UNIVERSIDADE FEDERAL DA PARAÍBA
CAMPUS II – AREIA-PB
CENTRO DE CIÊNCIAS AGRÁRIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA ANIMAL

MATEUS LACERDA PEREIRA LEMOS

EFEITOS DA ADMINISTRAÇÃO PROFILÁTICA PÓS-ECLOSÃO DE CEFTIOFUR E DA SUPLEMENTAÇÃO COM SANGUINARINA NA MICROBIOTA CECAL DE PINTINHOS

AREIA
2020

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Dissertação apresentada ao Programa de Pós-Graduação em Ciência Animal da Universidade Federal da Paraíba, como requisito parcial à obtenção do título de Mestre em Ciência Animal.

Orientador: Prof. Dr. Celso José Bruno de Oliveira.

AREIA
2020

Catalogação na publicação
Seção de Catalogação e Classificação

L557e Lemos, Mateus Lacerda Pereira.
Efeitos da administração profilática pós-eclosão de ceftiofur e da suplementação com sanguinarina na microbiota cecal de pintinhos / Mateus Lacerda Pereira Lemos. - Areia, 2020.
37 f. : il.

Orientação: Celso José Bruno de Oliveira.
Dissertação (Mestrado) - UFPB/Campus II.

1. Avicultura. 2. Cefalosporinas. 3. Microbioma. I. Oliveira, Celso José Bruno de. II. Título.

UFPB/CCA-AREIA

MATEUS LACERDA PEREIRA LEMOS

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Dissertação apresentada ao Programa de Pós-Graduação em Ciência Animal do Centro de Ciências Agrárias da Universidade Federal da Paraíba, como parte das exigências para a obtenção do título de Mestre em Ciência Animal. Área de Concentração Saúde Animal do Brejo Paraibano.

APROVADA EM 02/03/2020

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Dedicatória:

Ao meu pai,

Abraão Pereira Lemos

In memoriam ao meu primo,

Francisco Brasilino Lemos

AGRADECIMENTOS

Primeiramente a Deus, pois vem me concedendo a força de vontade e o sopro da vida diários para que eu me mantenha de pé...muitos foram os momentos durante esta jornada na pós-graduação nos quais o Senhor me confortou e me recarregou, então, tive coragem para chegar até aqui.

Exalto a minha família logo após: Abraão Pereira Lemos, Nevilúcia Lacerda Pereira Lemos e Tiago Lacerda Pereira Lemos. Vocês foram o meu pilar não somente no decorrer da minha estadia na universidade, mas também em toda a minha vida, amo vocês.

Posteriormente, agradeço ao meu orientador, o professor Celso José Bruno de Oliveira, por todo o conhecimento transmitido, pela disposição de recursos e na orientação desde o princípio até o fim do mestrado. Não obstante, agradeço também a Mauro de Mesquita Souza Saraiva por ter me fornecido o que serviu de fundamento para o meu projeto.

Agradeço a toda a equipe do Laboratório de Análises de Produtos de Origem Animal da UFPB, Campus II, pois o produto desta pesquisa é fruto de todo um trabalho em equipe. Em especial, destaco o pessoal que me auxiliou nos procedimentos de bancada, bioinformática e correção do artigo: Priscylla Carvalho, Núbia Vieira, Maylane Rayane e Elma Lima.

Devido à tutoria e ao acompanhamento no estágio docência, gostaria de agradecer à professora Suzana Aparecida Costa Araújo. Igualmente, não poderia esquecer do meu amigo Diogo Vieira Moura que me acompanhou nesta tarefa.

Apesar de não se fazerem presentes diretamente no meio acadêmico, eu gostaria de agradecer a Vinícius Tomé dos Santos Souza e a Hércules Camilo dos Santos, pois, além de colegas de profissão com quem tenho a oportunidade de compartilhar as minhas opiniões, são, antes de tudo, fiéis amigos. Lamento não poder citar o nome de todos aqui nesta breve lista, pois há muitos nomes de prezados (as) companheiros (as) que eu gostaria de mencionar e, infelizmente, seria inviável, porque as limitações deste texto fogem do escopo da minha gratidão e me obrigam a omiti-los.

Em suma, aconteceu que todos os participantes ativos da minha vida no ano de 2019 acabaram por impactar de modo profundo no meu desenvolvimento pessoal, inclusive pessoas cujas intenções não eram de prestar suporte à princípio. De um jeito ou de outro, vocês contribuíram com a construção da minha atual personalidade que a cada dia deixa de ser menos bruta e passa a ser mais lapidada. Obrigado!

RESUMO

A aplicação profilática de ceftiofur em frangos de corte recém-eclodidos é uma prática comum em vários incubatórios. Investigamos os efeitos dessa prática na microbiota cecal de pintinhos de 14 dias. Além disso, também investigamos os efeitos da suplementação dietética da sanguinarina como aditivo alimentar fitogênico na microbiota intestinal. As amostras de DNA foram extraídas do conteúdo cecal, amplificadas para as regiões v3-v4 do gene 16S rRNA microbiano e sequenciadas em uma plataforma ilumina Miseq. Após bioinformática a jusante e análises estatísticas, nossos resultados demonstraram que os tratamentos envolvendo tanto o ceftiofur como a sanguinarina aumentaram as proporções do filo Bacteroidetes e dos gêneros *Bacteroides* e *Megamonas* no ceco das aves, enquanto reduziram a abundância relativa do filo Firmicutes e da família *Lachnospiraceae*. A inferência gênica indicou um aumento nas vias metabólicas associadas à digestibilidade na microbiota de aves tratadas com ceftiofur. As mudanças de diversidade e abundância na comunidade bacteriana cecal de frangos de corte, desencadeados pelo ceftiofur, foram semelhantes às observadas nas aves que receberam dieta suplementada com sanguinarina. Tais descobertas apoiam relatos anteriores sobre os benefícios da sanguinarina na produtividade como uma alternativa para melhorar a saúde animal.

Palavras-chave: Avicultura. Cefalosporinas. Microbioma.

ABSTRACT

The prophylactic application of ceftiofur to newly hatched broiler chickens is a common practice in several hatcheries. We investigated the effects of this practice on the cecal microbiota of 14 days-old chicks. Furthermore, we also investigated the effects of the dietary supplementation of sanguinarine on the gut microbiota. DNA samples were extracted from cecal contents, amplified for thev3-v4 regions of the microbial 16S rRNA gene and sequenced in an Illumina Miseq platform. After downstream bioinformatics and statistical analyses, our results demonstrated that both treatments involving ceftiofur and sanguinarine treatments increased the proportions of the phylum Bacteroidetes, and the genus *Bacteroides* and *Megamonas* in the cecum of birds while reduced the relative abundance of the Firmicutes phylum and the *Lachnospiraceae* family. The functional prediction indicated an increase in metabolic pathways associated with digestibility in the microbiota of birds treated with ceftiofur. Diversity and abundance changes in the cecal bacterial community of broiler chickens triggered by ceftiofur were similar to those observed in the birds receiving sanguinarine-supplemented diet. Such findings support previous reports on the productivity benefits of sanguinarine as an alternative compound to improve gut health.

