addition, to better clarify the role of DE miRNAs on the regulation of genes, identified by the functional analysis, an *in vitro* luciferase validation test may also be performed.

In conclusion, further studies must be conducted to identify and validate miRNAs used for mastitis detection, especially subclinical forms, but an integrative approach between clinical evaluation, SCC, microbiology and miRNAs will always be the optimal strategy to control bovine mastitis.

CHAPTER 5: Conclusions

The main aim of this PhD thesis was to investigate the application of NGS technology as a useful tool to understand the role of AMs on host and to identify new putative biomarkers for AMs treatments and disease diagnosis. In order to reach this aim, NGS technology was applied in two different farming area: poultry and dairy cows farming. In both farming systems, AMs were widely used to prevent and treat infectious diseases. The new regulatory scenario required a substantial reduction of AMs use to control the arousal and spreading of AMR. For this reason, this thesis focused on poultry and dairy cow farming systems, both models strongly accused for AMs misuse.

In poultry farming, AMs use has been quite reduced after the ban of growth promoter use. AMs have been applied in the past as a preventive tool to ensure the gut health. Since the removal of growth promoters and the actual restriction in the AM prophylaxes, ensuring gut health and efficient growth performance has become one of the biggest challenges in poultry farming. One of the aims of this thesis was to investigate the role of AMs on the host, in particular on the intestinal barrier. Investigating the AMs effects on gut microbiota and host intestinal barrier allows to identify the mechanism underlying the intricate relationship between host, microbiota and AMs. In that way, it will be possible to find new solutions to ensure gut health and efficient growth performance, but also to discover pathophysiological mechanisms useful in the identification of valuable alternatives to AMs in the modulation of gut health. In addition, in this context there is also a need for the identification of putative biomarkers in order to identify the broilers treated with AMs. The introduction of new limitations in AMs use requires the implementation and the improvement of the diagnostic tools, available for AMs identification in food matrices. In this context, the present thesis firstly investigated the role of AMs at gut barrier level using NGS technology (16S metabarcoding), associated with histopathology, IHC and gene expression techniques. The results of this thesis suggest a clear influence of AMs on the gut microbiota, which is characterised by dysbiosis. Moreover, the identification of intestinal microscopic lesions and their association with microbiota alterations, supported by the statistical analysis, revealed a negative effect of AMs on the gut barrier of treated broilers. TJ proteins have been investigated as possible mediators in the modulation of the negative effects of the gut dysbiosis on the intestinal tissue. The application of IHC did not allow to identify differences in TJ proteins expression due to AMs treatments. However, qPCR results showed that claudin-1 can be significantly downregulated by the treatments. Considering the identification and validation of AMs treatment biomarkers, many potential molecular biomarkers were tested on liver and PM muscle samples of broilers treated with different AMs protocols. Unfortunately, data obtained in this study did not provide conclusive findings for the validation of these biomarkers. The additional treatment with doxycycline, administered to all animals of trial 3, is the biggest bias of this part of the study. Nevertheless, many statistical differences in the GM, intestinal lesions and TJ gene expression

results may confirm a role of preventive prophylaxis in the worsening of the observed findings. Additional studies will be necessary for both investigating more in deep the role of AMs at gut barrier level and also for a validation of the AMs treatment biomarkers. In particular, to better clarify the role of TJ proteins in the modulation of the gut barrier other imaging or molecular techniques can be applied. For instance, the application of immunofluorescence together with RNA-scope hybridisation in situ may allow to answer this question. Additionally, investigating the role of entero-hepatic circulation will allow to understand the regulation mechanisms between host and gut microbiota and it also may confirm the abovementioned biomarkers or identify other potential ones.

