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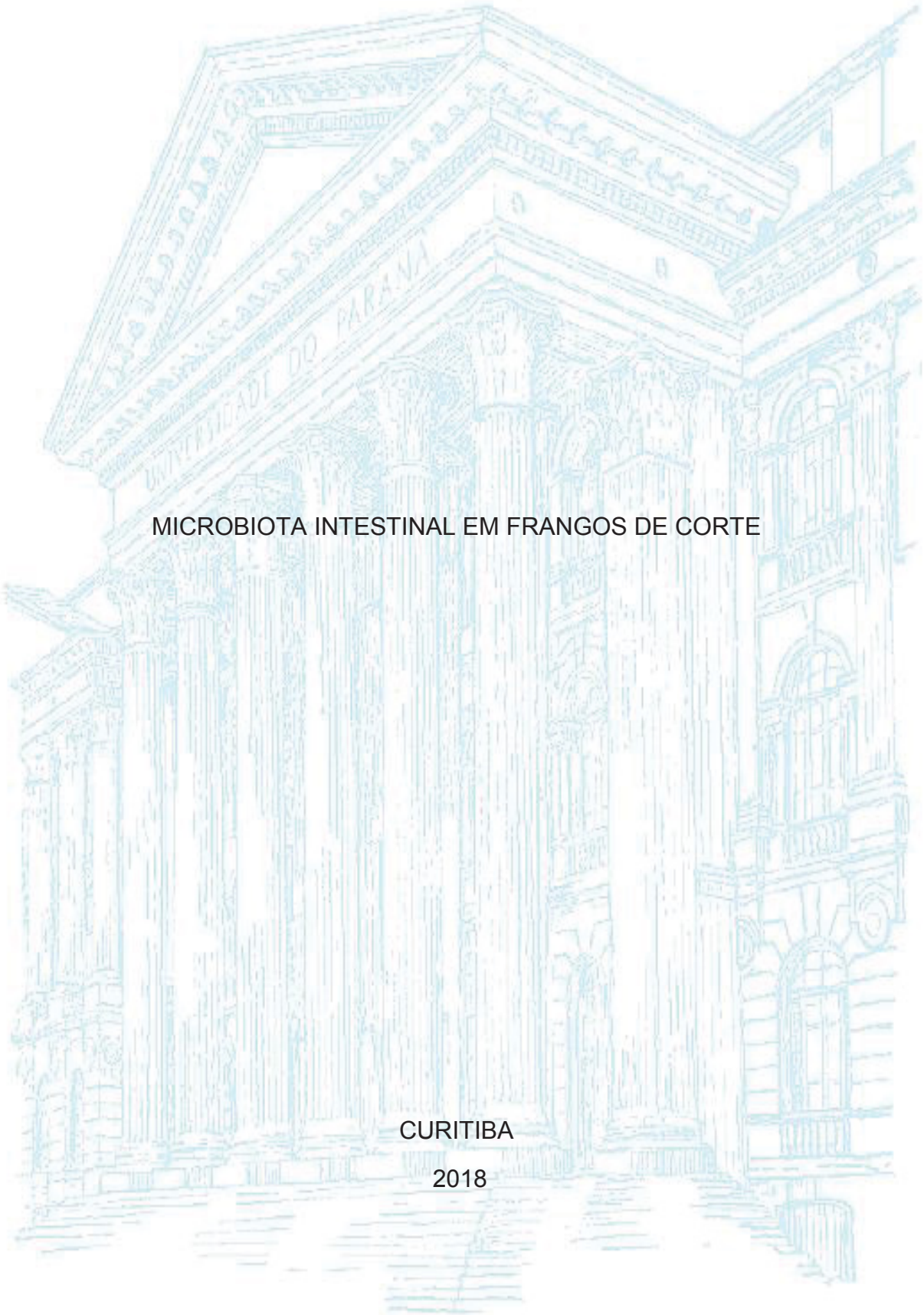

MICROBIOTA INTESTINAL EM FRANGOS DE CORTE

CURITIBA

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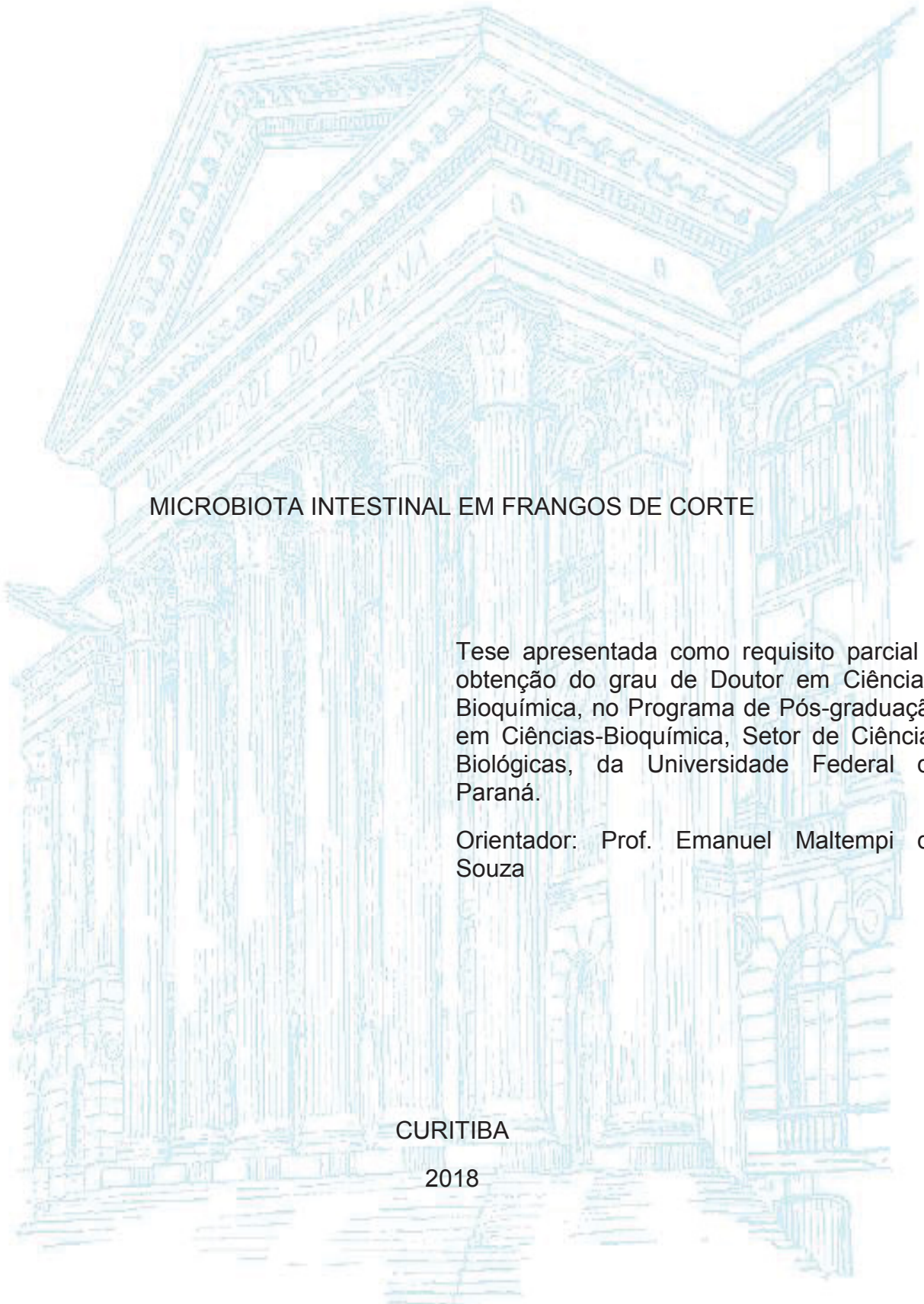

MICROBIOTA INTESTINAL EM FRANGOS DE CORTE

Tese apresentada como requisito parcial à obtenção do grau de Doutor em Ciências-Bioquímica, no Programa de Pós-graduação em Ciências-Bioquímica, Setor de Ciências Biológicas, da Universidade Federal do Paraná.

Orientador: Prof. Emanuel Maltempi de Souza

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIAS (BIOQUÍMICA) da Universidade Federal do Paraná foram convocados para realizar a arguição da Tese de Doutorado de **DANY ALBERTO MESA FIAGA**, intitulada: **MICROBIOTA INTESTINAL EM FRANGOS DE CORTE**, após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de Doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 18 de Maio de 2018.

EMANUEL MALTEMPI DE SOUZA(UFPR)
(Presidente da Banca Examinadora)

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CYNTIA MARIA TELLES FADEL PICHETH(UFPR)

MARIA BERENICE REYNAUD STEFFENS(UFPR)

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RESUMO

A avicultura industrial é um setor técnico e moderno que passou ao longo de várias décadas por um constante processo de aperfeiçoamento em vários níveis. Atualmente, a evolução continua em alguns segmentos, por exemplo: a genética, a nutrição de precisão, os programas de biossegurança e o bem-estar animal. Incluído no bem-estar estão o manejo de doenças infecciosas, melhora da imunidade e da saúde intestinal. Esta última tem tido grande destaque nos últimos anos devido aos avanços no conhecimento das relações entre a microbiota intestinal e os diversos sistemas e seus efeitos sobre o hospedeiro. A maioria do conhecimento gerado sobre microbiota é relativo à interação com humanos e os resultados são extrapolados para animais. Assim, este trabalho se propôs estudar a microbiota intestinal em frangos de corte utilizando sequenciamento de nova geração. Este estudo foi dividido em quatro capítulos: Capítulo I, uma mini revisão sobre “Bactérias intestinais produtoras de ácidos orgânicos” direcionada a aves domesticas. A revisão de literatura evidenciou que a microbiota intestinal é uma comunidade rica e complexa, composta por vários gêneros bacterianos com diferentes níveis de especialização na síntese de produtos como ácidos orgânicos. O capítulo II, intitulado “Cecal Microbiota in Broilers Fed with Prebiotics”, um artigo publicado na revista Frontiers in Genetics "Livestock Genomics" descreve mudanças na microbiota em frangos tratados com prebióticos derivados de leveduras. Embora o tratamento com os prebióticos mananoligossacarídeo e nucleotídeo não tenham resultado em ganho de peso, foi observado uma mudança na microbiota, com predominância de *Lactobacillus*, *Roseburia* e *Butyricimonas* que são gêneros considerados benéficos. Os resultados sugerem que prebióticos contribuem

positivamente para saúde animal provocando uma mudança na microbiota intestinal. No capítulo III, intitulado “Immunosuppression increases *Lactobacillus* in the intestinal microbiota in chickens” artigo que se encontra em fase de formatação e que será submetido na revista Microbiome, a microbiota intestinal de frangos imunossuprimidos pelo tratamento com ciclofosfamida foi analisada. Os resultados mostraram aumento de *Lactobacillus*, *Blautia* e *Faecalibacterium* em aves imunossuprimidas. Este resultado sugere que o aumento de gêneros associados com maior proteção da parede intestinal tem um efeito compensatório sobre a imunossupressão. O capítulo IV "Genomic comparison of *Salmonella* Heidelberg isolates show deletion of in the type III secretion system gene cluster and suggests cause for low invasiveness" trata da comparação genômica de estirpes de *Salmonella* Heidelberg isoladas da indústria avícola de corte. Artigo em formatação e que será submetido na revista DNA Research. Os resultados revelaram deleções de genes no sistema de secreção tipo III presentes na ilha de patogenicidade 1 de *Salmonella* Heidelberg. Este genótipo pode ser responsável pela diminuição da capacidade de invadir macrófagos em cultivo celular. Adicionalmente, foi observado a expansão clonal de *S.* Heidelberg na avicultura de corte do sul do Brasil. Dessa forma, os resultados aqui apresentados sugerem que o sequenciamento de nova geração é uma ferramenta de alta sensibilidade que permite avaliar as mudanças na microbiota intestinal em animais. Assim, como estudar e comparar genomas bacterianos.

Palavras-chave: 16S rRNA, Sequenciamento, *Salmonella*

ABSTRACT

The poultry industry is a modern and high technical sector that has spent in several decades a constant process of improvement on several levels. Currently, evolution remains in some segments, for example: genetics, precision nutrition, biosecurity programs and well-being. Well-being includes management of infectious diseases, immunity and intestinal health. This last one has gained great importance in the last years due to the great advances in the knowledge of the relations of the intestinal microbiota with the diverse systems and all its benefits with the host. However, the majority of the knowledge was generated in humans and the results extrapolated to animals. Thus, the research on intestinal microbiota in broilers using new generation sequencing tools was proposed as the subject for this work. This study was divided into four chapters: Chapter I, a review of “Acid-producing intestinal bacteria” directed to domestic birds. The chapter II, a scientific work entitled "Cecal Microbiota in Broilers Fed with Prebiotics" published in the journal Frontiers in Genetics "Livestock Genomics". The Chapter III, a scientific paper entitled “Immunosuppression increases *Lactobacillus* in the intestinal microbiota in chickens”. The chapter IV, a genomic comparison between strains of *Salmonella*, a normal resident of the intestinal microbiota of birds. Scientific work entitled "Genomic comparison of *Salmonella* Heidelberg isolates show deletion of in the type III secretion system gene cluster and suggests cause for low invasiveness". In the literature review it was observed that the intestinal microbiota is a complex bacterial community that produce metabolites such as organic acids. In scientific papers II and III it was observed that the intestinal microbiota can be modified with additives or drugs. In Chapter IV it was observed that changes in the genotype of *Salmonella*

Heidelberg lead to different phenotypes. In addition, a phenomenon of clonal expansion of S. Heidelberg was observed in the poultry industry of southern Brazil. The results presented here suggest that the next-generation sequencing is a tool of high sensitivity that allows to evaluate the changes of the intestinal microbiota in animals. Besides that, to study and to compare bacterial genomes.

Key-words: 16S rRNA, Sequencing, *Salmonella*

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INTRODUÇÃO

Atualmente, estamos passando pela "era do microbioma" (WALDOR et al., 2015). O corpo dos animais é povoado por trilhões de bactérias como, por exemplo, as do sistema gastrointestinal que aloja a "microbiota intestinal". O conjunto de todas as microbiotas de um organismo forma o "microbioma" (URSELL et al., 2012). Dentro do microbioma a microbiota intestinal tem mobilizado a maior atenção, isso porque a maioria das bactérias do corpo residem no intestino (SENDER et al., 2016) e tem um impacto na saúde geral do hospedeiro (MAROTZ e ZARRINPAR, 2016).

Dessa forma, pesquisas sobre obesidade humana trouxeram muita atenção para a relevância das bactérias intestinais. Experimentos demonstraram que a obesidade pode, dentre diversos fatores, ser determinada pela composição microbiana do intestino. Em outras palavras, não só a dieta afeta a microbiota, mas a própria bactéria pode determinar os resultados metabólicos no hospedeiro (LEY, 2010).

As implicações disso são imensas uma vez que a reversão de uma microbiota "obesa" para uma microbiota de fenótipo "magro" seria importante para a cura da obesidade (HARLEY e KARP, 2012). Para o nutricionista veterinário, esse raciocínio gera a ideia que um único elemento pode ser alterado para melhorar a produtividade animal. Assim, práticas de nutrição animal como a administração de aditivos alimentares (prebióticos, probióticos, ácidos orgânicos) são, obviamente, direcionadas para a alteração das populações microbianas do intestino.

No entanto, este é um campo incipiente pois, em primeiro lugar, as interações da microbiota com a dieta ou com aditivos são muito complexas. As intervenções mostram um impacto na microbiota (POLANSKY et al., 2016), mas também

dependem da composição bacteriana inicial do intestino. Portanto, os animais criados em diferentes condições responderão de maneira diferente à mesma intervenção (UPADRASTA et al., 2013). Adicionalmente, outros fatores influenciam essa interação, como por exemplo, idade e manejo dos animais (LU et al., 2003).

Até alguns anos atrás essas interações eram estudadas por métodos microbiológicos convencionais (CROSS et al., 2007). Mas, muitas bactérias intestinais não são propensas a cultivo em laboratório. Nos últimos anos, as interações da microbiota intestinal tem sido estudadas com a ajuda de tecnologias de análises genômicas (CALLAWAY et al., 2009; LI et al., 2017).

Para entender as mudanças na comunidade intestinal, uma parte do genoma dessas bactérias é sequenciada. Isso gera dados que permitem a identificação de praticamente todas as bactérias da amostra. O resultado desta técnica é uma longa lista de bactérias presentes e suas quantidades relativas. Com esses dados em mãos, podem ser realizadas análises sobre o impacto das intervenções, por exemplo, o uso de probióticos sobre a constituição da microbiota (BEIRÃO et al., 2018).

O objetivo deste trabalho, no capítulo I, foi revisar literatura sobre bactérias intestinais produtoras de ácidos e fazer uma análise genômica das vias metabólicas relacionadas à síntese do butirato. Caracterizar as mudanças da microbiota cecal de frangos de corte suplementados com prebióticos (capítulo II) e medicados com ciclofosfamida (capítulo III) mediante o sequenciamento massivo do gene ribossomal 16S rRNA. Adicionalmente, no capítulo IV, os genomas de sete estirpes de *Salmonella* Heidelberg isoladas da indústria de corte do sul do Brasil foram sequenciados e comparados.