Key words: Poultry. Cephalosporins. Microbiome.

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1 INTRODUÇÃO

A avicultura de corte é uma atividade agropecuária caracterizada por rápidos retornos financeiros no pós-investimento em razão de diversos fatores, como a exigência de pequenas áreas disponíveis e menos infraestrutura, possibilitando que países em desenvolvimento possam competir no mercado e, além disso, o consumo de carne de frango tem se mostrado crescente em decorrência de preços mais baixos em relação à carne bovina, maior aceitabilidade do que a carne suína em certas regiões do planeta e apresentar níveis elevados de nutrientes essenciais, como proteína de boa qualidade, minerais e baixo teor de gordura (FAO, 2013).

A avicultura brasileira desempenha um papel relevante no mercado internacional, sendo que o Brasil atingiu o posto de maior exportador de carne de frango no ano de 2019, com um total de 3,75 milhões de toneladas vendidas, superando os EUA, União Europeia e China. Adicionalmente tem ocorrido aumento na demanda interna em razão da restruturação da economia brasileira, maior expectativas no crescimento do PIB nacional e uma previsão para 2020 da diminuição das taxas de desemprego (USDA, 2019).

Assim como os demais setores do agronegócio, o sistema produtivo avícola apresenta desafios relacionados a questões ambientais, como o desmatamento de vegetações nativas para a construção de aviários e plantio de monoculturas base da alimentação animal, acúmulo de compostos nitrogenados e emissão de gases estufa (MOTTET; TEMPIO, 2017). Há também questões relacionadas ao bem estar animal, como a alta densidade populacional por m², exposição contínua à luz artificial, estresse térmico, locomoção restrita, anormalidades músculo esqueléticas (BESSEI, 2006), enfermidades infecciosas que afetam os animais ou até mesmo a seres humanos, principalmente relacionadas a zoonoses importantes com impacto em saúde pública (FILHO, L.A.F.; BALIAN, S. DE CARVALHO, 2009). Alguns estudos epidemiológicos demonstraram que alguns patógenos originalmente presentes no trato gastrointestinal de aves, como *Salmonella* spp., levaram à hospitalização de seres humanos através do consumo de ovos e carne de frango contaminados (CHAI *et al*., 2017). Outrossim, sabe-se que a diversidade microbiana do intestino dos animais é um dos principais fatores preventivos à manifestação clínica de enfermidades nas aves, principalmente diarreias e enterites (BORDA-MOLINA; SEIFERT; CAMARINHA-SILVA, 2018).

O sistema digestório é considerado um ecossistema complexo que promove uma relação simbiótica com o seu hospedeiro, exercendo influência direta sobre a digestibilidade dos alimentos, motilidade intestinal e até mesmo sobre a resposta imunológica; fatores estes que interferem no ganho de peso dos animais e, consequentemente, no sucesso da produção (ZHAO

et al., 2013). A suscetibilidade a infecções, em poedeiras e frangos de corte, causadas por *E. coli, Salmonella enterica, Clostridium perfringens, Mycoplasma gallisepticum* e várias famílias virais têm sido associadas à falta de estimulação da resposta imune do hospedeiro em razão de distúrbios na microbiota intestinal (CLAVIJO; FLÓREZ, 2018). Portanto, fica claro que anormalidades no microbioma intestinal, ou seja, a disbiose, estão correlacionados a processos inflamatórios, distúrbios metabólicos e má funcionalidade orgânica nos animais (KRISS *et al*., 2018). Uma das principais causas de disbiose é a administração de antibióticos, pois eles geram efeitos tóxicos nas populações bacterianas comensais (CLAVIJO; FLÓREZ, 2018).

A utilização de antibióticos na agropecuária foi amplamente difundida a partir dos anos 60, por representar soluções eficazes na melhoria da produção agropecuária intensiva, em destaque, sob a forma de promotores de crescimento (BROWN *et al*., 2017). No entanto, as abordagens profilática e como promotor de crescimento, dos antibióticos, têm sido restringidas no mundo inteiro (CHALMERS *et al*., 2017). No Brasil, a Instrução Normativa n° 9 de Maio de 2016 proibiu ouso do sulfato colistina e, mais recentemente, a Instrução Normativa n° 1 de Janeiro de 2020 proibiu o uso da tilosina, lincomicina, e tiamulina como aditivos promotores de crescimento na ração (MAPA, 2020).

Estudos epidemiológicos observacionais indicam que o uso indiscriminado de antibióticos na cadeia produtiva pecuária favorece processo de disseminação de genes de resistência a drogas antimicrobianas, logo o surgimento e disseminação de bactérias multirresistentes (ADEGOKE *et al*., 2016). Com base nisso, a Organização Mundial da Saúde tem preconizado o uso racional de antimicrobianos aplicados à produção animal, por meio de um sistema de classificação (importante, muito importante e crítico), no qual algumas classes de drogas, utilizadas estritamente na medicina humana, são desaconselhadas a serem usadas em animais de produção, por exemplo, β-lactâmicos da classe das cefalosporinas de terceira geração (WHO, 2017). Dentre as cefalosporinas, o ceftiofur em particular tem sido apontado como determinante no surgimento de cepas de *E. coli* multirresistentes isoladas de frangos de corte, de modo que países como o Canadá já baniram o seu uso profilático (CHALMERS *et al*., 2017).

A despeito destas recomendações, é recorrente o uso profilático extra-bula do ceftiofur em conjunto com a vacina de Marek nos incubatórios, no primeiro dia de vida dos pintinhos, a fim de prevenir quadros infecciosos precoces e aumentar as taxas de sobrevivência (BARON *et al*., 2014; BUSCAGLIA, 2013; SARAIVA *et al*., 2018).

Diante destes embargos, propostas de compostos alternativos às abordagens subterapêutica e profilática de antibióticos na produção animal estão em evidência, pois eles apresentam espectro bactericidas e imunomodulatórios equivalentes aos efeitos dos

antibióticos (CZAPLEWSKI, L. *et al*., 2016). Peptídeos bioativos, extratos de plantas, enzimas digestivas, bacteriófagos, pré- e probióticos, ácidos orgânicos e inibidores alvo de fatores de patogenicidade, como a inibição da formação de biofilme e outros fatores de virulência (CHENG *et al*., 2014).

