UNIVERSIDADE ESTADUAL PAULISTA - UNESP

CAMPUS DE JABOTICABAL

AVALIAÇÃO DE PROBIÓTICO COMERCIAL COMO ALTERNATIVA AOS ANTIBIÓTICOS PROMOTORES DE CRESCIMENTO, UTILIZANDO UM MODELO DE DESAFIO SANITÁRIO EM FRANGOS DE CORTE

Marllon José Karpeggiane de Oliveira

Zootecnista

2019

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Marllon José Karpeggiane de Oliveira

Orientadora: Profª. Drª. Nilva Kazue Sakomura

Coorientador: Dr. Juliano Cesar de Paula Dorigam

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AUTOR: MARLLON JOSÉ KARPEGGIANE DE OLIVEIRA
ORIENTADORA: NILVA KAZUE SAKOMURA
COORIENTADOR: JULIANO CESAR DE PAULA DORIGAM

Aprovado como parte das exigências para obtenção do Título de Mestre em ZOOTECNIA, pela Comissão Examinadora:

Profa. Dra. NILVA KAZUE SAKOMURA
Departamento de Zootecnia / FCAV / UNESP - Jaboticabal

Dr. NEI ANDRÉ ARRUDA BARBOSA
Nutrição Animal / Evonik - Senior Regional Marketing Manager - São Paulo/SP

Prof. Dr. LUCIANO HAUSCHILD
Departamento de Zootecnia / Faculdade de Ciências Agrárias e Veterinárias - UNESP - Câmpus de Jaboticabal

Jaboticabal, 27 de maio de 2019

DADOS CURRICULARES DO AUTOR

Marllon José Karpeggiane de Oliveira, nascido no dia 19 de março de 1992 em Itabira – MG, Brasil, filho de Wanda Maria da Aparecida Glória Oliveira e Carlos Roberto de Oliveira. Zootecnista pelo Instituto Federal de Educação, Ciência e Tecnologia de Minas Gerais, campus de Bambuí, Minas Gerais – título obtido em abril de 2017. Durante a graduação foi bolsista de monitoria em disciplinas dos cursos técnicos e superiores (2013) e bolsista de iniciação científica (2014-2015 FAPEMIG e 2015-2016, CNPq), sob a orientação do Prof. Dr. Adriano Geraldo. Iniciou o curso de mestrado pelo Programa de Pós-Graduação em Zootecnia em março de 2017 da Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal sob a orientação da Profª. Drª. Nilva Kazue Sakomura, sendo bolsista da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

“Sonhos determinam o que você quer. Ação determina o que você conquista”.

Aldo Novak

Aos meus amados pais Wanda e Carlos por sempre apoiarem minhas decisões e por sempre acreditarem que todos meus sonhos poderiam se tornar realidade.

Dedico

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CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o projeto intitulado "**Avaliação de probióticos comercias como alternativa aos antibióticos promotores de crescimento, utilizando um modelo de desafio sanitário em frangos de corte"**, protocolo nº 010300/17**,** sob a responsabilidade do Prof. Dr. Nilva Kazue Sakomura, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 06 de Julho de 2017.

Vigência do Projeto	21/08/2017 a 02/10/2017
Espécie / Linhagem	Cobb 500
Nº de animais	2.142
Peso / Idade	45g / 1 dia de vida
Sexo	Macho
Origem	Incubatório Pluma Agro Avícola – Descalvado - SP

Jaboticabal, 06 de julho de 2017.

Profª Drª Lizandra Amoroso
Coordenadora – CEUA

Faculdade de Ciências Agrárias e Veterinárias
Via de Acesso Prof. Paulo Donato Castellane, s/n - 14884-900 - Jaboticabal - SP - Brasil
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AVALIAÇÃO DE PROBIÓTICO COMERCIAL COMO ALTERNATIVA AOS ANTIBIÓTICOS PROMOTORES DE CRESCIMENTO, UTILIZANDO UM MODELO DE DESAFIO SANITÁRIO EM FRANGOS DE CORTE

RESUMO – Doenças entéricas se apresentam como um dos maiores problemas da produção avícola. Historicamente, antibióticos promotores de crescimento (APC's) têm sido usados para controlar essa situação. No entanto, as preocupações a respeito de resistência bacteriana a antibióticos têm levado a proibições regionais para reduzir o uso de APC's. Alternativamente, microrganismos administrados diretamente na dieta como por exemplo os probióticos, podem impactar positivamente sobre a microbiota intestinal, prevenindo problemas entéricos que, inevitavelmente culminam em perdas no desempenho das aves. Outro ponto importante a ser considerado quando se fala em desafio sanitário é o consumo de ração dos animais. A utilização de modelos matemáticos que consideram o fator desafio para modelagem do consumo ainda é escasso. Nesse sentido, essa dissertação foi conduzida com dois principais propósitos: 1) investigar os efeitos de *Bacillus amyloliquefaciens* CECT 5940 como probiótico (DFM) sozinho ou em associação com bacitracina metileno disalicilato (BMD) em frangos de corte sobre desafio de patógeno entérico. 2) modelar o consumo relativo de animais sobre condição de desafio sanitário e integrar esse modelo dentro de um modelo mecanicista de simulação, o Broiler Growth Model (BGM). Assim, permitindo a modelagem do desempenho de animais sob condição de desafio sanitário. Para isso, um total de 1.530 frangos de corte machos Cobb500 com um dia de idade foram casualizados em cinco tratamentos, com nove repetições de 34 aves cada. Tratamentos incluíram controle positivo (CP, dieta basal sem aditivos ou desafio); controle negativo (CN, dieta basal sem aditivos e com aves desafiadas); CN + 0,05 g de BMD / kg de ração; CN + 1g de probiótico / kg de ração (10^6 CFU *B. amyloliquefaciens* CECT 5940 / g de ração); e CN + 0,05 g de BMD + 1 g de probiótico / kg de ração. O desafio consistiu em gavagem oral com inóculo de *Eiméria máxima* e *Clostridium perfringens*. Ganho de peso corporal (GP), consumo de ração (CR), e conversão alimentar (CA) foram avaliados nos dias 21, 35 e 42. Conteúdo ileal e cecal foram coletados nos dias 21 e 28 para enumeração de *C. perfringens* utilizando-se a técnica de biologia molecular para amplificar e detectar sequencias de DNA de *C. perfringens* (reação em cadeia da polimerase em tempo real - qPCR). A saúde intestinal foi avaliada por escores. Uniformidade (U), rendimento de carcaça (RC), e rendimento de peito (RP), escore visual de cama e lesões de patas foram avaliados no dia 42. Para o desenvolvimento do modelo que prediz o consumo relativo de ração de animais sob condição de desafio sanitário, os tratamentos controle positivo e controle negativo foram considerados. Para isso, o consumo relativo diário (RFI) durante o período experimental foi calculado diariamente, dividindo-se o consumo atual de ração (AFI) de animais desafiados pela média do AFI de animais não desafiados. A partir desse consumo relativo, duas funções "broken line" (linear e quadrático) foram ajustadas, construindo um único modelo, o modelo segmentado (MS). Esse modelo (MS) foi integrado dentro do software BGM, permitindo a correção do output AFI. Em seguida, o novo AFI corrigido é usado como input para construir uma simulação do desempenho de frangos de corte, proposta para representar uma simulação do desempenho de animais sob condição de desafio. Para avaliar os resultados dessa predição, os outputs gerados na simulação foram comparados com os dados observados do experimento a campo. Como resultados, podemos perceber

que após 14 e 21 dias pós inoculação, aves no grupo desafiado tiveram menor CR e GP comparados ao grupo controle positivo (P < 0.05). Porém, os grupos recebendo probiótico, BMD ou a combinação deles, apresentaram melhor CA, RC, RP e menor incidência de lesão de patas e escore visual de cama, comparados ao controle negativo sem inclusão de aditivos (P < 0,05). Mortalidade não foi afetada pelos tratamentos (P > 0,05). Frangos de corte alimentados com dietas suplementadas com probiótico, BMD ou sua combinação apresentaram menor contagem de *C. perfringens* no conteúdo ileal aos 21 e 28 dias quando comparados ao CN sem aditivos (P < 0.05) e também mantiveram a saúde intestinal por garantir frequência de embalonamento, conteúdo anormal e inchaço de mucosa comparável ao grupo CP (P > 0.05). A partir do modelo segmentado integrado no BGM, pode-se notar que após 42 dias, o AFI acumulado observado (4581,16 g) foi maior que o consumo acumulado simulado em 5%. O peso corporal simulado nas idades 0, 14, 21, 28 e 35 dias foram 50, 502, 956, 1419 e 2126 g sendo menor que o peso corporal observado em 1%, 4%, 8%, 5% e 2% respectivamente. Por outro lado, o peso corporal simulado (2781 g) aos 42 dias foi maior que o peso corporal observado em 5%. O estudo indica que *Bacillus amyloliquefaciens* CECT 5940 é efetivo assim como BMD para fornecer desempenho similar e saúde intestinal em frangos de corte desafiados. Além do mais, os resultados indicam que o SM para predição do consumo relativo de ração de frangos de corte sobe condição de desafio sanitário pode ser integrado no BGM, permitindo construir uma simulação realística, conforme confirmado pelos dados observados do experimento de campo.

Palavras-chave: antibióticos, *Bacillus amyloliquefaciens, Clostridium perfringens*, *Eimeria maxima*

ABSTRACT – Enteric diseases appear one of the biggest problems of poultry production. Historically, antibiotics growth promoter (AGPs) have been used to control this framework. However, concerns about antibiotic resistance bacteria have led regional bans for reducing the use of AGPs. Alternatively, microorganisms directly supplemented in diets such as probiotics, may exhibit a positive impact on intestinal microbiota avoiding enteric problems that inevitably causes loss on broilers performance. Another important point to be considered when talking about sanitary challenge is the feed intake. The use of mathematical models that consider the challenge factor for modelling in this feed intake is still scarce. In this sense, this project was conducted with two main purposes: 1) to investigate the effects of *Bacillus amyloliquefaciens* CECT 5940 as a probiotic (DFM) alone or in combination with bacitracin methylene disalicylate (BMD) in broilers on enteric pathogen challenge. 2) Model the relative feed intake of animals under sanitary challenge condition and to integrate this model into the mechanistic simulation model, the Broiler Growth Model (BGM). Thus, allowing the modelling of performance of animals under sanitary challenge condition. For such, a total of 1,530-day-old male Cobb500 chicks, were randomly assigned to five treatments, with nine replicate pens with 34 birds each. Treatments included positive control (PC, basal diet without additives or challenge); negative control (NC, basal diet without additives and challenged birds); NC + 0.05g/kg BMD; NC + 1g/kg DFM (10^6 CFU *B. amyloliquefaciens* CECT 5940/g of feed); and NC + 0.05g/kg BMD + 1g/kg DFM. The challenge consisted of oral gavage with *Eimeria maxima* and *Clostridium perfringens* inoculum. Body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) were evaluated on days 21, 35 and 42. Ileal and cecal content were collected on days 21 and 28 for *C. perfringens* enumeration using a molecular biology technique to amplify and detect DNA sequences of *C. perfringens* (quantitative polymerase chain reaction - qPCR). The intestinal health was evaluated by scores. Uniformity (UN), carcass yield (CY), breast meat yields (BMY), footpad lesion and litter quality visual scores, were evaluated on day 42. For the development of the model that predicts the relative feed intake of broilers under sanitary challenge condition, the treatments positive control and negative control were taken. For such, the relative feed intake (RFI) during the whole experimental period was daily calculated, dividing the AFI of challenged broilers by the average of AFI of unchallenged broilers. From that RFI, two “broken line” function (linear and quadratic) were fitted, building a single model, the segmented model (SM). This model (SM) was integrated into the BGM, allowing the correction of AFI output. After that, the new corrected AFI is used as input for making a simulation of broilers performance, proposed to represent a performance simulation of broilers under sanitary challenge condition. To evaluate the predictions results, the outputs obtained from the simulation were compared with those observed data of the field experiment. As a result, it is possible to notice that after 14 and 21-days post-inoculation, birds in the challenged groups had significant lower FI and BWG compared to the PC group ($P < 0.05$). However, the groups receiving DFM, BMD or its combination presented better FCR, CY, BMY, UN and lower incidence of footpad lesion and litter quality visual scores, compared to the NC group without feed additives ($P < 0.05$). Mortality was not affected by treatments ($P > 0.05$). Broilers fed diet supplemented with DFM, BMD or its combination presented lower enumeration of *C. perfringens* in ileal content at 21 and 28 days compared to NC group without additives ($P < 0.05$) and also maintained gut health by keeping the frequency of ballooning, abnormal content and swollen mucosa

comparable to the PC group (P > 0.05). From the segmented model integrated at BGM, it is possible to notice that after 42 days, the accumulated AFI - observed (4581.16 g) was higher than AFIn in 5%. The simulated BW at 0, 14, 21, 28, 35 d were 50, 502, 956, 1419 and 2126 g, being lower than observed BW in 1%, 4%, 8%, 5% and 2% respectively. On the other hand, the simulated BW (2781 g) at 42 d was higher than observed BW in 5%, with no mean bias of prediction (p > 0.05). The study indicates that *Bacillus amyloliquefaciens* CECT 5940 is effective as BMD to provide similar performance and gut health in challenged broilers. Furthermore, the outcomes indicate that the SM for predicting the RFI of broilers under pathogen challenge can be integrated into BGM, allowing to make a realistic simulation, as confirmed by observed data of a field trial.

Keywords: *Clostridium perfringens,* direct-fed microbial, *Eimeria maxima*, necrotic enteritis, probiotic

CAPÍTULO 1 – Considerações gerais

1. Introdução

A intensiva exploração da atividade avícola, considerada como uma das mais tecnificadas atividades ligadas a exploração animal de interesse econômico (Clavijo e Flórez, 2018), tem levado ao aparecimento de distúrbios metabólicos de origem multifatorial, o que dificulta a sua compreensão e identificação. Os processos patológicos que envolve esses animais são causados pela rápida taxa de crescimento, que desencadeia falhas nas respostas fisiológicas ou sistémicas, tornando os animais susceptíveis a diversos patógenos, agravados principalmente pela baixa responsividade do sistema imunológico (Jaenisch et al., 2001; Rubio, 2018).

Antibióticos e quimioterápicos em dose subterapêutica são usados como promotores de crescimento (APC), contribuindo com a prevenção de enfermidades das aves principalmente no que se refere a doenças entéricas. Sua utilização é ampla em decorrência de seus benéficos efeitos para os animais, que podem ser fisiológicos, nutricionais e metabólicos (Anderson et al., 1999; Gaskins et al., 2002; Applegate et al., 2010).

Apesar de sermos encorajados a utilizar os antibióticos à campo devido sua contribuição para a produção animal, a sua ação não seletiva sobre a microbiota benéfica do trato gastrointestinal (TGI), bem como a emergência de bactérias resistentes e sua transferência a humanos (White et al. 2002; Marshall e Levy, 2011; M'Sadeq et al., 2015), fez com que a União Europeia em 2006 proibisse a utilização dos promotores de crescimento, conforme publicado pelo Official Journal of the European Union (2003).

Paralelamente à preocupação governamental, existe a pressão dos consumidores que tem se preocupado com a utilização da forma prudente dos antibióticos. Isso fez com que a pesquisa fosse estimulada e direcionada a buscar possíveis substitutos aos APC. Uma solução factível para tal é a utilização de aditivos como os ácidos orgânicos, o extrato de ervas e óleos essenciais e em maior ênfase os prebióticos e probióticos (Santana et al., 2011; Roberts et al., 2015; Naveenkumar et al., 2017).

Sabe-se que a condição de desafio sanitário pode ser parcialmente controlada por meio da utilização de APC ou utilizando a suplementação com aditivos conforme

citado anteriormente. Adicionalmente, conhecer o impacto dos diferentes desafios sobre o consumo de ração é de extrema importância para nutricionistas. Uma das formas de descrever o comportamento da ingestão alimentar durante o curso de doenças é mensurando o consumo de animais doentes e relativizá-los com o consumo de animais saudáveis (Sandberg et al., 2006).

Modelos de simulação são ferramentas úteis que auxiliam nutricionistas em suas tomadas de decisões. Entretanto devido a complexidade que envolve a condição de desafio, tais modelos, que geralmente são de característica mecanicista, não consideram tal fator. A proposta de inclusão de modelos empíricos dentro de modelos mecanicistas já estabelecidos (ex. Broiler Growth Model, BGM) se mostra como uma oportunidade para estabelecimento de um modelo de simulação que permita realizar simulações sob condição de desafio sanitário.

