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AVALIAÇÃO DE PROBIÓTICO PARA FRANGOS DE CORTE E PROBIÓTICO E CROMO ORGÂNICO PARA POEDEIRAS

Otoniel Félix de Souza

Santa Maria, RS
2023

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AVALIAÇÃO DE PROBIÓTICO PARA FRANGOS DE CORTE E PROBIÓTICO E CROMO ORGÂNICO PARA POEDEIRAS

Tese apresentada ao Curso de Pós-Graduação em Zootecnia da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutor em Zootecnia**.

Orientadora: Prof[a]. Dr[a]. Catarina Stefanello

Santa Maria, RS
2023

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Otoniel Félix de Souza

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“Se, encontrando a desgraça e o triunfo, conseguires, tratar da mesma forma a esses dois impostores”.

(Rudyard Kipling)

RESUMO

AVALIAÇÃO DE PROBIÓTICOS PARA FRANGOS DE CORTE E PROBIÓTICO E CROMO ORGÂNICO PARA POEDEIRAS

AUTOR: Otoniel Félix de Souza
ORIENTADORA: Catarina Stefanello

O presente estudo avaliou a utilização de probióticos comerciais, compostos por microrganismos do gênero *Bacillus*, e seus efeitos sobre o desempenho produtivo, qualidade de ovos e bioquímica sérica de matrizes de poedeiras, além do desempenho e microbiota fecal de frangos de corte. Para isso, foram realizados dois experimentos nas instalações do Laboratório de Avicultura da Universidade Federal de Santa Maria. No experimento I, o objetivo foi avaliar os efeitos da utilização de *Bacillus subtilis* PB6 (2.0×10^{11} UFC/g), propionato de cromo ou uma combinação dos dois aditivos sobre o desempenho, qualidade dos ovos e da casca do ovo, metabolizabilidade de nutrientes e bioquímica sérica de matrizes de poedeiras. Foram utilizadas 64 matrizes de poedeiras das raças Rhodes Island Red (n = 32) e Plymouth Rock White (n = 32) com 55 semanas de idade, alojadas em gaiolas individuais em galpão convencional de postura. As aves foram distribuídas em delineamento de blocos ao acaso, com 4 tratamentos e 16 repetições, com as aves utilizadas como unidade experimental. As dietas foram: controle; controle + probiótico (suplementada com *B. subtilis,* 500g/t); controle + CrProp (propionato de cromo, 50g/t); controle + *B. subtilis* + CrProp. Parâmetros produtivos e qualidade da casca do ovo, bem como parâmetros bioquímicos e cortisol no soro foram agrupados em períodos de 28 dias cada, e no período total de 55 a 70 semanas. A metabolizabilidade de nutrientes e energia foi determinada às 70 semanas de idade. Os dados foram submetidos à análise de variância pelo procedimento GLM do SAS, e quando significativas, as médias foram comparadas pelo teste Tukey (P<0,05). No período total, as galinhas alimentadas com as dietas controle + probiótico ou controle + probiótico + CrProp tiveram maior produção de ovos, massa de ovos, porcentagem de casca, espessura e resistência da casca (P<0,05). A metabolizabilidade da matéria seca, nitrogênio e energia foi maior quando as galinhas que receberam a dieta controle + probiótico + CrProp (P<0,05). Portanto, a suplementação de probiótico + CrProp em rações para matrizes de poedeiras de 55 a 70 semanas resultou em melhor utilização de nutrientes e melhor desempenho produtivo e qualidade de ovos. No experimento II, foi avaliado o probiótico *B. subtilis* BS-009, BS-020 e BS-024 (5×10^{11} esporos/g), comparado a um antibiótico promotor de crescimento. Foram utilizados 750 pintos, machos, Cobb 500 com 1 dia de idade, distribuídos em 5 tratamentos, com 6 repetições de 25 aves. Os tratamentos foram: Controle negativo (dieta sem aditivo e sem desafio); Controle positivo desafiado (CP) e com suplementação de 0,2 kg/t de probiótico; CP + 0,3 kg/t de probiótico; CP + 0,4 kg/t de probiótico e CP + 0,025 kg de flavomicina/t. Aos 14 dias, os frangos dos tratamentos desafiados receberam vacina comercial para coccidiose (10× a dose). No 19° dia, foi realizada gavagem oral de 1 mL/ave com inóculo de *Clostridium perfringens* ($3,1 \times 10^9$ UFC/mL). Nos dias 5 e 25 foram coletadas excretas para análise de microbioma. O desempenho produtivo foi avaliado semanalmente até os 42 dias. Não houve diferença significativa para as variáveis de desempenho entre os tratamentos. Entretanto, houve aumento na diversidade da microbiota fecal de bactérias ácido-láticas e do gênero *Clostidiales*, aos 5 e 25 dias. Dessa forma, a suplementação do probiótico contribuiu na manutenção do desempenho e composição da microbiota benéfica em frangos. Portanto, probióticos de diferentes cepas de *B. subtilis* contribuíram para a melhoria do desempenho e qualidade de ovos de poedeiras e do microbioma de frangos de corte.