A sanguinarina é um alcaloide benzofenantridínico derivado de raízes de plantas, como a *Macleaya cordata*, que tem potencial medicinal por suas propriedades antibacteriana (MIAO *et al.*, 2011), anti-inflamatória (WANG et al., 2017), antitumoral (GAZIANO, 2016) e antihelmíntica (HUANG *et al.*, 2020). Em ensaios *in vivo*, a suplementação com sanguinarina não demonstrou qualquer efeito colateral negativo aos animais, podendo ser utilizada como orexígeno e antimicrobiano alternativo aos promotores de crescimento convencionais (KOSINA *et al*., 2004). Por serem derivados de plantas, são também chamados de fitogênicos ou fitobióticos, já que apresentam efeitos modulatórios sobre a microbiota intestinal, resultando em maior ganho de peso, melhor performance e profilaxia de doenças, porém, sem os riscos de favorecer o surgimento de microrganismos multirresistentes (YADAV; JHA, 2019).

Considerando a importância da interação comensal entre a microbiota intestinal e seu hospedeiro, tornaram-se necessárias novas ferramentas para a sua avaliação laboratorial que permitissem detectar múltiplos microrganismos, uma vez que a microbiologia convencional se baseia numa identificação limitada frente à dificuldade de se reproduzir determinadas condições de cultivo, portanto, técnicas de sequenciamento de DNA de última geração, como a amplificação do gene que codifica a subunidade 16S do rRNA e o método *Shotgun*, possibilitaram contornar tais limitações (MORGAN; HUTTENHOWER, 2012). O gene que codifica a subunidade 16S do rRNA é único de procariotos, no entanto, há possui regiões de ampla variação, ou hipervariáveis, constituindo os alvos para diferenciação taxonômica e, assim, caracterizar comunidades microbianas (MORGAN; HUTTENHOWER, 2012).

Devido aos dados gerados serem compactos, faz-se necessária uma conversão em arquivos virtuais, uso de computadores de rápido processamento e disponibilidade do recurso de diversos *softwares* que possibilitem gerenciar as análises de bioinformática, ou seja, pela abordagem do rRNA 16S são: estudos ecológicos de diversidade microbiana (alfa, beta), análises composicionais (abundâncias relativa e diferencial), predição gênica e, por fim, a distribuição das sequências em redes de dados públicos, integralizando assim a pesquisa científica no âmbito global (KUCZYNSKI *et al*., 2012).

Destaca-se, neste cenário, a importância dos estudos da microbiota intestinal de animais de produção animal.

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2 CAPÍTULO I - EFEITOS DA ADMINISTRAÇÃO PROFILÁTICA PÓS-ECLOSÃO DE CEFTIOFUR E DA SUPLEMENTAÇÃO COM SANGUINARINA NA MICROBIOTA CECAL DE PINTINHOS

Effects of the post-hatch prophylactic administration of ceftiofur and dietary sanguinarine supplementation on the cecal microbiota of broilers

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Running title: Ceftiofur-mediated changes in broiler´s cecal microbiome

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Section: Microbiology and Food Safety

ABSTRACT

The prophylactic administration of ceftiofur to newly hatched chicks is a common practice in some hatcheries worldwide in order to mitigate early gastrointestinal infections caused by *Enterobacteriaceae*. In spite of the crucial role of the gut microbiome for the broiler chicken´s health, there is still limited information on how the microbial composition is affected by such procedure. We investigated the effects of prophylactic application of ceftiofur on the cecal microbiota of 14 days-old broilers fed regular or sanguinarine-supplemented diets. DNA samples were extracted from cecal contents, amplified for the v3-v4 regions of the microbial 16S rRNA gene and sequenced in a high throughput sequencing platform (Illumina Miseq). After downstream bioinformatics and statistical analyses, our results demonstrated that both ceftiofur and sanguinarine treatments similarly increased the proportions of the phylum Bacteroidetes, and the genera *Bacteroides* and *Megamonas* while reduced the relative abundance of Firmicutes and *Lachnospiraceae* in the ceca of the birds. Such changes are probably associated with increased carbohydrate fermentation processes favoring the production of Shorty-Chain Fatty Acids (SCFA). This was also corroborated by the functional prediction findings which suggest an increase in some metabolic pathways associated with digestibility in broilers receiving ceftiofur. Considering that antimicrobial stewardship in animal production systems is strongly needed to mitigate the threat of antimicrobial resistance, our findings show that supplementation with a phytogenic feed additive can lead to a similar microbial composition in the ceca of commercial broiler chickens, suggesting that the use of alternative products could lead to functional modifications without increasing pressure for antimicrobial resistance.

Keywords: broiler, cecal microbiota, ceftiofur, sanguinarine, 16S rRNA.

2.1 INTRODUCTION

The poultry industry stands out as one of most successful agricultural sectors, supporting the increasing global demand for affordable and high-quality protein. For some countries, such as United States and Brazil as leading chicken-meat exporters, the poultry agribusiness represents an importance sector of their economy (Wen et al., 2019). The sector's success stems from competitive advantages over the production of proteins of other species (beef, for instance), such as high food efficiency, lower production costs, and more accessible prices to consumers (FAO, 2019).

For more than half a century, antibiotics have contributed significantly to the increase of the animal productivity, either in terms of antimicrobial growth promoters, or therapeutically or prophylactic to treat or prevent infectious diseases that compromise production (Singh et al., 2012; Cox et al., 2014; Brown et al., 2017). Efficient antibiotic therapy is and will continue to be a crucial resource in the broiler industry warranting the proper treatment of infections (Sharma et al., 2016).

On the other hand, the emergence of antimicrobial resistant avian pathogens has imposed a challenge for the poultry industry (Bortolaia et al., 2016). In recent decades, there has been an intense global debate about the real impact of the use of antibiotics in animal production on the public health, not only about the emergence and spread of zoonotic resistant pathogens (Bortolaia et al., 2016; Bueno et al., 2018) but also about putative risks associated with the spread of resistance genes to humans through the food chain, such as chicken meat, or some direct and indirect contact with environmental sources contaminated by waste of farming systems, where antimicrobials are intensively used (Maron et al., 2013; Panzenhagen et al., 2016; Wall et al., 2016; Costa et al., 2017; Xiong et al., 2018). The presence of commensal bacteria in foods represents a public health concern, as they can serve as vehicles carrying resistance genes that could be ultimately transferred to human bacteria by horizontal transfer mechanisms (Lerner et al., 2017).

Epidemiological studies indicate a direct positive association between the use of some classes of antibiotics in animal production and increased antimicrobial resistance among pathogens causing infections in humans (Tang et al., 2017). The regulations of several countries, including the USA and Brazil, has restricted the use of antimicrobial substances in intensive animal production. In other regions, such as the European Union, the use of antibiotics as growth promoters has been banned for decades (Maron et al., 2013; Costa et al., 2017).