Todos os capítulos foram elaborados seguindo as diretrizes da Associação Brasileira de Normas Técnicas (ABNT).

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CAPÍTULO I – BACTÉRIAS INTESTINAIS PRODUTORAS DE ÁCIDOS ORGÂNICOS – REVISÃO BIBLIOGRÁFICA

BACTÉRIAS INTESTINAIS PRODUTORAS DE ÁCIDOS ORGÂNICOS – REVISÃO BIBLIOGRÁFICA

(***Acid-producing intestinal bacteria – Review***)

RESUMO

A microbiota intestinal é uma rica e complexa comunidade de microrganismos na qual, as bactérias são o grupo predominante e especializado no metabolismo de diferentes substratos, assim como na produção de diversos metabólitos. Entre essas especializações, se encontram as bactérias que sintetizam ácidos orgânicos como o lático e ácidos graxos de cadeia curta como o acetato, propionato e butirato. Adicionalmente, tem se observado que estes ácidos compartilham vias metabólicas para sua síntese e por outro lado possuem propriedades benéficas na saúde intestinal e do hospedeiro, por exemplo, o ácido lático atua como um bactericida impedindo dessa forma a colonização por patógenos; já o butirato atua como fonte de energia nas células do intestino. Assim, o objetivo desta revisão foi determinar um pequeno grupo de bactérias produtoras destes ácidos no intestino, revisar suas informações na literatura e fazer uma análise genômica das vias metabólicas envolvidas na síntese de butirato. Na revisão foi observado que muitas das discussões geradas para justificar o enriquecimento de alguns gêneros bacterianos foi baseada nas propriedades benéficas do butirato. Pelo contrário, a análise demostrou que as vias metabólicas para síntese do butirato nas espécies analisadas ainda não são claras.

Palavras-chave: *Faecalibacterium*, butirato, biodiversidade.

ABSTRACT

The intestinal microbiota is a rich and complex community of microorganisms where Bacteria are dominant. In this community there are groups specialized in metabolism of different substrates, as well as in production of several metabolites such as, lactic acid and short chain fatty acids (acetate, propionate and butyrate). Additionally, metabolic pathways for the synthesis of these acids overlap partially share and they have beneficial effects on intestinal health. For example, lactic acid acts as a source of energy for the intestine cells. In this chapter the capacity of gut bacteria to produce butyrate was inferred from genomic analyses. The results compared to published works. Although a large proportion of published works explain the enrichment of some bacterial genera on the beneficial properties of butyrate, genomic analysis showed that the classic metabolic pathways for butyrate synthesis in these genera are not present.

Key-words: *Faecalibacterium*, butyrate, biodiversity.

INTRODUÇÃO

Entre 500-1000 gêneros bacterianos são habitantes naturais do trato gastrointestinal em humanos, em que os filos *Firmicutes* e *Bacteriodetes* são dominantes e modulam expressivamente este ambiente. Além das bactérias, leveduras, vírus e bacteriófagos também influenciam o ambiente, embora em menor proporção (GIANLUCA et al., 2014).

Nesta interação, o hospedeiro fornece fontes de energia através da ingestão de alimentos e, em troca, as bactérias do intestino (microbiota intestinal) colaboram com o metabolismo de carboidratos não digeríveis, com a resistência à colonização de agentes patogênicos (LEE e HASE, 2014) e com a síntese de metabólitos como, ácido lático, hormônios, vitaminas e ácidos graxos de cadeia curta (em inglês *short-chain fatty acids* ou SCFAs).

Os SCFAs são um subgrupo de ácidos com cadeias carbônicas curtas, tendo como principais representantes o ácido acético, propiônico e butírico. Tanto SCFAs como ácido lático são sintetizados no ceco das aves como produtos da fermentação bacteriana (PAN e YU, 2014).

Por outro lado, a microbiota intestinal e seus metabólitos tem uma interação constante e balanceada com outros sistemas, por exemplo: 1. com o sistema imunológico do animal, modulando assim a resposta imune local e sistêmica; 2. com o próprio sistema gastrointestinal, participando da motilidade e regulando o metabolismo de nutrientes (SOMMER e BACKHED, 2013).

Adicionalmente, o equilíbrio desta interação pode ser perturbado por uma série de eventos externos, tais como infecções gastrointestinais e mudanças na dieta (DE FILIPPO et al., 2010; PERUMBAKKAM et al., 2014). Assim, esse desbalanço da interação, ou disbiose, é caracterizado por mudanças na composição da microbiota intestinal (JOOSSENS et al., 2011; HANDL et al., 2013). Por exemplo, tem sido observada a diminuição significativa de *Faecalibacterium* em humanos com distúrbios intestinais (MIQUEL et al., 2013).

Neste capítulo gêneros e/ou espécies bacterianas relacionadas com a síntese de ácido lático e SCFAs em humanos foram identificadas. Adicionalmente, a presença desses organismos foi evidenciada em aves domésticas com base na literatura.

Bactérias intestinais produtoras de ácidos

Existem várias revisões de literatura que abordam diversos aspectos da microbiota intestinal em aves domésticas (KOHL, 2012; POURABEDIN e ZHAO, 2015; WAITE e TAYLOR, 2015), embora sua participação na saúde animal não tenha sido documentada. Por isto, gêneros e/ou espécies cuja composição varia frequentemente em casos de disbiose em humanos foram selecionados (*Lactobacillus, Ruminococcus torques, Blautia, Faecalibacterium* e *Subdoligranulum*) e sua presença e função em aves domésticas foi analisada.

Lactobacillus

O gênero *Lactobacillus* é constituído por bacilos Gram-positivos que pertencem ao filo *Firmicutes*, são anaeróbios facultativos, tem um genoma de entre 2-3 Mega bases, não formam esporos e são o principal representante do grupo de bactérias produtoras de ácido láctico (FARNWORTH, 2005).

Bactérias deste gênero tem sido usadas como probióticos na nutrição de animais de produção e seus benefícios na saúde intestinal são bem documentados (ANADÓN et al., 2006). *Lactobacillus* são conhecidos pela produção de ácido lático, que tem ação bactericida (FARNWORTH, 2005), bem como pelos efeitos diretos no intestino, ativando componentes celulares do sistema imune inato como células epiteliais intestinais, células natural killer (NK) e monócitos (HALLER et al., 2001). Além dos benefícios diretos dos metabólitos de *Lactobacillus* no intestino, tem-se observado uma relação desse gênero bacteriano com ganho de peso em humanos (MILLION et al., 2011).

No caso de animais de produção, em uma pesquisa de meta-análise realizada por MILLION et al. (2012), foram identificados 51 trabalhos com animais de granja em que as espécies *Lactobacillus acidophilus, Lactobacillus fermentum* e *Lactobacillus ingluviei* foram relacionadas com ganho de peso. Ao contrário, as espécies *Lactobacillus plantarum* e *Lactobacillus gasseri* foram relacionadas com perda de peso. Assim, os efeitos benéficos deste gênero bacteriano parecem estar relacionados com a espécie. De forma geral, tem se levantado alguns mecanismos de ação dos *Lactobacillus* no intestino (TANNOCK, 2004), sendo um deles o mecanismo de exclusão competitiva, no qual, gêneros bacterianos dependentes de

um mesmo substrato não podem existir simultaneamente. É aceito que o aumento da colonização do trato gastrointestinal por *Lactobacillus* pode proteger o hospedeiro de agentes patogênicos, simplesmente por ocupar mais espaços dentro do intestino (SCHLEIFER, 2007). ZHANG et al. (2007), observaram que a suplementação oral de frangos com várias estirpes de *Lactobacillus* reduziram a colonização do ceco por *Salmonella*.

Outro mecanismo que poderia explicar efeitos benéficos seria a produção de bacteriocinas como a reuterina, um potente antimicrobiano produzido por *Lactobacillus reuteri,* que induz o aumento da expressão de genes envolvidos na resposta ao estresse oxidativo em células de *E. coli* (SCHAEFER et al., 2010). Curiosamente, observou-se que a interação com *E. coli* estimulou a produção e secreção de reuterina por *L. reuteri*, indicando que o contato com outras bactérias no intestino aumenta a produção de reuterina. Assim, a reuterina inibe o crescimento bacteriano de um grande número de bactérias (SCHAEFER et al., 2010).

Ruminococcus torques

Ruminococcus torques são cocos Gram-positivos que pertencem ao filo *Firmicutes*, são anaeróbios estritos, tem um genoma de ~3 Mega bases e são encontrados no intestino de seres humanos saudáveis como integrantes do grupo bacteriano associado à mucosa (ECKBURG et al., 2005). *R. torques* é uma bactéria mucolítica e tem sido sugerido que o aumento populacional desproporcional desta espécie no intestino poderia estar relacionado com a doença inflamatória intestinal (PNG et al., 2010) e com o autismo (WANG et al., 2013).

Em crianças autistas a permeabilidade intestinal está aumentada (D'EUFEMIA et al., 1996) e isso poderia representar um possível mecanismo para o aumento da passagem através da mucosa intestinal de peptídeos derivados de alimentos ou bactérias com efeitos sistêmicos subsequentes (D'EUFEMIA et al., 1996). Nesse sentido, *R. torques* ao ser enriquecido no intestino leva a maior degradação de muco intestinal e consequentemente a alterações na permeabilidade e na barreira mucosa do intestino (WANG et al., 2013).

Por outro lado, no caso de animais de produção as informações referentes a esta espécie são limitadas. Em trabalho de cultivo anaeróbico a partir de amostras de conteúdo cecal de galinhas poedeiras com 12 semanas foi observado que 10% das colônias bacterianas recuperadas pertenciam à espécie *R. torques* (LAN et al., 2002). Sequências dessa espécie também foram identificadas no ceco de frangos de corte ao 42 dias de idade (TOROK et al., 2011). Assim, o aumento dessa espécie no intestino de aves poderia ser explicado pelo seu potencial mucolítico. Tem-se observado que a produção de muco varia em pintinhos durante os primeiros sete dias de vida como resultado da colonização bacteriana do intestino, sugerindo alteração da expressão gênica do hospedeiro em resposta à microbiota intestinal (FORDER et al., 2007). Além disso, a retenção de nutrientes entéricos favorece a colonização por bactérias comensais e patogênicas que usam muco como substrato, enfraquecendo assim a proteção proporcionada pela mucosa intestinal (DEPLANCKE et al., 2002).

Outra hipótese que poderia explicar o aumento desta espécie no intestino de aves seria a inflamação intestinal de origem alimentar (KOGUT, 2017). A busca

constante por alimentos de baixo custo para alimentação animal levou à inclusão nas rações de algumas matérias primas que contêm polissacarídeos não-amiláceos (PNAs), por exemplo derivados de trigo, cevada e centeio, entre outros (CHOCT et al., 1996). Assim, o aproveitamento desses compostos pelas aves é limitado à fermentação microbiana, ocorrendo principalmente no ceco. Uma das consequências da presença de PNAs é o aumento da viscosidade intestinal que por sua vez reduz a capacidade digestiva e a absorção de nutrientes (CHOCT et al., 1996). Dessa forma, parece que o intestino das aves nos sistemas modernos de produção entra em um círculo vicioso, no qual a retenção de compostos como PNAs influenciam a homeostase intestinal e, juntamente com um excesso de nutrientes persistentes levam a inflamação crônica, disfunção intestinal e mudanças na microbiota, com características muito semelhantes às doenças metabólicas intestinais humanas (KOGUT, 2017).

Blautia

O gênero *Blautia* agrupa cocos Gram-positivos que pertencem ao filo *Firmicutes*, são anaeróbios estritos, possuem um genoma de ~3 Mega bases e são considerados habitantes normais do intestino de humanos (LAWSON e FINEGOLD, 2015). A diminuição de bactérias deste gênero no intestino tem sido relacionada com o diabetes tipo 1 em crianças, quando comparado a crianças saudáveis (MURRI et al., 2013). O aumento significativo do gênero foi observado nas fezes de pessoas com uma alimentação rica em cevada integral. Isso sugere que o gênero possa se beneficiar com a produção de hidrogênio, produto da fermentação

intestinal provavelmente induzida pelo consumo de grãos integrais (MARTÍNEZ et al., 2012).

Por outro lado, em camundongos foi observado o aumento significativo na abundância da espécie *B. obeum* após a infecção por *Vibrio cholerae*, o agente causal da cólera, associado à melhoria no quadro clínico dos camundongos, mostrando assim um possível efeito benéfico desta espécie contra enteropatógenos (HSIAO et al., 2014). Por sua vez, em aves domésticas, POLANSKY et al. (2016), observaram a expressão de proteínas de *Blautia*, como acetiltransferases e enzimas necessárias para a produção de ácido propiônico e ácido butírico no ceco de frangos de corte de sete dias de idade.

Subdoligranulum

Trata-se de um gênero de cocos Gram-negativos que pertencem ao filo *Firmicutes*, são estritamente anaeróbicos e tem um genoma de ~3,2 Mega bases. O grupo fermenta glucose e alguns outros carboidratos, os principais produtos do metabolismo são ácidos butírico e lático, juntamente com pequenas quantidades de ácidos acético e succínico (HOLMSTRØM e LAWSON, 2015). É um gênero bacteriano habitante normal do trato intestinal e tem sido observado o aumento significativo nas fezes de pacientes com diabetes tipo 2 quando comparado a pacientes sadios (ZHANG et al., 2013). Nesse sentido, esses resultados são contraditórios, uma vez que o gênero *Subdoligranulum* é um produtor de SCFAs, os quais tem sido relacionados com benefícios para a saúde do hospedeiro, entre eles,

resistência a doenças cardiovasculares, redução de doenças inflamatórias intestinais e redução de diabetes tipo 2 (LATTIMER e HAUB, 2010).

Em animais de produção, foi observada a expressão de butirato quinase no ceco de frangos de corte (POLANSKY et al., 2016), uma enzima envolvida na síntese de ácido butírico. Esse ácido desempenha um papel importante na fisiologia intestinal, servindo como fonte de energia para as células epiteliais do íleo (VAN DER WIELEN et al., 2000). Talvez essa função dos SCFAs explique a abundância do gênero no intestino de aves (POLANSKY et al., 2016). Adicionalmente, nas fezes de pintainhas de postura de sete e vinte e um dias de idade foi observada uma alta prevalência deste gênero (BEIRÃO et al., 2018) que poderia ser explicada pela maior necessidade de butirato pelas células intestinais de aves jovens em crescimento (VAN DER WIELEN et al., 2000). Na fase adulta (depois dos 4 meses de idade) o butirato não é mais necessário (VIDENSKA et al., 2014) uma vez que a digestão de polissacarídeos complexos por outros gêneros representantes do filo *Bacteroidetes* resultam na produção tanto de propionato como de butirato, o que pode ser mais vantajoso no equilíbrio de nutrientes no intestino (POLANSKY et al., 2016).

Faecalibacterium

O gênero *Faecalibacterium* é um dos principais produtores de butirato no intestino (RUSSELL et al., 2011). Dentro do gênero, a espécie mais estudada é *F. prausnitzii*, um bacilo Gram-negativo, imóvel, estritamente anaeróbico, com um genoma de ~3 Mega bases (DUNCAN et al., 2002).

Esta espécie é um comensal ativo, abundante na microbiota intestinal humana e animal de indivíduos saudáveis e que representa mais de 5% das bactérias totais no intestino. Mudanças na abundância de *F. prausnitzii* foram ligadas à disbiose em vários transtornos humanos (MIQUEL et al., 2013). Além dos efeitos benéficos indiretos desse comensal pela produção de butirato, tem-se observado efeitos anti-inflamatórios diretos em um modelo celular *in vitro*. Metabólitos secretados por *F. prausnitzii* foram capazes de bloquear a ativação do fator de transcrição NF-κB, assim como de estimular a produção da citocina pro-inflamatória IL-8 pelas células epiteliais, resultando numa resposta anti-inflamatória (SOKOL et al., 2008). Outras atividades anti-inflamatórias atribuídas ao gênero foram observadas em camundongos com colite induzida, através do aumento de metabólitos tais como o ácido salicílico que por sua vez diminuíram a produção de IL-8 (MIQUEL et al., 2015).

Adicionalmente foi observada diminuição significativa de *F. prausnitzii* e um aumento significativo de *R. torques* em pacientes com doença de Crohn quando comparados a pacientes saudáveis (JOOSSENS et al., 2011). Esses resultados sugerem a falta de capacidade de produção de butirato em conjunto com a degradação do muco como explicações fisiopatológicas para a doença (JOOSSENS et al., 2011).

Por outro lado, em frangos de corte foi observada uma associação da espécie *F. prausnitzii* com um dos perfis microbianos relacionados ao maior ganho de peso (TOROK et al., 2011). Além disso, foi observada alta prevalência deste gênero no ceco de frangos de corte de 14 dias de idade criados em cama de maravalha. Foi

também observada uma diminuição significativa deste gênero nas aves tratadas com um prebiótico a base de nucleotídeos (MESA et al., 2017).