Frente aos grandes problemas enfrentados pela cadeia avícola com a proibição do APC, e da necessidade de integrar fatores sanitários dentro dos modelos mecanicistas de simulação, esse trabalho tem como objetivos principais: verificar se a suplementação dietética com o probiótico a base de *Bacillus amyloliquefaciens* CECT 5490 para animais desafiados com patógeno entérico pode sustentar similar desempenho aos animais suplementados com APC; e, desenvolver um modelo empírico para predizer o consumo relativo de ração de animais sob condição de desafio e integrar esse modelo dentro do modelo mecanicista BGM, criando assim uma nova ferramenta para simulação em condição de desafio sanitário.

2. Revisão de literatura

2.1 Probióticos

2.1.1 Definição

A definição para o termo probiótico não é recente. Possivelmente utilizado pela primeira vez em 1954 por Vergio (1954), teve sua definição melhor estabelecida em 1965, por Lilly e Stillwell (1965), sendo ela: “substância produzida por um microrganismo que é capaz de estimular o crescimento de outro microrganismo”. Diversas outras definições são propostas por diferentes autores (Parker, 1974; Fuller, 1989), que caracterizam o termo da sua maneira baseando-se em diferentes

princípios. Porém, com o decorrer do tempo, o termo vem se evoluindo. A definição usualmente adotada é proposta por Reid et al. (2003) que define probióticos como "microrganismos vivos, que quando administrados em quantidades adequadas, confere benefícios à saúde do hospedeiro".

2.1.2 Mecanismos de ação

Os probióticos possuem diferentes mecanismos de ação. Entre eles o mais discutido é a resistência a colonização, fenômeno amplamente descrito como exclusão competitiva (EC). Nesse sistema, probióticos saturam os sítios de fixação de patógenos na mucosa, tornando-os limitados de tal maneira que, culminam na exclusão de bactérias patogênicas do trato gastrointestinal - TGI (Rubio, 2018). Entre os fatores responsáveis pela EC desencadeados pela adição de um microrganismo probiótico estão: 1) redução do pH intestinal devido ao aumento na produção de ácidos graxos de cadeia curta e média pelos microrganismos probióticos, reduzindo a velocidade de multiplicação e o número de algumas bactérias patogênicas (Kabir, 2009; Schneitz, 2005; Servin, 2004; Blajman et al., 2015), associado a ação lipofílica exercida por esses ácidos sobre a parede bacteriana. Os ácidos graxos causam trocas na permeabilidade de membranas e alteram as atividades de algumas proteínas essenciais para mantença das funções celulares (Saito e Tomioka, 1988); 2) disputa por sítios de adesão ao epitélio do TGI, sem os quais microrganismos patogênicos são carreados para fora do organismo hospedeiro (Collado et al., 2005); 3) competição por nutrientes no TGI, o que pode reduzir a população de microrganismos patogênicos (Schneitz, 2005; Mountzouris et al., 2010); 4) produção de bacteriocinas, também chamadas de pequenos peptídeos antibióticos (PPA), que variam de 30 a 100 sequencias de aminoácidos e possuem ação contra uma ou um grupo de bactérias. Os PPAs possuem o papel de matar ou inibir uma grande variedade de organismos e células, incluindo bactérias gram-positivas e negativas, vírus, protozoários, parasitas e fungos. Portanto, os mecanismos de ação de alguns microrganismos probióticos tem sido atribuído à produção de PPAs (Klaenhammer, 1988; Oscáriz e Pisabarro, 2001; Singh et al., 2015).

Alguns probióticos são capazes de modular as respostas imunes, reduzindo a inflamação induzida por patógenos através de vias de sinalização (Kim e Ho, 2010; M'Sadeq et al., 2015). Essa modulação ocorre por meio de interações entre células

imunes presentes no trato gastrointestinal dos animais e microrganismos probióticos ou seus metabólitos. Através das células dendríticas, os probióticos são apresentados como antígenos e desencadeiam respostas celulares por meio de células T, induzindo a produção e liberação de citocinas como IL-10 e TGF-β, e células B, que se diferenciam em células secretoras de anticorpos (exemplo Ig-A), contribuindo para a defesa da mucosa (Delcenserie et al., 2008; Ng et al., 2009 ;Lemme-Dumit et al., 2018). Pode ocorrer ainda por intermédio de receptores de membrana tipo Toll (TLR) encontrados em células epiteliais que são responsáveis por desencadear a produção de citocinas protetoras que aumentam a regeneração celular, e prevenindo apoptose celular (Ng et al., 2009).

A imunomodulação desencadeada pelos probióticos está ligada basicamente à sua capacidade de aumentar a produção de citocinas anti-inflamatória (principalmente IL-10) e diminuir a produção de citocinas pro-inflamatórias como por exemplo TNF-α e IL-1 (Ng et al., 2008; Waititu et al., 2014; dos Reis et al., 2017; Luan et al., 2018). Cao et al. (2012) demonstraram que a suplementação de um probiótico a base de *Lactobacillus fermentum* cepa 1.2029, reduziu a inflamação causada pela presença de alfa toxina produzida por *Clostridium perfringens*, por meio de modificações nos níveis de expressão de citocinas e TLR.

Os probióticos são capazes ainda de melhorar as funções da barreira intestinal, favorecendo o seu hospedeiro por meio de dois principais mecanismos. O primeiro por intermédio do aumento da expressão gênica de MUC2 – mucina, em células caliciformes (Aliakbarpour et al., 2012; Majidi-Mosleh et al., 2016; Huang et al., 2018; Gadde et al., 2017a,b; Luan et al., 2018). A mucina quando associada a água tem a capacidade de formar um biofilme glicoproteico responsável por lubrificar o epitélio intestinal e conferir proteção à camada mucosa (Oliveira-Sequeira et al., 2008). Dessa maneira, diminuindo a possibilidade de adesão de microrganismos patogênicos. O segundo é atribuído a uma maior expressão gênica de proteínas de junção intracelular como por exemplo JAM2, ZO1, occludin, claudin-2, claudin-3 (Rajput et al., 2013; Song et al., 2014; Gadde et al., 2017a,b; Luan et al., 2018), garantindo maior estabilidade entre células da parede intestinal, amenizando a possibilidade de translocação bacteriana (dos Reis et al., 2017). Dessa maneira, exercendo papel importante na conservação da arquitetura do citoesqueleto celular.

Vale ressaltar que diferentes cepas possuem diferentes mecanismos de ação, não sendo universal para todos os tipos de probióticos. Portanto, podem existir

diferenças nos seus efeitos junto ao hospedeiro, principalmente no que tange a imunomodulação.

2.1.3 Probióticos na avicultura de corte

Os probióticos na alimentação animal vem sendo amplamente utilizada para diferentes espécies como alternativa aos antibióticos promotores de crescimento. Diversos estudos vêm sendo conduzidos na avicultura, e tem apresentado resultados satisfatórios de seus efeitos. Os diferentes tipos de probióticos têm demonstrado potencial para substituir os AGP's, seja em modelo experimental de desafio sanitário (Zhang et al, 2016; Hayashi et al., 2018) ou apenas pela suplementação (Mountzouris et al., 2007; Wu et al., 2018; Manafi et al., 2018), contribuindo na maioria das vezes com melhorias no desempenho animal, principalmente na conversão alimentar.

Além da utilização dos probióticos como potenciais substitutos dos agentes promotores de crescimento, sua inoculação em ovos embrionários vem sendo estudado. Alguns pesquisadores (Pender et al., 2017 e Triplett et al., 2018), relatam que inoculando um probiótico a base de *Lactobacillus* no 18º dia de incubação não afeta a eclodibilidade dos ovos e se tem um efeito positivo quando se avalia o peso corporal dos animais ao sexto dia. Pesquisas com inoculação de probióticos em ovos tem contribuído para elucidar os conceitos de programação inicial de desenvolvimento de frangos de corte bem como tem demonstrado potencial para ajudar a contornar problemas enfrentados pela moderna produção avícola, como por exemplo as desordens metabólicas (Rubio, 2018). Ademais aos efeitos de trabalho com probióticos *in ovo,* o estudo desenvolvido por Nakphaichit et al., (2011) demonstrou que a administração de probiótico na primeira semana de vida tem efeito residual positivo até a sexta semana, aumentando a diversidade e abundância de Lactobacillus e reduzindo significativamente a presença de patógenos quando comparados a um grupo controle.

Entretanto, alguns critérios são considerados para caracterizar um probiótico como potencial aditivo. Antes de tudo, a cepa não pode ser patogênica a espécie hospedeira, e nem aos humanos; deve estar livre de genes de resistência a antibióticos; deve ser resistente a sais biliares e ácidos gástricos (Merrifield et al., 2010; M'Sadeq et al., 2015). É desejável ainda que seja capaz de aderir e/ou crescer dentro do muco intestinal; colonizar a superfície epitelial intestinal; apresentar

propriedades antagónicas em relação a um ou mais agentes patogénicos chave; produzir enzimas digestivas extracelulares relevantes ou produzir vitaminas; modular a resposta imune do hospedeiro e alterar as suas atividades microbianas, tendo ainda seus benefícios comprovados por meio de experimentos *in vitro* e *in vivo* (Patterson e Burkholder, 2003; Clavijo e Flórez, 2018).

Apesar de diversos trabalhos demonstrarem a eficiência e viabilidade da utilização de probióticos na avicultura, alguns resultados da literatura são conflitantes (Majidi-Mosleh et al., 2016; Wu et al., 2018). Fatores como as cepas utilizadas, condições de preparação dos probióticos, idade, via de administração, estado imunológico e linhagem do animal, além dos fatores ligado ao ambiente, podem também influenciar na resposta à suplementação com probióticos (Fuller, 1975; Otutumi et al., 2012; Clavijo e Flórez, 2018).

Nesse contexto fica claro que a somatória dos fatores acima mencionados pode acarretar grandes diferenças na efetividade do produto, podendo levar a resultados controversos.

2.1.4 Classificação e tipos de probióticos

Os probióticos tem sido classificado como aditivos alimentares funcionais. Ou seja, sua função é ir além dos efeitos nutricionais tradicionais. São responsáveis por influenciar as funções corporais afim de melhorar a saúde do animal, estendendo seus efeitos a níveis fisiológicos e psicológicos (Al-Khalaifah et al., 2018). Em resumo, podem contribuir para a melhoria do bem-estar animal principalmente em situações de estresse (Rahimi e Khaksefidi, 2006; Ghareeb et al., 2008; Sohail et al., 2010; Sohail et al., 2011; Ibrahim et al., 2018).

Esse aditivo pode ainda ser classificado em duas categorias distintas. A primeira de espécies colonizadoras, como é o caso do *Lactobacillus* e *Enterococcus spp.* e a segunda como espécies não colonizadoras, de trânsito livre, como é o caso dos *Bacillus spp*. e *Saccharomyces cerevisiae* (Huyghebaert et al., 2011). Existe ainda a classe de fungos que também são considerados probióticos potenciais para uso na avicultura. Como exemplo cito o *Acremonium charticola* que possui forte atividade antioxidante, contribuindo para melhoria do sistema imune (Sugiharto et al., 2015). Tem-se ainda a classe de leveduras, que começa a ser estudada como potencial aditivo probiótico (Al-Seraih et al., 2015).

As bactérias do ácido láctico incluem os lactobacilos (*Lactobacillus lactis, L. acidophilus, L. plantarum, L. brevis, L. fermentum, L. casei, L. bulgaricus, L. rhamnosus, L paracasei, L. jensenii, L reuteri, L. johnsonii, L. helveticus, L. gasseri), Enterococcus faecium* SF68 e *E. faecalis, Streptococcus salivarius e S. thermophilus, Pediococcus acidilactici* e espécies de Leuconostoc e Lactococcus (Barbosa et al., 2011).

As bactérias bifidobactérias incluem *Bifidobacterium bifidum, B. longum, B. breve, B. infantis, B. animalis, B. adolescentes.* Tem-se ainda o grupo das *Escherichia coli* EMO e *Nissle, Bacillus subtilis e Bacillus toyoi.* Já as leveduras incluem *Saccharomyces boulardii e S. cerevisiae* (Barbosa et al., 2011).

Holzapfel (2001) propõem uma classificação diferente para probióticos, conforme descrito a seguir.

Espécies de Lactobacillus	Espécies de Bifidobacterium	Outras bactérias ácidos láticas	Bactérias não láticas
		Enterococcus faecalis	*Bacillus cereus* var. toyoi
L. acidophilus	*B. adolescentis*	*Enterococcus faecium*	*Escherichia coli* strain nissle
L. amylovorus	*B. animalis*	*Lactococcus lactis*	*Propionibacterium freudenreichii*
L. casei	*B. bifidum*	*Leuconstoc mesenteroides*	*Saccharomyces cerevisiae*
L. crispatus	*B. breve*	*Pediococcus acidilactici*	*Saccharomyces boulardii*
L. delbrueckii subsp. Bulgaricus	*B. infantis*	*Sporolactobacillus inulinus*	
L. gallinarum	*B. lactis*	*Streptococcus thermophilus*	
L. gasseri	*B. longum*		
L. johnsonii			
L. paracasei			
L. plantarum			

L. reuteri

L. rhamnousus

Fonte: Adaptado de Holzapfel, (2001).

2.1.5 Cepas probióticas e suas características

Atualmente, existem muitos microrganismos utilizados como probiótico na produção animal, visto que estes possuem características que atendem aos requisitos mínimos dessa classe de aditivos, trazendo benefícios ao hospedeiro (Otutumi et al., 2012). Contudo, podem existir diferenças na eficácia entre as diferentes espécies de microrganismos probióticos, ou mesmo em cepas de mesma espécie (Latorre et al., 2016). Abaixo são descritas as características de algumas cepas comerciais utilizadas como base de probióticos disponíveis para alimentação animal.

A cepa *Bacillus amyloliquefaciens* CECT 5940 é um esporo que quando germinado, é capaz de produzir enzimas protease e amilase, que ajudam na degradação de proteínas e amido. É também capaz de produzir enzimas como celulases e xilanases. Sobretudo, favorecem a produção de ácido láctico por bactérias da flora intestinal, abaixando o pH do intestino ou produzindo substâncias inibitórias (bacteriocinas) que controlam parte da população de bactérias patogênicas (Diaz, 2007; Ahmed et al. 2014).

A cepa *Bacillus subtilis* DSM 17299 que pertence ao grupo dos *Bacillus subtilis*, são capazes de consumir oxigênio do trato gastrointestinal a adicionalmente produzem enzimas como a catalase e a subtilisina, o que pode significar aumento na digestibilidade de nutrientes e melhoria no desempenho animal. São capazes ainda de secretar enzimas como protease, amilase e lipase (Santoso et al., 2001; Deniz et al. 2011; Reis et al., 2017).

A cepa *Bacillus subtilis* PB6 já é conhecida por secretar surfactinas como atividade antimicrobiana. Essas substâncias têm capacidade emulsionante, além de propriedade antibacteriana, antiviral e antitumoral (Vollenbroich et al., 1997; Teo & Tan, 2005)

2.2 Enterite necrótica

A tendência de substituir os antibióticos usados como promotores de crescimento por aditivos alternativos, vem como consequência da legislação que entrou em vigor na Europa no ano de 2006 (Castanon, 2007). Adicionalmente a esse fato, temos a grande pressão do mercado consumidor, que demanda por animais que sejam criados sem a utilização de antibióticos promotores de crescimento. Decorrente das novas exigências impostas neste processo, incidências de doenças de grande impacto econômico na avicultura, tornaram-se mais emergentes. Neste contexto, podemos destacar a enterite necrótica que tem importância expressiva sobre as perdas econômicas, estimadas em mais de $ 6 bilhões por ano. Nesse valor são considerados o prejuízo causado pela redução do consumo bem como os tratamentos veterinários em lotes acometidos pela enfermidade (Wade e Keyburn, 2015).

Os primeiros relatos sobre a enterite necrótica foram descritos por Parish (1961) na Inglaterra. Essa doença acomete frangos entre duas e seis semanas de vida, e está altamente correlacionada com os microrganismos do gênero *Clostridium.* As bactérias desse gênero são anaeróbias, fermentativas, gram-positivos e produtoras de esporos (Dahiya et al., 2006; Robert e Porter Jr., 1998).

Algumas espécies de Clostridium são encontradas na população de bactérias comensais da microbiota intestinal dos animais sem causar problemas quando estão com uma população abaixo de 10^4 UFC/g de digesta (Robert e Porter Jr., 1998; Clavijo e Flórez, 2018; Bhogoju et al., 2018). Acima disso, decorrente dos diversos fatores que levam a proliferação desses microrganismos, a população atinge um número de 10^7até10^9 UFC/g de digesta, resultando no aparecimento clínico da enterite necrótica (Dahiya et al., 2006).