In some poultry hatcheries, ceftiofur, a third-generation cephalosporin belonging to the β-lactam group, is mixed with the Marek's disease vaccine and off-label administered in a single dose to post-hatch chicks (Baron et al., 2014). The mechanisms behind the beneficial effects of this practice are unknown. The gut commensal microbiome is responsible for the rapid development of the intestinal epithelium, as well as for its proper functionality. Modulation of specific commensal microbial species can favor animal performance by improving feed conversion (Kogut, 2019). On the other hand, the recurrent use of antibiotics can cause imbalances in the bacterial community and, as a consequence, predisposition to various conditions, mainly when they occur in the early stages of development *(*Yassour et al., 2016).

Furthermore, cephalosporins are known to induce the bacterial conjugation process of resistance genes, a phenomenon observed *in vitro* among several pathogenic microorganisms, e.g., *Salmonella enterica* and *Escherichia coli* originating from commercial poultry (Mo et al., 2017; Campos et al., 2018). Resistance to cephalosporins represents a major public health problem, as they are listed among the Highest Priority Critically Important Antimicrobials (**HPCIA**) classes of drugs by the World Health Organization (WHO) (Scott et al., 2019).

Considering the major challenges represented by antimicrobial resistance and the consequent limitation of the use of antibiotics, the animal industry has been exploring alternatives to conventional drugs, such as digestive enzymes, probiotics, prebiotics, organic acids, and phytogenic compounds, in order to reduce losses in productivity and improve animal health (Gadde et al., 2017). These substances can act directly modulating the intestinal microbiota and improving performance (Salaheen et al., 2017). Among these alternative compounds, sanguinarine, a bioactive phytogenic alkaloid, has been used as an alternative food additive in the commercial broiler industry (Hassan et al., 2018).

Sanguinarine is a benzophenanthridine alkaloid with anti-inflammatory (Wang et al., 2017), antibacterial (Miao et al., 2011), antitumoral (Gaziano, 2016) and antihelminthic properties (Huang et al., 2020). However, there is not much information available on the potential effects of these compounds on the gut microbial community of broiler chickens. Comparative analysis using 16S rRNA metagenomics (high-throughput sequencing of amplified hypervariable regions of the microbial 16S rRNA gene) is an interesting approach to understand changes in microbiomes and has been used as an efficient method for microbial ecology studies for over a decade (Gill et al., 2006).

The aim of this study was to investigate putative changes in the cecal microbiota of 14-day-old chicks triggered by the post-hatch administration of ceftiofur. Additionally, we

investigated possible changes in the microbiota resulting from the dietary supplementation of sanguinarine.

2.2 MATERIAL AND METHODS

2.2.1 *Experimental design*

The experimental proposal was submitted and approved (protocol 6513240218) by the Animal Use Ethics Committee of the Federal University of Paraiba (**CEUA / UFPB**), accredited by the National Council for Animal Experimentation Control (**CONCEA**).

The experiment was carried out according to a completely randomized design, with 5 repetitions for each of the following treatments: NC (negative control: birds receiving Marek vaccine suspended in 0.2 mL sterile saline solution subcutaneously at the first day post-hatch); PHYTO (birds receiving Marek vaccine only fed the sanguinarine-based commercial product Sangrovit, 50g / ton); ATB (birds receiving 0.2mL of Marek vaccine diluted in a sodium ceftiofur solution 0.2 mg / mL subcutaneously); and MIXED (birds receiving Marek vaccine plus sodium ceftiofur subcutaneously and also fed sanguinarine-supplemented diet).

The animals were fed corn-soybean meal diet without the addition of antibiotics. After 14 days post-hatch, the animals were euthanized the left ceca sampled by scraping to obtain feces and intestinal mucous membrane.

2.2.2 *DNA extraction and sequencing library preparation*

Total DNA was extracted using a commercial kit (PowerSoil DNA Isolation Kit, Qiagen) according to the manufacturer's protocols. The V3-V4 region of the microbial 16S rRNA gene was amplified by PCR (95°C for 3 min, followed by 25 cycles at 95°C for 30’s, 55°C for 30’s and 72°C for 30’ s and a final extension to 72 for 5 min) using the primers 341F: 5' -TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG - 3 ' and 785R: 5'- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C - 3 '. The PCR reactions were performed following the recommendations of the 16S rRNA gene library preparation protocol for the Illumina Miseq system. The amplicons were visualized in 1.5% agarose gel with the aid of a UV transilluminator (Carestream Molecular Imaging Software, USA). The amplification products were purified using Ampure beads (Beckman Coulter, USA) according to the instructions of the Illumina protocol.

The purified PCR products were quantified by fluorometry (Qubit2.0, Life Invitrogen) before library preparation, which was performed according the recommended protocol using the indexing kit Nextera XT Set A (Illumina California, USA) The sequencing was performed using the Illumina V2 kit (2 × 250 cycles) on a MiSeq platform (Illumina).

2.2.3 *Sequencing data processing*

The raw demultiplexed paired-end sequences were downstream processed using QIIME 2 platform v.19.10 (Bolyen et al., 2019). Reads were joined, selected by the maximum and minimum sizes (200-500 bp), filtered according to a minimum Phred score of 20 and re-replicated through VSEARCH (Rognes et al., 2016). Chimeric sequences were removed using UCHIME (Edgar et al., 2011). Operational Taxonomic Unit (**OTU**) identification was performed by the *De Novo* clusterization method with 99% similarity between the centroid groups. The sequences were aligned by Mafft (Katoh, 2002) and then used for the construction of the phylogenetic tree by Fasttree2 (Price et al., 2010). The visualization of the taxonomic composition in its different levels, relative abundance, and *alpha* diversity, were performed by the Phyloseq v.1.8.2 package (McMurdie and Holmes, 2013) in R v.3.5.7. The taxonomic classification was attributed using the Naïve Bayes method on the trained database of SILVA version 132 with 99% for region V3-V4 (Quast et al., 2012).

The assessment of *alpha* diversity was made by the indexes Chao1 and Shannon, which estimate richness and evenness of the microbial communities, respectively. For the *beta* diversity, the base matrix was determined considering all pairs of samples by the Unifrac method (Lozupone and Knight, 2005) in its qualitative unweighted metric variant. Visualization was performed by Principal Component Analysis (PCoA) using the QIIME 2 visualization platform. The functional predictions found in the intestinal microbial community based on taxonomic information were carried out by the phylogenetic investigation platform of communities through the reconstruction of unobserved states from Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (**PICRUSt**) (Langille et al., 2013).