CONCLUSÕES

A revisão de literatura evidenciou que muitas das discussões geradas nos trabalhos científicos estão baseadas nos efeitos benéficos dos SCFAs, em especial do butirato. Muitos destes argumentos surgem de trabalhos *in vitro*, que observaram atividades enzimáticas ou produção de SCFAs por uma estirpe em um determinado meio de cultivo.

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CAPITULO II - CECAL MICROBIOTA IN BROILERS FED WITH PREBIOTICS

(***Frontiers in Genetics "Livestock Genomics"***)

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CECAL MICROBIOTA IN BROILERS FED WITH PREBIOTICS

RESUMO

Na produção de frangos de corte são utilizados alguns aditivos na ração, entre eles, os prebióticos. Estes são moléculas que induzem o crescimento de bactérias no intestino que são benéficas para o hospedeiro, por exemplo, *Lactobacillus* e *Bifidobacterium*. O uso de prebióticos pode prevenir infecções por patógenos e promover melhor desempenho em aves. O objetivo deste estudo foi avaliar através do sequenciamento do gene 16S rRNA as mudanças da microbiota cecal devido ao uso dos prebióticos mananoligossacarídeo (Mos) e nucleotídeos (Nuc) na alimentação de frangos de corte. Foram avaliadas as mudanças em dois tempos de coleta (14 e 35 dias). Uma mudança transitória na microbiota intestinal foi observada entre os 14 e 35 dias de idade. O tratamento com prebióticos promoveu mudanças na estrutura da comunidade bacteriana do ceco. Adicionalmente, uma menor biodiversidade foi observada nos grupos tratados. Embora a biodiversidade tenha sido reduzida; o uso de prebióticos promoveu o aumento de gêneros bacterianos benéficos. O uso de Nuc como prebiótico aumentou significativamente a contagem de sequências de *Lactobacillus* e diminuiu significativamente a contagem de sequências de *Escherichia-Shigella*. Por outro lado, o uso de Mos aumentou significativamente a contagem de sequências de *Butyricimonas* e *Roseburia*, ambos os gêneros conhecidos como bactérias produtoras de ácido butírico. Estes dados mostraram que a microbiota cecal foi influenciada tanto pela suplementação prebiótica como pelo tempo entre coletas.

Palavras-chave: *Lactobacillus*, *Roseburia*, mananoligossacarídeo.

ABSTRACT

In poultry production, several additives are used in the ration, such as prebiotics. These are molecules that induce the growth of bacteria beneficial for the host in the gut (e.g. *Lactobacillus, Bifidobacterium*). In chickens, the use of prebiotics can prevent illness from pathogens and promote better performance. The aim of this study was to evaluate through sequencing of 16S rRNA gene the shifts of the cecal microbiota due to the use of different prebiotics, mannan-oligosaccharide (Mos) and nucleotide (Nuc) in the feed of broilers, at two sampling times (14 and 35 days). A transitory change in the gut microbiota was observed at 14 days of sampling, but differences were clearer at 35 days. The prebiotics induced shifts in the community structure of the microbiota in the cecum, and a lower diversity was observed in the treated groups. Although the diversity was reduced; the use of prebiotics promoted the increase of known beneficial bacterial genera. The use of Nuc as prebiotics significantly increased the count of sequences of *Lactobacillus* and significantly decreased the count of sequences of *Escherichia-Shigella*. On the other hand, the use of Mos significantly increased the count of sequences of *Butyricimonas* and *Roseburia*, both genera known as butyrate producing bacteria. These data showed that the cecal microbiota was influenced by both time and the prebiotic supplementation.

Key-words: *Lactobacillus*, *Roseburia*, mannan-oligosaccharide.

INTRODUCTION

Prebiotics are typically fibrous compounds that pass undigested through the upper part of the gastrointestinal tract and stimulate the growth or activity of advantageous bacteria that colonize the bowel and contribute to the well-being of their host (GIBSON and ROBERFROID, 1995). Some of the most widely used prebiotics in the poultry industry are fructo-oligosaccharides, mannan-oligosaccharides, galacto-oligosaccharides and beta-glucans (HUYGHEBAERT et al., 2011).

Selective fermentation of some prebiotics has been shown to induce changes in the composition and/or activity of the gastrointestinal microbiota, improving the health of the host (GIBSON et al., 2004). Like this, ZHENPING et al. (2013) showed increased growth performance, enhanced endocrine metabolism, and improved immune function in broiler chickens after in-feed supplementation with xylo-oligosaccharides prebiotics. Additionally, changes in enteric bacteria in the cecum (SPRING et al., 2000) and improved intestinal morphology have been observed in broilers fed with dietary mannan-oligosaccharide (BAURHOO et al., 2009).

Because the microbiota can be modified (KHORUTS et al., 2010; BORODY and KHORUTS, 2012) it constitutes an attractive target for therapeutic manipulation. However, successful outcome of such manipulations require a better understanding of the interactions between the host and its microbiota (HAMILTON et al., 2013).

The composition and diversity of chicken intestinal microbiota were previously investigated using cultivation-based methodologies (FERNANDEZ et al., 2002). However, the use of DNA-based molecular biology techniques, such as

metagenomics and new generation DNA sequencing, allowed new opportunities to characterize uncultivable members of intestinal microbiota (GONG et al., 2002) shedding light on the composition and temporal spatial location of the microbial population in broiler's intestine. Nevertheless, the knowledge of the structure, interactions and functions of the intestinal microbiota is still limited and fragmented (OAKLEY and KOGUT, 2016).

In this work the effect of dietary supplement with prebiotics derived from yeast wall (mannan-oligosaccharide and nucleotide) on the cecal microbiota of broilers was evaluated by the massive parallel sequencing of the 16S rRNA gene.

MATERIALS AND METHODS

Experimental design and sampling

Thirty female newborn chicks (Hubbart®) were placed on wood shavings litter for 35 days. Water and feed were given *ad libitum*. At arrival, chicks were randomly divided in groups for three different types of treatment (10 birds per treatment): treatment without additives in the feed (Neg), treatment with the prebiotics mannan-oligosaccharide (Mos), or nucleotides (Nuc). 200 g/ton of each prebiotic were incorporated in the ration from the first day until the 35th day. Briefly, the prebiotics used in this work were extracted from biomass derived from the sugar and beer industry. These by-products contain abundant in *Saccharomyces cerevisiae* yeast. The raw-matter was treated by industrial autolysis, obtaining two fractions. The first, composed mainly for yeast wall, was rich in mannan-oligosaccharides. The second

fraction contained soluble yeast extract and was abundant in nucleotides (CHAUD and SGARBIERI, 2006). All animal procedures were approved by the Animal Experimentation Ethics Committee of the Federal University of Paraná (authorization CEUA-Bio UFPR 898/2015). At 14 and 35 days of age, the chicks' body weights were recorded and samples were collected for DNA extraction, purification and sequencing. Immediately after euthanasia, the abdominal cavity was exposed and the cecum was dissected from the other intestinal sections. The cecum from each bird was cut open, and the contents were collected in a sterile 2-ml tube, stored on ice and later frozen and stored at −80°C until use. In total, 24 samples for cecal contents were collected (four chickens per treatment at 14 and 35 days of age).

DNA extraction, 16S rRNA gene amplification and sequencing

Genomic DNA from each sample was isolated from 200 mg of cecal luminal content using PowerFecal® DNA Isolation Kit (MO BIO laboratories, Inc.). The variable V4 region of 16S rRNA gene was amplified using the universal primers 515F and 806R (CAPORASO et al., 2011) and KlenTaq Master Mix (Sigma). The PCR conditions used were 94°C for 3 min; 18 cycles of 94°C for 45 s, 50°C for 30 s and 68°C for 60 s; followed by 72°C for 10 min. The amplicons were quantified with Qubit using HS dsDNA kit (Invitrogen), diluted to 500pM and pooled. Then, 16pM of pooled DNA were sequenced using MiSeq reagent 500V2. Sequencing was performed using an Illumina MiSeq® sequencer (Illumina) obtaining paired-end reads of 250 bp as described (CAPORASO et al., 2011). The dataset were submitted in NCBI site under the BioSample accession code SAMN07211773 (http://www.ncbi.nlm.nih.gov/biosample/7211773).

Diversity analysis

Sequencing data were analysed with the QIIME pipeline (CAPORASO et al., 2010). Since the Illumina output ranged from approximately 41,000 to 172,499 reads, the read number was re-sampled to 41,800 reads per sample, allowing for the diversity comparisons. Sequences were quality filtered and identified at the phyla and genus levels using the open-reference OTU method implemented in QIIME and the SILVA database (123 release) (YILMAZ et al., 2013; RIDEOUT et al., 2014). Basic diversity analysis (OTU number and Unifrac-distances) was conducted using QIIME, and the OTU table exported to R for further analyses.

Statistical analysis

Statistical analyses were performed using the Stats package included in R software (R-CORE-TEAM, 2015). First, data was explored using multivariate tools. Weighted unifrac-distances previously obtained from QIIME were used to sample ordination using PCA and cluster analyses. Since the samples showed a non-parametric distribution by the Shapiro Wilk test, the genera previously identified were compared according to the prebiotic treatments using the Kruskal Walli's test. Only statically significant results were reported ($P < 0.05$).

RESULTS

The use of the prebiotics Mos and Nuc resulted in no changes in the body weight of the birds in 14 or 35 days (not shown). However, they did cause several changes in the gut microbiota. Using multivariate analysis, small changes in the microbiota were observed at 14 days, but differences were clearer after 35 days(Table 1). At 14 days, *Firmicutes* was the most predominant phylum in all treatments and, on average,

accounted for 84.5% of identified sequences, followed by *Proteobacteria* (7.9%) and *Tenericutes* (4.3%).

Table 1 - Most prevalent bacterial genera in broiler's cecum microbiota. Data is shown by treatment (%, mean and standard error, n = 4). The K.W columns show the result of statistical comparison between treatments (Neg, Mos, and Nuc) for each genus by the Kruskal Wallis test. Same uppercase letters indicate no statistical difference in genus abundance between treatments. The column Time shows statistical comparison between the two time points by the Tukey test. Asterisk (*) indicates significant difference between sampling times (P < 0.05). Control group (Neg), mannan-oligosaccharide (Mos) and nucleotide (Nuc).

Genera	Treatment	14 day	K.W	35 day	K.W	Time
Faecalibacterium	Neg	21,52 ± 3,79	A	6,1 ± 1,03	A	*
	Mos	25,7 ± 2,97	A	1,66 ± 0,46	B	*
	Nuc	13,62 ± 3,63	B	1,34 ± 0,11	B	*
Bacteroides	Neg	0		10,87 ± 1,35	B	*
	Mos	0		17,70 ± 4,52	B	*
	Nuc	0		35,91 ± 1,88	A	*
Lactobacillus	Neg	8,38 ± 2,16	A	3,44 ± 0,93	B	*
	Mos	8,62 ± 2,02	A	3,51 ± 1,17	B	*
	Nuc	2,97 ± 0,72	B	6,79 ± 1,10	A	*
Streptococcus	Neg	9,75 ± 1,25	A	3,28 ± 1,36	B	*
	Mos	3,88 ± 4,22	B	5,90 ± 0,38	A	*
	Nuc	1,83 ± 1,93	B	5,60 ± 0,82	A	*
Lachnoclostridium	Neg	5,38 ± 1,22		3,12 ± 0,45	A	*
	Mos	4,34 ± 0,27		1,59 ± 0,28	B	*
	Nuc	5,19 ± 1,16		3,01 ± 0,11	A	*
Anaerotruncus	Neg	0,97 ± 0,17	B	6,30 ± 0,29	A	*
	Mos	1,73 ± 0,26	A	3,52 ± 1,02	B	*
	Nuc	2,22 ± 0,41	A	5,93 ± 0,30	A	*
Ruminiclostridium 5	Neg	3,25 ± 0,33	C	2,37 ± 0,14	A	
	Mos	5,08 ± 0,78	B	0,87 ± 0,30	B	*
	Nuc	6,47 ± 1,56	A	0,75 ± 0,02	B	*
Subdoligranulum	Neg	7,22 ± 2,30	A	1,63 ± 0,22	A	*
	Mos	1,52 ± 0,41	B	0,41 ± 0,11	B	
	Nuc	7,52 ± 2,92	A	0,12 ± 0,01	B	*
Parabacteroides	Neg	0		0	A	
	Mos	0		11,40 ± 4,38	B	*
	Nuc	0		0	B	
Escherichia-Shigella	Neg	4,15 ± 1,49	A	0,43 ± 0,13	A	*
	Mos	3,37 ± 2,05	A	0,20 ± 0,14	B	*
	Nuc	2,68 ± 2,53	B	0,11 ± 0,01	B	*
Alistipes	Neg	0		4,90 ± 0,35	A	*

Mos	0	4,13 ± 1,18	A	*
Nuc	0	0,88 ± 0,24	B	*

After 35 days, *Firmicutes* remained the most predominant phylum in cecum in all treatments and, on average, accounted for 61.4% of all the bacterial sequences, followed by *Bacteroidetes* (29%), *Proteobacteria* (4.5%), and *Tenericutes* (4.2%). Interestingly, the phylum *Bacteroidetes* was only detected at 35 days.

In another taxonomic level, 665 bacterial genera were identified using the SILVA database (release 123). At 14 days, the most predominant genera were *Faecalibacterium*, accounting for 20.3% of all cecal bacterial sequences. The second genera were *Lactobacillus* with 6.65% of bacterial sequences. The third most predominant genus was *Subdoligranulum* accounting for 5.42% of the cecal sequences (Table 1). At 35 days, *Bacteroides* was the most predominant genus in the cecum, accounting for 21.5% of the cecal sequences. The second genera were *Anaerotruncus*, accounting for 5.59% of the sequences. The third most predominant genera were *Streptococcus*, accounting for 4.93% of all cecal bacterial sequences. Among, the most abundant bacterial genera, an increase in the count of sequences at 35 days was observed in *Bacteroides, Anaerotruncus* and *Alistipes*. In an opposite way, the genera *Faecalibacterium, Lactobacillus, Streptococcus, Lachnoclostridium, Ruminiclostridium 5, Subdoligranulum* and *Escherichia-Shigella* decreased at 35 days, when compared at 14 days.

Moreover, Principal Component Analysis (PCA) of OTUs representation clearly showed the age explained most of the differences (66.4%), followed by treatment (15.9%) (Figure 1).

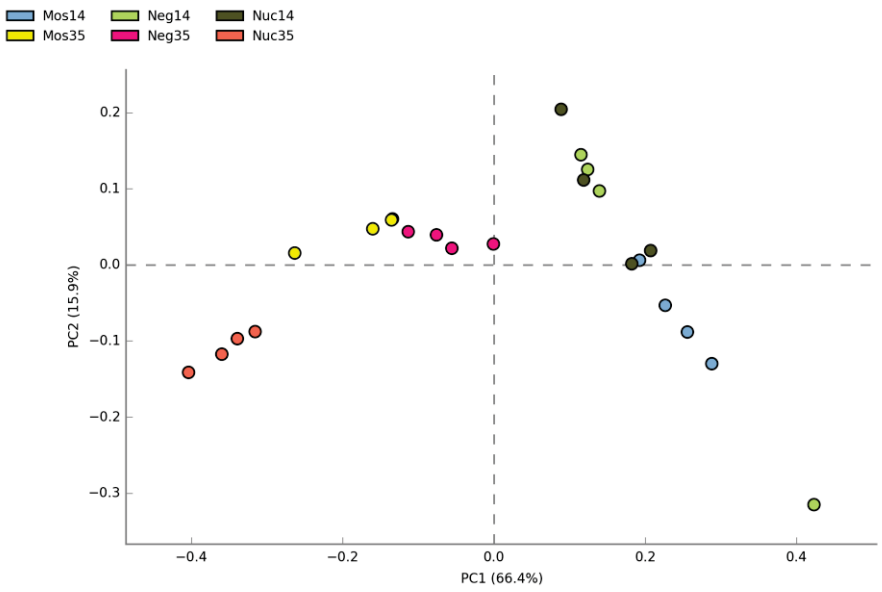

Figure 1 - Principal Component Analysis (PCA) of broiler's cecal bacterial community fed with yeast-derived prebiotics. Control group (Neg) mannan-oligosaccharide (Mos) and nucleotide (Nuc) are shown. Samples were compared according to the out composition at the genus level as identified by the SILVA database (SILVA 123 release) using the Unifrac distance weighted method.

Finally, the supplementation with both prebiotics significantly decreased the Observed OTUs index at 35 days, when compared to the negative group. This index is a species richness estimator, which estimates the total number of species present in a community (Figure 2).