Esses microrganismos são capazes de produzir cinco tipos de toxinogênicos classificados como A, B, C, D, E, e são diferenciados de acordo com a produção de quatro diferentes toxinas principais: Alpha, Beta, Epsilon, Iota (Robert e Porter Jr. 1998; Shojadoost et al., 2012). Porém, segundo Shojadoost et al. (2012), foram recentemente descobertas novas toxinas (Beta2, NetB, TpeL), fazendo-se necessário um novo esquema de classificação.

A enterite necrótica é comumente causada pela toxinogênio A e C (Robert e Porter Jr. 1998; Dahiya et al., 2006) através da toxina de sorotipo alfa que tem propriedade de fosfolipase C (Rehman et al. 2006) e esfingomielinase (Keyburn et al.,

2006). Essa fosfolipase é capaz de penetrar na membrana celular pelo seu domínio C-terminal, e pelo N-terminal desempenhar a função enzimática de hidrólise de fosfolipídios, fazendo com que se separe as frações polar e apolar formando di-acil-glicerol e ácido fosfatídico. Em consequência desse evento temos a lise da membrana celular (Sakurai et al., 2004).

A sua ocorrência está altamente correlacionada com infecções por coccídeos em destaque para a *Eimeria brunetti* e a *Eimeria máxima* sendo agravada pela ocorrência da coccidiose (Cooper e Songer, 2009; Moore, 2016)). Em geral, *Eimeria spp.* tem a capacidade de danificar a mucosa intestinal, levando ao extravasamento do plasma sanguíneo dentro do intestino (Moore, 2016). Em resposta a inflamação causada pela coccidose, há um aumento da produção de muco pelas células caliciformes que estão distribuídas por toda mucosa do trato gastrointestinal (Ohland e MacNaughton, 2010). O muco, associado a todo plasma extravasado, podem ser utilizados como substrato pelo *Clostridium perfringens* (CP), favorecendo a sua proliferação (Silva et al., 2015).

2.2.1 Fatores que predispõem a enterite necrótica

A enterite necrótica não é causada apenas pela presença da cepa patogênica do *Clostridium perfringens* (Flores-Díaz et al. 2016; Moore et al., 2016). Para sua emergência, fatores predisponentes precisam atuar individualmente ou associados, seja para culminar em casos subclínicos quanto em casos clínicos (M'Sadeq et al., 2015; Moore, 2016). A severidade desta enfermidade depende da intensidade com que os fatores ocorrem. Dentre os fatores ligados a nutrição, pode-se destacar a inclusão de cereais com altos níveis de polissacarídeos solúveis não amiláceos (Annett et al., 2002; Timbermont et al., 2011; Barekatain et al., 2013; Latorre et al., 2015) e a inclusão de proteína de origem animal - principalmente da farinha de peixe (Drew et al., 2004; Fernandes da Costa et al., 2013; Stanley et al., 2014b). A associação desses fatores à coinfecção entérica causadas por agentes patogênicos como a *Eimeria* por exemplo, aumentam as chances de ocorrência da enterite necrótica (Wu et al., 2014; Zhou et al., 2017). Outros fatores que muito contribuem são: contaminação ambiental do local de alojamento; condições fisiológicas no que se refere ao pH gástrico e intestinal; estresse, principalmente relacionado a troca de dieta e alta densidade nos alojamentos e a ocorrência de doenças (Allaart, et al. 2013).

A atuação conjunta de todos os fatores mencionados, favorecem a proliferação do *Clostridium perfringens*, que encontra condições ideais à sua multiplicação e posteriormente, após fixarem na mucosa intestinal, induzem à sua necrose (Rehman et al. 2006; Prescott et al. 2016; Józefiak et al. 2016).

A utilização de antibióticos no tratamento contra bactérias patogênicas também deve ser considerada. Esses antibióticos podem ter efeitos antibacterianos não seletivo que se estendem além do patógeno alvo. Dessa maneira, podem desequilibrar a microbiota intestinal, favorecendo a seleção de microrganismos resistentes. Partindo desse ponto de vista, ocorre uma proliferação de microrganismos resistentes a essas drogas, considerados como não desejáveis de tal forma que podem superar a microbiota intestinal normal (Allaart et al. 2013).

2.3 Fisiologia do sistema digestivo e sua associação com a integridade intestinal

O epitélio intestinal compreende umas das principais barreiras contra patógenos, e é parte integral da resposta imune inata no intestino (Carter et al., 2009). Além de ser a primeira linha de defesa contra vários organismos invasores, ela se comunica extensivamente com a microbiota residente (Gadde et al., 2017a).

As aves possuem um sistema digestivo completo com a maior parte do processo de digestão química ocorrendo no intestino delgado, que por sua vez é segmentado em três porções, denominadas duodeno, jejuno e íleo (Sousa et al., 2015; Clavijo e Flórez, 2018). Este é o principal seguimento para a digestão enzimática e absorção de nutrientes.

Estruturalmente, o trato gastrointestinal é recoberto por uma túnica mucosa que influencia diretamente no processo de absorção dos nutrientes (Macari e Furlan, 2005; Fernandes et al. 2017). Para que todo o mecanismo de digestão seja efetivo, é indispensável que esta túnica esteja sempre íntegra e em intensa renovação (Leandro et al., 2017). Em condições normais, a manutenção deste epitélio é responsável por um elevado custo energético, estimado em aproximadamente 20% da energia bruta absorvida pelo animal (Maiorka et al., 2002). Isso apoia a importância de manter sua integridade, já que as perturbações que levam a processos lesivos, desencadeiam um redirecionamento da energia que seria destinada à produção, para a reconstituição do epitélio intestinal (turnover celular). No aspecto econômico, isso acarretaria em um

aumentando expressivo sobre os custos de produção da atividade, logo diminuindo receitas.

Analisando a túnica mucosa intestinal a nível microscópico, é possível notar a presença de pequenas projeções denominadas vilosidades ou vilos. Estas, por sua vez possuem a função de aumentar a superfície de absorção do intestino (Boleli et al., 2002). Esses vilos são compostos por conjunto de células epiteliais, sendo as principais as células caliciformes, os enterócitos, as células de Paneth e as células enteroendócrinas que desempenham diferentes funções no processo absortivo (Kim e Ho, 2010).

Os vilos estão distribuídos por toda extensão do intestino delgado e grosso, com seu tamanho diretamente relacionado com o número de células epiteliais que o compõe (Sousa et al., 2015). Quanto maior o seu número, maior o tamanho das vilosidades e por consequência, maior a capacidade absortiva do seguimento intestinal na presença de nutrientes, decorrente da elevada superfície de contato para absorção (Macari e Maiorka, 2000; Hosseini et al., 2017). Alterações morfofisiológicas podem acontecer quando animais são submetidos a coinfecção com diferentes microrganismos tais como *Eimeria* e *Clostridium perfringens* (Wu et al., 2016) e *Salmonella* (Abudabos et al., 2018).

A produção animal como um todo, está intimamente ligado as questões morfofuncionais do sistema digestório. Qualquer alteração nesse sistema, pode colocar à prova o sucesso da atividade. Apesar de ser uma tarefa difícil é preciso atentar-se para esse ponto. Prejuízos sobre o epitélio intestinal, sem dúvidas, irá desencadear um efeito direto sobre o desempenho animal, com piora nos seus índices zootécnicos.

2.4 Microbiota do trato digestório

O trato gastrointestinal é composto por um grande conjunto de microrganismos: fungos, bactérias e protozoários, que coletivamente são chamados de microbiota (Reis et al. 2016; Sender et al., 2016). Dentre os principais filos que compõem essa microbiota, temos *Firmicutes, Bacteroidetes, Proteobacteria e Actinobactérias*, com predominância dos gêneros *Clostridium, Ruminococcus, Lactobacillus* e *Bacteroides* (Lu et al., 2003b; Wei et al. 2013; Rubio, 2018). Diversos fatores podem influenciar na colonização inicial do TGI, bem como alterar sua composição ao longo da vida do

animal. Como influência inicial vale destacar o manejo pós eclosão, representado pela manipulação humana e de caixas de nascimento e transporte, e tipo de cama ao alojamento (Stanley et al., 2013). Além disso, sexo, idade, localização no trato gastrointestinal, infecção por organismos patogênicos, tipo dieta, uso de antibióticos ou aditivos, ou ainda questões ambientais também interferem na dinâmica de colonização (Santana et al., 2011; Clavijo e Flórez, 2018; Oakley et al., 2018). Desse modo a colonização por cada espécie ocorrerá em função da sua adaptabilidade (Albuquerque et al., 2005; Boleli e Thimotheo, 2017).

A colonização do intestino delgado e ceco ocorrem durante os primeiros 2 a 4 dias após a eclosão, e sua densidade máxima é alcançada até o 5º dia de vida. Porém, com o decorrer do tempo a composição dessa microbiota geralmente se altera. Após a primeira semana de vida, há uma predominância de Lactobacilos no intestino delgado, enquanto o ceco é colonizado principalmente por bactérias anaeróbias como por exemplo a *Escherichia coli*, *Bacteroides* e *Bifidobactérias*, com um menor número de anaeróbios facultativos. A microbiota adulta das aves é estabelecida dentro de duas semanas de idade (van der Wielen et al., 2000; Amit-Romach et al., 2004).

Através de relações comensais e mutualísticas com o hospedeiro, a microbiota tem participação efetiva na fisiologia e nos processos digestivos (Oakley et al., 2014; Stanley et al., 2014a; Gadde et al., 2017a). No trato gastrointestinal degradam compostos dietéticos, gerando nutrientes essenciais que são utilizados pelo seu hospedeiro, tais como amino ácidos, obtidos a partir de fontes de nitrogênio não específicas (Metges, 2000), e vitaminas (LeBlanc et al., 2013) e ácidos graxos de cadeia curta (Lan et al., 2005; Tellez et al., 2006) como resultado do seu metabolismo. Atuam também no metabolismo de sais biliares reduzindo o efeito bactericida destes compostos, decorrente de sua propriedade detergente (Ranjitkar et al., 2016). Essa interferência no metabolismo pode exercer influência sobre a composição da microbiota intestinal, modulando positivamente a composição dietética, aumentando a utilização de energia e nutrientes (Lan et al., 2005; Rubio, 2018). Vale ressaltar também o seu papel no desenvolvimento do sistema imune e proteção primária contra invasores patogênicos, decorrentes da sua fixação na superfície luminal da mucosa (Mulder et al., 2009; Boleli e Thimotheo, 2017; Clavijo e Flórez, 2018).

Além dos fatores mencionados anteriormente, a variação genética influência no estabelecimento da microbiota do trato digestório, já que animais de diferentes linhagens possuem diferentes mecanismos de lidar com patógenos ao eclodirem

(Schokker et al., 2015). Ademais, intervenções dietéticas logo no início de vida dos animais como por exemplo: uso de dietas por fase com diferença na composição química e física, uso de aditivos ou antibióticos afetam a relação entre a microbiota e células da mucosa gastrointestinais. Como consequência pode ocorrer uma mudança do desenvolvimento imunológico (Schokker et al., 2017).

Diante da importância da microbiota para o sistema orgânico animal, manter seu equilíbrio pode ser tratado como uma ferramenta chave para um bom desenvolvimento da atividade avícola. Isso porque, quando em condições ideias, proporcionam performance adequadas as aves. Em condições de desequilíbrio (disbiose) podem favorecer a ação de microrganismos patogênicos (Boleli e Thimotheo, 2017). Dessa forma, qualquer alteração na composição da microflora do animal refletirá em efeitos benéficos ou prejudiciais à saúde do animal (Lu et al., 2003a).

2.5 Aspectos gerais sobre modelagem na produção animal

Um dos principais motivos da utilização de modelos matemáticos na produção animal é a resolução de problemas práticos encontrados à campo através de inferências quantitativas e predições (St-Pierre, 2015). Para isso, a replicação das condições de criação (por exemplo, manejo e nutrição) devem ser bem estabelecidas de tal maneira que permita que os animais sejam criados em condições bem próximas das encontradas em galpões comerciais. Reproduzindo essa realidade e dependendo do objetivo, por meio de pequenos grupos é possível modelar a criação em diferentes situações/cenários. Dessa maneira, essa ferramenta no geral, pode auxiliar produtores e nutricionistas em suas tomadas de decisões. Com isso, grande parte dos avanços nessa área se deve ao interesse da indústria (iniciativa privada), com uma exploração menor por parte de instituições públicas (Oviedo-Rondón, 2015).

2.6 Características dos modelos matemáticos

Os modelos matemáticos em sistemas biológicos podem ser classificados de acordo com suas características. Conforme France e Thornley (1984) sugere, os estes podem ser divididos em três pares: empíricos e mecanicistas, determinísticos ou estocásticos, dinâmicos ou estáticos.

Modelos empíricos são modelos que descrevem relações matemáticas entre duas variáveis: dependente e independente, porém não é capaz de explicar qualquer processo biológico envolvido. Já os modelos mecanicistas também descrevem relações entre essas duas variáveis, contudo considera as vias que representem os processos biológicos envolvidos na resposta (Zoons et al., 1991).

Modelos de estrutura determinística, são modelos que descrevem o animal médio do sistema de produção, entretanto sem considerar suas variações, ou seja, desconsideram as probabilidades de destribuição. Ao contrário, modelos estocásticos que consideram a probabilidade de distribuição dentro do próprio modelo. Em síntese, a estocasticidade considera características incertas dos sistemas biológicos, entendidas como variações. (Zoons et al., 1991; St-Pierre, 2015).

Modelos dinâmicos consideram o tempo como variável, enquanto modelos estáticos representam eventos de um dato tempo, ou para a média de um período (Zoons et al., 1991).

2.7 O modelo matemático ideal

É esperado que um bom modelo de predição de acordo com suas características, seja: mecanicista, estocástico e dinâmico. Entretanto, é evidente que devido à grande complexidade dos sistemas biológicos de crescimento, isso se torna uma tarefa extremamente difícil. Após a idealização de qualquer modelo seja ele qual for, é preciso entender que este ainda sim irá possuir limitações de uso, mesmo apresentando vantagens de aplicação expressiva. Zoons et al. (1991) relata que tal complexidade se dá não apenas as grandes interações que acontecem entre o crescimento e o ambiente, mas também no detalhamento da sequência dos eventos envolvidos nesse processo, que podem ser casuais, ou mutuamente controlados.

Por tanto, na concepção dos estudos desenhados para o desenvolvimento de modelos matemáticos, é preciso ter em mente quais são os problemas do mundo real que possivelmente serão resolvidos com a pesquisa. Em outras palavras, é preciso determinar com propriedade qual a finalidade do estudo bem como quais serão as limitações de acordo com o interesse dos envolvidos. Essa definição é importante para garantir a aplicabilidade do modelo.

2.8 Desenvolvimento de modelos

É certo que no processo de desenvolvimento de modelos matemáticos, quanto maior o número de variáveis envolvidas que possam contribuir com as respostas biológicas, maior será o seu nível de acurácia. A complexidade disso se estende conjuntamente com o número de parâmetros que são utilizados para tal. Vale destacar que nem sempre o acréscimo em número de variáveis contempladas nos modelos, necessariamente culmina no aumento da precisão ou acurácia das respostas. Ou seja, cada variável implementada dentro do modelo deve ser sistematicamente avaliada quanto a sua inclusão. O interessante é começar o desenvolvimento de modelos a partir dos mais simples, e passo a passo estabelecer a inclusão de novas variáveis considerando sua contribuição na sua variável resposta (Rivera-Torres, 2015).

De forma bem resumida e superficial o desenvolvimento de modelos matemáticos se baseia em: i) definição do problema que precisa ser resolvido, ii) condução de experimentos delineados de forma estratégica que sejam capazes de fornecer dados que deem condições de responder o que se definiu como objetivo dentro da sua problemática, iii) avaliação estatística dos dados e criação de modelos ou ajustes de modelos conhecidos, baseando-se em conceitos teóricos previamente envolvidos por trás de cada um, iv) validação através de avaliações de acurácia (exatidão na predição), precisão (variabilidade do conjunto de dados), robustez (adaptação a perturbações) e flexibilidade, por meio de comparações entre outputs e valores observados. Por meio dessas avaliações é possível identificar quais são as limitações do modelo proposto (St-Pierre, 2015).