2.2.4 *Statistical analyses*

Alpha diversity indices were assessed using the paired Kruskall-Wallis test, while the dissimilarity between treatments was assessed using the Permutational Multivariate Analysis of Variance (**PERMANOVA**) at 5% probability. Differential abundance analysis to identify significant differences in specific OTUs between treatments was performed by means of

Linear Discriminant Analysis Effect Size (**LDA**) method using the LEfSe program (Segata et al., 2011).

After the PICRUSt gene prediction, the significance, and visualization of the results were evaluated using the platform of statistical analysis of taxonomy and functional profiles (Statistical Analysis of Taxonomic and Functional Profiles - STAMP) (Parks et al., 2014).

2.3 RESULTS AND DISCUSSION

2.3.1 *16S rRNA sequencing products*

Rarefaction was performed to 24,500 reads per sample before downstream analyses. The mean length of the reads was 448.95 base pairs ranging from 207 to 497 bp. It was possible to detect 84, OTUs after the *De Novo* clusterization approach of 99% between the centroid region, corresponding to 322 different taxa according to SILVA v. 132 classifiers.

2.3.2 *Diversity analyses*

In terms of *alpha* diversity, the negative control group (NC) presented the highest values for Shannon's diversity index (Fig. 1-A) and the lowest ones for the Chao1 index (Fig. 1-B). The opposite pattern was observed in the groups PHYTO, ATB, and MIXED. However, no statistical differences were observed ($P < 0.05$).

The cecal microbiota of commercial chickens exposed to antibiotics seems to reach maximum richness and evenness at 14 days post-hatch, earlier to 18 days reported in free-range or birds not previously exposed to antibiotics (Ocejo et al., 2019).

In terms of *beta* diversity, the different groups were clustered apart, as shown in the PCoA plot (Fig. 1-C), where NC group was most dissimilar to MIXED. Less dissimilarity was observed between NC and PHYTO. Our findings corroborate previous studies revealing increased dissimilarities between antimicrobial-exposed birds and their respective control (non-exposed) groups (Costa et al., 2017). This microbial shift caused by the antibiotic use has already been observed in other species, such as steers (Foster et al., 2019) and piglets (Ruczizka et al., 2019). Considering that antibiotic-driven changes in the microbiota might be associated with a decreased adaptive immune system stimulation, compromising some basic sanitary protocols, such as vaccination (Yitbarek et al., 2019), the practice of administering ceftiofur with Marek´s disease vaccine should be revisited.

Sanguinarine is associated with the prevention of gastrointestinal diseases, e.g., necrotic enteritis related to gut dysbiosis in broilers (Xue et al., 2017). No negative collateral effects have been observed in chickens fed sanguinarine, which is partially absorbed in the intestines metabolized in the liver (Hu et al., 2019).

2.3.3 *Compositional analyses*

The microbial compositional structure of the cecum of 14-days broilers under the different experimental groups are shown in Figure 2-A. The phyla Firmicutes (83.4%/NC, 55.5%/PHYTO, 57.26%/ATB, and 50.77%/MIXED) and Bacteroidetes (12.40%/NC, 43.50%/PHYTO, 40.98%/ATB, and 47.14%/MIXED) comprised the main taxa at this taxonomic level. In comparison with the control group, all treatments led to a reduction in the Firmicutes abundance while increased Bacteroidetes. Increased Bacteroidetes abundance was also observed in birds receiving antibiotics as growth promoters (Mancabelli et al. 2016). In contrast, non-antibiotic exposed free-range chickens presented higher abundances of Firmicutes (Ocejo et al., 2019). Changes in the Firmicutes/Bacteroidetes ratio in the gut microbiota could lead to dysbiosis and therefore predispose gastrointestinal diseases (Le Roy et al., 2019).

Lachnospiraceae (42.62%/NC, 34.37%/PHYTO, 26.40%/ATB and 28.78%/MIXED), *Ruminococcaceae* (28.11%/NC, 13.63%/PHYTO, 25.75%/ATB and 14.83%/MIXED), *Bacteroidaceae* (12.64%/NC, 43.04%/PHYTO, 41.68%/ATB and 47.90%/MIXED), and *Enterobacteriaceae* (4.04%/NC, 0.95%/PHYTO, 1.72%/ATB and 1.91%/MIXED) comprised the major bacterial families, as shown in Fig. 2-B. Decrease in *Lachnospiraceae* triggered by both sanguinarine and ceftiofur could be considered as an undesirable result because this family is associated with BWG, improved digestion and also considered an indicator of healthy gut microbiota in broiler chickens (Apajalahti and Vienola, 2016).

Bacteroides (19.37%/NC, 53/11%/PHYTO, 52.91%/ATB, and 59.69%/MIXED), and *Ruminococcus* (27.66%/NC, 23.91%/PHYTO, 13.34%/ATB, and 14.63%/MIXED) comprised the main microbial genera (Figura 2-C). According to Johnson et al. (2017), higher *Bacteroides* abundance is associated with increased polysaccharide fermentation and the production of SCFA, which contribute to the rapid development in broiler chickens (Zheng et al., 2019).

Our findings indicate that both sanguinarine supplementation in the diet and ceftiofur administration led to similar effects in terms of structural composition of the cecal microbiome at the end of the initial production phase (14 days), corroborating studies which

based on the administration of different antibiotics as previous experimental designs (Chen et al., 2019). The most significant advantage of the phytobiotics relies on the fact that no pressure for antimicrobial resistance has been triggered by this product (Yadav and Jha, 2019).

There were significantly higher abundances of *Ruminoclostridium*, *Anaerotruncus*, and *Ruminoclostridium* in NC, whereas increased abundances of *Megamonas* and e *Veillonellaceae* were observed in ATB (Figure 3-A). The higher abundance of *Ruminiclostritidium* in NC suggests that this microbiota treatment could be more efficient in terms of fibrous material digestion, since some species belonging to this genus such as *Ruminiclostridium cellulolyticum* can metabolize cellulose (Ravachol et al., 2016). However, the higher abundance of *Anaerotruncus* in NC may suggest a high SCFA production, since the main byproducts of its metabolism are acetic and butyric acids (Lawson, 2015). In this case, the absence of antimicrobial drugs may favor not only fiber digesting bacteria such as *Ruminococcus* but also organisms involved in carbohydrate fermentation.

On the other hand, the higher abundance of *Megamonas* observed in ATB may be associated with improved carbohydrate digestion and SCFA production, similarly to *Bacteroides* (Polansky et al., 2016). *Veillonellaceae* seem to be responsive to dietary calcium as they are more abundant in animals under calcium supplementation (Tilocca et al., 2016) and involved in bone formation disorders in broiler chickens, such as tibial dyschondroplasia (Tong et al., 2018).