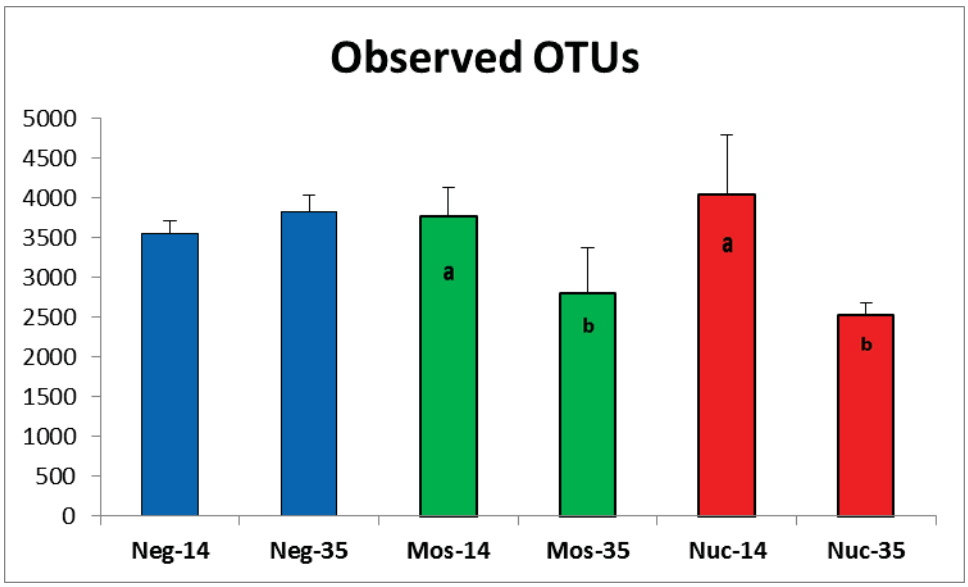

Figure 2 - Observed OTUs index of different bacterial groups in cecum at 14 and 35 days. Different letters mean statistical difference in genera abundance between sampling times by the Tukey test (*P<0.05*). Data is shown by treatment (%, mean and standard error, n=4). Control group (Neg), mannan-oligosaccharide (Mos) and nucleotide (Nuc).

DISCUSSION

In this work we evaluated the cecal microbiota of chickens treated with prebiotics using high throughput 16S rRNA gene sequencing. The present study demonstrates that prebiotic treatment for 35 consecutive days caused shifts in the microbial community structure in the cecum in chickens. As seen by traditional microbiology by some authors (FERNANDEZ et al., 2002; BAURHOO et al., 2007; OWENS et al., 2008). In addition, our results show that in the first two weeks of life the broiler's cecum undergo a process of alteration before reaching more stable microbiota (DANZEISEN et al., 2011).

At level of phylum, *Firmicutes*, *Bacteroidetes* and *Proteobacteria* were the most abundant groups. Similarly to the results observed by MANCABELLI et al. (2016). At a deeper level, at genus level, the bacterial profiles are highly variable among the chicken intestinal microbiota studies (WEI et al., 2013). In this study, at 35 days, *Bacteroides* was the most predominant genera in the cecum (21.5%). The second genera were *Anaerotruncus* (5.59%) and the third most predominant genera were *Streptococcus* (4.93%). In contrast, the cecal microbiota of broilers with 25 days without treatment was primarily composed for *Lactobacillus* (24.38%), *Clostridium* (20.13%) and *Bacteroides* (15.83%) (STANLEY et al., 2012). On the other hand, VIDENSKA et al. (2013) observed that the cecal microbiota of chickens was dominated by representatives of the family *Lachnospiraceae* followed by *Ruminococcaceae* and *Enterobacteriaceae*. Maybe, the difference between works can be different due to the effect of the diet, water origin, management of birds and treatment, among others (TOROK et al., 2008). Moreover, differences in technical methodologies for sample collection and processing could contribute to differences in gut microbiota composition results (KENNEDY et al., 2014). These complexities make it difficult direct comparison between studies.

The prevalence of *Firmicutes* at 14 days might be explained by the higher need of butyrate by the young chicken growing intestine (VAN DER WIELEN et al., 2000). Since in adult chickens maximal butyrate production is no longer required, digestion of complex polysaccharides by representatives of *Bacteroidetes* results in the production of both propionate and butyrate, which might be a more advantageous balance of nutrients (POLANSKY et al., 2016). In agreement with this assumption,

(POLANSKY et al., 2016) observed a gradual increase of *Bacteroidetes* counts in the cecum from the third week of life of chickens.

Overall, we observed a decrease in the count of OTUs and in the diversity in the cecum in chickens treated with Mos and Nuc prebiotics. This result indicates that the prebiotics promote enrichment of specific bacterial groups in the cecum, decreasing community diversity (COX and PAVIC, 2010; GAGGÌA et al., 2010). Although the prebiotics Mos and Nuc caused a decrease the richness and the diversity in the cecum, the abundance the some genera such as, *Lactobacillus*, *Butyricimonas*, and *Roseburia* is a benefic result, because these genera are known for producing short-chain fatty acids (SCFAs) and have shown beneficial effects on the host's development and health (PRYDE et al., 2002; FARNWORTH, 2005; LOMAN and TAPPENDEN, 2016).

CONCLUSIONS

In conclusion the composition of cecal microbiota is not constant and develops over time in chickens. Moreover, prebiotic supplementation significantly affected the microbial community structure in the cecum in broiler chickens with most significant shifts at 35 days. Although the prebiotics Mos and Nuc caused a decrease the richness and the diversity in the cecum, the prevalence of beneficial bacteria increased in both treatments. Nuc increased the count of sequences of *Lactobacillus* and decreased the count of sequences of *Escherichia-Shigella*, while Mos significantly increased the count of sequences of *Butyricimonas* and *Roseburia*.

AUTHOR CONTRIBUTIONS

MN provided the prebiotics. DM and DL analysed the data and performed the numerical and statistical analyses. DM and ES designed the work and wrote the manuscript. DL and LMC processed the sequence raw data. CS and LFC designed the protocol. DM performed DNA extraction from samples. EB performed amplicons library preparation and sequencing. FP and ES supervised the work.

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CAPITULO III - IMMUNOSUPPRESSION INCREASES *LACTOBACILLUS* IN THE INTESTINAL MICROBIOTA IN CHICKENS

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RESUMO

A produção de aves é uma indústria muito intensa. Devido ao número considerável de animais criados por um produtor, mesmo pequenas variações na produtividade levam a importantes resultados econômicos. Assim, a microbiota intestinal das aves está sob intenso escrutínio devido ao seu potencial como fator controlável que pode melhorar os resultados de produção. Dados recentes em humanos indicam que a imunossupressão também está correlacionada com mudanças na microbiota intestinal. No entanto, a relação entre imunossupressão e microbiota intestinal não foi estudada em frangos de corte. Assim, pesquisamos as correlações entre células imunes e a microbiota intestinal pelo sequenciamento massivo do gene ribossomal 16S rRNA em frangos imunossuprimidos com ciclofosfamida. Os resultados mostraram correlações entre células imunológicas periféricas e a microbiota intestinal. Surpreendentemente, observou-se um aumento significativo na abundância de *Lactobacillus* no ceco das aves imunossuprimidas, além de apresentarem maior quantidade do filo *Firmicutes*, entre outras alterações na microbiota. Essas mudanças indicam o papel da imunidade no controle da microbiota em aves de produção. Adicionalmente, estes dados são úteis para comparar com a microbiota de animais que sofrem de imunossupressão por outras causas.

Palavras-chave: Frango de corte, imunossupressão, *Lactobacillus*, Bursa cloacal

ABSTRACT

Poultry production is a very intensive industry. Due to the substantial number of animals being raised by any one producer, even small variations in productivity lead to important economical outcomes. The intestinal microbiota of birds is under intense scrutiny due to its potential as a controllable factor that might improve production results. Recent data in humans indicate that immunosuppression is also correlated with shifts in the intestinal microbiota. However, the relationship between immunosuppression and intestinal microbiota has not been studied in chickens. Thus, we investigated the correlations between immune cells and intestinal microbiota by the massive parallel sequencing of the 16S rRNA bacterial gene in immunosuppressed chickens with cyclophosphamide. The results showed correlations between peripheral immune cells and intestinal microbiota. An increase in the abundance of intestinal *Lactobacillus* in the immunosuppressed chickens was observed. These birds also had higher bacterial diversity and were enriched for *Firmicutes*, among other alterations in the microbiota. These shifts indicate the role of immunity in controlling the microbiota of poultry. This data will be useful for comparison with the microbiota of animals suffering from immunosuppression from other causes.

Key-words: Broiler, Immunosuppression, *Lactobacillus*, Cloacal bursa

INTRODUCTION

Poultry production is one of the largest food industries worldwide. In farms, the birds frequently endure conditions that can lead to immunosuppression, such as high mycotoxin concentrations in feed (AWAD et al., 2013) and infectious agents that target immune cells, such as infectious bursal disease virus (IBDV) (LASHER and SHANE, 1994). As a consequence, immunosuppressed chickens perform poorly, therefore affecting economic returns (HOERR, 2010).

In addition, recent data indicate that immunosuppression is also correlated with characteristic changes in the intestinal microbiota in humans, likely due to damages to gut-associated lymphoid cells and tissues (LOZUPONE et al., 2013). This is important because the intestinal microbiota not only reflects the immune changes, but also shapes the states of health and disease; for some immune-mediated conditions, alterations of the intestinal microbiota seem to be crucial aspects of the disease (FUJIMURA et al., 2010; LOZUPONE et al., 2013).

Thus, the intestinal microbiota seems to interact directly with the immune system of the host, contributing to maintaining the integrity of the epithelial barrier and stimulating local and systemic immune interactions (SOMMER and BACKHED, 2013). These biological interactions are beginning to be studied in poultry science. For example, LUO et al. (2013) observed an increase in immune proteins and changes in the intestinal microbiota in chickens treated with a probiotic, while OAKLEY and KOGUT (2016) showed a correlation between cytokines and intestinal microbiota in chickens.

However, the relationship between immunosuppression and intestinal microbiota has not been studied in chickens. To understand this interaction, we investigated the correlations between immune cells and intestinal microbiota by the massive parallel sequencing of the 16S rRNA gene in immunosuppressed chickens.

MATERIALS AND METHODS

Experimental design and sampling

Eighty male newborn chicks (Cobb®) were divided in two groups (40 birds per group): Control and Cyclophosphamide-treated. Cyclophosphamide (3mg/kg) was administered subcutaneously during the first four days of life, following previously published guidelines for inducing immunosuppression in chickens (RUSSELL et al., 1997). Water and feed were given *ad libitum* throughout the experiment. Birds were housed for 49 days in isolators with HEPA-filtered air flow.

At 10, 21, 35, 42 and 49 days of age, eight blood samples per treatment were collected for immune cell measurement by flow cytometry (total leukocytes, B lymphocytes, CD4 and CD8 T lymphocytes and phagocytic cells (monocytes and heterophils)).

Antibody responses were assessed by immunization of chickens with an inactivated Newcastle disease vaccine (Ceva®) at 30 days of age. Hemagglutination inhibition was used to assess antibody titers against Newcastle disease virus (NDV) at 30, 35, 42 and 49 days of age. At 10, 21, 30, 35, 42 and 49 days of age the cloacal bursa was extracted and weighed in eight birds per treatment following euthanasia.

Bursal lesions were assessed by histopathology at these time points. Additionally, samples of cecal contents were collected for DNA extraction, purification and sequencing. Immediately after euthanasia, the abdominal cavity was exposed and the cecum was dissected from the other intestinal sections. Cecal contents were collected in sterile 2-ml tubes, stored on ice and later stored at -20°C until processing. In total, eight samples of cecal contents were collected (four chickens per treatment at 35 days of age).

All animal procedures were approved by the Animal Experimentation Ethics Committee of the Federal University of Paraná (authorization CEUA-Bio UFPR 018/2017).

Flow cytometry

Whole blood samples was processed according to a no-lyse-no-wash modified protocol from STABEL et al. (2000). Briefly, 50 µl of whole blood were incubated for 30 minutes at 37°C with antibodies against one of the following chicken cellular markers: CD4, CD8 or B lymphocytes (Southern Biotechnology). All samples were also stained for CD45, used for gating the leukocyte population. Samples were fixed with 1% paraformaldehyde for 30 minutes at 4°C. Subsequently, the samples were diluted with PBS to a final volume of 2 ml. Phagocytic cells and their activity was assessed using the pHrodo® FITC reagent (Thermo Scientific). 50 µl of pHrodo were added to 50 µl of whole blood for 30 min at 37ºC. Cells were then stained for CD45 as described in STABEL et al. (2000), and cells were assessed for the number of green-fluorescence events (phagocytic cells) and the intensity of fluorescence (thus inferring phagocytic activity). Phagocytic cells were further discriminated regarding

cell granularity (side scatter). High granularity fluorescent cells are herein named as phagocytic heterophils, and low granularity fluorescent cells are referred to as phagocytic monocytes. For absolute quantification of leukocytes, CountBright beads (Thermo Scientific) were added to the tube. Samples were read in a FACSCalibur® flow cytometer Becton Dickinson equipped with an argon laser.

NDV hemagglutination inhibition and bursal histopathology

Antibody responses to NDV were assessed by ELISA, using commercial kits (Idexx Laboratories). Samples of the bursa were processed routinely for histology and stained with hematoxylin and eosin. Twenty fields per treatment were scanned under a light microscope (Olympus BX41 Olympus USA) at 40x magnification. Bursal histopathology analyses were performed by a trained veterinarian through the subjective conventional method. The fields were classified in to depletion scores that vary from 1 to 4 (score 1 = depletion <25%; score 2 = depletion 25-50%; score 3 = depletion 50-75%, score 4 = depletion >75%).

DNA extraction, 16S rRNA gene amplification and sequencing

Genomic DNA from each sample was isolated from 150 mg of cecal luminal content using the ZR Fecal DNA MiniPrep Kit® (Zymo Research, Inc.). The variable V4 region of 16S rRNA gene was amplified using the universal primers 515F and 806R (CAPORASO et al., 2011) and KlenTaq Master Mix (Sigma). The PCR conditions used were 94°C for 3 min; 18 cycles of 94°C for 45 sec, 50°C for 30 sec

and 68°C for 60 sec; followed by 72°C for 10 min. The amplicons were quantified with Qubit using HS dsDNA kit (Invitrogen), diluted to 500 pM and pooled. Then, 16 pM of pooled DNA were sequenced using MiSeq reagent 500V2. Sequencing was performed using an Illumina MiSeq® sequencer (Illumina) obtaining reads of 250 bp as described (CAPORASO et al., 2011).

Diversity analysis

Sequencing data were analysed with the QIIME 1.9.1 pipeline (CAPORASO et al., 2010). Sequences were filtered by quality and identified at the phylum and genus levels with the open-reference operational taxonomic unit (OTU) method implemented in QIIME and using the SILVA database (128 release) (YILMAZ et al., 2013; RIDEOUT et al., 2014). The Illumina reads output were re-sampled to 12.671 per sample, allowing for direct diversity comparison. The file table_even.biom containing the OTUs identified in QIIME was converted to spf format using the script biom_to_stamp.py available through the Microbiome Helper website (https://github.com/mlangill/microbiome_helper/wiki) (COMEAU et al., 2017). It was transferred to the statistical program STAMP (Statistical Analysis of Metagenomic Profiles) (PARKS et al., 2014) for basic diversity analysis such as proportion of sequences per genus and PCA plot.

Statistical analysis

To compare bacterial genera abundance between treatments, Welch's t-test (*P < 0.05*) and corrected Bonferroni test was used. Both analyses were included in the STAMP software. Biodiversity richness was compared with Tukey´s test (*P < 0.05*). Immune cell and numbers were compared with the Kruskal Wallis test (*P < 0.05*). Bursa weight, lymphoid depletion and antibody response to NDV were also analysed by Kruskal Wallis test (*P < 0.05*). Pearson's correlation analysis and non-metric multidimensional scaling (NMDS) plot were performed using the psych and vegan package included in R software (R-CORE-TEAM, 2015). Only statistically significant results were reported (*P < 0.05*).

RESULTS

Administration of cyclophosphamide caused a marked decrease in the number of peripheral blood leukocytes. Total leukocyte counts were decreased by about 65% at 42 days (*P* < 0.05, Figure 3A). B lymphocyte counts were significantly decreased by about 80% at 35 days and 65% at 49 days of age (Figure 3B). Counts of other leukocytes in blood (CD4 and CD8 T lymphocytes, phagocytic monocytes and phagocytic heterophils) were also reduced in the group treated with cyclophosphamide (Figure 3C-F). In addition, the cloacal bursa, which is the primary site for B lymphocyte development in birds, was significantly reduced in the chickens treated with cyclophosphamide (Figures 4A-C). In histopathological analysis, the Bursa showed marked lymphocyte depletion (Figure 4D). The effect of treatment also

translated into lower antibody responses to NDV vaccination at 42 days of age (Figure 4E).