On the other hand, the dairy cows farming system is coping with different consequences for the new AMs restrictions. Here, the ban of AMs prophylaxes was the biggest issue, which were previously adopted in some dairy farms as preventive measures in the dry-off period after lactation. For this reason, there is an urgent need to support new studies with the aim of identifying new biomarkers for an early diagnosis of mastitis. In this way, an early identification of sick dairy cows (especially subclinical forms) will allow the application of preventive pharmacological treatments and efficient biosecurity measures. Moreover, the isolation of those subclinically sick will allow first to restrict the pathogens spreading and indirectly also to reduce the overall amount of AMs used to treat mastitis in the dairy herd. In this study, conducted applying smallRNA-seq, the role of miRNAs vehiculated by EVs has been investigated in the modulation of mastitis and in their application as potential early predictors of subclinical mastitis. A panel of 4 miRNAs, highlighted by the differential and functional analyses, has been presented in this study. According to the functional analysis, their function is mainly related to immune and inflammatory functions and most likely their regulation is attributable to mastitis pathogenesis. One possible limitation of this study may be the limited representation of different bacterial pathogens in the sampled group. It is well-known that according to the mastidogen microorganisms miRNome profile can be differently modulated. However, considering the previously presented comparison of this study with literature, it is possible to emphasise the strong points of this study mainly related to the large sample size and the selection of dairy cows naturally affected by mastitis. Further studies will be conducted to first confirm the role of these miRNAs during mastitis and then validate them as new biomarkers. For example, a new sampling will possibly be conducted on a different set of dairy cows with a better representation of mastidogen pathogens. The validation study will be also completed in order to understand the activity of these miRNAs on mRNA targets. The identification of the silenced mRNA targets will allow to better understand the consequences of these miRNAs using a more reliable bovine model than the functional results obtained using human databases.

In conclusion, it is possible to confirm that NGS technology has been a useful choice in the investigation here presented. The results of this PhD thesis confirm that NGS is an affordable approach in the identification of potential biomarkers of AMs treatments and disease diagnosis, but also in the investigation of the relationship between host, pathogen and drug. The main advantages are related to the fast time needed for the analyses, the big amount of data achievable with NGS and the reliability of results obtained with those developed techniques. The application of NGS can be for sure a powerful tool to find new solutions and support the new requirements derived from the veterinary scenario related to AMR and AMs use. However, the optimal situation for NGS application will always be its association with other techniques, such as histopathology, IHC, microbiology and other routine diagnostic tests.

ANNEX A: experimental designs

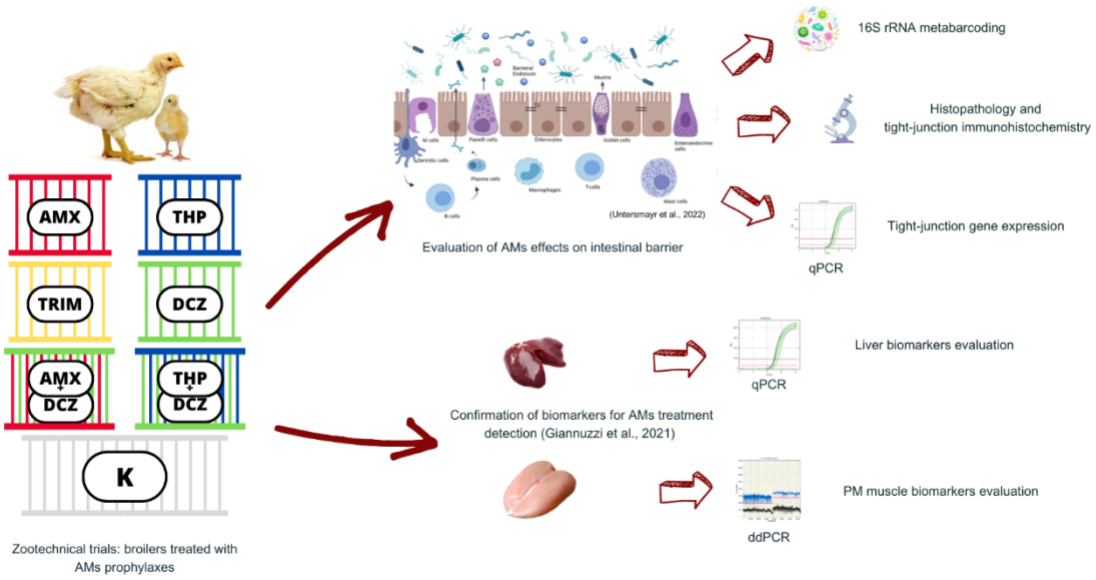

Figure 1. Graphical representation of the experimental design in the poultry farming research line.