Palavras-chave: *Bacillus subtilis*, digestibilidade, frango de corte, microbioma, propionato de cromo, qualidade de ovo.

ABSTRACT

EVALUATION OF PROBIOTICS FOR BROILER CHICKENS AND PROBIOTICS AND ORGANIC CHROMIUM FOR LAYING HENS

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ADVISOR: Catarina Stefanello

The present study evaluated commercial probiotics, composed of microorganisms from *Bacillus*, and their effects on the productive performance, egg quality, and serum biochemistry of layer breeders, as well as growth performance and fecal microbiota of broiler chickens. For this, two experiments were performed in the Poultry Science Laboratory at Federal University of Santa Maria. In experiment I, the objective was to evaluate the effects of using *Bacillus subtilis* PB6 (2.0 x 10^{11} CFU/g), chromium propionate, or a combination of both on performance, egg and eggshell quality, nutrient metabolizability, and serum biochemistry of layer breeders. Sixty-four hens of Rhodes Island Red (n = 32) and Plymouth Rock White (n = 32) at 55 weeks of age, housed in individual cages in a conventional laying house, were used. Birds were distributed in a randomized block design, with 4 treatments and 16 replicates, with birds used as the experimental unit. The diets were: control; control + probiotic (supplemented with *B. subtilis*, 500g/ton); control + CrProp (chromium propionate, 50g/ton); control + *B. subtilis* + CrProp (supplemented with both additives). Productive parameters and eggshell quality as well as biochemical parameters and serum cortisol, were grouped into periods of 28 days each and from 55 to 70 weeks. Nutrient and energy metabolizability were determined at 70 weeks of age. Data were submitted for analysis of variance using the SAS GLM procedure, and when significant, means were compared by the Tukey test ($P<0.05$). In the total period, hens fed the control + probiotic or control + probiotic + CrProp diets had greater egg production, egg mass, shell percentage, shell thickness, and shell strength ($P<0.05$). The metabolizability of dry matter, nitrogen, and energy was higher in hens fed the control + probiotic + CrProp ($P<0.05$). In conclusion, the diet probiotic + CrProp supplementation for layer breeders from 55 to 70 weeks increased the digestibility of nutrients and improved the productive performance and egg quality. In experiment II, a *probiotic B. subtilis* BS-009, BS-020, and BS-024 (5 x 10^{11} spores/g) was evaluated, compared to an antibiotic growth promoter. A total of 750 male Cobb 500 1-day-old chicks were distributed to 5 treatments, with 6 replicates of 25 birds. The treatments were: Negative control (basal diet without additives or challenge); Challenged positive control (CP) and supplemented with 0.2 kg/ton of probiotic; CP + 0.3 kg/ton of probiotic; CP + 0.4 lg/ton of probiotic and CP + 0.025 kg of flavomycin/ton. At 14 days, broilers of challenged treatments received a commercial cocci vaccine (10x dose). On day 19, birds were orally gavaged with 1 mL/bird of *Clostridium perfringens* (3.1 x 10^9 cfu/mL). O day 5 and 25, excreta were collected for microbiome analysis. Growth performance was evaluated weekly until day 42. There was no significant difference in performance variables between treatments. However, there was an increase in the diversity of fecal microbiota for acidic bacteria and Clostidiales at 5 and 25 days. Therefore, probiotic supplementation contributed to maintain performance and for the beneficial composition of microbiota in broilers. In conclusion, probiotics from different *B. subtilis* strains contributed to improve performance and egg quality of laying hens as well as microbiome of broiler chickens.

Keywords: *Bacillus subtilis*, broiler, chromium propionate, digestibility, egg quality, microbiome.