In order to better compare our results with previous reports using conventional microbial analyses, the relative abundance of OTU counts classified as *Escherichia-Shigella* and *Lactobacillus* taxa were selected and compared across the groups (Figure 3-A). The highest abundance of *Lactobacillus* was observed in the ATB group, which is associated with a better protection against gut pathogens (Clavijo and Flórez, 2018). Although this genus is not very abundant in chickens caecum (Johnson et al., 2018), our findings suggest that the prophylactic use of ceftiofur favored *Lactobacillus* growth at the end of the initial production phase, which can possibly contribute to the benefits observed under field conditions (Pereira et al., 2019).

Lower abundances of *Enterobacteriaceae* in response to both ceftiofur and sanguinarine treatments might have been caused by their bactericidal effects. A massive reduction in *Enterobacteriaceae* groups within 24 hours after the administration of β-lactam antibiotic compounds has already been reported (Schokker et al., 2017). Our findings indicate that such reduction caused by the administration of ceftiofur can last until 14 days in broiler

chickens. Although a decreased *Enterobacteriaceae* population is considered positive effect of the antimicrobial treatment, the prophylactic use of ceftiofur could be a driver for selection of antibiotic-resistant bacteria. Indeed, increased number of Extended Spectrum Beta Lactamase (**ESBL**) *E.coli* strains from chicken gut has been associated with the post-hatch administration of ceftiofur (Saraiva et al., 2018). Further studies on the dynamics of resistance determinants in the microbiota of broiler chickens under supplementation of phytogenic feed additives are warranted.

2.3.4 *Gene prediction*

It was possible to detect significant differences ($P < 0.05$) between NC and ATB in terms of functional prediction performed by PICRUSt (Figure 4). The prophylactic use of ceftiofur increased functions related to the metabolism of alanine, aspartate, glutamine, and glutamate, as well as the transcription machinery indicating intense protein metabolic routes. The interaction between the gut microbiota and its host is crucial to guarantee appropriate digestion and promote BWG in birds (Pan and Yu, 2014). The findings observed in this study indicate that ceftiofur increased metabolic pathways associated with higher protein synthesis, and therefore stimulating enterocyte development and increased nutrient absorption (Qi et al., 2018; Xue et al., 2018).

The difference between the microbiota of NC and ATB groups for butanoate production by the Phosphatransferase System (**PTS**) is probably due to the higher abundances of fiber degrading bacteria such as *Ruminococcus* and *Ruminiclostridium*, which is associated with the ability to forage and the establishment of healthy gut commensal bacteria (Hou et al., 2016).

2.4 CONCLUSION

Microbial shifts in the cecum of broiler chickens at the end of the first production phase (14 days) caused by sanguinarine added to feed were similar to those triggered by the off-label administration of ceftiofur post-hatch. In view of the challenges imposed by the emergence of antimicrobial resistance and the need to reduce the use of HCIA in livestock, the use of non-antibiotic compounds could be used as an alternative to module the microbiota of commercial broiler chickens in intensive production systems. Our finding warranty further investigation on the mechanisms associated with intestinal microbial modulation.

Funding

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES, financing code 001).

Conflict of Interest

The authors declare that there is no conflict of interest.

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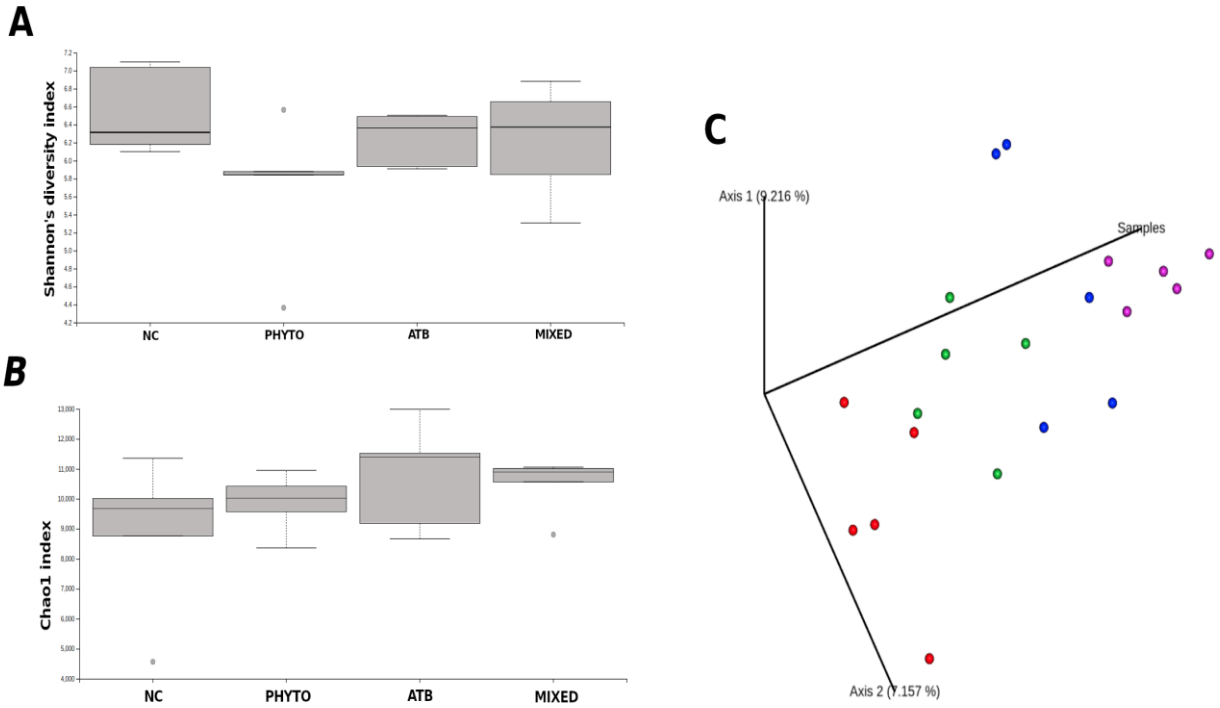

Figure 1. Boxplots showing alpha diversity measured by Shannon (A) and Chao1 (B) diversity indexes for each treatment group: NC (negative control), PHYTO (sanguinarine supplementation), ATB (prophylactic use of ceftiofur), and MIXED (sanguinarine and ceftiofur). 3D PCoA plot from an unweighted Unifrac distance matrix showing the dissimilarities (beta-diversity) across the different groups (C): NC (red), ATB (blue), PHYTO (green), MIXED (purple).