Um dos mais promissores usos de modelagem no sistema agropecuário está associado a utilização de modelos matemáticos para criação de algoritmos de controle em tempo real dentro da produção (Fisher, 2015). A integração de mensurações físicas de peso corporal, consumo de ração e água, juntamente com variáveis ambientais, contribui para um controle extremo do sistema produtivo. De acordo com os inputs do sistema, todo o manejo envolvido torna-se integrado com o intuito de atender os objetivos da produção, previamente estabelecidos pelo produtor, como por exemplo a maximização do desempenho do lote de acordo com a demanda comercial para aquele dado momento. Dessa maneira, aumentando a eficiência e a lucratividade da atividade (Oviedo-Rondón, 2015).

2.9 Lacunas nos modelos atuais

Alguns modelos mecanicistas de simulação são amplamente utilizados por nutricionistas bem como produtores dentro da atividade avícola. Dois dos principais softwares disponíveis são amplamente utilizados devido sua simplicidade de uso e da excelente acurácia dos resultados de predição. O primeiro é o Broiler Growth Model desenvolvido por Sakomura et al., (2019, em processo de patente), e o segundo, o EFG Broiler Growth Model (EFG software, 2015). Ambos modelos são utilizados para predição de desempenho, composição corporal e exigências de nutrientes para frangos de corte. Entretanto tais softwares levam em consideração para o desenvolvimento dos seus modelos de predição, condições não limitantes de criação (expressão máxima do potencial genético pelos animais). Dessa maneira, não conseguem predizer o desempenho animal em condições variadas, em que a expressão do potencial genético do animal possa ser comprometida, no qual destaco condições de desafio patogênico. Dessa maneira, integrar alguma ferramenta dentro desses modelos de simulação que permita realizar a simulação de desempenho sob condição de desafio é um trabalho importante a ser desenvolvido.

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CAPÍTULO 2 – *Bacillus amyloliquefaciens* CECT 5940 alone or in combination with antibiotic growth promoters improve performance in broilers under enteric pathogen challenge

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Bacillus amyloliquefaciens CECT 5940 alone or in combination with antibiotic growth promoters improve performance in broilers under enteric pathogen challenge

Marllon José Karpeggiane de Oliveira,[*] Nilva Kazue Sakomura,[*,1] Juliano Cesar de Paula Dorigam,[†] Kiran Doranalli,[†] Letícia Soares,[*] Gabriel da Silva Viana,[*]

* Department of Animal Science, Universidade Estadual Paulista "Julio de Mesquita Filho," FCAV/UNESP, Jaboticabal, São Paulo, Brazil 14884-900

[†] Evonik Nutrition & Care GmbH, Hanau, Germany.

[1] Corresponding author – nilva.sakomura@unesp.br

Scientific Section: Immunology, Health, and Disease

ABSTRACT A study was conducted to investigate the effects of *Bacillus amyloliquefaciens* CECT 5940 as a direct-fed microbial (DFM) alone or in association with Bacitracin Methylene Disalicylate (BMD) in broilers under enteric pathogen challenge. A total of 1,530-day-old male Cobb500 chicks, were randomly assigned to five treatments, with nine replicate pens with 34 birds each. Treatments included positive control (PC, basal diet without additives or challenge); negative control (NC, basal diet without additive and challenged birds); NC + 0.05g/kg BMD; NC + 1g/kg DFM (10^6 CFU *B. amyloliquefaciens* CECT 5940/g of feed); and NC + 0.05g/kg BMD + 1g/kg DFM. The challenge consisted of oral gavage with *Eimeria maxima* and *Clostridium perfringens* inoculum. Body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) were evaluated on days 21, 35 and 42. Ileal and cecal content were collected on days 21 and 28 for *C. perfringens* enumeration by qPCR and the intestinal health was evaluated by scores. Uniformity (UN), carcass (CY) and breast meat yields (BMY) were evaluated on day 42. After 14 and 21-days post-inoculation, birds in the challenged groups had significant lower FI and BWG compared to the PC group ($P < 0.05$). However, the groups receiving DFM, BMD or its combination presented better FCR, CY, BMY, UN and lower incidence of footpad lesion and litter quality visual scores, compared to the NC group without feed additives ($P < 0.05$). Mortality was not affected by treatments ($P > 0.05$). Broilers fed DFM, BMD or its combination presented lower *C. perfringens* in ileal content at 21 and 28 days compared to NC group without additives ($P < 0.05$) and also maintained gut health by keeping the frequency of ballooning, abnormal content and swollen mucosa comparable to the PC group ($P > 0.05$). The study indicates that *Bacillus amyloliquefaciens* CECT 5940 is effective as BMD to provide similar performance and gut health in challenged broilers.

Key words: *Clostridium perfringens*, direct-fed microbial, *Eimeria maxima*, necrotic enteritis, probiotic

INTRODUCTION

Clostridium perfringens is a gram-positive bacteria that naturally occur in intestinal microbiota without compromising gut health (Timbermont et al., 2009; Miller et al., 2010; Prescott et al., 2016; Bhogoju et al., 2018). However, the high dietary inclusions of protein sources of animal origin such as fishmeal (Drew et al., 2004; Fernandes da Costa et al., 2013; Stanley et al., 2014) and cereals with high non-starch polysaccharides content (Annett et al., 2002; Timbermont et al., 2011; Barekatain et al., 2013; Latorre et al., 2015) or enteric coccidial infections might result in overgrowth of *C. perfringens* population as well as the increase in bacterial toxin production that may lead to necrotic enteritis (Keyburn et al., 2010; Yan et al., 2013; Zhou et al., 2017). Necrotic enteritis (**NE**) is an avian disease responsible for economic losses of approximately $ 6 billion (US) annually (Wade and Keyburn, 2015). Clinical signs of NE include feed consumption depression, ruffled feathers, severe necrosis of the intestinal tract, diarrhea, and a sudden rise in flock mortality, whilst subclinical challenge with *C. perfringens* is characterized by a less severe depression in voluntary feed intake and damages to the intestinal mucosa (Porter, 1998; Van Immerseel et al., 2009; Cooper et al., 2013; Du et al., 2015).

Under commercial conditions, subtherapeutic doses of antibiotics have been included in feed in order to prevent the overgrowth of potential pathogenic bacteria such as the mentioned *C. Perfringens* and also as a growth promoter (**AGP**). However, the application of subtherapeutic doses of antibiotics for a long period may increase antimicrobial resistance in pathogens of importance for animal and human health and this concern led to regional bans in order to reduce AGPs use in poultry feed (Dibner and Richards, 2005; M'Sadeq et al., 2015; Lekshmi et al., 2017). Such framework inevitably led the industry to look for valid alternatives that support adequate broiler´s health and performance like AGPs.

Probiotic, also known as direct fed microbial (DFM), is a potential replacement for AGPs (Patterson and Burkholder, 2003; M'Sadeq et al., 2015; Clavijo and Flórez, 2018) and can also

help to decrease the dietary supplementation of AGP when supplied together. Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host (Reid et al., 2003). *Bacillus* species have been shown to be viable probiotics, whose benefits for host includes the suppression of pathogen colonization (Wu et al., 2018), improvements on growth performance (Rathnapraba et al., 2018), nutrient digestibility (Sen et al., 2012; Hossain et al., 2015), modulation of immune responses (Paszti-Gere et al., 2012; Gadde et al., 2017), and maintenance of gut integrity (Al-Baadani et al., 2016; Jayaraman et al., 2017). *Bacillus amyloliquefaciens* CECT 5940 is a probiotic used in poultry feeds due to the positive effects on pathogenic bacteria such as *C. perfringens* or *E. coli* (Diaz, 2007; Lei et al., 2015).

Even though several research efforts have been focused on investigating the benefits of *Bacillus amyloliquefaciens* on broiler growth performance, few reports were conducted to achieve such purpose under the condition of an enteric pathogen challenge. Given this background, we hypothesized that dietary supplementation of *Bacillus amyloliquefaciens* CECT 5940 in challenged-broilers could support similar growth rates to diets containing AGP. Therefore, the purpose of the current study was to evaluate the effects of supplementation with *Bacillus amyloliquefaciens* CECT 5940 on performance, carcass traits and gut health of broilers under enteric pathogen challenge.

MATERIALS AND METHODS

Ethical Committee Statement

The experimental protocol used in the current study was previously approved by the Ethics Committee of Animal Care and Use of the Universidade Estadual Paulista "Julio de Mesquita Filho," FCAV/UNESP, Jaboticabal, São Paulo, Brazil (protocol Nº 010300/17).

Bird Husbandry and Experimental Design

A total of 1,530-day-old male Cobb500 chicks with initial body weight of 50.2 ± 0.1 g were used in this study. The study was conducted in a completely randomized design with five treatments and nine pen replicates with 34 birds each. Birds were raised in pens (2.0m x 1.5m) with the floor covered by wood shavings. All pens were equipped with nipple drinkers and tubular feeders, which provided *ad libitum* access to feed and water for the entire 42-d-feeding trial.

Diets and Experimental Treatments

A basal diet, composed mainly of corn and soybean meal, was formulated to meet the nutritional requirements (Rostagno et al., 2017) of broilers in the starter (day 1-13), grower I (day 14-21), grower II (day 22-35) and finisher (day 36-42) phases. From the basal diet (Table 1), five treatments were produced: 1) positive control (**PC**, basal diet without additives or challenge); 2) negative control (**NC**, basal diet without additive and challenged birds); 3) NC + 0.05g/kg Bacitracin Methylene Disalicylate (**BMD**); 4) NC + 1g/kg DFM (1 x 10^6 CFU *B. amyloliquefaciens* CECT 5940/g of feed); and 5) NC + 0.05g/kg BMD + 1g/kg DFM.

Challenge Model

The enteric pathogen challenge model used in this study was designed based on a subclinical *in vivo* model described previously (Onrust et al., 2018) with some minor modifications. The method combines a *C. perfringens* challenge with a coccidial challenge. With the purpose of inducing the onset of a *C. perfringens* infection, a mild *Eimeria maxima* infection was used as a trigger. At 17 days, all birds in the challenged groups were individually inoculated by oral gavage with 1 ml of *Eimeria maxima* inoculum (approximately 3.85 × 10^4 sporulated oocysts) and, afterwards, inoculated with 1 ml of broth fresh culture of *C. perfringens* (approximately 2.5 × 10^6 CFU) per bird on days 18, 19 and 20.

The birds were inoculated with the strain of *Clostridium perfringens* ATCC 13124 (alpha-toxin producer), which was kindly provided by the National Institute of Quality Control in Health (INCQS) of the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil). Briefly, the strain was grown in Brain Heart Infusion broth on agar (BHI; Oxoid) under anaerobic conditions at 37 ˚C for 72 h. The culture of *C. perfringens* was placed on the tubes containing BHI and incubated according to Boarini et al. (2015) and PCR was used to confirm the presence of phospholipase C (*plc* gene; GenBank accession number KY584046). Whole suspended culture was then centrifuged under 5,000 × g for 10 minutes at 4 ˚C, the bacterial pellets were collected and concentred to 10^{-7} using the McFarland® Standards. The bacterial count was made as described by Boarini et al. (2015) and final concentration of *C. perfringens* inoculum was 2.5 × 10^6 CFU/mL. The inoculum of *Eimeria maxima* sporulated oocysts was obtained by a commercial laboratory (Jaboticabal, São Paulo, Brazil) at the concentration of 3.85 × 10^4 oocyst/ml.

Performance and Carcass Trait Measurements

Live body weight and feed intake (**FI**) were recorded at 21, 35, and 42 days for further calculation of body weight gain (**BWG**) and feed conversion ratio (**FCR**). The cause, date and weight of dead birds were recorded daily. Mortality was used to correct FCR. All birds were weighed individually to determine flock uniformity on day 42. Three broilers, whose weight represented the average BW of each pen were selected for carcass evaluation (total carcass yield and breast meat yield). Broilers were euthanized, bled, scalded (60 ˚C/120 s), plucked and eviscerated. After removing neck, head and feet, carcasses were weighed (**CW**) so that the carcass yield could be calculated (CW/live body weight). Subsequently, breast weight (**BW**) was measured to calculate breast meat yield (BW/CW).

Foot Pad Dermatitis and Litter Quality Measurements

Foot pad dermatitis (**FPD**) was scored on the same three euthanized birds used for carcass trait measurements. Chickens were individually scored using the 5-point Welfare Quality recommended scale (Welfare Quality, 2009). The scores ranged from 0 (no evidence of pododermatitis) to 4 (severe pododermatitis). At day 28, litter quality was evaluated. For such, the assessment within the pen was done from the litter floor at each four corners and the values of each treatment represents the mean of 36 observations (4 corners x 9 replicates). Litter samples were visually scored on a scale of 0 to 5 as follows: 0 = Dry friable; 1 = Dry with very fine texture; 2 = Sticky on compression or crumbles; 3 = Clod on compression; 4 = Wet; 5 = Drops of water come out on compression.

Intestinal Sample Collection and Genomic DNA Extraction

On days 21 and 28, two birds were randomly selected from each group, euthanized to collect ileal and cecal contents. The digesta content of ileum (from Meckel's diverticulum to the ileo-cecal junction) and caeca from two broilers per pen were collected aseptically, pooled and stored in a germ-free universal collector at −80 ˚C separately for further analysis.

The homogenate of the ileum and caeca content from each pool (a total of 9 samples per treatment) was weighed and recorded (45 mg ± 10 mg). Bacterial Genomic DNA was extracted directly from the pooled of ileal and cecal content using commercial reagents (NewGene), following manufacture's specifications (Simbios Biotecnologia, Cachoeirinha, RS, Brazil). Briefly, each pool was mixed with 1250 μL of lysis solution (5 M guanidine thiocyanate, 0.1 M Tris-HCl [pH 6.4]), vortexed vigorously and incubated at 60 ˚C (± 5 ˚C) for 10 min. After centrifugation (10,000 × g, 1 min), 500 μL of the supernatant was transferred to a tube containing 20 μL of a silica suspension. After agitation and centrifugation (10,000 × g, 1 min), the pellet was rinsed with 150 μL of solution A (5 M guanidine thiocyanate, 0.1 M Tris-HCl [pH 6.4]), then, with solution B (75% ethanol), and lastly, with solution C (absolute ethanol).

After last rinsed, the silica was dried at 60 ˚C for 10 min and total DNA was eluted in 50 µL of EL buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA).

Real-time PCR Assay (qPCR)

The *plc* gene was targeted using the primers and probe as described by Abildgaard et al. (2010) based on *plc* gene sequences from 60 different strains of *C. perfringens* isolated from broilers as described in Table 2. The TaqMan probe was labelled on the 5′ end with the fluorescent 6-carboxyfluorescein (FAM) and with non-fluorescent quencher dyes at the 3′ end. Bacterial populations were quantified by absolute quantitative real-time PCR. The samples were amplified by commercial NewGene CPRAmp Master Mix (Simbios Biotecnologia, Cachoeirinha, RS, Brazil). The reactions were performed in a total of 30 µL volume, with 2.0 µL of extracted DNA, 1.5 U of Taq DNA Polymerase, and final concentration of 1.5 mM MgCl2, 0.25 µM of each primer and 0.125 µM of the probe. All real-time Taqman PCRs were performed on StepOne Plus (Applied Biosystems), and thermocycler conditions were: one cycle of 3 min initial denaturation at 95 ˚C, and 40 cycles of denaturing at 95 ˚C (15 s) and annealing/extension at 60 ˚C (1 min). Data was collected at all temperature steps and analyzed using the StepOne™ Software v2.0.2 (Applied Biosystems). For quantification, a double-stranded (**ds**) DNA fragment (gBlock® Gene Fragment, Integrated DNA Technologies, Iowa, USA) was designed containing target regions of alpha-toxin-encoding gene (*plc*) amplified by the primers described above and designed based in *C. perfringens* strain ATCC 13124 alpha-toxin (*plc* gene; GenBank accession number KY584046). A 10-fold serial dilution of gBlock® was used to generate a standard curve (**SC**) between log standard concentrations (4.0×10^5 to 4.0×10^1 copies per reaction) and quantification cycle ($\mathbf{C_q}$) values. Samples that showed signals crossing the threshold line in both replica until C_q value of 40, and presented a characteristic sigmoid curve were regarded as positive. To limit of detection (**LOD**) and conversion of PCR cycle threshold values to bacterial cell numbers, a standard curve was constructed using a DNA

extracted from the pure culture of *C. perfringens* (ATCC 13124). The DNA was diluted using the serial dilution method from 2.41×10^7 cell/ml until 2.41×10^0 and subjected to the real-time PCR procedure. A plot of C_q versus log cell numbers was created and PCR efficiency was calculated from the slope of this graph using the equation $E = 10^{(-1/slope)} - 1$ (Rasmussen, 2001). For all amplification assay this graph of calibration curve, the y intercept and r^2 were determined.