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1 INTRODUÇÃO

A nutrição avícola busca de maneira eficiente produzir proteína animal de qualidade por meio do adequado ajuste das exigências nutricionais, formulação de dietas e utilização de aditivos, em geral buscando custo mínimo. Desse modo, aves destinadas à produção comercial foram selecionadas para alta produtividade, e esse potencial genético deve ser atendido sem comprometer o bem-estar animal (BRYDEN et al., 2021), de modo que a nutrição representa uma ferramenta importante para manter em equilíbrio essa relação.

De maneira geral, a maioria das condições de manejo e criação das aves apresenta certo grau de complexidade, estando a ave exposta a inúmeros desafios no ambiente de criação. Dentre esses desafios, a presença de microrganismos patogênicos é uma realidade na produção avícola, podendo resultar em problemas sanitários com perdas na produção (SNEERINGER et al., 2015). Desse modo, ao longo de décadas, a prática rotineira do uso de antibióticos promotores de crescimento (APCs) na ração de animais de produção foi utilizada, com o objetivo de prevenir doenças e proporcionar ganhos no desempenho animal (CASTANON, 2007). No entanto, atualmente existe uma pressão global para remoção de APCs na ração dos animais, o que tem sido adotado atualmente pelas agroindústrias no Brasil. A proibição é motivada por uma série de fatores, incluindo medidas legislativas, pressão do público consumidor e razões éticas (COWIESON; KLUENTER, 2019).

A proibição do uso de APCs na produção animal oportuniza um vasto campo de estudos sobre aditivos que sejam favoráveis à saúde intestinal das aves, como uso de probióticos. De acordo com NEIJAT et al. (2019) existe um interesse científico e comercial para o desenvolvimento de soluções alternativas para beneficiar a saúde animal e promover segurança alimentar em sistemas avícolas. Assim, a utilização de probióticos na indústria avícola tem sido estudada para aumentar a eficiência alimentar, proporcionar uma microbiota intestinal saudável, melhorar a taxa de crescimento e inibir o desenvolvimento de microrganismos patogênicos (JENI et al., 2021). Neste contexto, a maioria dos resultados de pesquisa e utilização de probióticos a campo tem sido realizada em frango de corte, já os estudos que avaliam a eficácia de probióticos em rações para aves de postura são limitados. Dessa forma, é oportuno realizar mais pesquisas para desenvolver novos conhecimentos, permitindo embasar estratégias nutricionais para superar os fatores limitantes na produção de aves.

A utilização de cromo orgânico como aditivo em rações para aves tem sido oportunizada através dos efeitos positivos do aditivo para reduzir estresse por calor na

produção de frangos de corte. Pesquisas já exploraram o efeito do cromo em poedeiras, matrizes e frangos de corte, para diferentes objetivos, suplementado de diferentes fontes e níveis, o que tem demonstrado que o cromo apresenta propriedades antioxidantes, que ajudam a atenuar os efeitos negativos do estresse oxidativo, enquanto contribui para o metabolismo de lipídios, proteínas e ácidos nucleicos (RAJALEKSHMI et al., 2014; FARAG et al., 2017; SPEARS et al. 2019).

Em frangos de corte, as pesquisas e a utilização do cromo orgânico são mais frequentes, comumente avaliado em lotes submetidos a estresse por calor (DALOLIO, et al., 2018). Em galinhas poedeiras, a suplementação de propionato de cromo nas rações demonstrou benefícios no aumento da produção de ovos, eficiência alimentar e qualidade dos ovos, níveis de colesterol do ovo e cortisol sérico (MA et al., 2014; CHEN et al., 2021). Embora os probióticos e o cromo apresentem diferentes funções principais quando suplementados em rações para aves, esses aditivos têm algumas ações semelhantes no controle do estresse, melhorando o estado de saúde e o sistema imunológico das aves, o que pode resultar em melhorias no desempenho das aves.

Probióticos contendo diferentes cepas de microrganismos estão entre os aditivos mais utilizados na avicultura atual, e avaliar esses aditivos para poedeiras e para frangos de corte também é uma necessidade. Desse modo, estudar os efeitos de probióticos e a combinação de aditivos é um tema atual e que requer mais dados por meio da realização de pesquisas científicas.

2 OBJETIVOS

2.1 GERAL

Avaliar a suplementação do probiótico *Bacillus subtilis* PB6 e cromo orgânico para matrizes de poedeiras e *B. subtilis* BS-009, BS-020 e BS-024 para frangos de corte.