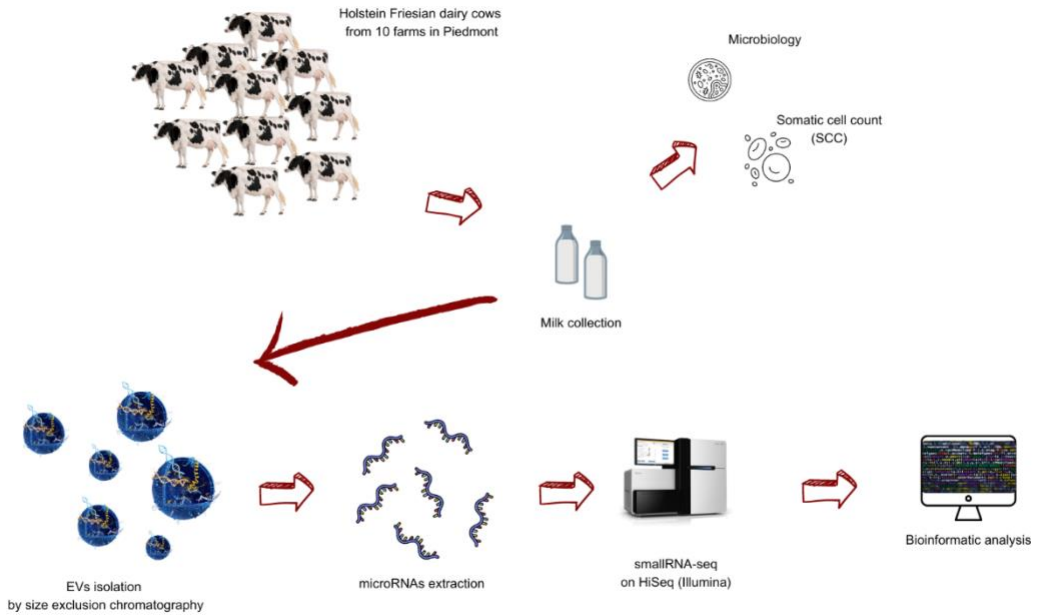

Figure 2. Graphical representation of the experimental design in the dairy cows research line.