Intestinal Evaluation

On days 21 and 28, two broilers per pen were randomly selected (18 birds/treatment) and euthanized for intestinal health analysis. The intestinal condition was analysed according to Teirlynck et al. (2011) where it is attributed values of 1 (presence) or 0 (absence) for each one of the seven parameters evaluated: 1) ballooning in the gut; 2) gut mucosa sloughed; 3) significant swollen or redness of the serosal or mucosal or both surface of the gut; 4) thin or fragile intestinal wall; 5) abnormal appearance of the contents in the lumen (excessive slime, water, gas, greasy aspect or mixture of these); 6) presence of undigested food particles, and 7) muscle tone.

The evaluations were represented as 'frequency of occurrence' (**% F_i**), defined as the percentage in which each specific variable occurred on all observations. It was calculated as follows: % F_i = (N_i / N) x 100, where: **N_i** = number of positive observations containing variable i and, **N** = the total number of observations.

Statistical Analysis

Data were analyzed as a one-way ANOVA using the GLM procedure of SAS software 9.4 (SAS Inst. Inc., Cary, NC). Each pen was considered as experimental units. Means were compared using Student-Newman-Keuls multiple range test where appropriate. The evaluation of the intestinal health was analyzed by the non-parametric Kruskal-Wallis test, followed by

Dunn-Bonferroni, as the data were not normally distributed. All statements of significance are based on the 0.05 level of probability.

RESULTS

Performance, Carcass Traits and Uniformity

Broiler performance responses are detailed in Table 3. There were no significant differences for BWG and FCR between treatments on day 21 ($P > 0.05$). However, FI was significant lower in the PC and DFM groups compared to NC group without feed additives ($P < 0.05$), but not significantly different from the groups receiving BMD or its combination with DFM ($P > 0.05$). On days 35 and 42, BWG and FI were significantly lower in the challenged groups receiving additives or not comparable to the PC group ($P < 0.05$). On the other hand, FCR was improved in the groups receiving DFM, BMD or its combination compared to the NC group without additives, but still significantly higher than the PC group ($P < 0.05$). As presented in Table 4, broilers fed DFM, BMD or its combination had better uniformity, carcass and breast meat yields compared to the NC group without feed additives ($P < 0.05$) and values similar to the PC group ($P > 0.05$).

Foot Pad Dermatitis and Litter Quality

The effects of experimental treatments on FPD and litter quality scoring are detailed in Table 5. Compared with all the experimental treatments, the incidence of FPD was higher ($P < 0.05$) in challenged broilers of NC group. Irrespective of whether supplemented with DFM or BMD alone or both in association, diets containing the feed additives assessed herein supported similar performance to that exhibited by unchallenged group. At day 28, litter quality scores were lower ($P < 0.05$) in challenged groups receiving DFM or its combination with BMD

compared to the challenged group without feed additives or the challenged group receiving only BMD.

Clostridium perfringens Enumeration by qPCR

In Table 6 the results for *C. perfringens* enumeration are presented. *Clostridium perfringens* measured in ileal content at days 21 and 28 (1 and 8 days post-infection, respectively) were significantly lower in the groups receiving DFM, BMD, or its combination as compared to the NC group without feed additives ($P < 0.05$) and the values were similar to those of the PC group ($P > 0.05$). There were no differences for *C. perfringens* enumeration in the cecal content at days 21 ($P = 0.060$) or 28 ($P = 0.076$), but only tendency to be lower in the treatment groups compared to NC group.

Gut Health

The parameters evaluated at 21 and 28 days for intestinal health and their frequency of occurrence are presented in Table 7. The challenge significantly increased the abnormal content in the challenged group compared to the PC group ($P < 0.05$). In addition, the other gut health traits assessed (thin or fragile intestinal walls, ballooning in the gut, sloughed mucosa and swollen or red serosal and mucosal surface) were not significantly different between treatments ($P > 0.05$). Both lack in muscular tone or presence of undigested feed in the colo-rectum segment were not observed in the birds during the evaluation.

The frequencies of thin intestinal wall and sloughed mucosa were not significantly different between dietary treatments ($P > 0.05$) at 28 days of age. The frequencies observed for abnormal content and swollen gut mucosa in the NC group without feed additives were higher compared to the groups receiving DFM, BMD or its combination ($P < 0.05$), but was not different from the PC group ($P > 0.05$). On the other hand, the observed frequencies for

ballooning in the challenged group receiving only BMD was not significantly different from the PC group without feed additives ($P > 0.05$).

DISCUSSION

The present study evaluated the performance of broilers fed DFM, BMD and its combination under an enteric pathogen challenge. Although we've used the challenge model with *Eimeira* and *C. perfringens*, our evaluation was aimed at understanding the framework of disturbances in the microbiota that occur in a model close to the subclinical condition of necrotic enteritis. The whole effects addressed below which took into account challenged broilers, must be understood as the sum of effects caused by the inoculation of both microorganisms, since the control groups used for comparation do not include challenged broilers with neither *Eimeria* nor *Clostridium* separately. The treatment control groups used here are widely described in the literature in trial conducted aiming induction of necrotic enteritis (Qing et al., 2017; Wu et al., 2018; Hofacre et al., 2019).

The results present in Table 3 supports the hypothesis that *B. amyloliquefaciens CECT* 5940 can provide similar performance in broilers fed diets containing AGP, here represented by BMD. Up to day 21 there was no significant effect of the challenge models used, but on days 35 and 42, the drop in the performance was evident compared to the PC group. The poor weight and feed efficiency usually occur from 4 to 9 days post-inoculation with *Eimeria*.

Although BWG and FI were not significantly improved with the supplementation of DFM, BMD or its combination on days 35 and 42 compared to NC group without feed additives, there was an improvement in FCR. Jayaraman et al. (2013) inducing broilers to necrotic enteritis disease and providing supplemented diets with DFM based on Bacillus spp., did not observed differences in BWG at 35 days, but noticed a better FCR. However, broilers fed diets supplemented with feed additives did not had similar performance as observed in the PC group. The same outcomes were observed by Wang et al. (2017) in which broilers of the

NC group had the same FCR as observed in broilers challenged and fed diet with probiotic, both being worse when compared to those broilers of PC group.

The reason behind this is that the applied challenge model might have been strong enough to partially prevent the total recovery of the birds in such time, mainly characterized by the big drop in feed intake. Generally, a compensatory growth can be observed after the challenge period (Arczewska-Włosek and Świątkiewicz, 2013). However, no improvement in BWG at 35 or 42 d among challenged broilers was observed. Thus, it was not substantial in the study proposed herein.

It is interesting to observe that the better FCR indicated that the birds were more efficient to use the nutrients for muscle deposition and this might have resulted in the significant better uniformity, carcass and breast meat yields observed in the groups receiving DFM, BMD or its combination and these results were comparable to the PC group (Table 4). It is also reasonable to consider that different enzymes compounds are produced by *Bacillus amyloliquefaciens* CECT 5940 such as cellulase, protease, amylase and xylanase (Diaz, 2007). Thus, the inclusion of *B. amyloliquefaciens* may have a positive effect on nutrient digestibility and absorption, due to the presence of exogenous enzymes in the intestinal lumen (Farhat-Khemakhem et al., 2017).

The results presented in Table 5 indicate that supplementation with *Bacillus amyloliquefaciens* CECT 5940 alone or in combination with BMD provided lower wet litter scores as observed in the PC group. Consequently, the supplementation of *Bacillus amyloliquefaciens* CECT 5940 also reduced the footpad dermatitis scores. Our outcomes are in line with Whelan et al. (2018) which reports that broilers fed diet supplemented with *Bacillus subtilis* or AGP had lower mean score of footpad dermatitis in comparison to broilers of negative control group. Although there was a difference in strains used in our trial compared with the author aforementioned, we believe that the similarity on results are due the mechanism of action most commonly shared by the most of *Bacillus spp.* which refers to the competitive

exclusion. Through this mechanism, the population of *C. perfringens* have diminished in the ileum of broilers in both trials helping to keep gut integrity. This might have contributed for better nutrient utilization and is frequently related to better litter quality (Lei et al., 2015). Furthermore, poor quality of litter can increase incidence of FPD, which in turn are observed in necrotic enteritis (Timbermont et al., 2011).

The supplementation of the diets with DFM, BMD or its combination was also effective in reducing *C. perfringens* counts observed in the ileum of the birds on days 21 and 28 (Table 6). A trend ($P < 0.10$) for reduction in *C. perfringens* was also observed in cecum on days 21 and 28; however, the high diversity of the cecal microbiota may contribute for a more stable environment (Bjerrum et al., 2006; Pourabedin and Zhao, 2015; Blajman et al., 2017; De Cesare et al., 2017). The mechanisms of inhibition of antibiotics are well known, but *Bacillus amyloliquefaciens* CECT 5940 have the different mode of action to inhibit the action of *C. perfringens* by modulating quorum sensing system, i.e. by interrupting communication between these pathogenic bacteria (Ortiz et al., 2016). Other mechanisms may involve the production of secondary metabolites such as lactic acid (Diaz, 2007) and bacteriocins (Mantovani et al., 2011).

The effect of the challenge on performance was not evident until 21 days, but the necropsy done in this age indicated an increase in the presence of abnormal content compared to the PC group (Table 7). According to Teirlynck et al. (2011) abnormal content is characterized by excess of mucus with presence of some blood and gases which indicates an initial appearance of dysbiosis. After 7 days, the results observed on day 28 showed that the supplementation of *Bacillus amyloliquefaciens* CECT 5940 in combination or not with BMD significantly decreased the frequency of abnormal content and the swollen mucosa compared to the NC group without additives. Either swelling or redness are common and classical signs of inflammation process due to toxins produced by *C. perfringens* (Guo et al., 2015). The

supplementation of DFM alone or combined with BMD, but not DFM alone, significantly reduced the frequency of ballooning compared to the NC group without additives. Ballooning is a classical consequence of dysbiosis, being characterized by the visible enlargement in gut diameter and the presence of liquid, slimy or gases (Pattison, 2002; De Gussem, 2007). Previous findings also found that broilers challenged with *Eimeria* species and *Clostridium perfringens* exhibited an increase in ballooning in the gut (Jayaraman et al., 2013). Therefore, the reduction in frequencies of abnormal content, ballooning and inflammation could be related to the decrease in the population of *C. perfringens* with the supplementation of *B. amyloliquefaciens* CECT 5940 as observed in Table 6.

In conclusion, dietary supplementation of *B. amyloliquefaciens* CECT 5940 can totally or partially replace AGPs in the diets of broiler chickens due to its beneficial effects in improving FCR, reducing CP in ileum, improved uniformity, carcass and breast meat yield during an enteric pathogen challenge. Therefore, these improvements on the performance may be attributable to better intestinal health and litter quality.

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Table 1 - Composition (%) of the basal experimental diet.

Ingredients (%)	Starter Day 1–13	Grower I Day 14–21	Grower II Day 22–35	Finisher Day 36–42
Corn	55.53	62.71	66.10	65.84
Soybean meal (45%)	35.68	29.19	25.98	25.50
Meat and bone meal (43%)	3.00	3.00	3.00	3.00
Soybean oil	3.34	2.94	3.08	3.87
Dicalcium phosphate	1.00	0.85	0.59	0.59
Salt (NaCl)	0.47	0.47	0.47	0.47
Choline Chloride (60%)	0.10	0.10	0.10	0.10
Vitamin Premix [1]	0.05	0.05	0.05	0.05
Mineral Premix [2]	0.05	0.05	0.05	0.05
DL- Methionine (99%)	0.32	0.26	0.23	0.23
L-Lysine (54.6%)	0.32	0.28	0.27	0.22
L-Threonine (98.5%)	0.09	0.07	0.07	0.05
L-Valine (96.5%)	0.06	0.02	0.01	0.01
Nutritional composition (%)				
Metabolizable Energy, kcal/kg	3050	3100	3150	3200
Calcium	0.90	0.84	0.76	0.76
Av. Phosphorus	0.58	0.54	0.48	0.48
Sodium	0.21	0.21	0.21	0.21
Potassium	0.85	0.76	0.71	0.70
Crude Protein	23.68 (24.17)	21.15 (21.50)	19.90 (19.64)	19.60 (18.50)
Lysine	1.40 (1.45)	1.21 (1.27)	1.12 (1.12)	1.08 (1.04)
Methionine + cystine	1.01 (0.95)	0.90 (0.85)	0.84 (0.80)	0.83 (0.77)
Threonine	0.94 (0.92)	0.83 (0.81)	0.78 (0.74)	0.76 (0.71)
Valine	1.13 (1.12)	0.99 (0.98)	0.92 (0.89)	0.91 (0.87)
Isoleucine	0.97 (0.97)	0.85 (0.85)	0.80 (0.77)	0.78 (0.75)
Arginine	1.55 (1.57)	1.36 (1.37)	1.26 (1.22)	1.24 (1.19)
SID[3] lysine	1.26 (1.31)	1.09 (1.14)	1.01 (1.01)	0.97 (0.93)
SID methionine + cystine	0.91 (0.86)	0.81 (0.77)	0.76 (0.72)	0.75 (0.70)
SID threonine	0.80 (0.78)	0.70 (0.68)	0.66 (0.63)	0.64 (0.60)
SID valine	1.00 (0.99)	0.87 (0.86)	0.81 (0.78)	0.80 (0.76)
SID isoleucine	0.86 (0.76)	0.76 (0.76)	0.71 (0.68)	0.70 (0.67)
SID arginine	1.41 (1.43)	1.23 (1.24)	1.14 (1.10)	1.12 (1.07)

Analyzed content in parenthesis.

[1] Provided per kg of vitamin premix: Folic Acid (mín) 1600 mg; Vitamin B_5 - Pantothenic acid (mín) 24.96 g; Biotin (mín) 80 mg; Butyl hydroxide toluene 100 mg; Niacin (mín) 67.20 g; Selenium (mín) 600 mg; Vitamin A (mín) 13440000 UI; Vitamin B_1 (mín) 3492 mg; Vitamin B_{12} (mín) 19200 mcg; Vitamin B_2 (mín) 9600 mg; Vitamin B_6 (mín) 4992 mg; Vitamin D_3 (mín) 3200000 UI; Vitamin K_3 (mín) 2880 mg.

[2] Provided per kg of mineral premix: Copper (mín) 15 g; Iron (mín) 90 g; Iodine (mín) 1500 mg; Manganese (mín) 150 g; Zinc (mín) 140 g. [3] SID, standardized ileal digestible (value in parenthesis are calculated using total AA analyzed and the SID coefficients from AMINODAT® 5.0).

Table 2 - Oligonucleotides used in this study.

Target	Name	Type	Sequence 5'-3'
Clostridium perfringens (*plc* gene)	398F	Forward primer	CTA GAT ATG AAT GGC AAA GAG GAA ACT A
	475R	Reverse primer	AAC ATT GCA GGA TGA TAT GGA GTA GTA TCT AT
	430T	TaqMan probe	CAA GCT ACA TTC TAT CTT GGA GAG GCT ATG CAC TAT TT

Table 3 – Effects of supplementation with *Bacillus amyloliquefaciens* CECT 5940 probiotic (DFM), Bacitracin methylene disalicylate (BMD) or its combination on performance parameters of challenged broiler.

Treatment	Day 1-21			Day 1-35			Day 1-42			
	[1]BWG (kg)	[2]FI (kg)	[3]FCR $_{adj..}$ (kg/kg)	BWG (kg)	FI (kg)	FCR $_{adj.}$ (kg/kg)	BWG (kg)	FI (kg)	FCR $_{adj..}$ (kg/kg)	Mortality (%)
PC [4]	0.996	1.295^{b}	1.300	2.408^{a}	3.566^{a}	1.481^{c}	3.052^{a}	4.838^{a}	1.586^{c}	3.648
NC [5]	0.998	1.317^{a}	1.320	2.083^{b}	3.337^{b}	1.602^{a}	2.740^{b}	4.670^{b}	1.704^{a}	5.730
NC + BMD [6]	0.993	1.301^{ab}	1.310	2.100^{b}	3.270^{b}	1.557^{b}	2.716^{b}	4.521^{b}	1.664^{b}	4.167
NC + DFM [7]	0.990	1.292^{b}	1.305	2.076^{b}	3.253^{b}	1.567^{b}	2.697^{b}	4.501^{b}	1.669^{b}	4.169
NC + BMD + DFM	1.000	1.301^{ab}	1.307	2.111^{b}	3.300^{b}	1.563^{b}	2.744^{b}	4.563^{b}	1.664^{b}	5.092
SEM	0.015	0.016	0.015	0.049	0.066	0.020	0.084	0.120	0.023	3.219
P-value	0.767	0.049	0.181	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.709

Means in the in the same column followed by different superscript differ significantly ($P < 0.05$, ANOVA, Student–Newman–Keuls test).