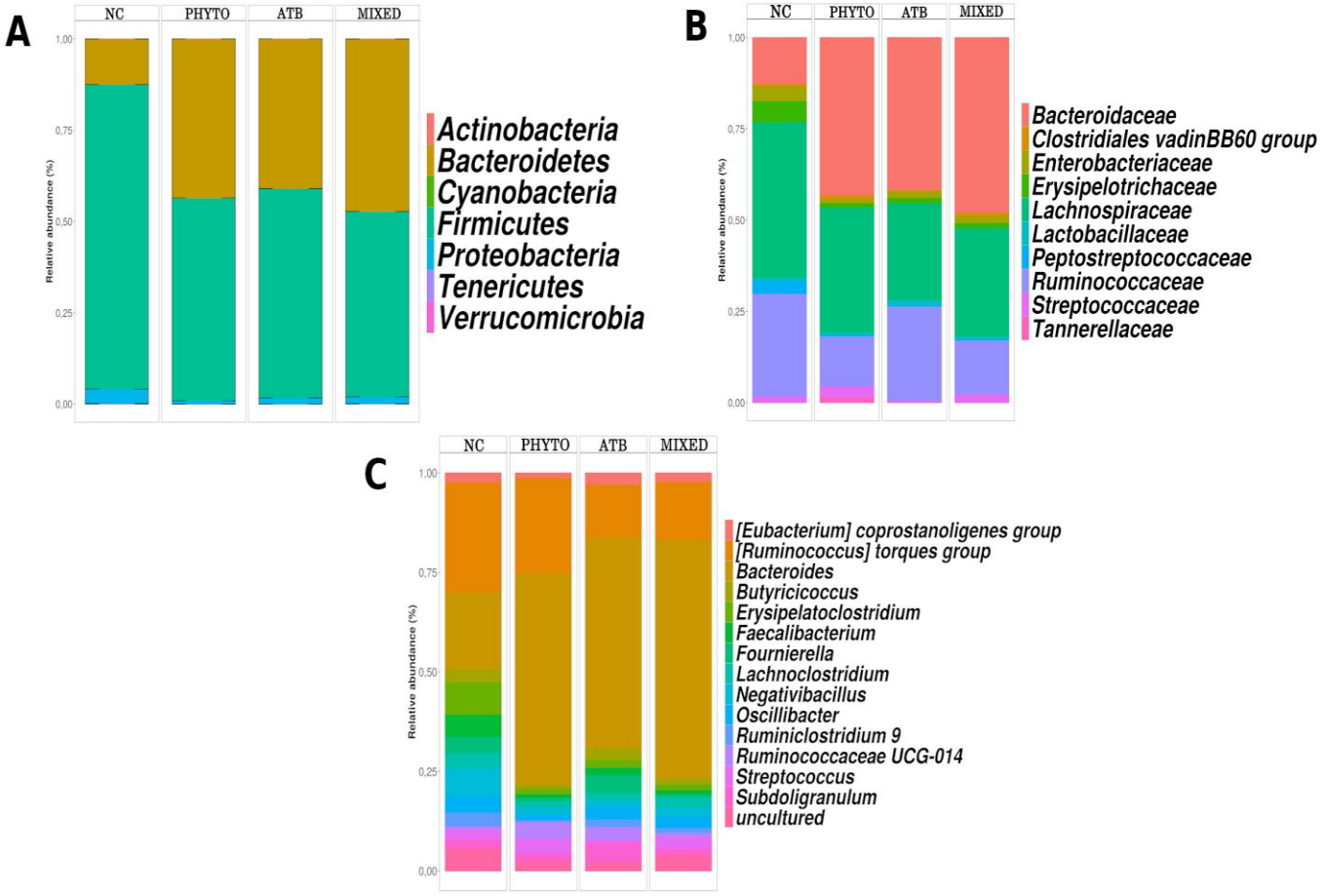

Figure 2. Relative abundances of the 7 most prevalent phyla (A), the 10 most prevalent families (B), and the 15 most prevalent genera (C) across the four treatment groups: NC (negative control), PHYTO (sanguinarine supplementation), ATB (prophylactic use of ceftiofur), and MIXED (sanguinarine and ceftiofur).

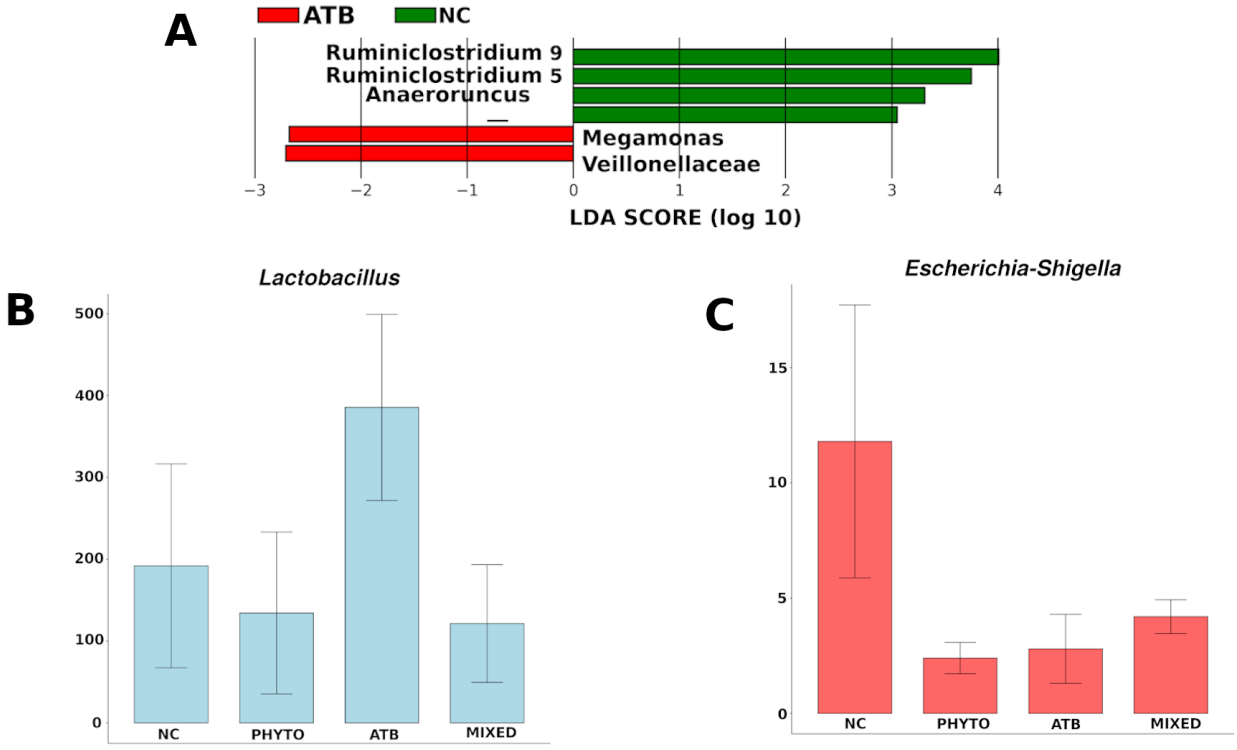

Figure 3. Operational Taxonomic Units (OTUs) showing statistically significant ($P < 0.05$) differential abundances between NC (control group) and ATB (broilers receiving ceftiofur post-hatch) assessed by Lefse (A). Relative abundance of *Lactobacillus*-associated OTUs among the different treatment groups (B). Relative abundance of *Enterobacteriacea*-associated OTUs among the different treatment groups (C).