2.2 ESPECÍFICOS

Avaliar os efeitos do *B. subtilis* PB6, propionato de cromo e a combinação desses aditivos sobre o desempenho produtivo, qualidade de ovos, metabolizabilidade de nutrientes e bioquímica sérica de matrizes de poedeiras de 55 a 70 semanas de idade.

Avaliar os efeitos do probiótico *B. subtilis* BS-009, BS-020 e BS-024 sobre o desempenho produtivo e o microbioma fecal de frangos de corte de 1 a 42 dias e submetidos a desafio intestinal.

3 REVISÃO BIBLIOGRÁFICA

3.1 INTRODUÇÃO DOS ANTIBIÓTICOS PROMOTORES DE CRESCIMENTO NA AVICULTURA

O segmento da avicultura moderna conhecido nos dias de hoje foi construído ao longo de décadas, fundamentado em um modelo tecnificado com conceitos teóricos e científicos nas premissas da nutrição, seleção genética e automação do sistema produtivo, impulsionado pela demanda de proteína animal pelo mercado conumidor. Assim, criou-se um sistema intensivo, caracterizado pela prática de criação animal em granjas e cadeia produtiva integrada em larga escala.

A implantação do sistema de produção avícola intensivo resultou na necessidade de estabelecer medidas profiláticas para o controle sanitário dos plantéis avícolas. Desse modo, com a descoberta dos antibióticos e verificada sua atuação sobre o controle e prevenção de contaminações microbianas intestinais, passaram a ser utilizados amplamente nas rações para animais de produção, em pequenas doses e para atuação como promotores de crescimento (GOETTING; LEE; TELL, 2011).

A descoberta dos antibióticos ocorreu por volta da década de 1920, alcançando um grande marco na cadeia produtiva avícola global, uma vez que a utilização desses aditivos em doses sub terapêuticas na produção animal resultou em melhora no desempenho, promovendo maior taxa de crescimento e maior eficiência alimentar, além de eliminar microrganismos patogênicos que pudessem competir com bactérias benéficas por nutrientes no trato digestivo das aves (CASTANON, 2007; GADDE et al., 2017). O primeiro antibiótico promotor de crescimento (APC) oficialmente licenciado para inclusão em rações de aves foi a sulfaquinoxalina, utilizado para o controle de coccidiose.

Considerando o período pós-guerra, grandes empresas farmacêuticas emergiram como principais produtoras de antibióticos sintéticos e biológicos. Nesse cenário, o elo entre empresas farmacêuticas e de rações, permitiu que pesquisas fossem desenvolvidas para testar substâncias medicamentosas em rebanhos de produção, a fim de possibilitar a retomada da produção animal dos países afetados pela guerra (KIRCHHELLE, 2018). No entanto, com o passar das décadas a prática rotineira e indiscriminada da adição de APCs em doses sub terapêuticas nas rações passou a ser questionada na nutrição animal, em decorrência dos riscos associados ao surgimento de resistência bacteriana aos princípios ativos dos antibióticos e possível presença de resíduos nos alimentos de origem animal (LAXMINARAYAN; HEYMANN, 2012; MUND et al., 2017).

A exposição contínua de animais à doses baixas de APCs e profiláticos atrelado ao cenário ao qual são administrados na produção animal, possibilita condições ideais para o surgimento de resistência bacteriana (YOU; SILBERGELD, 2014). Ademais, a presença de resíduos de antibióticos nos produtos de origem animal é considerada um problema para a saúde humana, visto que, alguns substâncias residuais estão associadas a problemas cancerígenos, reações alérgicas e desequilíbrio da microbiota intestinal (CHEN; YING; DENG, 2019).

Reconhecidas as implicações associadas ao uso dos APCs na produção animal, entidades internacionais como a Organização Mundial da Saúde (OMS) e Comité Econômico e Social Europeu (CESE), atestaram para a proibição do uso de algumas substâncias desde 1997 e 1998, respectivamente (CASTANON, 2007). Nesse sentido, no Brasil à medida que surgem casos comprovados de resistência microbiana, APCs são proibidos de comercialização. Seguindo as recomendações de órgãos e entidades internacionais, o Brasil, por meio da Instrução Normativa n° 1, 13 de janeiro de 2020, do Ministério da Agricultura Pecuária e Abastecimento (MAPA), determinou a proibição do uso de aditivos melhoradores de desempenho que contenham os antibióticos tilosina, lincomicina e tiamulina (BRASIL, 2020). Nessa perspectiva, estudos buscam alternativas nutricionais frente ao uso dos antibióticos na produção avícola, como óleos essenciais e ácidos orgânicos (BRENES; ROURA, 2010; KALMENDAL et al., 2016), por exemplo. Um dos aditivos nutricionais mais bem estudados na avicultura moderna consiste no uso dos probióticos (AL-KHALAIFAH, 2018; ALAGAWANY et al., 2018).