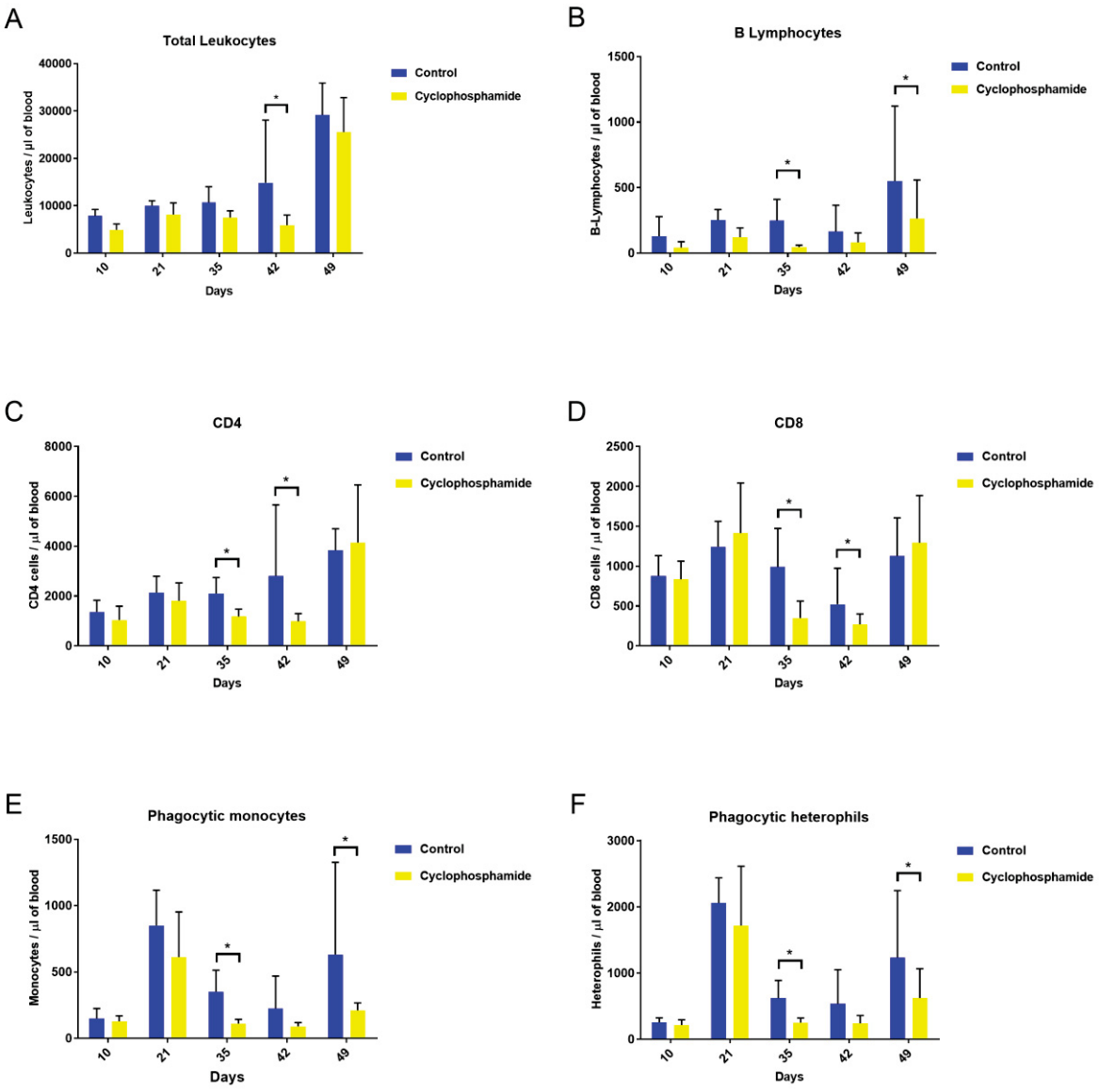

Figure 3 - Peripheral blood leukocytes were reduced by cyclophosphamide treatment. (A) Total leukocyte numbers. (B) B lymphocyte counts. Count of CD4 (C) and CD8 (D) T lymphocytes, phagocytic monocytes (E) and heterophils (F) were determined by flow cytometry. Data are representative of 8 samples/group. Values represent the number of cells per µL of blood (average ±

SD). Asterisks indicate statistical differences between groups at a given time point by Kruskal Wallis test ($P < 0.05$).

To evaluate the effect of administration of cyclophosphamide on the cecal microbiota, the V4 variable region of the 16S rRNA gene was sequenced in an Illumina Miseq® sequencer at 35 days of age of birds, when the effect of cyclophosphamide on immune cells was evident. The cecal content of 4 birds from each treatment was collected, the metagenomic DNA purified and used as template for 16S rRNA gene amplification. The number of reads ranged from 12.671 to 143.650 in the libraries. For further analyses, the reads of each library were scaled to the smallest library size of 12.671 reads. Principal component analysis of the libraries showed a distinct separation according to treatment and that the treatment (PC1) explained 93.5% of the differences between treatments (Figure 5).

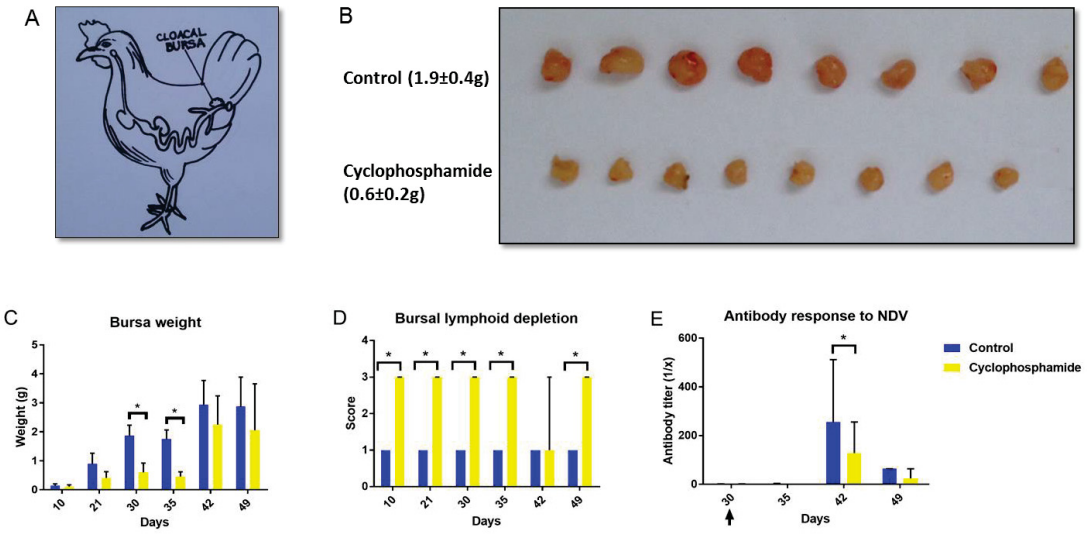

Figure 4 - Cyclophosphamide treatment reduced bursal weight and caused lymphoid depletion. (A) Anatomical localization of the cloacal bursa in birds. (B) Representative images of cloacal bursa at 35 days. Weight and standard deviation are shown, n=8. (C) Bursa weight throughout the experiment. Data are representative of 8 samples/group (average ± SD). (D) Lymphoid depletion scores of the

bursa, assessed by histopathology. Data are representative of 8 samples/group (median ± SD). (E) Antibody response to NDV, assessed by ELISA. The arrow indicates the day of vaccination. Asterisks indicate statistical differences between groups at a given time point by Kruskal Wallis test ($P < 0.05$).

In addition, cyclophosphamide shifted the composition of cecal microbiota. In all samples, 16 bacterial phyla were identified by searching against the SILVA database (release 128). Among these phyla, *Firmicutes* was the most abundant in both treatments, accounting for, on average, 93.4% of all cecal bacterial sequences. At genus level, 256 bacterial genera were identified using the SILVA database (release 128). Five genera (*Firmicutes* phyla) were statistically different in abundance between treatments by Welch's t-test and corrected with Bonferroni test ($P < 0.05$, Figure 6). Among these five bacterial genera, three were more abundant in the cyclophosphamide treatment (*Lactobacillus, Blautia* and *Faecalibacterium*), while two were more abundant in the control treatment (*Enterococcus* and *Weissella*) (Figure 6). Moreover, the administration of cyclophosphamide significantly ($P < 0.05$) increased the richness of the cecal community, indicated by the number of observed OTUs (Figure 7).

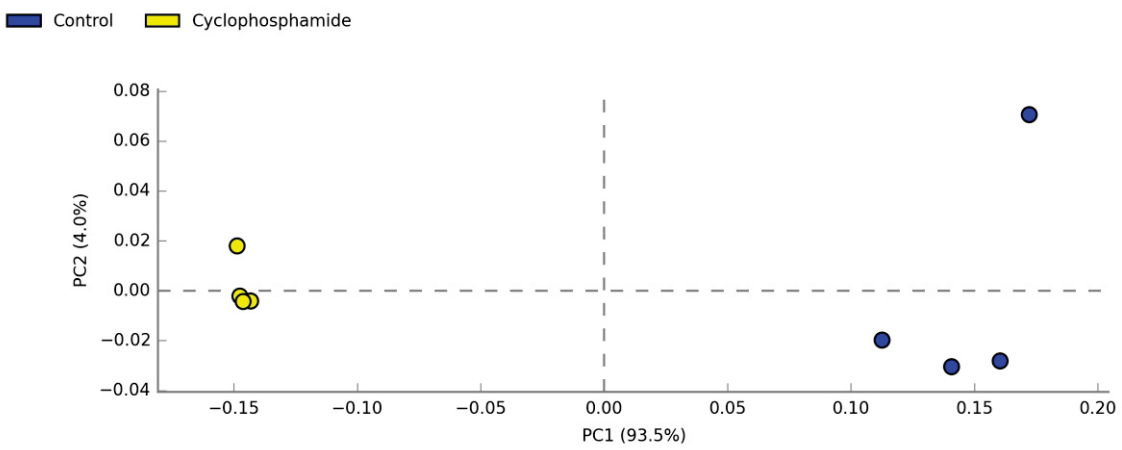

Figure 5 - Cyclophosphamide induced major alterations in microbiota composition. Comparison of cecal microbiota composition between treatments. Principal component analysis (PCA) of microbiota community by Bray-Curtis distance. PC1 is likely to represent the effect of the treatment over microbial populations.

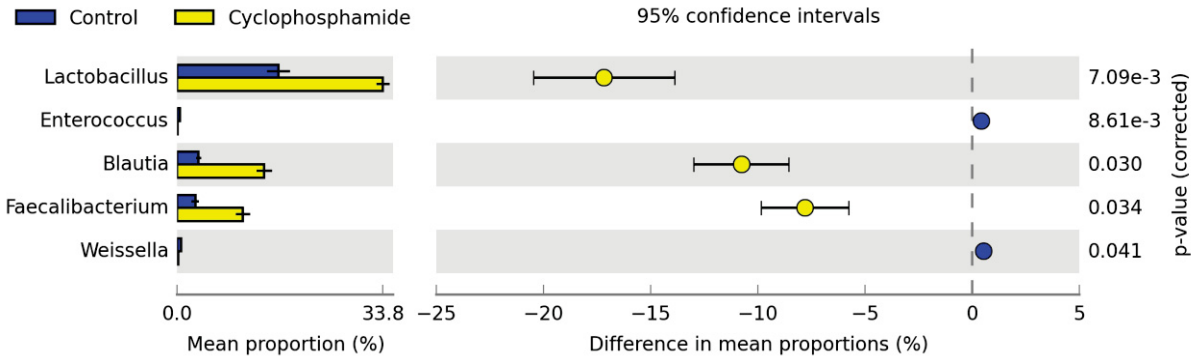

Figure 6 - Abundance of five bacterial genera was altered by cyclophosphamide. Statistical differences between treatments by Welch's t-test ($P < 0.05$) and corrected with Bonferroni test. Data represents mean proportion in % and standard deviation per treatment, n=4.

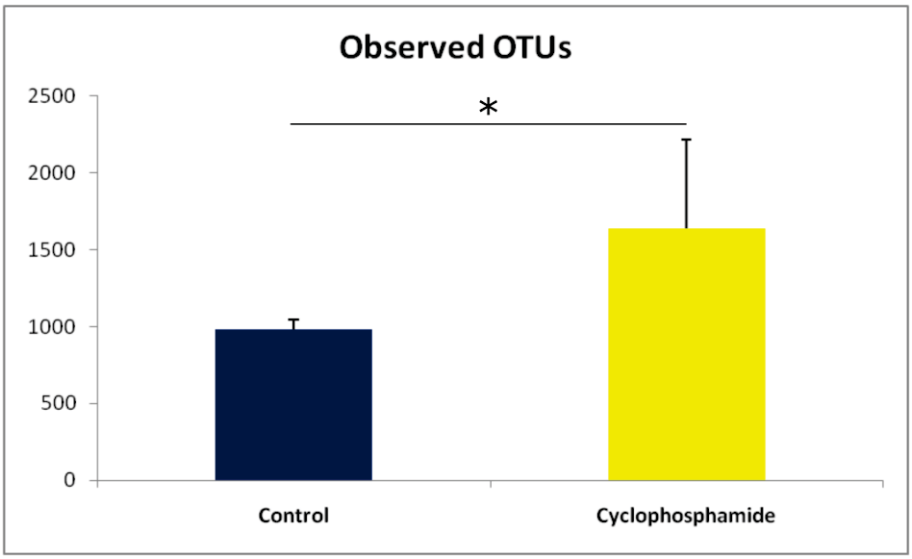

Figure 7 - Cyclophosphamide treatment increased cecal microbiota richness. Asterisk indicates statistical difference in the number of observed OTUs between groups by the Tukey test *(P* < 0.05). Graph represents mean and standard error, n=4.

The Pearson's correlation was also used to evaluate the relationship between immune cells and microbiota. Pearson's correlation revealed significant correlations (*P* < 0.05) between treaments and microbiota. In the control treatment, there was no significant positive or negative relationship between immune cells and microbiota (Figure 8A). Interestingly, in the cyclophosphamide treatment, there was significant negative correlations between B lymphocytes and the abundance of *Lactobacillus* plus *Faecalibacterium*; between CD8 lymphocytes and *Blautia*; and, between monocytes and prevalence of *Lactobacillus, Enterococcus* plus *Faecalibacterium* (Figure 8B).

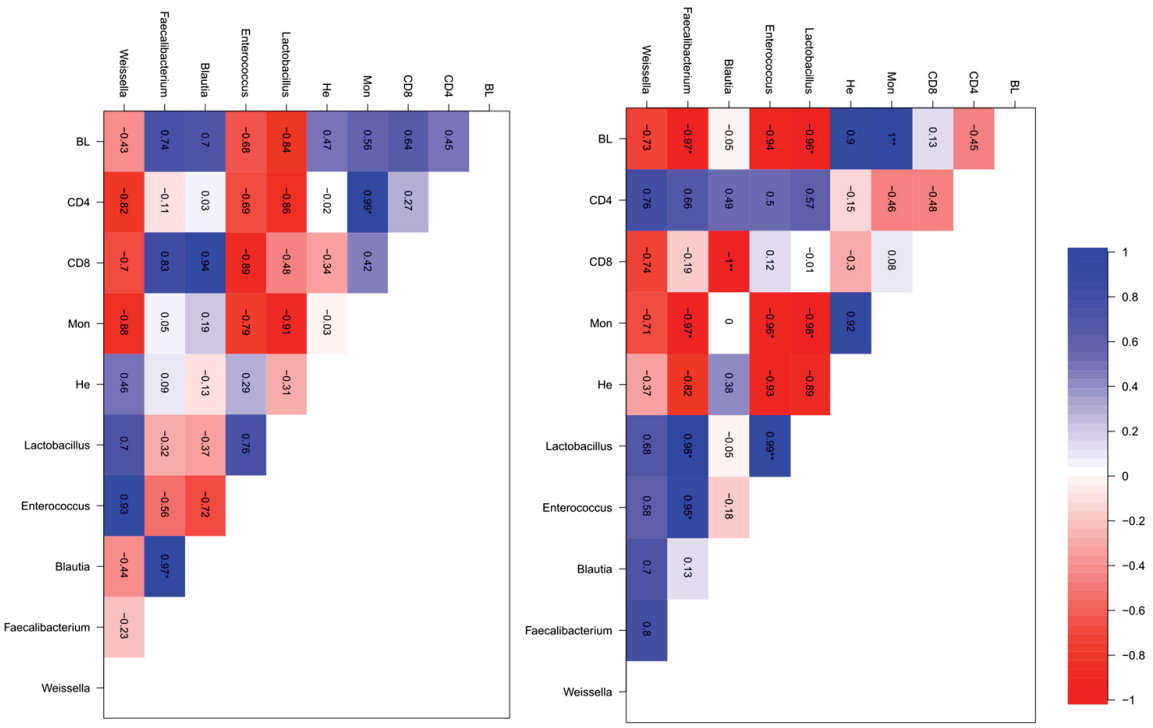

Figure 8 - Several bacterial genera correlated negatively with immune cell counts in the cyclophosphamide group. Correlation matrix showing Pearson's correlation coefficient between cecal bacterial genera and immune cells in the control group (A) or in the cyclophosphamide-treated group (B). Blue indicates a positive and red indicates a negative correlation (see bar on the right of graph). The "R" value is shown for each correlation inside the boxes. The asterisk indicates a significant correlation between variables ($P < 0.05$). BL = B lymphocytes, CD4 and CD8 = T lymphocytes, Mon = Monocytes, He = Heterophils. n=4.