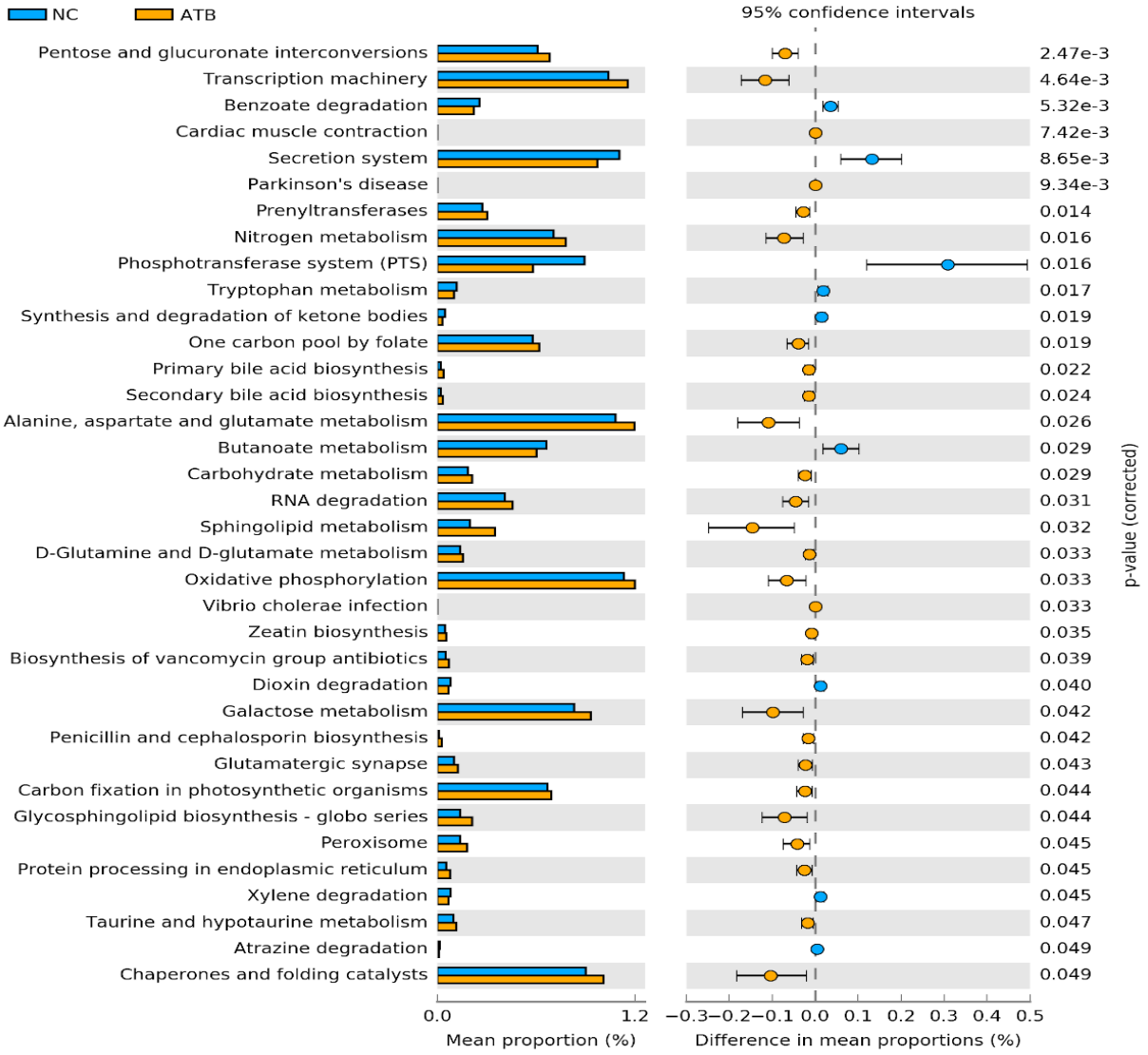

Figure 4. Gene prediction results from PICRUSt showing KEGG metabolic pathways at the third hierarchical level between ATB (ceftiofur) and NC (negative control).

3 **CONSIDERAÇÕES FINAIS**

O microbioma intestinal pode ser considerado como um importante órgão interativo, uma vez que ele é capaz de exercer fortes influências sobre os processos fisiológicos de seu hospedeiro. Portanto, um microbioma intestinal estável, rico e equilibrado implica em um animal saudável; do contrário, aqueles que são instáveis, pobres e desequilibrados podem implicar na manifestação de vários distúrbios, não somente gastrointestinais, mas também metabólicos, comportamentais e imunológicos. Os antibióticos são amplamente utilizados nos sistema produtivos intensivos, porém, mormente os problemas da indução da pressão de seleção por genes de resistência e surgimento de microrganismos multirreristentes, faz-se necessária uma maior busca por tais substitutos na agropecuária. Nossa investigação pela abordagem de metagenômica do rRNA 16S revelou que a aplicação profilática injetável do ceftiofur em pintinhos pós-eclosão desencadeia alterações na microbiota cecal, detectáveis aos 14 dias de vida. Em termos de diversidade ecológica, as alterações provocadas pelo ceftiofur foram similares ao efeito do fitogênico sanguinarina, administrado como aditivo alimentar na ração das aves. De acordo com a análise de abundância diferencial, as alterações causadas pelo ceftiofur foram marcadas por aumentos de alguns táxons (*Bacteroides*, *Megamonas*) e redução de outros (*Lachnospiraceae*, *Ruminococcus*, *Ruminiclostridium*).

A análise de inferência gênica e funcional revelou ainda que a modulação da microbiota intestinal, causada pelo ceftiofur, está associada a rotas metabólicas potencialmente relacionadas com o ganho de peso das aves.

Neste cenário, a sanguinarina demonstrou ser uma substância alternativa eficaz ao uso profilático do ceftiofur na modulação da microbiota intestinal em prol da saúde animal, justificando a melhoria da perfomance das aves associada à administração destas substâncias.