3.2 MICROBIOTA DO TRATO GASTROINTESTINAL

3.2.1 Resistência microbiana aos antibióticos

As aves de produção comercial, frangos de corte e galinhas poedeiras, apresentam em seu trato digestório uma comunidade de microrganismos complexa e amplamente variada, denominada microbiota intestinal (AKINYEMI et al., 2020). A microbiota comensal do trato gastrointestinal das aves desempenha importante papel na manutenção da saúde intestinal através de mecanismos moduladores e funções fisiológicas, fundamentais para homeostase intestinal (CARRASCO; CASANOVA; MIYAKAWA, 2019).

Os microrganismos benéficos podem atuar na fermentação intestinal de substratos dietéticos (carboidratos e proteínas) não digeridos ou não absorvidos pelo animal,

convertendo em monossacarídeos e ácidos graxos de cadeia curta, melhorando posteriormente a absorção intestinal desses nutrientes (NEIJAT et al., 2019).

No passado, acreditava-se que a colonização do trato digestório das aves pela população microbiana estava relacionada unicamente aos fatores externos: ração, água, cama e ambiente de criação. Atualmente, acredita-se que a colonização do trato digestório pode ocorrer muito antes da exposição da ave ao ambiente externo, por meio da transmissão vertical, derivada da matriz para o embrião, através do processo de formação do ovo no oviduto (DING et al., 2017). Adicionalmente, a microbiota do trato gastrointestinal de pintos recém eclodidos pode ser originada de contaminantes fecais ou ambientais, aderidos na casca dos ovos, durante o processo de incubação (DONALDSON et al., 2017).

A comunidade microbiana presente no trato digestório das aves pode sofrer constante mudança ao longo do ciclo de desenvolvimento do animal, podendo ser influenciada pela genética, fatores ambientais, sexo, características do indivíduo, órgão, idade do animal, dieta e uso de antimicrobianos (YADAV; JHA, 2019). Assim, tendo em vista que cada órgão do sistema digestivo desempenha funções importantes para o processo digestivo e absorção de nutrientes. Os microrganismos também desempenham funções independentes em cada um dos órgãos, e tem sido sugerido que existe uma diferença significativa na composição taxonômica nos diferentes órgãos do trato digestivo, podendo ser considerados ecossistemas separados, apesar de estarem fortemente interligados (VAN DER WIELEN et al., 2002).

A microbiota gastrointestinal tem uma das maiores densidades celulares para qualquer ecossistema e as aves apresentam uma quantidade de 10^7 a 10^{11} bactérias por grama de conteúdo intestinal (APAJALAHTI; KETTUNEN; GRAHAM, 2004). As bactérias presentes no hospedeiro são divididas em três tipos: bactérias dominantes ($>10^6$ UFC/g amostra), bactérias subdominantes (10^3 a 10^6 UFC/g amostras) e bactérias residuais ($<10^3$ UFC/g amostra) (YADAV; JHA, 2019).

Os diferentes segmentos do trato digestivo das aves são colonizados essencialmente por bactérias do gênero gram-positivas, majoritariamente anaeróbio facultativos (YADAV; JHA, 2019). A região do papo, moela e duodeno compartilham microbiota semelhante, dominada pelo gênero *Lactobacillus* (99% em algumas aves), *lactobacillus, Clostridiaceae, Bifidobacterium, Enterobacteriaceae, Enterococcus, coliforms* e *Streptococcus,* (SEKELJA et al., 2012). O jejuno também apresenta uma maior quantidade de Lactobacillus, principalmente *L. salivarius* e *L. aviarius* (FENG et al., 2010).

No intestino delgado e grosso, o ambiente torna-se favorável ao crescimento de microbiota diversa. Na região do íleo encontra-se a presença de *Lactobacillus* (dominante),

Candidatus Arthromitus, Enterococcus, Escherichia coli/shigella e *Clostridium* XI (MOHD SHAUFI et al., 2015; POURABEDIN; ZHAO, 2015). Já os cecos, são o segmento do trato digestivo que apresentam maior densidade e diversidade microbiana (*Lactobacillus, clostridium, Bifidobacterium*) quando comparado com o trato gastrointestinal superior nas aves (POURABEDIN; ZHAO, 2015; STANLEY; HUGHES; MOORE, 2014).