ANNEX B: summary results

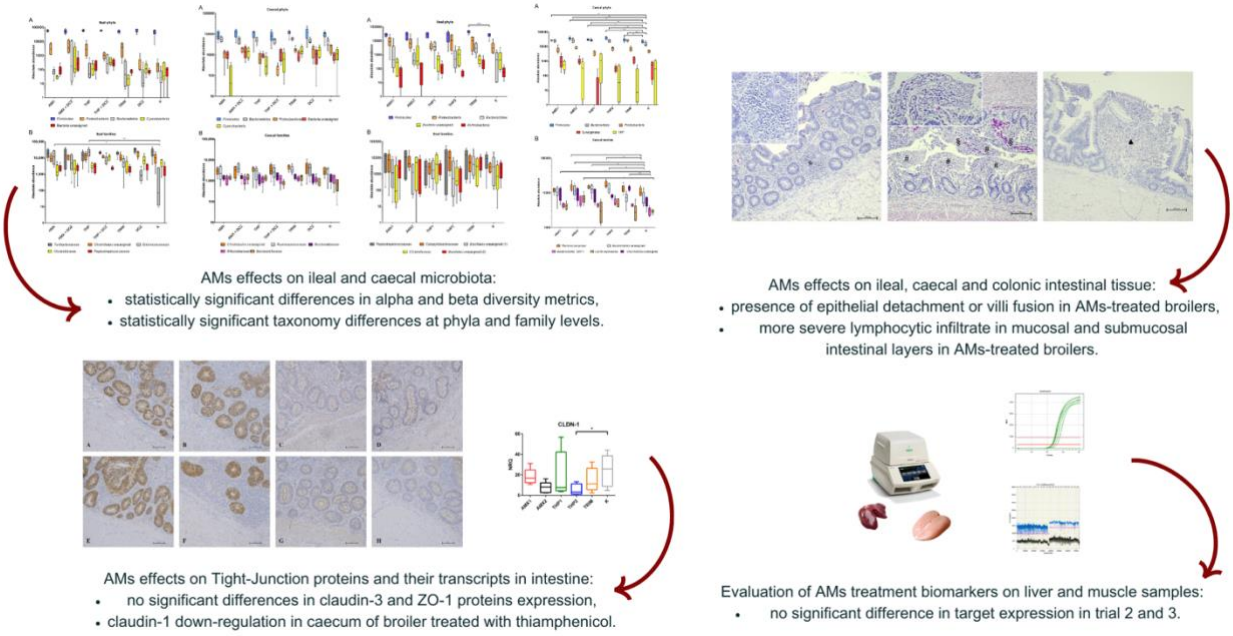

Figure 1. Graphical representation of the experimental design in the poultry farming research line.

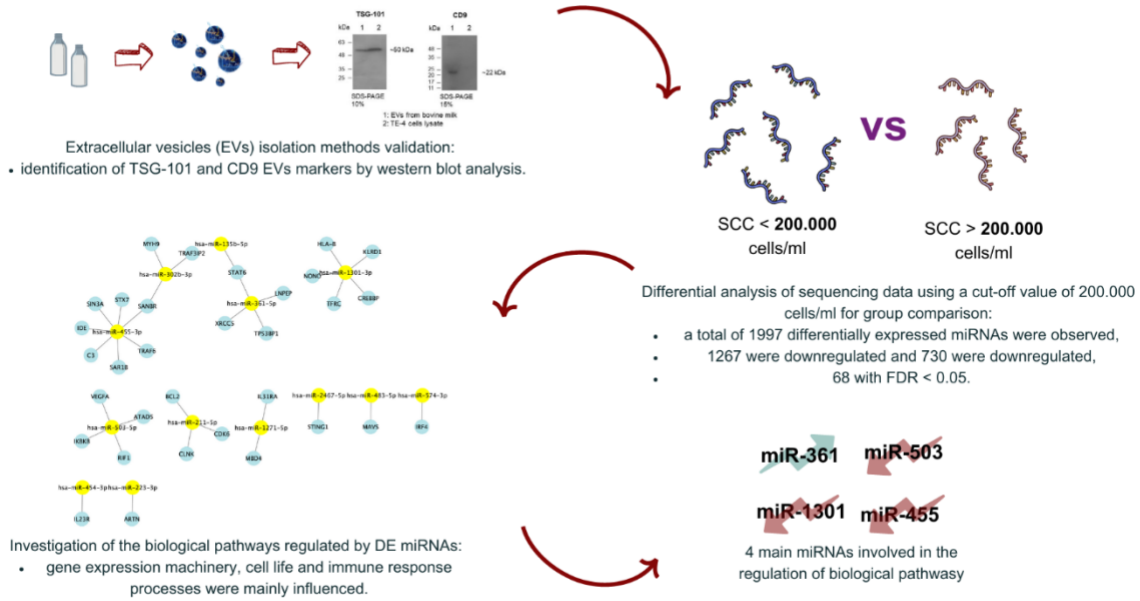

Figure 2. Graphical representation of the experimental design in the dairy cows research line.

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