Additionally, to represent the behaviour of the variables in a multivariate system, we used a non-metric multidimensional scaling (NMDS) plot. This analysis represents the pairwise dissimilarity between objects in a two-dimensional space, in this case, the cecal microbiota and the immune cells (plotted as vectors). NMDS results confirmed the clustering of the treatments in two groups with different bacterial genera composition. The first, the control treatment, with higher abundance

of *Enterococcus* and *Weissella* genera, and the second, the cyclophosphamide treatment with higher abundance of *Lactobacillus, Blautia* and *Faecalibacterium* genera (Figure 9). In addition, the vectors representing the immune cells were in the opposite direction to the cyclophosphamide treatment, showing an inverse correlation of these parameters with bacteria present in high quantities in this treatment (Figure 9), as seen in Pearson's correlation (Figure 8B).

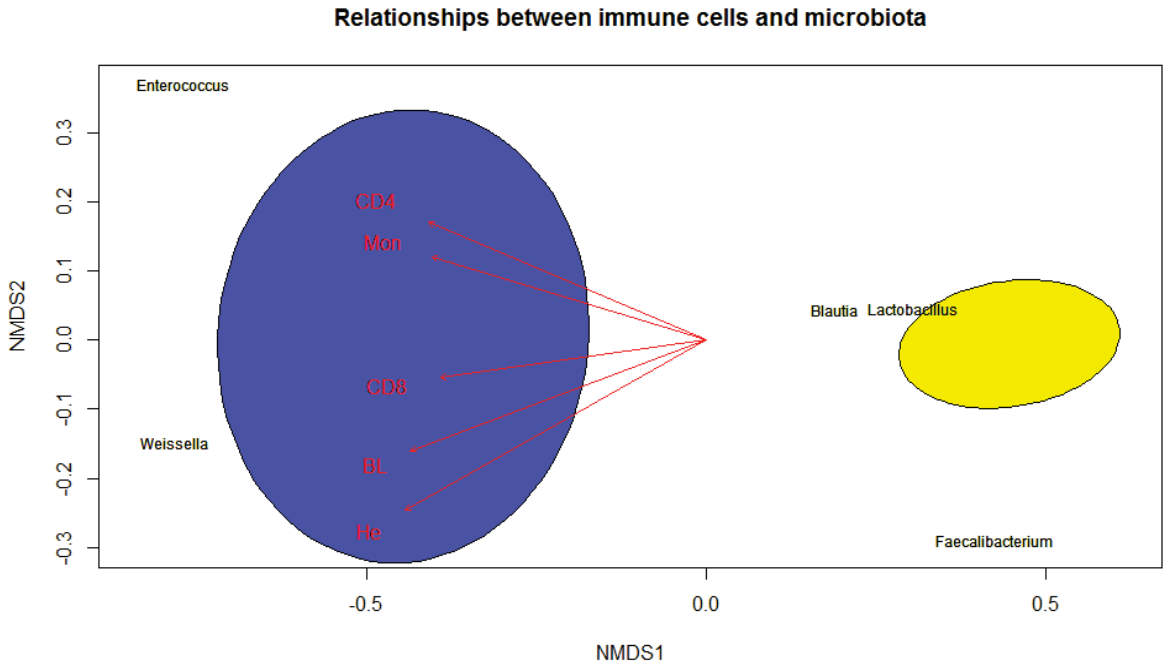

Figure 9 - Non-metric Multidimensional Scaling (NMDS) plot. The ellipses encompass different treatments, the control group in blue and the cyclophosphamide group in yellow. Bacterial genera are show in black and immune cells in red vectors. The direction of the vectors indicates an inverse relationship with bacteria present in high quantities in the cyclophosphamide group.

DISCUSSION

Bacterial genera found in the present study are, overall, in agreement with previous reports of the intestinal microbiota in chickens (WEI et al., 2013; MESA et al., 2017). The cyclophosphamide treatment however led to significant changes in the cecal microbiota which was correlated to the induced immunosuppression. The bacterial genera that were increased in the cecum cyclophosphamide treatment are generally associated with beneficial effects. For example, *Blautia* is an important producer of propionate, a central source of energy for enterocytes of cecum and ileum. *Lactobacillus* produces lactic acid and its beneficial effects for poultry have been widely reported, including higher productivity (SMIRNOV et al., 2005; TOROK et al., 2011). Likewise, *Faecalibacterium* is a major producer of butyrate in the intestine (RUSSELL et al., 2011), another important short-chain fatty acid with beneficial effects to the host.

Changes in the composition of the microbiota can have an indirect effect of immune suppression by cyclophosphamide. In normal conditions, intestinal B lymphocytes are activated and differentiated in Peyer's patches (PASPARAKIS et al., 1997). B lymphocytes migrate to the intestinal lamina propria and secrete IgA, which is transported through the intestinal epithelium into the lumen (MACPHERSON et al., 2008). There, IgA protects the mucosal epithelium, preventing invasion by pathogens (WIJBURG et al., 2006). This antibody isotype also modulates the composition of the intestinal microbiota (LYCKE and BEMARK, 2012) by binding to commensal bacteria in the intestinal lumen and enabling their transport to the lamina propria, where the bacteria interact with phagocytes (MOWAT, 2003). This process of luminal sampling

of commensal bacteria by the immune system induces tolerance to the intestinal microbiota, while also keeping it in check. For instance, mucosal antibodies generated against *Proteobacteria* alter the maturation of the entire microbiota (MIRPURI et al., 2014).

Immunosuppression with cyclophosphamide led to significant decrease of several circulating leukocyte subsets in chicken. The data allow speculating about changes in local immune responses that may have affected the luminal sampling and thus the cecal microbiota. B lymphocytes and CD4 T lymphocytes were diminished in immunosuppressed animals. Accordingly, the decrease in numbers of these cells decreased serum antibody production, as seen in a lower antibody response to NDV. Although not measured here, it is expected that local IgA production was also decreased, which could lead to alteration in the microbiota (MACPHERSON et al., 2000; WEI et al., 2011). In addition, phagocytic cells were also impaired by cyclophosphamide. A reduced number of phagocytic cells and CD4 T lymphocytes would lead to a decrease in the immune sampling in the intestinal lumen, thus, possibly leading to disruption of the microbial balance (Figure 10). Supporting this interpretation, the Pearson's correlation in the cyclophosphamide treatment showed a significant negative correlation between B lymphocytes and *Lactobacillus*. Additionally, cecal biodiversity increased in birds treated with cyclophosphamide, indicating that the reduction in luminal sampling may have allowed the expansion of certain bacterial genera in the intestine.

On the other hand, in mice treated intraperitoneally with cyclophosphamide it was observed significantly increased in the intestinal permeability and enrichment of

Escherichia coli and *Pseudomonas* (YANG et al., 2013), but not of *Lactobacillus.* Moreover, XU and ZHANG (2015), observed that families *Lachnospiraceae*, *Lactobacillaceae* and *Staphylococcaceae* were significantly more abundant in fecal samples of mice treated with cyclophosphamide when compared with control group using massive 16S rRNA gene sequencing. Therefore, our results agree, we observed enriched of *Blautia* and *Lactobacillus* genera. The genus *Blautia* belong of family *Lachnospiraceae* and the genus *Lactobacillus* belong to *Lactobacillaceae* family.

Additionally, increase of intestinal permeability allow bacterial translocate through the gut mucosa resulting in systemic dissemination (VAISHNAVI, 2013). In normal chickens, defense mechanisms such as innate immunity, mechanical mucosal barrier, and colonization resistance prevent the translocation of endogenous bacteria from the intestine (VAN VLIET et al., 2009). Intestinal colonization resistance is defined as the resistance to colonization by ingested bacteria or inhibition of overgrowth of resident bacteria normally present at low levels within the intestinal tract (VAN VLIET et al., 2009). The mechanism is maintained by the beneficial effect of predominantly anaerobic resident microbiota (e.g. *Lactobacillus*) in the intestine, which, by their sheer numbers, provide resistance toward invasion (STECHER and HARDT, 2008).

In this sense, NAKAMOTO et al. (2017) observed inexplicable enrichment of *Lactobacillus* and increase of intestinal permeability in the gut of mice treated with concanavalin A, a potent immunosuppressant at high dose (MARKOWITZ et al., 1969). In addition, the authors observed that *Lactobacillus* activated IL-22 production

by gut lymphoid cells that elicited to decreased intestinal permeability and better mucosal barrier function. Thus, the increase of *Lactobacillus* observed in this work could suggest a possible mechanism of colonization resistance that promote health and counteracts the deleterious effects of cyclophosphamide in the intestine.

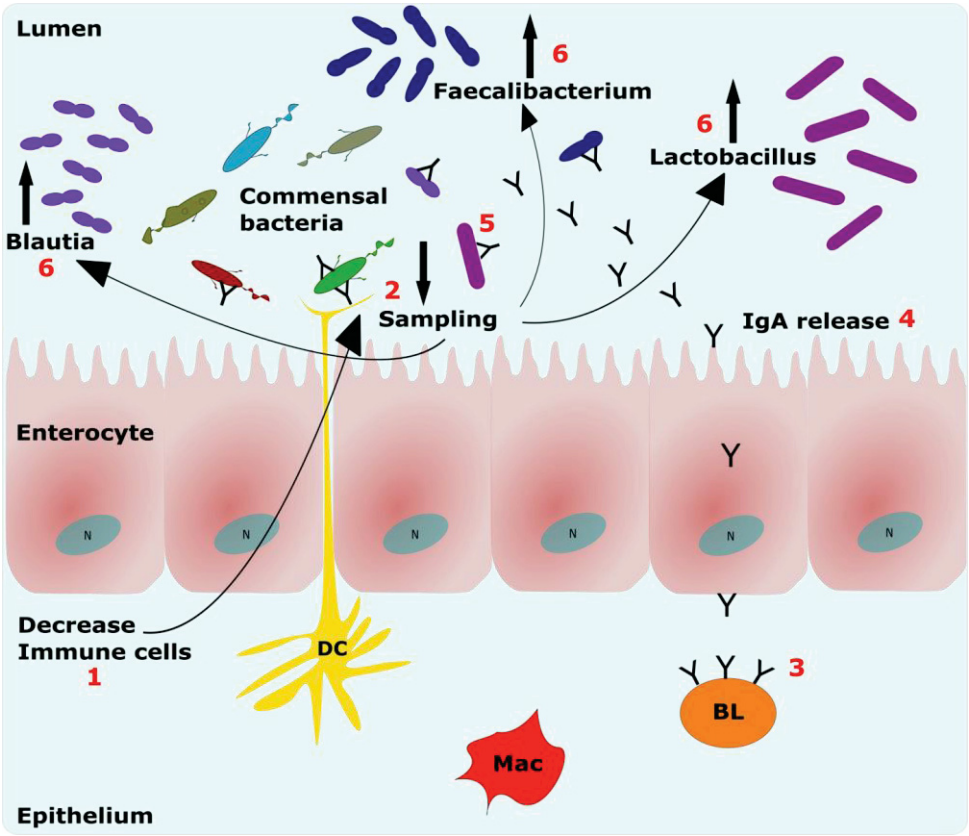

Figure 10 - Proposed mechanism for the changes in the microbiota following cyclophosphamide treatment. 1. decrease in immune cells in the epithelium leads to 2. decrease in sampling of luminal contents by phagocytes. As consequence, 3. there is a decrease in the number of B lymphocytes in the lamina propria and lower antibody production that leads to 4. lower IgA release in the lumen by enterocytes. Thus, there is looser control of commensal bacteria by IgA, leading to 5. In this way, the luminal sampling also is decreased, leading to 6. disruption of intestinal balance, thus, some

predominant groups such as *Lactobacillus* grow due to loss of control by the immune system. BL=B lymphocytes; Mac=Macrophages; DC=Dendritic cell; IgA= Immunoglobulin A.

CONCLUSIONS

Administration of cyclophosphamide affected the overall composition of the cecal microbiota, where it caused an increase in concentration of *Lactobacillus, Blautia* and *Faecalibacterium*. These effects are possibly due to direct or indirect mechanisms. It remains to be determined if the enriched genera can lead to variations of metabolic profiles associated with potential beneficial effects to the host. Finally, it is interesting to observe how a systemically administered drug elicited shifts in the local intestinal microbiota. The detail mechanism that lead to these changes in the microbiota deserves further investigation.

AUTHOR CONTRIBUTIONS

DM and BCBB analysed the data and performed the numerical and statistical analyses. DM and BCBB wrote the manuscript. DM and ES wrote the discussion. DM and LMC processed the sequence raw data. LS, BCBB and LFC designed the protocol. EB performed DNA extraction from samples, amplicons library preparation and sequencing. FP and ES supervised the work.

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CAPÍTULO IV – GENOMIC COMPARISON OF *SALMONELLA* HEIDELBERG ISOLATES SHOW DELETION OF IN THE TYPE III SECRETION SYSTEM GENE CLUSTER AND SUGGESTS CAUSE FOR LOW INVASIVENESS

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RESUMO

A infecção por *Salmonella* é uma das doenças transmitidas por alimentos mais prevalentes no mundo, a maior parte do tempo associada ao consumo de alguns alimentos de origem animal contaminados, como carne de frango e ovos. Assim, o diagnóstico e o seguimento epidemiológico dos surtos são fundamentais para o controle de novos surtos. Durante anos, as investigações epidemiológicas de *Salmonella* dependeram da classificação dos isolados bacterianos pelo sorotipo. Atualmente, as tecnologias de sequenciamento de nova geração permitiram estudos de epidemiologia molecular baseada em sequenciamento do genoma completo. Dessa forma, o objetivo deste estudo foi sequenciar e comparar os genomas de sete estirpes de *Salmonella* Heidelberg isoladas da produção de frango de corte. Os genomas apresentaram deleções gênicas no sistema de secreção tipo III (T3SS) codificado na ilha de patogenicidade 1 em 4/7 das cepas e a presença do sistema de secreção tipo IV codificado em plasmídeo em todas as cepas 7/7. Além disso, a invasão *in vitro* de macrófagos foi reduzida nas estirpes com deleções no T3SS. Este achado é particularmente interessante uma vez que se abre a possibilidade de que nem todas as estirpes de *Salmonella* Heidelberg sejam patógenos.

Palavras-chave: Invasão, macrófagos, patogenicidade.

ABSTRACT

Salmonella infection is one of the most prevalent foodborne diseases in the world, most of the time associated with the consumption of contaminated animal foods, such as chicken meat and eggs. The diagnostic and epidemiologic tracking of *Salmonella* outbreaks are fundamental for the disease control. For years, epidemiological investigations of *Salmonella* have depended on the classification of bacterial isolates by serotype. Currently, Next-Generation Sequencing (NGS) technologies allow epidemiologic research based on whole genome sequencing (WGS) with great advantage and expanding possibilities in this area. The aim for this study was to sequence and compare the genomes of seven strains of *Salmonella* Heidelberg isolated from poultry meat production industry. The genome analyses showed deletions of genes of the type III secretion system (T3SS) in the pathogenicity island 1 in 4/7 of strains and the presence of type IV secretion system encoded in a plasmid in all strains 7/7. In addition, *in vitro* invasion of macrophages cells was significantly reduced in the strains with deletions in the T3SS. The lower virulence of these isolated may explain their resilience in poultry meat supply chain. Moreover, the findings are of particular interest since they open the possibility that not all naturally occurring *Salmonella* Heidelberg are pathogenic.

Key-words: Invasion, macrophages, pathogenicity.

INTRODUCTION

One of the most important concerns in the poultry industry is the control of Salmonellosis. The occurrence of an outbreak can jeopardize the credibility and cause major financial losses, since *Salmonella* contamination is a potential cause of human food poisoning (MÜRMANN et al., 2008).

The control of Salmonellosis is not an easy task and depends on variables such as the strain involved, the host, the environment and geographical features (BARROW and METHNER, 2013). The enormous diversity of *Salmonella* serotypes and their different survival mechanisms are points of difficulty in combating this microorganism (GANTOIS et al., 2006). In addition, *Salmonella* spp. show high resilience in the environment with wide distribution in the ecosystems and, particularly, great adaptability in the poultry production environment (KOTTWITZ et al., 2013).

Between 2013 and 2014 there was a significant increase in notifications of the European Union "Rapid Alert System for Food and Feed (RASFF)" to poultry products from Brazil. The most prevalent strain identified in Brazilian meat chicken in this period was *Salmonella* Heidelberg (RASFF, 2015).

Salmonella enterica subsp. *enterica* serotype Heidelberg is a common food-borne pathogen, that ranks fourth among the *Salmonella* serotypes isolated from human sources between 1970 and 2009 in the United States (CDC, 2012). During this period, *Salmonella* Heidelberg has been identified as the causative agent in more than 100 outbreaks, which were mostly attributed to the consumption of poultry or egg-related food (CHITTICK et al., 2006).

On the other hand, epidemiological investigations of *Salmonella* that infect humans and animals depends on the classification of bacterial isolates by serotype (ECHEITA et al., 2002) which can be insufficient to access their virulence. Nowadays, comparative genomics is a powerful tool to identify factors that might explain the high prevalence and virulence of strains and improve the understanding of infections due to *Salmonella* (LEEKITCHAROENPHON et al., 2014).

The purpose of this work was to compare the genome sequences of *Salmonella* Heidelberg isolates from broilers of south Brazil, identify genetic differences and correlate with *in vitro* capacity of cellular invasion.