O estabelecimento e atuação da microbiota comensal no sistema digestório contribui para a disponibilidade de nutrientes (ácidos graxos, aminoácidos, compostos nitrogenados e vitaminas) que de maneira direta ou indireta são importantes para o metabolismo animal (CLAVIJO; FLÓREZ, 2018).

A fração do alimento que não é aproveitada no trato digestório superior das aves, é direcionada para a porção distal, servindo de substrato para fermentação bacteriana. As bactérias intestinais degradam frações indigestíveis do alimento, como polissacarídeos não amiláceos e proteínas de baixa solubilidade, resultando em unidades simples de monossacarídeos e aminoácidos, substratos importantes para a fermentação e produção de ácidos graxos de cadeia curta (acetato, propionato e butirato) e amônia (REHMAN et al., 2007; PAN; YU, 2014).

A síntese de ácidos graxos de cadeia curta através da fermentação bacteriana confere redução no pH do lúmen intestinal, inibe o crescimento de bactérias patogênicas, modula secreções biliares e pancreáticas, fornece energia para a proliferação de células epiteliais (enterócitos), auxilia na manutenção da integridade da parede celular (produção de muco) e viabiliza uma maior capacidade de absorção intestinal das aves (MROZ et al., 2006).

A presença de microrganismos patogênicos *(Salmonella, E. coli e Clostridium* spp.) no trato digestório das aves é uma ocorrência natural, no entanto, uma situação de desequilíbrio da população intestinal pode alterar a proporção de bactérias benéficas e patogênicas. Esse evento pode desencadear infecções intestinais, como colibacilose, enterite necrótica e outras doenças que são de grande preocupação na avicultura, levando a enormes perdas econômicas (SNEERINGER et al., 2015).

Para solucionar esse problema, a indústria ao longo dos anos fez uso da utilização de antibióticos, uma alternativa viável para o controle de microrganismos patogênicos do trato digestório das aves, além de atuar na melhora do desempenho animal. No entanto, a exposição contínua ao uso de APCs na produção animal, tem desencadeado aumento na pressão de seleção de cepas bacterianas resistentes (DAVIS et al., 2011).

Um antibiótico deve apresentar um modo de ação seletivo, atuando apenas sobre o microrganismo patógeno, mas não no hospedeiro. O antibiótico deve atuar em estruturas e

funções microbianas distintas daquelas encontradas no hospedeiro, através dos processos de inibição da síntese de proteínas, da síntese de parede celular, de ácidos nucléicos e provocar danos à membrana plasmática (TORTORA, 2017).

A resistência a antibióticos pelas bactérias patogênicas de humanos e animais pode ser desencadeada e compartilhada através de mecanismos de transformação e conjugação, que podem ser rapidamente disseminados dentro das comunidades microbianas (DIARRA; MALOUIN, 2014).

Dessa forma, a resistência pode ser expressão através de propriedades intrínsecas ou adaptativas, sendo a primeira uma propriedade genética estável, codificada no cromossoma do DNA e compartilhada por todos os membros do gênero. No entanto, a propriedade adquirida ocorre quando há uma mudança no DNA bacteriano, de modo que um novo traço fenotípico pode ser expresso (BEZERRA; et al., 2017).

3.3 *BACILLUS* COM AÇÃO PROBIÓTICA

Em termos conceituais os probióticos podem ser definidos como microrganismos vivos que quando administrados em quantidades adequadas, conferem um efeito benéfico a saúde do hospedeiro (AL-KHALAIFAH, 2018; FULLER, 1989). Muito embora, os probióticos já existam há algumas décadas, o uso desse aditivo na nutrição animal vem apresentando maior destaque em decorrência da preocupação crescente do público consumidor com relação à segurança alimentar e qualidade dos produtos de origem animal (VOHRA; SYAL; MADAN, 2016).