[1] BWG - Body weight gain.

[2] FI - Feed intake.

[3] $FCR_{adj.}$ - Feed conversion ratio adjusted for mortality.

[4] PC - Positive control (basal diet without additives or challenge).

[5] NC - Negative control (basal diet without additive and challenged birds receiving 3.85×10^4 sporulated oocysts of *Eimeria maxima* + 2.5×10^6 CFU of *Clostridium perfringens*).

[6] BMD - Bacitracin methylene disalicylate added in 0.05g/kg feed.

[7] DFM – direct fed microbial added 1 g/kg feed (1×10^6 CFU/g of *B. amyloliquefaciens* CECT 5940).

Table 4 - Effects of supplementation with *Bacillus amyloliquefaciens* CECT 5940 probiotic (DFM), Bacitracin methylene disalicylate (BMD) or its combination on body weight uniformity, carcass and breast meat yield of challenged broiler at 42 d of age.

Treatment	Body weight uniformity (%)	Carcass yield (%)	Breast meat yield (%)
PC [1]	91.715[a]	77.029[a]	37.453[a]
NC [2]	78.944[b]	75.360[b]	35.640[b]
NC + BMD [3]	88.951[a]	76.271[a]	37.413[a]
NC + DFM [4]	86.295[a]	76.439[a]	37.250[a]
NC + BMD + DFM	84.895[a]	76.763[a]	36.659[a]
SEM	4.737	0.786	0.870
P-value	0.001	0.011	0.003

Means in the in the same column followed by different superscript differ significantly (P < 0.05, ANOVA, Student–Newman–Keuls test).
[1] PC - Positive control (basal diet without additives or challenge).
[2] NC - Negative control (basal diet without additive and challenged birds receiving 3.85 x 10^4 sporulated oocysts of *Eimeria maxima* + 2.5 x 10^6 CFU of *Clostridium perfringens*).
[3] BMD - Bacitracin methylene disalicylate added in 0.05g/kg feed.
[4] DFM – direct fed microbial added 1 g/kg feed (1 x 10^6 CFU/g of *B. amyloliquefaciens* CECT 5940).

Table 5 – Effects of supplementation with *Bacillus amyloliquefaciens* CECT 5940 probiotic (DFM), Bacitracin methylene disalicylate (BMD) or its combination on Foot pad dermatitis (FPD) and visual litter quality scoring of challenged broiler at 42 d of age.

Treatment	FPD (from 0 to 4)	Litter quality visual scoring [**] (from 1 to 5)
PC [1]	0.250[b]	1.167[b]
NC [2]	1.532[a]	1.594[a]
NC + BMD [3]	0.629[b]	1.667[a]
NC + DFM [4]	0.734[b]	1.179[b]
NC + BMD + DFM	0.584[b]	1.219[b]
SEM	0.447	0.301
P-value	<0.001	<0.001

Means in the in the same column followed by different superscript differ significantly (P < 0.05, ANOVA, Student–Newman–Keuls test).
[1] PC - Positive control (basal diet without additives or challenge).
[2] NC - Negative control (basal diet without additive and challenged birds receiving 3.85 x 10^4 sporulated oocysts of *Eimeria maxima* + 2.5 x 10^6 CFU of *Clostridium perfringens*).
[3] BMD - Bacitracin methylene disalicylate added in 0.05g/kg feed.
[4] DFM – direct fed microbial added 1 g/kg feed (1 x 10^6 CFU/g of *B. amyloliquefaciens* CECT 5940).

Table 6 - Effects of supplementation with *Bacillus amyloliquefaciens* CECT 5940 probiotic (DFM), Bacitracin methylene disalicylate (BMD) or its combination on *Clostridium perfringens* enumeration (*plc* gene copies/g digesta) in cecal and ileal contents of broilers.

Treatment	Ileum (log_{10} gene copies/g digesta)		Cecum (log_{10} gene copies/g digesta)	
	21 days	28 days	21 days	28 days
PC [1]	5.36[b]	5.25[b]	5.84	5.71
NC [2]	5.85[a]	5.70[a]	6.11	5.92
NC + BMD [3]	5.07[b]	4.82[b]	5.49	5.35
NC + DFM [4]	5.24[b]	4.99[b]	5.68	5.43
NC + BMD + DFM	5.17[b]	4.90[b]	5.53	5.40
SEM	0.420	0.355	0.481	0.487
P-value	0.003	< 0.001	0.060	0.076

Means in the in the same column followed by different superscript differ significantly ($P < 0.05$, ANOVA, Student–Newman–Keuls test).

[1] PC - Positive control (basal diet without additives or challenge).

[2] NC - Negative control (basal diet without additive and challenged birds receiving 3.85×10^4 sporulated oocysts of *Eimeria maxima* + 2.5×10^6 CFU of *Clostridium perfringens*).

[3] BMD - Bacitracin methylene disalicylate added in 0.05g/kg feed.

[4] DFM – direct fed microbial added 1 g/kg feed (1×10^6 CFU/g of *B. amyloliquefaciens* CECT 5940).

Table 7 - Frequency* of intestinal health evaluation measurements of broilers under challenge conditions fed diets supplemented with *Bacillus amyloliquefaciens* CECT 5940 probiotic (DFM), Bacitracin methylene disalicylate (BMD) its combination at 21 and 28 days of age.

Treatment	Abnormal content		Thin or fragile intestinal walls		Ballooning in the gut		Sloughed mucosa		Swollen or red (serosal/mucosal surface)	
	21 d	28 d	21 d	28 d	21 d	28 d	21 d	28 d	21 d	28 d
PC [1]	1 (1%) [b]	11 (9%) [ab]	3 (2%)	1 (1%)	3 (2%)	6 (5%) [b]	11 (9%)	9 (7%)	3 (2%)	11 (9%) [ab]
NC [2]	15 (12%) [a]	18 (14%) [a]	2 (2%)	3 (2%)	7 (6%)	16 (13%) [a]	9 (7%)	15 (12%)	2 (2%)	16 (13%) [a]
NC + BMD [3]	13 (10%) [a]	6 (5%) [b]	2 (2%)	5 (4%)	7 (6%)	10 (8%) [ab]	11 (9%)	12 (10%)	4 (3%)	6 (5%) [b]
NC + DFM [4]	16 (13%) [a]	7 (6%) [b]	1 (1%)	6 (5%)	8 (6%)	6 (5%) [b]	8 (6%)	13 (10%)	4 (3%)	6 (5%) [b]
NC + BMD + DFM	16 (13%) [a]	7 (6%) [b]	3 (2%)	6 (5%)	7 (6%)	6 (5%) [b]	8 (6%)	10 (8%)	3 (2%)	6 (5%) [b]
P-value	<0.0001	<0.0001	0.845	0.217	0.464	0.0012	0.7395	0.2368	0.906	0.0008

Values in the in the same column with different superscripted letters differ significantly ($P < 0.05$) by the non-parametric Kruskal-Wallis test followed by Dunn-Bonferroni test.
[*] Number of positive observations are presented followed by the frequency in parenthesis.
[1] PC - Positive control (basal diet without additives or challenge).
[2] NC - Negative control (basal diet without additive and challenged birds receiving 3.85×10^4 sporulated oocysts of *Eimeria maxima* + 2.5×10^6 CFU of *Clostridium perfringens*).
[3] BMD - Bacitracin methylene disalicylate added in 0.05g/kg feed.
[4] DFM – direct fed microbial added 1 g/kg feed (1×10^6 CFU/g of *B. amyloliquefaciens* CECT 5940).

CAPÍTULO 3 – Modelling feed intake of broilers under pathogen challenge

Este capítulo é apresentado de acordo com as normas da revista Animal.

Modelling feed intake of broilers under pathogen challenge

M. J. K. Oliveira[1], M. P. Reis[1], L. Hauschild[1], J. C. P. Dorigam[2] and N. K. Sakomura[1]

[1] *Department of Animal Science, Universidade Estadual Paulista "Julio de Mesquita, Filho", FCAV/UNESP, Jaboticabal, São Paulo 14884–900, Brazil*

[2] *Evonik Nutrition & Care GmbH, Hanau, 63067, Hessen, Germany*

Corresponding author: Nilva Sakomura. Email: nilva.sakomura@unesp.br

Short title: Actual feed intake of challenged broilers

Abstract

Through relative feed intake (**RFI**) it makes possible to model the actual feed intake (**AFI**) of broiler under pathogen challenge. Thus, the present study aimed: 1) to develop a model for predicting the RFI of broilers challenged with *Eimeria maxima* and *Clostridium perfringens*; 2) to integrate this model into a mechanist simulation model (Broiler Growth Model, **BGM**); 3) to evaluate the simulation outcomes and check whether the inclusion of this model into the BGM allows to predict broilers performance under a pathogen challenge. Initially, the RFI was calculated by dividing the value of AFI of broilers challenged with *Eimeria maxima* and *Clostridium perfringens* by the value of AFI of unchallenged broilers. From RFI estimated, two broken line function (linear and quadratic) were fitted, building a segmented model (**SM**). Lastly, the SM was integrated into the BGM, being used to correct the AFI outputs. Then, BGM uses the corrected AFI as input for making a simulation of broilers performance. It is expected that the outcomes represent broilers under pathogen challenge. The outputs

obtained from the simulation, accumulated AFI (**AFIa**) and BW were taken in order to compare with those observed data of a field experiment. The outcomes were analysed by residual analysis. The results showed that after 42 days, the observed AFIa (4581 g) was higher than simulated AFIa in 5%. The simulated BW at 0, 14, 21, 28, 35 d were 50, 502, 956, 1419 and 2126 g, being lower than observed BW in 1%, 4%, 8%, 5% and 2% respectively. On the other hand, the simulated BW (2916 g) at 42 d was higher than observed BW in 5%, with no mean bias of prediction ($p > 0.05$). The outcomes indicate that the SM proposed in this study for predicting the RFI of broilers under pathogen challenge might be included into BGM, allowing to make a realistic simulation, as confirmed by observed data.

Keywords: *Eimeria maxima*, Challenge model, *Clostridium perfringens*, Performance, Prediction

Implications

The poultry industry has been dealing with several enteric diseases mainly due to the withdrawal of antibiotics growth promoters of the diets in some countries. This framework implies in negative impact in the feed intake of those animals. Using a mechanistic model to predict the feed intake of broilers under pathogen challenge would help poultry nutritionists to adjust the fed program. Furthermore, it makes possible to predict the impact of the pathogen challenge on broilers performance.

Introduction

Biological models have been used for simulations in order to predict deviations in production system by considering different scenarios in poultry industry. Simulation models allow predicting performance in different environmental conditions (Emmans,

1987; Gous, 2007). Models provide important information that could help in making management decisions such as the optimal age for slaughtering where the profit is highest, or deciding nutritional programs (Sakomura *et al*., 2015; de Lima *et al*., 2018).

Mechanistic models such as Broiler Growth Model (**BGM**), developed at UNESP by Sakomura *et al*., (2019, in registration process), and EFG Broiler Growth Model (EFG software, 2015) have been used in poultry industry with the purpose of predicting performance, body composition, and nutrient requirements for chickens. These models allow to predict the desired amount of a given feed the bird needs to consume each day to express its potential, as well as actual feed intake (Sakomura *et al*., 2015; EFG software, 2015). Actual feed intake (**AFI**) is defined as the real amount of feed consumed by animal, considering factors inherent to animals (genotypes, sex, age, bulk capacity), environment (disease, management, stocking density, temperature, lighting), and diet (composition and feed form), which in turn may constrain the feed intake (Emmans, 1997; Abdollahi *et al*., 2018; Bendezu *et al*., 2018).

One of the important factors that need to be included in simulation models is associated with the exposure of broilers to diseases. In general, diseases may exhibit influences in prediction accuracy of the model, especially in prediction of AFI. Knowing about actual feed intake has shown great importance, since the reduction in growth may be explained in part by the reduction in feed intake (Sandberg *et al*., 2007). The AFI is also a key point to help nutritionist in the process of diet formulation. Subclinical disease for instance, may decrease the feed intake by 15 to 20% (Kyriazakis and Sandberg, 2006). On the other hand, Black *et al*. (1986) report that depending on severity and the extend of the challenge, feed intake may be reduced to zero. Integrating the effect of sanitary challenge into any mechanistic model has been a difficult task for researchers. Thus, the development of empirical approach although

not provide information on the biological mechanisms involved enable better prediction of broilers response under sanitary challenge. Sandberg *et al.* (2006) reports that AFI can be predicted empirically from the relative feed intake (**RFI**).

Given this background, we hypothesized that actual feed intake of broilers under pathogen challenged could be modelled empirically and integrated into a mathematical model used to estimate broiler response and nutrient requirements. Therefore, the purpose of the current study was to develop a model for predicting the RFI of broilers challenged with *Eimeria maxima* and *Clostridium perfringens* and include this model on Broiler Growth Model allowing prediction of broilers performance (actual feed intake and body weight) under a pathogen challenge condition.

Material and methods

Database

The data used here is part of the study from Oliveira *et al*. (2019) and consist of two treatment groups with 9 replicates of 34 broilers each as follows: 1) Positive control group (**PC**) where broilers received a basal diet without any feed additives and no challenge, and 2) the negative control group (**NC**) where broilers received the same basal diet, but including the challenged. In this study, the challenge consisted in inoculating once a day 3.85 x 10^4 sporulated oocysts / bird of *Eimeria maxima* at 17 days of age and 2.5 x 10^6 *CFU* / bird of *Clostridium perfringens* on days 18, 19 and 20. In total, the data of 306 Cobb500 chickens were collected between the periods from 1 to 42 days of age. Dietary nutritional composition and feed program was reported by Oliveira *et al*., (2019). The response variables collected were AFI (g/bird/day) correct by mortality daily measured from 1 to 42 d, accumulated feed intake (**AFIa, g/bird/day**) and body weight (**BW**, **g**) in different ages (0, 14, 21, 28, 35 and 42 d).

Estimating relative feed intake

Relative feed intake (%) was daily calculated during the whole experimental period (1-42 d). For such, the AFI of NC group was divided by the average of AFI of PC group, as the formula bellow.

$$RFI = (AFI_{NC} / AFI_{PC}) \times 100 \quad \text{[eq. 1]}$$

Modelling the relative feed intake

A segmented model (**SM**) was proposed for modelling the daily values of RFI during pathogen challenged. This model is divided in four segments, consisted by two broken line function (Robbins *et al*. 2006): the linear broken line (**LBL,** 1st and 2nd segment) and quadratic broken line (**QBL,** 3rd and 4th segment). The first segment represents the RFI of broilers before the challenge, where the RFI is equal to L_1 (eq. 2). The second segment describes RFI after the challenge, when the RFI starts to decrease and achieve the lowest value of RFI (eq. 3). The third segment represented by a quadratic curve, describes the recovery of RFI (eq. 4). The fourth segment indicates the complete recovery of feed intake, where the RFI is equal L_2 (eq. 5). The segmented model is represented mathematically by the equations:

$$Y = L_1 \quad \text{for } X < R_1 \ (1^{st} \text{ segment}) \quad \text{[eq. 2]}$$

$$Y = L_1 + U_1 \times (R_1 - X) \quad \text{for } R_1 \geq X \leq 24 \ (2^{nd} \text{ segment}) \quad \text{[eq. 3]}$$

$$Y = L_2 + U_2 \times (R_2 - X)^2 \quad \text{for } 24 > X \leq R_2 \ (3^{rd} \text{ segment}) \quad \text{[eq. 4]}$$

$$Y = L_2 \quad \text{for } X > R_2 \ (4^{th} \text{ segment}) \quad \text{[eq. 5]}$$

where, Y is the dependent variable (RFI of broilers under pathogen challenge), X is the independent variable (age of broilers), L_1 and L_2 represent the plateau of responses (1st and 4th segment), U_1 and U_2 represent the slopes of the second segment and the third segment respectively, R_1 and R_2 represent the inflection point in X axis for the

first and fourth segments respectively. A value of 24, was found as the age at which the drop in RFI was maximum, being used in the equations 3 and 4.