MATERIALS AND METHODS

Bacterial strains, DNA extraction, PCR and sequencing

Forty-one strains of *Salmonella* Heidelberg were isolated and kindly supplied by one important poultry meat industry. The strains were representatives from different locations of the meat poultry industry and isolated from 2014 to 2017 in the south of Brazil (Table 2) and serotyped by traditional microbiology. From those, seven strains were randomly selected for whole genome sequencing and genomic comparison.

Table 2 - Salmonella Heidelberg isolates. Table shows the year, the state and the strain isolation source. PR=Paraná state; SC=Santa Catarina state; RS=Rio Grande do Sul state. Strains in red were selected for whole genome sequencing. The column hilA shows the presence or absence of hilA gene by end-point PCR ("+" indicates presence and "–" absence).

Strain	Isolation year	State	Sample	*hilA*
1	2014	PR	Meat	-
2		PR	Shed	-
3		PR	Shed	+
4		PR	Litter	-
5		**SC**	**Ceca**	-
6		SC	Litter	+
7		SC	Feed	+
8		RS	Slaughterhouse	-
9		RS	Feed	-
10	2015	PR	Meat	+
11		PR	Litter	-
12		PR	Feed	-
13		PR	Shed	-
14		PR	Slaughterhouse	+
15		SC	Meat	-
16		SC	Litter	+
17		SC	Feed	-
18		SC	Slaughterhouse	+
19		RS	Meat	-
20		RS	Litter	+
21		RS	Feed	-
22		RS	Slaughterhouse	-
23	2016	PR	Feed	+
24		PR	Slaughterhouse	-
25		PR	Feed	+
26		SC	Meat	-
27		SC	Litter	+
28		SC	Feed	-
29		SC	Slaughterhouse	+
30		RS	Meat	-
31		RS	Litter	+
32		RS	Feed	-
33		RS	Feed	-

34		RS	Slaughterhouse	+
35	2017	**PR**	**Litter**	+
36		**SC**	**Litter**	-
37		**PR**	**Litter**	+
38		**SC**	**Litter**	-
39		**PR**	**Litter**	+
40		PR	Feed	+
41		**PR**	**Slaughterhouse**	-
Total with Deletion:				**23/41**

Genomic DNA (gDNA) was extracted as described (SOUZA et al., 1991), and used as template for a PCR reaction with Y1 and Y3 primers for amplification of the 16S rRNA gene (CRUZ et al., 2001). The amplicons were enzymatically prepared for sequencing with ExoI/SAP and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing in an ABI3500xL sequencer. Sequences generated were assembled with CAP3 (HUANG and MADAN, 1999) and compared with NCBI's database by BLASTn (ALTSCHUL et al., 1990).

hilA amplicon (497 bases) was amplified using the primers Forward 5'CTGCCGCAGTGTTAAGGATA, and Reverse 5'CTGTCGCCTTAATCGCATGT (GUO et al., 2000) in a 25-µl final reaction volume. PCR cycling was: 95°C by 2 min; 30 cycles of 95°C by 30 seg, 55°C by 30 seg and 72°C by 45 seg; followed of 72°C for 10 min. The PCR products were analysed by electrophoresis (1% agarose, 1X TAE buffer), stained with ethidium bromide solution and visualized on an ultraviolet light trans-illuminator.

The gDNA of the seven strains selected (Table 2) were quantified with Qubit, diluted and used for the construction of genomic DNA sequencing libraries with the

Illumina NexteraXT kit, according to the manufacturer's recommendations. The libraries were quantified and quality verified with Bioanalyzer. The libraries were diluted to 500pM and pooled. This pool was quantified by qPCR using the Kapa Biosystems kit, and 17.5pM of pooled libraries were sequenced in the Illumina MiSeq with 500V2 kit in pair-end, generating paired reads of 250 base pairs from each DNA fragment.

Bacterial strains and growth conditions for invasion assay

The invasion assay was performed with the following strains: *Salmonella* Muenchen (S.M) as positive control, *Salmonella* Heidelberg strains (5, 35, 36, 37 and 38) and *Escherichia coli* ATCC 25922 as negative control. These strains were grown in 9 ml of buffered peptone broth at 37ºC, 240 rpm for 16-18h (MCWHORTER et al., 2015).

Cell culture and invasion assay

To obtain a cell monolayer for invasion assay, $5x10^5$ chicken macrophage-like (HD11) cells were seeded onto 35mm dish plates, incubated in Dulbecco's Modified Eagle's medium (DMEM; Invitrogen) and supplemented with 10% foetal calf serum in 5% CO_2 at 37°C for 48h. The culture medium of each plate was removed and fresh medium containing the bacterial strains at a multiplicity of infection (MOI 20:1) was added. After 2h, the cells were gently washed with sterile PBS (20ml) and fresh medium with antibiotics (100μg/ml spectinomycin plus 50μg/ml kanamycin) was

added and incubated for 30min at 37°C. Then, the excess of antibiotics was washed and the macrophages incubated at 4°C for 16h, followed by cell washing with sterile PBS (20ml) and lysis with 1% Triton X-100 for 20min. Cell extracts were homogenized and a 10-fold serial dilutions were prepared in buffered peptone broth (10g/L bacteriological peptone/5g/L NaCl). Finally, 100 µl of the serial dilutions were plated on XLT4 agar, a selective and differential media for *Salmonella* species. The *E. coli* control was plated on Brilliant Green Agar.

Plates were incubated for 24 hours at 37ºC, before colony forming units (CFU) were counted. The capacity of invasion among strains were compared by Tukey´s test ($P < 0.05$).

Genome assembly, annotation and serotyping

On average, 6,795,728 ± 163,643 reads were obtained, giving a 35 ± 3-fold coverage for the seven strains sequenced. The quality of raw reads was checked by FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/). The sequence data were *de novo* assembled using CLC Genomics Workbench program (CLC bio, Aarhus, Denmark), version 7. The assemblies were performed with the parameters: kmer 25, minimum size 0.5 with similarity 0.8. Following genomic assembly (Table 3), the genome sequences were submitted to the RAST server annotation pipeline (AZIZ et al., 2008) to identify putative coding sequences (CDS) and provide an initial automatic annotation. When necessary annotations were manually curated using Artemis (Sanger Institute, Cambridge, UK) and BLAST (ALTSCHUL et al., 1990). The CDS manually analysed were related to pathogenicity, invasion, virulence,

biofilm synthesis, antibiotics and heavy metals resistance. In order to confirm the serotype of the *Salmonella* strains, the reads were deposited in the SeqSero 1.0 server (ZHANG et al., 2015). This server classifies the *Salmonella* strains based on the "Kauffmann-White-Le Minor Scheme", by the identification of antigens O and H related genes. The seven strains were classified as *Salmonella* Heidelberg.

Table 3 - Summary of genomic assembly of the different strains of Salmonella Heidelberg.

Strain	Size (Mb)	Contigs
5	4,94	1
35	5,13	568
36	5,19	733
37	5,13	420
38	5,21	932
39	5,55	2030
41	5,54	1354

Comparative genomic and phylogenetic analysis

The genomes and the T3SS operons were compared against a reference genome using the BLAST Ring Image Generator (BRIG) program (ALIKHAN et al., 2011), that use the Basic Local Alignment Search Tool (BLAST). The reference genome was isolated from turkey meat in USA*, Salmonella* Heidelberg SL476 (NCBI Assembly ASM2070v1). Additionally, for the phylogenetic analyses, the genomes were imported into Mauve (version. 2.0) program (DARLING et al., 2004) to conduct multiple alignment between strains, subsequently, sequences with single nucleotide polymorphism (SNPs) were extract and exported to MUSCLE (EDGAR, 2004) for the alignment of SNPs sequences. Thereafter, the alignment file was export to

phylogenetic program MEGA7 (KUMAR et al., 2016) for the construction of phylogeny. The tree was constructed with Neighbor-joining method (SAITOU and NEI, 1987) and a bootstrap (FELSENSTEIN, 1985) of 1000. *Salmonella* Gallinarum 9R was used as an outgroup strain.

RESULTS

Comparative genomic analysis

Over 50% (23/41) of the isolates did not present the *hilA* gene (Table 2). On average the seven sequenced genomes were 4,859,687 bp and contained 4,797 ± 45 genes. The comparative analyses showed the presence of several mobile elements such as phages and transposons (Figure 11). Moreover it was observed the absence of an operon (~16 genes) for the synthesis of colanic acid in strains 35 and 37, the absence of formate dehydrogenase gene (*fdn*) in the strains 5, 36, 38, 39 and 41, and partial deletion of the pathogenicity island I, which encodes a type III secretion system (T3SS), in the strains 5, 36, 38 and 41.

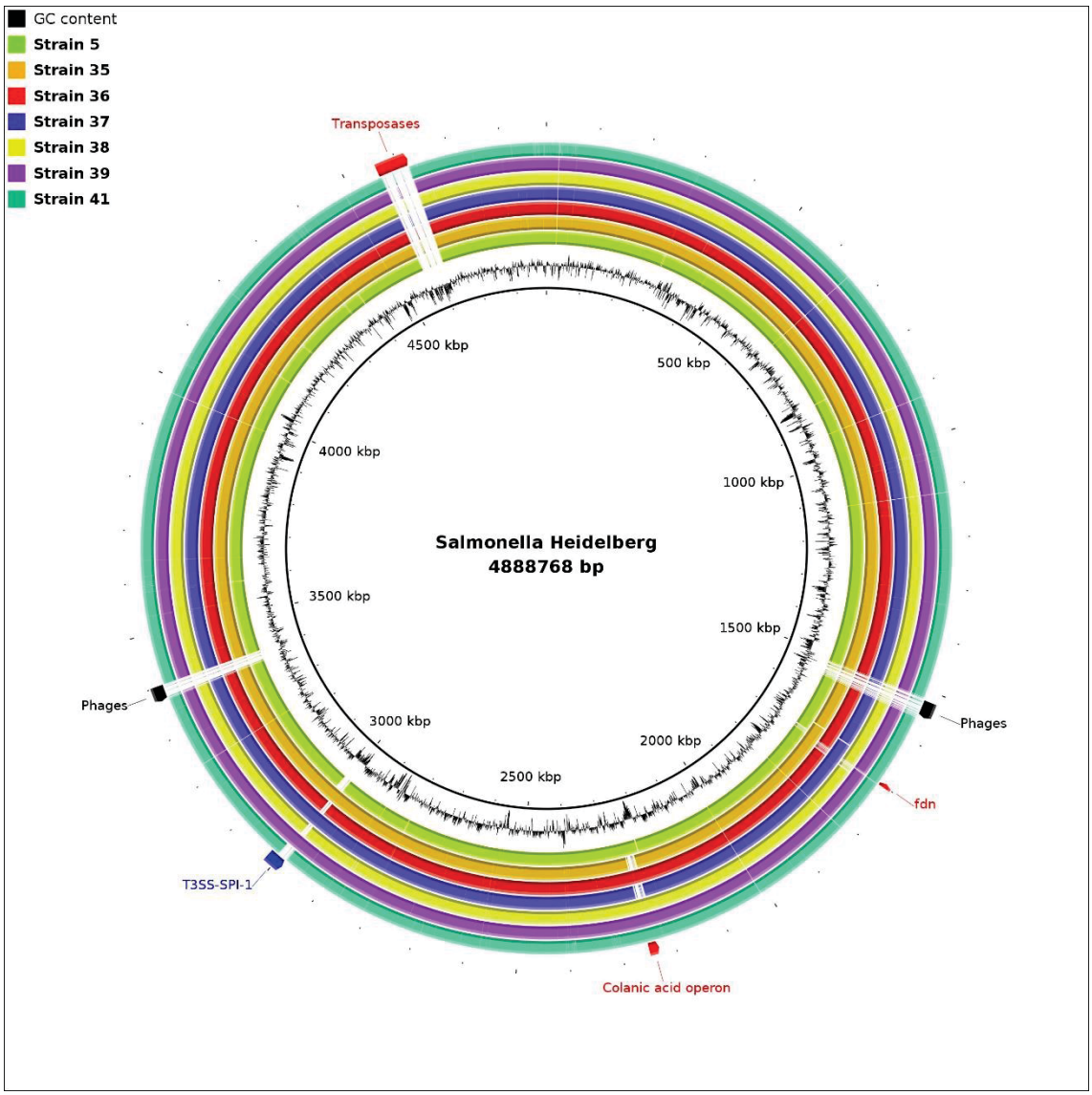

Figure 11 - Genomic comparison shows high similarity between *Salmonella* Heidelberg strains. Each ring represent the genome of one strain. *Salmonella* Heidelberg SL476 (NCBI Assembly ASM2070v1) was used as genome of reference. Gaps in the rings means absence of the region in the target genome. T3SS-SPI-1 = Type III secretion system encoded in the pathogenicity island 1, fdn = formate dehydrogenase.

To identify genes missing in Pathogenicity Island 1 (SPI-1), this genomic region of all isolates were compared (Figure 12). Several genes of the T3SS of the 5

strains were lost, among which genes for crucial effector proteins (*sipA, sipB, sipD*) and the genes essential for the transcription factors such as, *hilA* and *hilD*. Interestingly, in the case of strain 5 the whole SPI-1 was replaced by an *E. coli* transposase.

On other hand, the analyses showed that all strains shared a large collection of genes in the chromosome involved in survival in the environment, such as the colistin resistance operon, biofilm formation operon and the type III secretion system (T3SS) encoded in the pathogenicity island 2, among others. In addition, in all strains possessed genes for type IV secretion system (T4SS), tetracycline resistance and beta-lactamase C resistance located on a plasmid, as well as, an operon (~7 genes) related to mercury resistance (Table 4).

Table 4 - Genes related with resistant to the environment and pathogenicity. In the column are show the strains identifiers, in the row a number of genes observed for this function. Full T3SS = strains with type III secretion system encoded in the pathogenicity island 1 complete.

	5	35	36	37	38	39	41
Function	**Genes**	**Genes**	**Genes**	**Genes**	**Genes**	**Genes**	**Genes**
Alpha-fimbriae operon	4	4	4	4	4	4	4
Attachment invasion locus protein	4	4	4	4	6	4	5
Bacteriocin production	6	6	7	6	8	7	7
Beta-fimbriae operon	5	5	5	5	5	5	5
Beta-lactamase class C	2	2	2	2	2	2	2
Biofilm production	9	9	9	9	9	9	9
Cold shock protein	5	5	5	5	5	5	5
Fimbriae Sfm	7	7	8	8	7	8	8
Fimbriae Stc	3	3	3	3	3	3	3
Fimbriae Stf	9	8	9	10	8	10	10
Fimbriae Yad	8	8	8	9	9	8	8
Flagellar biosynthesis	41	41	41	41	41	41	41

Fosfomycin resistance	1	1	1	1	1	1	1
Heat shock protein	6	6	9	7	6	9	9
Invasin	5	6	5	7	5	5	5
Lipopolysaccharide	17	17	18	17	16	18	18
Macrolide resistance	2	2	2	2	2	2	2
Mercuric resistance	6	6	6	7	7	7	11
Multidrug resistance transporter	38	38	39	36	31	36	36
Negative regulator of flagellin synthesis	2	2	2	2	2	2	2
Phage protein	98	95	85	103	90	89	94
Polymyxin or colistin resistance	10	10	11	11	10	10	10
Streptomycin resistance	2	2	2	2	2	2	2
Tellurite resistance	3	3	3	3	3	3	3
Tetracycline resistance	1	1	1	1	1	1	1
Type 1 pilus	8	8	8	8	9	9	9
Type 4 pilus	10	10	12	12	10	10	11
Type I secretion system	7	7	8	6	8	8	8
Type III secretion system*	47	68	52	68	52	68	49
Type IV secretion system	10	10	8	9	7	9	9
Type VI secretion system	11	11	13	14	18	14	14
Virulence factor	7	7	6	8	6	7	8
Total	390	408	396	430	393	416	409
*Obs:		Full T3SS		Full T3SS		Full T3SS	