Atualmente, os probióticos apresentam uma ampla variedade de espécie de microrganismos como bactérias e fungos, formados por cepas de *Bacillus, Lactobacillus, Bifidobacterium, Streptococcus* e *Saccharomyces* (DOWARAH; VERMA; AGARWAL, 2017). Tais microrganismos apresentam atividade capaz de reduzir bactérias prejudiciais (*Salmonella typhimurim, Staphylococcus aureus, Escherichia coli* e *Clostridium perfringens*) (PALAMIDI et al., 2016; AL-KHALAIFAH, 2018). Nesse sentido, cepas de alguns *Bacillus* sp. têm sido utilizadas como aditivos dietéticos em rações para animais. O gênero *Bacillus* sp. representa um grupo de bactérias gram-positivas, aeróbicas ou anaeróbicas facultativas formadoras de endósporos. A capacidade de formar esporos é benéfica e permite seu armazenamento por longo prazo, sem causar perdas de viabilidade em comparação com aqueles que contêm bactérias não formadoras de esporos (MINGMONGKOLCHAI; PANBANGRED, 2018).

Estudos envolvendo espécies de *Bacillus sp*. para fins medicinais, datam de 1958. No entanto, o interesse científico por espécies de *Bacillus sp.* como probióticos, ocorreu apenas nas últimas duas décadas, sendo as espécies mais extensivamente estudadas nos últimos anos *Bacillus subtilis, Bacillus clausii, Bacillus cereus, Bacillus coagulaans* e *Bacillus licheniformis* (CUTTING, 2011).

A utilização de *B. subtilis* como aditivo é uma realidade na avicultura, dada sua característica favorável ao crescimento e manutenção da microbiota intestinal benéfica, capacidade de adaptação e sobrevivência à passagem pelo trato digestório das aves, além de sua aplicabilidade no processamento industrial de rações (TEO; TAN, 2007). Essas características são especialmente importantes para aquelas bactérias destinadas ao uso em rações, nas quais teriam que sobreviver a altas temperaturas durante o processo de peletização e, após a ingestão, ação lítica dos sais biliares no trato intestinal da ave (TEO; TAN, 2006).

Dentre as várias cepas de *B. subtilis*, o *B. subtilis* PB6 representa uma cepa natural, isolada do intestino de poedeiras saudáveis. Estudos realizados in vitro com o *B. subtilis* PB6, apontam para a produção de um composto biologicamente ativo com ação antimicrobiana, que são as surfactinas. As surfactinas são lipopeptídeos cíclicos anfipáticos pertencentes à família de peptídeos não ribossômicos, com propriedades emulsificantes, antibacterianas, antivirais e antitumorais (JAYARAMAN et al., 2013). Já o aditivo probiótico formado pela combinação das cepas de *B. subtilis* BS–009, BS–020 e BS–024, apresenta propriedades para formar endósporos altamente resistentes, além de sintetizar compostos antimicrobianos e diferentes enzimas exógenas.

Para que possam exibir efeitos benéficos no hospedeiro, as bactérias probióticas devem ser capazes de sobreviver, colonizar e persistir temporariamente no trato gastrointestinal do animal. Para isso, a cepa probiótica deve apresentar capacidade de produzir esporos, ser termoestável, não apresentar patogenicidade à espécie hospedeira e tolerar as condições ácidas do trato digestivo (MINGMONGKOLCHAI; PANBANGRED, 2018). Diante desse cenário, a abordagem de aditivos probióticos na nutrição animal apresenta-se como uma alternativa em relação ao uso dos antibióticos (quimioterápicos e fármacos), proporcionando benefícios ao hospedeiro, como: inibição de microrganismos patógenos do trato digestivo, imunoestimulação, aproveitamento dos nutrientes e melhora nos parâmetros produtivos (PARK et al., 2016; VOHRA; SYAL; MADAN, 2016).

3.4 MECANISMOS DE AÇÃO DOS PROBIÓTICOS

O efeito benéfico dos microrganismos probióticos pode ser representado por mecanismos de ação, por meio dos quais, pode inibir o crescimento e proliferação de comunidades bacterianas indesejáveis no trato digestivo do animal hospedeiro. Os mecanismos de ação incluem competição por sítios de adesão no epitélio intestinal ou exclusão competitiva, competição por nutrientes, produção de bacteriocinas, ácidos graxos voláteis e modulação do sistema imune (KHAN; NAZ, 2013).

3.4.1 Exclusão competitiva

O mecanismo de exclusão competitiva desempenhado pelas bactérias probióticas, diz respeito à ocupação física dos sítios de adesão no epitélio intestinal, evitando a formação de colônias de bactérias patogênicas. Tal processo ocorre em locais específicos de adesão, como vilosidades intestinais e criptas, além de provocar a síntese e excreção de mucinas (MUC2 e MUC3) pelas células caliciformes que inibem a aderência de bactérias enteropatogênicas (CHICHLOWSKI et al., 2007).