Data were analysed as a one-way ANOVA using the GLM procedure of SAS software (SAS Inst. Inc., 2013). Each pen was considered as experimental units. AFI of each day, in the same treatment at different pen were analysed. The broken line regression function (linear and quadratic) were computed by the NLIN procedure and taken together as a single model, the SM.

Evaluation of the model

To evaluate the results, the estimated RFI by the model for broilers under challenge condition was compared with the data observed in trial. The outcomes were evaluated by residual analysis as described by St-Pierre (2003). The residuals (**ER**) were calculated: ER = observed data – predicted data. To assess the SM, the residuals were plotted against the predicted data, according to the following model:

$$ER = b_0 + b_1 (Y_p - \bar{Y}_p) + \varepsilon \quad \text{[eq. 6]}$$

where, ER is the residual value, b_0 and b_1 are estimated parameters of the linear regression, Y_p is the predicted value of RFI, $\bar{Y}_p$ is the mean of all predicted values of RFI and ε is the error of the regression analysis. The coefficient of determination ($\mathbf{R^2}$) was used to ascertain the predictive performance of the model for the RFI measured and RFI predicted values.

Integrating the predicted RFI by Segmented Model into Broiler Growth Model

Broiler Growth Model (**BGM**) have been developed in Brazil by Nutrition and Modelling research group of Universidade Estadual Paulista (UNESP). The model follows the theoretical assumptions developed over time by Emmans (1981, 1987,

1994, 1999) and its general structure follows the proposal of Hauschild *et al*., 2015. Based on growth rate potential (genotype's growth, estimated using the Gompertz function), BGM estimates the feed intake and nutrient requirements of an animal. External effects on broilers responses such as environmental condition and feed (diet composition) are considered model inputs. In order to making available a new tool for simulating broilers performance under a pathogen challenge condition, the RFI estimated in this study was included in BGM. Thus, it allows to estimate a new actual feed intake (**AFI_n, g/bird/day**) under a specific pathogen challenge (*Eimeria maxima* and *Clostridium perfringens*). For that, an adjusted AFI during the pathogen challenge period is proposed:

$$AFI_n = AFI_i \times RFI_i \quad \text{[eq. 7]}$$

where AFI_n is the new actual feed intake, considering the SM used for predicting the RFI in sanitary challenge, AFI_i is the actual feed intake output for each i day, without considering the aforementioned model, which in turn represents health broilers, and the RFI_i is the relative feed intake for each i day, estimated by SM. Thus, considering the new tool, the BGM might simulate performance of broilers challenged with *Eimeria maxima* and *Clostridium perfringens.*

Evaluation of broilers performance challenged with Eimeria maxima *and* Clostridium perfringens *using the Broiler Growth Model*

The accuracy of the responses obtained from BGM, were evaluated based on its simulation in comparison with the observed data from Oliveira *et al*. (2019). For the simulation, the input sets required by the model are described below. 1) Genetic parameters: protein deposition ratio, lipid / protein ratio at maturity, feather maturity protein, maturity protein, feather protein deposition ratio, allometric growth coefficients.

These parameters represent the Cobb500 strain (default at BGM), the same genotype used by Oliveira *et al*. (2019). 2) Environmental variables (daily relative humidity, % and air temperature, ^{o}C). 3) Feed program: starter 1-13 d, crude protein (**CP, %**) = 24.17, metabolizable energy (**ME, kcal/kg**) = 3050; grower I 14-21 d (CP = 21.50, ME = 3100); grower II 22-35 d (CP = 19.64, ME = 3150) and finisher 36-42 d (CP = 18.50, ME = 3200). Both, environmental and feed program variables were set up to follow the same observed in the trial.

The outputs obtained from the simulation: AFI, AFIa and BW were used for comparing with the observed data. The outcomes were analysed by residual analysis as described by St-Pierre (2003).

Results

Segmented model

The estimated parameters for LBL and QBL function and fitted models to RFI are detailed in Table 1 and Table 2, respectively. The SM model includes both LBL and QBL function and the graphical representation is shown in Figure 1. At the first part of the SM, where LBL was fitted, the inflexion point indicates when the RFI starts to decrease (age > 20 d). At 24 d, the minimum RFI was observed. This represents a range of four days of decreasing RFI. In the second part of the SM where QBL was fitted, the inflexion point indicates when the RFI returns to normal (age ≥ 27 d). The QBL represents the part of the segmented model in which occurs the recovery in RFI.

Figure 2 (a) shows the regression between residuals (i.e., observed minus predicted RFI) and predicted values centred of RFI of broilers (**CRFI**) under sanitary challenge condition. The independent variable RFI was centred around the mean predicted value before the residuals were regressed on the predicted values. The slope of that regression represents the linear bias (**LB**) and the intercept represents the mean

bias (**MB**). Analysing the bias of prediction of RFI, the LB and MB were 0.0813 (± 0.032) and 1.342 % (± 0.303) respectively. The LB was statistically significant, likewise, the MB ($P < 0.05$). A relationship between the observed and predicted RFI applying the SM is shown in Figure 2 (b). The coefficient of determination ($R^2 = 0.76$) indicates that data were well adjusted.

Simulation at Broiler Growth Model

Figure 3 shows the AFI from 1 to 42 d considering the segmented model for correcting the AFI, described at equation [7]. It is important to highlight that the behaviour of simulated data using SM was the same of those data observed over the period studied, as expected. It reinforces the applicability of the SM in correcting the AFI at BGM.

Figure 4 (a) shows the relationship between residuals (i.e., observed minus predicted AFI) and predicted values centred of AFI of broilers (**CAFI**) under sanitary challenge condition. The independent variable AFI was centred around the mean predicted value before the residuals were regressed on the predicted values. Analysing the bias of prediction of AFI, the MB (5.263g ± 0.756) is significant statistically ($P < 0.001$), however, the LB (0.0099 ± 0.013) is not significant statistically ($P = 0.46$). The Figure 4 (b) shows a great R^2 (0.94) for the relationships between the measured and predicted values obtained using the SM.

Figure 5 (a) shows the simulated AFIa, observed AFIa and also the BW (observed and simulated) of broilers challenged with *Eimeria maxima* and *Clostridium perfringens* from 1 to 42 d. After 42 d, observed AFIa (4581 g) was higher than simulated AFI_a in 5%. The simulated BW at 0, 14, 21, 28, 35 d were 50, 502, 956, 1419 and 2126 g, being lower than observed BW in 1%, 4%, 8%, 5% and 2%, respectively.

229 On the other hand, the simulated BW (2916 g) at 42 d was higher than observed BW
230 in 5%. When observed BW of unchallenged broilers and simulated BW of challenged
231 broilers are compared, it is possible to notice that up to 14 days of age, there is no
232 difference in performance of broilers belonging to both groups. However, from 21d,
233 challenged broilers showed impairments in body weight. At 21, 28, 35 and 42 d,
234 challenged broilers had -8%, -17%, -13% and -6% BW, respectively.

235 Figure 6 (a) shows the relationship between residuals (i.e., observed minus
236 predicted BW) and predicted values centred of BW of broilers (**CBW**) under sanitary
237 challenge condition. The independent variable BW was centred around the mean
238 predicted value before the residuals were regressed on the predicted values. The MB
239 (15.26 g ± 10.68) is not significant statistically ($P = 0.16$), however, the LB (-0.039 ±
240 0.011) is significant statistically ($P < 0.001$). A higher R^2 (0.99) for the relationships
241 between the measured and predicted values for BW in simulation is shown in Figure 6
242 (b).

243

244 **Discussion**

245 According to St-Pierre (2015), the development of models involves several
246 steps, such as: i) definition of the problem that demands to be solved; ii) run
247 experiments properly designed, allowing collect data to answer specifics questions
248 defined as problematic; iii) statistical analysis and building/adjust of models based on
249 concepts involved behind in their theoretical concepts and lastly, iv) data evaluations
250 compering observed and predicted data. In the current research, an attempt was made
251 considering the aforementioned steps. Firstly, we define the problem that need to be
252 solved in simulation model, which in turn is related with the inclusion of pathogen
253 challenge factors. Secondly, we used the data of challenged and unchallenged broilers
254 obtained from Oliveira *et al.,* (2019) to develop a segmented model for estimating the

relative feed intake of broilers challenged with *Eimeria maxima* and *Clostridium perfringens*. Finally, the segmented model was integrated into the BGM, and data evaluations was conducted in order to compare the observed and predicted data.

The graphical representation of the relative feed intake shown in Figure 1, represents the segmented model fitted to the observed data. Although that model does not describe mechanistically the sanitary effect over the animal response because of the complexity, it may be useful to represent it empirically. Sandberg et al. (2006) suggested the use of relative feed intake as a useful tool for representing the feed intake of broilers during an infection framework. The fitted model allows to confirm that pathogen challenge have direct effects in appetite and consequently on feed intake.

Feed intake modification is an expected response to pathogen exposure, which have been widely described by a feed intake reduction, and hence performance depression (Remus *et al.,* 2014; Oliveira *et al.,* 2019). Feed intake reduction and impairment in growth performance depends on the pathogens load and type in the host (Kyriazakis and Houdijk, 2007), and if the host was previously challenged. Considering those peculiarity, the extension of reduction in feed intake as well as in broiler performance may vary. In the current study, shifts in relative feed intake were observed from four days after inoculating with *Eimeria maxima* and from one day after inoculating with *Clostridium perfringens*. The extension of reduction in relative feed intake lasted 4 d, whilst the extension of recovery lasted 3 d. Alnassan *et al*. (2014) reports that the depression of feed intake occurs five days after coccidia infection and it is intensified by *Clostridium perfringens* infection. In our study, the depression of feed intake occurred four days after *Eimeria maxima* inoculation. However, the fast recovery was substantially equal (4 d). Sakkas *et al*. (2018), observed the same behaviour, although broilers were challenged only with *Eimeria.*

Changes in feed intake are frequently triggered by activation of the innate immune response, which in turn leads to the development of an acute-phase response mediated by cytokines (Pastorelli *et al.,* 2011). Consequently, maintenance functions change, and an indirect effect might be notice in performance variables of broilers, such as growth. The impairments in performance are observed often when broilers are challenged and can be attributed to the requirements of extra amount of nutrients to support immune functions. Such functions include either, the replenishment of tissues or the synthesis of proteinaceous components of defence lines against pathogens such as mucus production, cytokines (IL-1-alpha, IL-6, IL-8, and TNF), acute phase proteins and immunoglobulins (Le Floc'h *et al.,* 2004; Kyriazakis and Sandberg, 2006; Terada *et al.,* 2018).

The composition of amino acids used to biosynthesis of these aforementioned components differ markedly from that required for body protein accretion (Le Floc'h *et al.,* 2004; Kyriazakis and Sandberg, 2006; Gottardo *et al.,* 2017; Fernandes *et al.,* 2018). In this sense, nutrient whose are diverted to support immune functions, instead of body protein accretion leads to impairment in live weight, because that function is prioritized over the growth function. Thus, the nutrient that would be destined for growth is allocated for other functions (Kyriazakis and Sandberg, 2006). Although the proposed model at this moment does not consider the effect of activation of the innate immune response induced by sanitary challenge on amino acids metabolism, it estimates amino acids requirements accounting for the reduced feed intake. Even though this approach does not provide information on the biological mechanisms involved in the observed performance changes, it enables estimates nutrient requirements according to the actual feed intake and growth of a broiler flock.

Variations between observed body weight and simulated body weight of challenged broilers in the Broiler Growth Model were lower than 5%. Thus, we used data of simulated body weight (challenged broilers) and observed body weight (unchallenged broilers), to compare the relative body weight. Since 40% of the growth rate reduction performance responses of chickens challenged by *Clostridium spp.* can be explained by feed intake reduction (Remus *et al.,* 2014), we used the relative feed intake to discuss about the body weight.

When the relative feed intake started to reduce at 21 d (4 d after the beginning of the sanitary challenge), it is already possible to observe losses in relative body weight (-8%), possibly explained by activation of the innate immune response. When the highest drop in feed intake was observed at 24 d, the next evaluation of the relative body weight at 28 d shows the most difference (-17%). It indicates that, even when relative feed intake had recovered, the relative body weight remains impaired, corroborating with some authors (Emmans and Fisher 1986; Emmans and Oldham, 1988) that report that the time loss in protein deposition (assumed here as body weight) cannot be recovered. However, Arczewska-Włosek and Świątkiewicz (2013) report that after the challenge period, a compensatory growth might be observed.

In the current research, along of the relative body weight evaluations period, after 28 d when the relative feed intake starts to remain in a plateau, the relative body weight at 35 and 42 d was -13% and -6% respectively, indicating that over the time, an increase trend in relative body weight can be observed, evidenced by diminishing in differences between the body weight of broilers challenge and unchallenged. Sedeik *et al.* (2019) also report a compensatory growth during the 2nd week post infection with parasitic pathogens, such as *Eimeria* with no difference in feed intake between broilers challenged and unchallenged. Although we did not evaluate the carcass composition,

329 we believe that improvements in body weight can be associated with increase in fat
330 deposition.

331 When the relative feed intake starts to recover, it is an indicative that the host
332 already overcome the negative effects of the pathogen. In other words, from that
333 moment, the recognition by the innate immune system host has been happened and
334 the acquired immune system is enabled. Thus, to recognize and eliminate pathogens
335 that enter into the host, the innate and acquired immunity work together to produce the
336 responses to the invasion (Swaggerty *et al.,* 2019).

337 Our results describe the effect of the pathogen challenge on feed intake through
338 a segmented model. The purpose of investigating changes in actual feed intake under
339 sanitary challenge becomes a first step for progress in models to warrant more
340 accurately feed intake predictions. When sanitary challenge is considering in order to
341 predict feed intake, due its complexity, several factors should be considered. In this
342 sense Sandberg *et al*. (2006) report a general model to predict the effects of subclinical
343 pathogen challenges during exposure to pathogen on relative feed intake, making a
344 detailed approach considering degree of infection, dose and virulence. Although we
345 proposed here a simple model, we believe that both models could be used to correct
346 the actual feed intake in simulation models. Applying the segmented model to correct
347 the actual feed intake in BGM make it possible to perform realistic simulation of broiler's
348 performance as observed in Figure 5.

349 We are aware that, the results of the simulation made in the BGM represents
350 the response for the challenge applied in the trial described in Oliveira *et al*. (2019). In
351 order to further improve the model, we recommend other studies using other
352 pathogens challenge models with different age periods, diets, inoculum concentration
353 and other pathogenic strain to correct the actual feed intake of broilers. Thus, new

options of different pathogen challenge, instead of *Eimeria* and *Clostridium*, could be implemented into the BGM.

In conclusion, the segmented model proposed in this study for predicting relative feed intake of broilers challenged with *Eimeria maxima* and *Clostridium perfringens* can be included into Broiler Growth Model, allowing prediction of broilers performance (actual feed intake and body weight) through a simulation. Since changes in actual feed intake obtained using the segmented model were able to predict shifts in body weight of broilers like observed in field trial, it confirms the applicability of this tool into the Broiler Growth Model.

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Declaration of interest

None.

Ethics statement

The experimental protocol used in the current study was previously approved by the Ethics Committee of Animal Care and Use of the Universidade Estadual Paulista "Julio de Mesquita Filho", FCAV/UNESP, Jaboticabal, São Paulo, Brazil (protocol N◦ 010300/17).

Software and data repository resources

None of the data were deposited in an official repository.

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Table 1 *Estimated parameters for linear broken line (LBL) and quadratic broken line (QBL) of the segmented model fitted to estimate relative feed intake of broilers under enteric disorder challenge* [1]

Models	Mean	Standard Error	Min	Max	*P*-value
LBL [2]					
Parameters					
U [3]	0.126	0.007	0.112	0.140	***
R [4]	20.137	0.162	19.814	20.461	***
L [5]	1.006	0.008	0.989	1.022	***
QBL [6]					
U	-0.067	0.013	-0.093	-0.042	***
R	26.440	0.227	25.985	26.894	***
L	0.955	0.010	0.935	0.974	***

[1] Inoculation with 3.8 x 10^4 sporulated oocysts of Eimeria maxima (at 17 days) and 2.5x10^6 CFU of Clostridium perfringens (at 18, 19 and 20 days).

[2] LBL = Linear broken line.

[3] U is the slope of the broken line function.

[4] R is the breakpoint of the broken line function.

[5] L is the maximum response.

[6] QBL = Quadratic broken line.