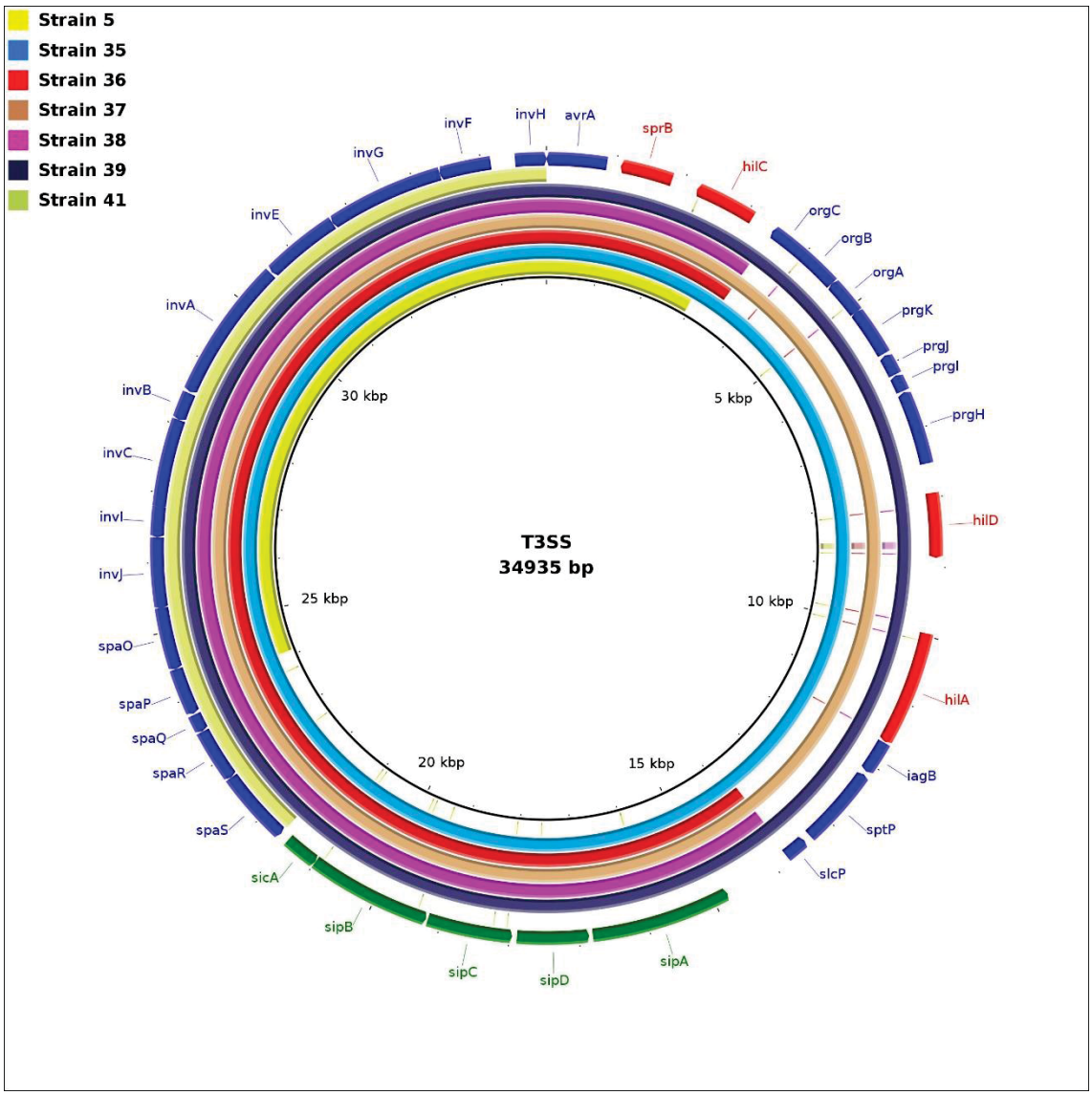

Figure 12 - Genomic comparison of T3SS between SH strains. Each ring represent the T3SS of one strain. For system of reference was used the strain *Salmonella* Heidelberg SL476 (NCBI Assembly ASM2070v1). Gaps in the rings means absence this gene in the system. The external ring show the names of genes. Transcriptional regulator genes in red; effector proteins in green; structural proteins in blue.

Phylogenetic analysis

To infer the phylogenetic relationship between the strains, the concatenated sequences for single nucleotide polymorphism (SNPs) were extracted from the genomes. The phylogenetic analysis shows the clustering of strains 35 and 37 in a clade with a common ancestral that is shared with the strain 39. In addition, strains 36 and 38 also were grouped in a clade and share the same ancestral with the strain 5. On the other hand, the reference strain SL476 and strain 41 were placed more distantly from the other strains in the tree, showing more remote divergence (Figure 13).

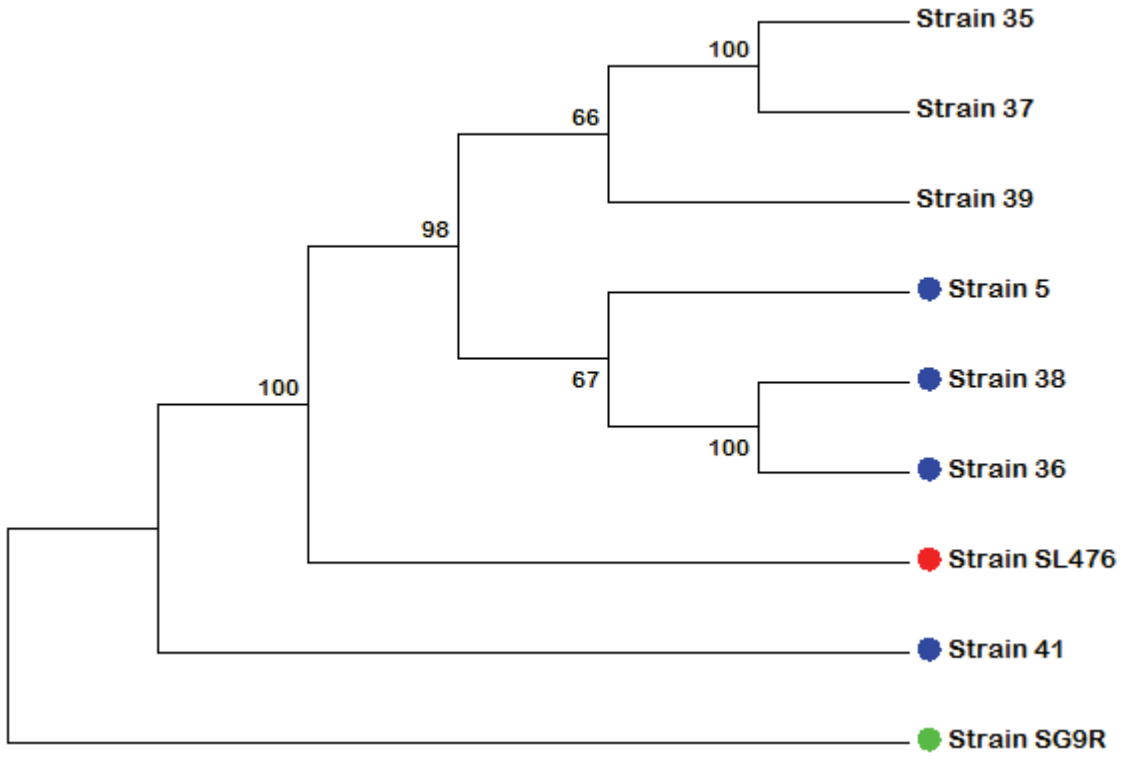

Figure 13 - Evolutionary relations between strains. The evolutionary history was inferred using 9 nucleotide sequences of 43,408 bases (SNPs) and Neighbor-Joining method with a bootstrap of 1000 replicates. Bootstrap values are shown next to the branches. The evolutionary distances were computed using the p-distance method. The reference, *Salmonella* Heidelberg strain SL476, is

marked in red, the outgroup, *Salmonella* Gallinarum 9R in green and the strains with deletions in T3SS-SP1 in blue.

Invasion assay

In vitro invasion assays showed a decreased invasion capacity of the strains with deletions in the T3SS. Strains 5 and 36, with deletions in the T3SS showed significant difference in the invasiveness when compared to the control strain (*Salmonella* Muenchen). Strains 37 and 39 that have a complete T3SS showed decreased invasion, but not significant. The positive control, showed a good capacity of invasion (Figure 14).

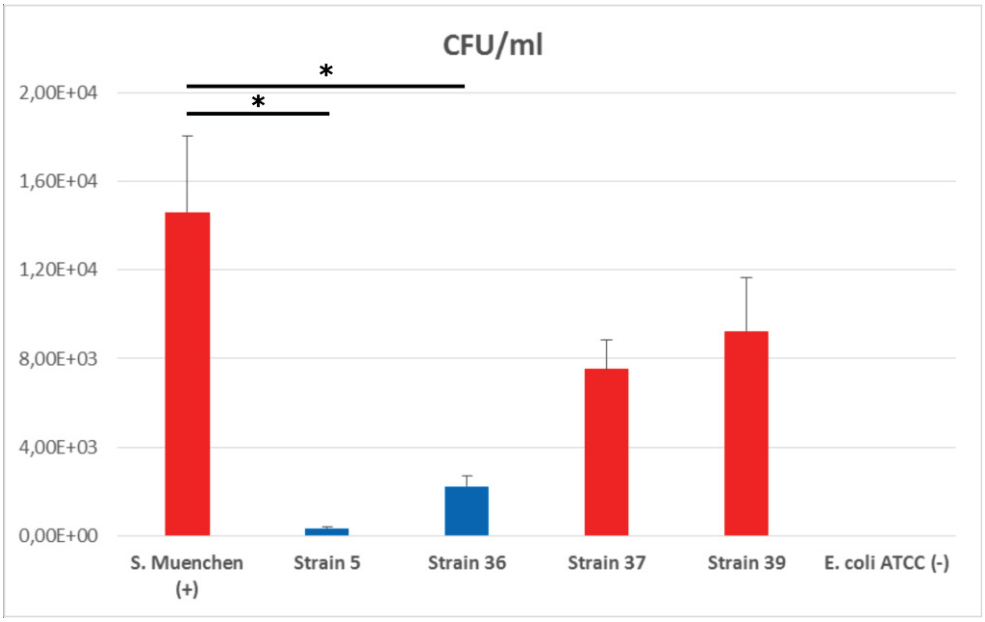

Figure 14 - Deletions of T3SS genes decrease in vitro invasiveness of *Salmonella* Heidelberg strains. The plot shows the invasiveness of different strains of S. Heidelberg in chicken macrophage like cell line (HD11). In blue strains with deletions in T3SS. Invasiveness values are expressed in CFU/ml. Data represent the mean and the standard deviation of two independent experiments. Asterisk means statistical difference between strains by the Tukey test ($P < 0.05$).

DISCUSSION

Genomic comparison of *Salmonella* Heidelberg isolated from the Brazilian poultry meat industry showed a high prevalence of deletions of genes in the T3SS of pathogenicity island 1 (SPI-1). SPIs are elements of horizontal acquisition with the hallmark lower G+C content (MARCUS et al., 2000). In *Salmonella,* these virulence genes are located in *Salmonella* pathogenicity islands (SPIs). T3SS-SPI-1 encode T3SS structural and effector proteins, as well as transcriptional regulators, widely recognized and responsible by the invasion in eukaryotic cells (LARA-TEJERO and GALÁN, 2009; LU et al., 2010); this genomic region is the primary responsible for eukaryotic cells invasion capacity *Salmonella* (KUBORI and GALÁN, 2002).

Several studies have shown that T3SS mutants have lower capacity for cell invasion (GALÁN and CURTISS, 1989; ZHOU et al., 1999). However, this observation is based on mutant strains constructed in the laboratory. In this work, naturally occurring strains lack key genes of SPI-1. In agreement with *in vitro* results all tested strains lacking SPI-1 genes also have a significantly lower invasion capacity.

Previous studies have shown that pathogenicity islands can be excised from the bacterial chromosome, either spontaneously or in response to certain stimuli (HUGO et al., 2013). These excisable pathogenicity islands often possess genes coding for mobility-related proteins, such as integrases and transposases, all of which show similarity to proteins found in phages (WILLIAMS, 2002). Similar results

were observed by QUIROZ et al. (2011), who found an unstable pathogenicity island (not T3SS) in *Salmonella enterica* Serovar Enteritidis.

Loss of SPI-1 in S. Heidelberg isolates may be related to adaption to the environment (NIETO et al., 2016). The loss of virulence genes, associated with acquisition of environmental resistance genes, would allow *Salmonella* circulation in the poultry supply chain without being challenged by the immune system of the host. Thus, low virulence strains can gain access and colonize several locations such as litter in farms, feed factories and slaughterhouses, potentializing their survival. Indeed, clonal expansion of *Salmonella* Heidelberg has been observed in poultry production in southern Brazil (CAMPOS et al., 2017).

Bacteria can accumulate mutations to adapt to their environment. For example, a bovine strain of *Salmonella* Heidelberg B182 deficient in DNA repair mechanism had increased rate of bacterial diversification facilitating niche expansion. The phenotype was related to a 12 bp deletion in the *mutS* gene, leading to increased adherence to epithelial cells (LE BARS et al., 2012). A condition opposite to observed here, but that could offer advantages to survive in the environment.

On the other hand, the strains with T3SS deletions such as 5 and 36 still had invasion capacity although much reduced. This observation suggests that other bacterial systems may be involved in cell invasion. A candidate could be the plasmid-borne T4SS present in all seven strains sequenced. The T4SS is a bacterial conjugation system adapted to deliver DNA, effector proteins and toxins to host cells (CHRISTIE and VOGEL, 2000). Furthermore, this system has been related to significant roles in pathogenesis such as intracellular replication (O'CALLAGHAN et

al., 1999) and virulence (BOSCHIROLI et al., 2002). In addition, *Salmonella* Heidelberg strains containing T4SS-carrying plasmid also had enhanced ability to invade and survive in intestinal epithelial and macrophage cell lines (GOKULAN et al., 2013). Thus, the plasmid-borne T4SS could partially compensate *in vitro* cells invasion capacity in SPI-1 deleted isolates.

Interestingly, among 4 strains defective in T3SS, only 36 and 38 had the same deletion in SPI-1, suggesting that distinct events led to the same phenotype. The phylogenetic tree constructed using SNPs sequences, is agreement with this conclusion, since the deleted strains are not present in a unique branch, since strain 41 bearing a SPI-1 deletion separated from the cluster containing strains with and without deletions.

Moreover, strains 35 and 37 lacked the operon for colanic acid synthesis. Colanic acid is a polymer of glucose, galactose, fucose and glucuronic acid that forms a protective capsule on the bacterial cell surface (STEVENSON et al., 1996). Studies have shown that the synthesis of colanic acid increased biofilm formation in bacteria (HANNA et al., 2003). The presence of this operon could suggest a mechanism of adaptation and survival, especially in environments such as the slaughterhouse where cleaning and disinfection are continuous.

CONCLUSIONS

Salmonella Heidelberg strains carrying natural deletions in SPI-1, which contains genes for a T3SS, were identified by genome sequencing of isolates from

the poultry meat supply chain in southern Brazil. Further, PCR assay of 41 isolates showed loosed of *hilA* in about 56% of strains. *hilA* is a key transcriptional regulator of T3SS operon, thus confirming the loss of a functional SPI-1 is widely spread among *Salmonella* Heidelberg isolates in the poultry industry. Overall, the results suggest that loss of SPI-1 may be advantageous by increasing the resilience in the environment and that this the mutation may have disseminated in the poultry meat industry in the south of Brazil by a process of clonal expansion in the last years. Additionally, we observed that these deletions lead to a decrease in the invasion of macrophages *in vitro,* thus opening the possibility that not all naturally occurring *Salmonella* Heidelberg are pathogenic.

AUTHOR CONTRIBUTIONS

DM, RC and LMC assembled and analysed the genomes. DM and ES wrote the manuscript. FR, SS and SZ performed the invasion assay. ECM and LFC designed the protocol. EB and MTS performed DNA extraction from samples, amplicons library preparation and sequencing. FP and ES supervised the work.

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CONSIDERAÇÕES FINAIS

A microbiota intestinal é uma vasta e diversa comunidade bacteriana na qual existem complexas relações entre seus habitantes e as células intestinais do hospedeiro. Essas relações não se limitam unicamente ao ambiente intestinal. Assim, a microbiota e seus metabólitos produzidos tem um impacto sistémico, modulando o sistema imune, estimulando o sistema nervoso e contribuindo no balanço geral da homeostase. O melhor entendimento desta complexa relação é um campo das ciências em auge devido a suas implicações na saúde do hospedeiro. Apesar do entusiasmo e da importância, ainda estamos longe de entender integralmente o sistema, por um lado porque muitas destas bactérias não são facilmente cultiváveis em laboratório e por outro lado devido às limitações em quanto às plataformas de sequenciamento massivo de nova geração. Talvez, num futuro não muito distante as plataformas permitam sequenciar toda a unidade ribossomal (5S, 16S e 23S) permitindo uma maior acurácia na taxonomia. Enquanto isso, pesquisas nesta área empregando adicionalmente outras ciências ômicas (transcriptômica, proteômica e metabolômica) são necessárias para conhecer mais sobre este ambiente. Pode ser citado, por exemplo, estudos de biodiversidade e sua relação com o sistema imunológico, com a resistência a doenças, com a resistência a patógenos, entre outras várias relações também importantes.

Por outro lado, fica também aberta a possibilidade de estudar os mecanismos moleculares que levam ao enriquecimento de gêneros bacterianos em situações como a imunossupressão e inflamação intestinal. Maior esclarecimento sobre estes

aspectos poderiam nos conduzir a manipular e selecionar grupos de bactérias responsáveis pela produção de moléculas benéficas para o intestino.

Finalmente, é evidente que o sequenciamento genômico é uma ferramenta de grandíssima utilidade que permite a identificação e classificação de cepas bacterianas com alta precisão. No nosso caso particular de cepas de *Salmonella*. Acreditamos que a acessibilidade dos sistemas de produção agropecuária a esse tipo de tecnologias possibilitarão estudos de epidemiologia molecular baseados no genoma, que por sua vez permitirão rastrear patógenos e tomar medidas preventivas.

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