Table 2 *Models fitted to relative feed intake (RFI, %) in function of the age (days) for broilers under enteric disorder challenge* [a]

Models	Equation
LBL [b]	
For Age ≤ 20	RFI_{fall} [d] = 1.0
For 20 > Age ≤ 24	RFI_{fall} = - 0.126 × Age + 3.5409
QBL [c]	
For 24 > Age < 27	$RFI_{rec.}$ [e] = 0.955 - 0.067 × (26.44 - Age) ^ 2
For Age ≥ 27	$RFI_{rec.}$ = 0.955

[a] Inoculation with 3.8 x 10^4 sporulated oocysts of *Eimeria maxima* (at 17 days) and 2.5x10^6 CFU of *Clostridium perfringens* (at 18, 19 and 20 days).

[b] LBL = Linear broken line.

[c] QBL = Quadratic broken line.

[d] RFI_{fall} = Falling in relative feed intake.

[e] RFI_{rec} = Recovery in RFI.

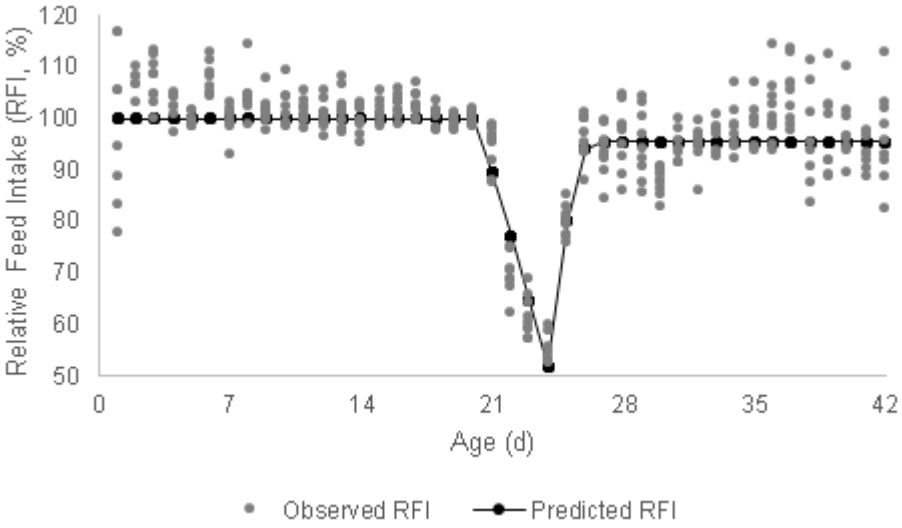


Figure 1 Graphical representation of segmented model fitted and observed relative feed intake (RFI) of broilers under pathogen challenge condition.

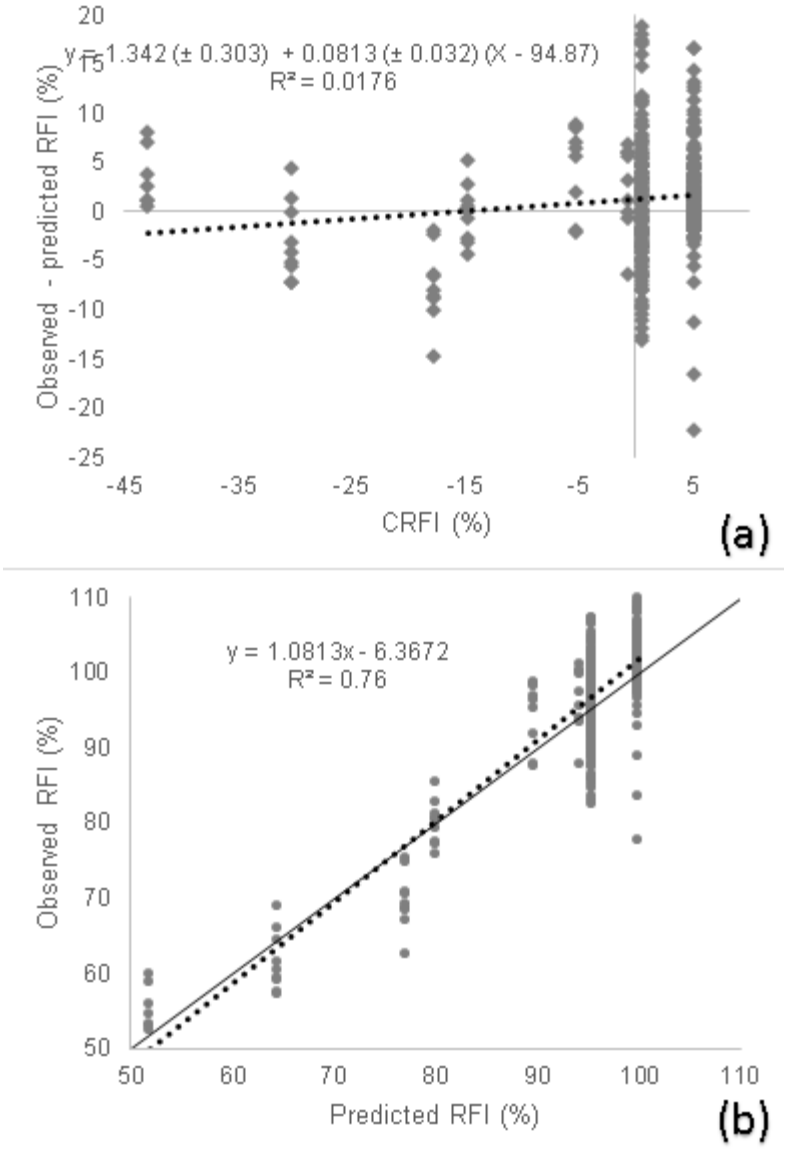
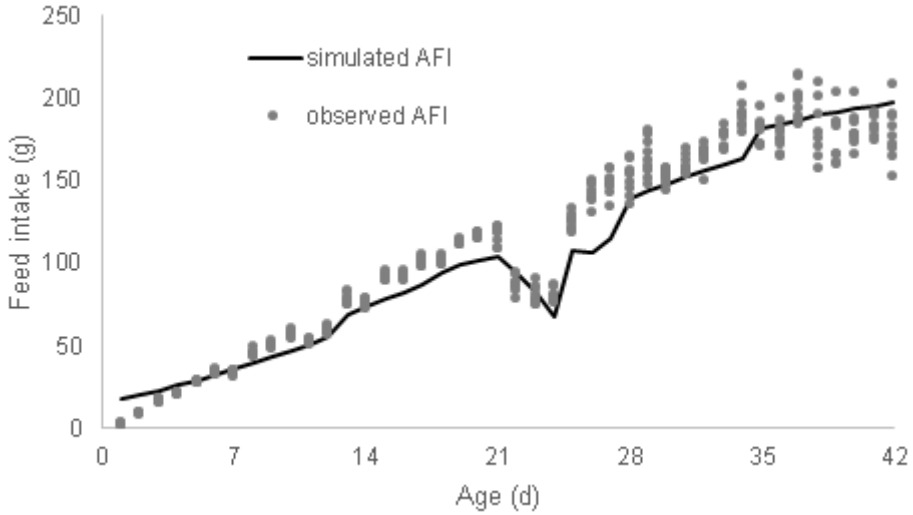


Figure 2 (a) Plot of observed minus predicted relative feed intake (RFI) vs. predicted values centred of RFI of broilers (CRFI) under sanitary challenge condition. The independent variable predicted RFI was centred around the mean predicted value before the residuals were regressed on the predicted values. **(b)** Plot of predicted vs. observed values of RFI of broilers under sanitary challenge condition. Observed data are from Oliveira *et al*. (2019). Equality line has intercept 0 and slope 1. Regression line (.....); line of equality (——).



561

562 **Figure 3** Graphical representation of both, simulated actual feed intake (AFI) at Broiler
563 Growth Model and observed AFI obtained of broilers under sanitary challenge
564 condition, from 1 to 42 d of housing.

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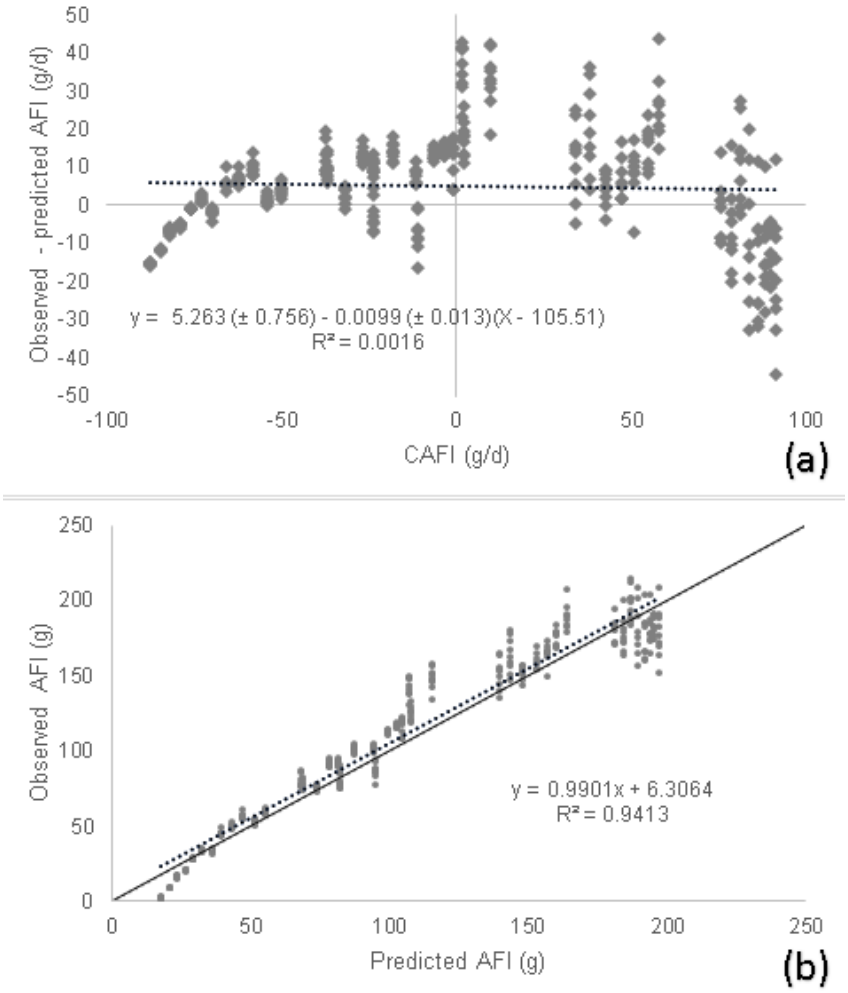

581

582 **Figure 4 (a)** Plot of observed minus predicted actual feed intake (AFI) vs. predicted
583 values centred of AFI of broilers (CAFI) under sanitary challenge condition. The
584 independent variable predicted AFI was centred around the mean predicted value
585 before the residuals were regressed on the predicted values. **(b)** Plot of predicted vs.
586 observed values of AFI of broilers under sanitary challenge condition. Observed data
587 are from Oliveira *et al*. (2019). Equality line has intercept 0 and slope 1. Regression
588 line (.....); line of equality (——).

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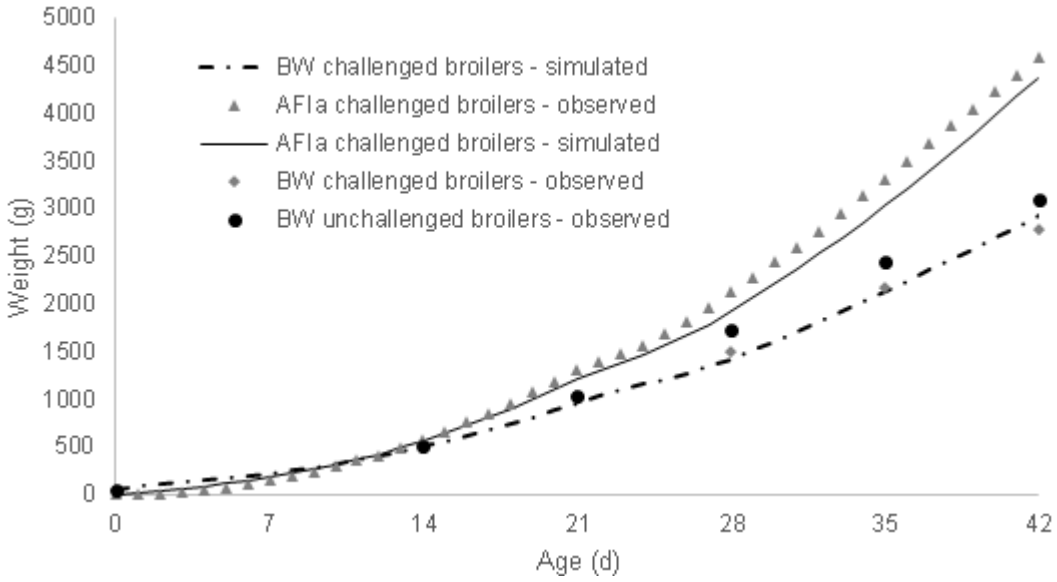

590

591 **Figure 5** Graphical representation of accumulated actual feed intake (AFIa) simulated
592 and observed and BW (simulated and observed) obtained of broilers under sanitary
593 challenge condition, from 1 to 42 d of housing. Simulation done at Broiler Growth
594 Model.

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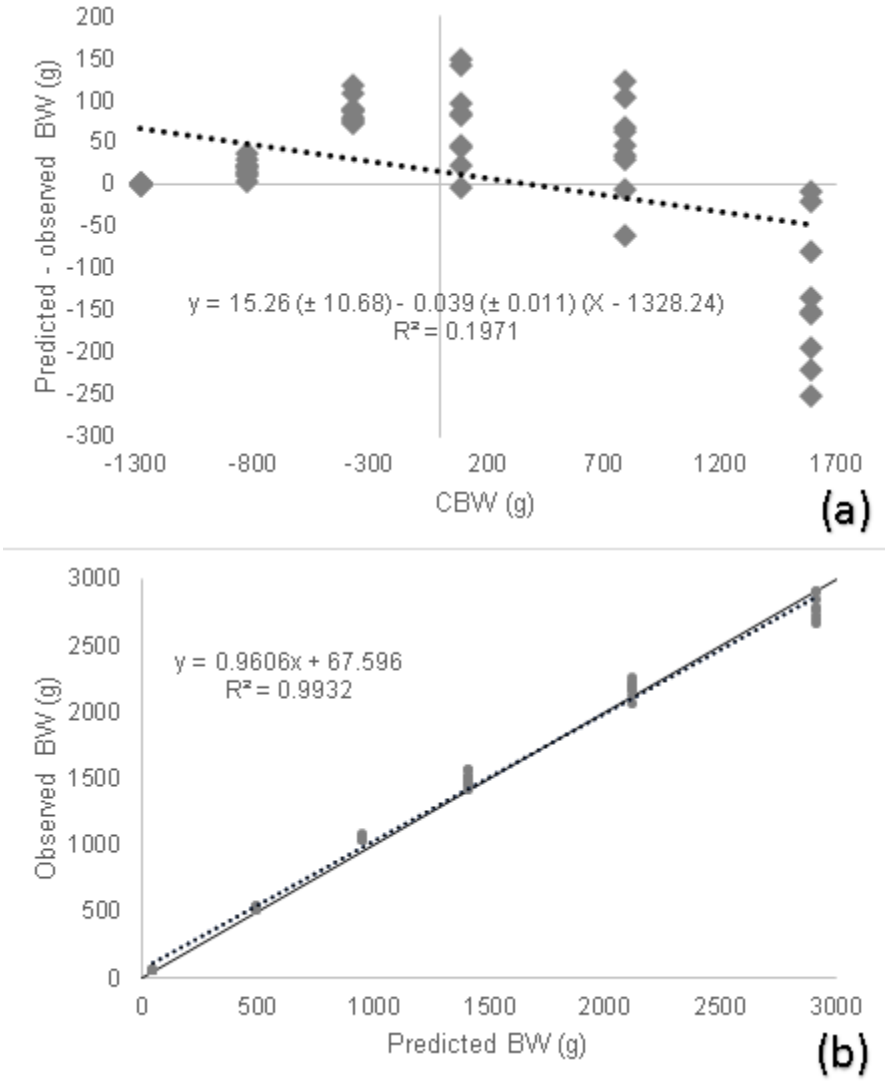

596

597 **Figure 6 (a)** Plot of observed minus predicted body weight (BW) vs. predicted values
598 centred of BW of broilers (CBW) under sanitary challenge condition. The independent
599 variable predicted BW was centred around the mean predicted value before the
600 residuals were regressed on the predicted values. **(b)** Plot of predicted vs. observed
601 values of BW of broilers under sanitary challenge condition. Observed data are from
602 Oliveira *et al*. (2019). Equality line has intercept 0 and slope 1. Regression line (.....);
603 line of equality (——).

604