A superfície da mucosa intestinal é revestida por uma camada epitelial simples, formada por células absortivas e caliciformes, revestida pelo glicocálice. A camada de glicocálice apresenta consistência viscosa, composta por glicolipídeos, glicoproteínas (mucina), imunoglobulinas e eletrólitos. Sua principal função é proteger o epitélio intestinal contra danos mecânicos e proteger o hospedeiro contra infecções bacterianas (BRON; VAN BAARLEN; KLEEREBEZEM, 2012; MONTEAGUDO-MERA et al., 2019).

A aderência das bactérias à mucosa intestinal, pode ser possível através de proteínas de ligação com especificidade à superfície de membrana da mucosa ou através das fimbrias (MONTEAGUDO-MERA et al., 2019). As fimbrias são estruturas de superfície que fornece às bactérias um vínculo com seu ambiente externo, possibilitando interagir e se ligar a células do hospedeiro (HOSPENTHAL; COSTA; WAKSMAN, 2017). Através das lectinas presentes em sua constituição, as fimbrias são capazes de reconhecer oligossacarídeos específicos dos sítios de ligação da parede intestinal, essas estruturas fornecem às bactérias uma vantagem para colonização das superfícies da mucosa (HOSPENTHAL; COSTA; WAKSMAN, 2017).

Ademais, as bactérias probióticas podem desencadear um efeito biológico, através da síntese de substâncias inibitórias por meio da fermentação de nutrientes não absorvidos no intestino delgado (REVOLLEDO; FERREIRA; MEAD, 2006). A produção de ácidos graxos voláteis pelo metabolismo das bactérias probióticas, resulta em menor pH intestinal, condição

essa adversa para o crescimento de microrganismos patogênicos, *Salmonella* e *Escherichia coli* (BERMUDEZ-BRITO et al., 2012). A produção de metabólitos bacterianos desempenha papel fundamental, não apenas contra bactérias patogênicas, mas podem também regular o estabelecimento e interação de comunidades bacterianas benéficas (NAVA et al., 2005).

3.4.2 Competição por nutrientes

A competição por nutrientes pode ser um dos mecanismos de resistência à colonização de patógenos no trato gastrointestinal. As bactérias probióticas presentes no intestino, utilizam os nutrientes presentes no lúmen, reduzindo de modo significativo a quantidade de nutrientes para as bactérias patogênicas (BAJAJ; CLAES; LEBEER, 2015). Isso ocorre quando as bactérias probióticas utilizam para sua nutrição os nutrientes dos ingredientes que foram parcialmente degradados pelas enzimas digestivas normais dos animais ou que foram intencionalmente adicionadas à ração (SAKOMURA et al., 2014).

3.4.3 Estímulo ao sistema imune

Um intestino saudável é de grande importância para o metabolismo animal, não apenas pela função de absorção de nutrientes, mas também como barreira de defesa inata contra a maioria dos patógenos intestinais (BRON; VAN BAARLEN; KLEEREBEZEM, 2012). Embora a relação entre os microrganismos probióticos e a imunomodulação não esteja totalmente elucidada, a aderência de algumas bactérias probióticas, pelo menos temporária, à mucosa gastrointestinal pode ser necessária para estimular o sistema imunológico do hospedeiro (HEMARAJATA; VERSALOVIC, 2013).

A atuação dos probióticos na resposta imunológica aumenta a atividade dos macrófagos, elevando os níveis de anticorpos locais, induzindo a regulação de interferon e células natural killers (DOWARAH; VERMA; AGARWAL, 2017). Os probióticos podem interagir com os receptores de reconhecimento padrão, como os receptores Toll-Like de células dentríticas e macrófagos por meio de padrões moleculares associados aos patógenos (PAMPs) que estão presentes na superfície da célula bacteriana ou que podem ser secretados no ambiente extracelular (MONTEAGUDO-MERA et al., 2019). Ademais, os probióticos podem ser capazes de fortalecer a barreira intestinal, mantendo o complexo de junções célula–célula impermeáveis e induzindo a produção de mucina. A imunomodulação mediada por probióticos favorece a secreção de citocinas por vias de sinalização, como fator nuclear kappa B (NF-kB) e das proteínas quinases ativadas por mitógenos (MAPK), que também podem

afetar a proliferação e diferenciação de células imunes (como células T) ou células epiteliais (THOMAS; VERSALOVIC, 2010).

3.5 IMPLICAÇÃO ZOOTÉCNICA DO USO DE PROBIÓTICOS NA AVICULTURA

A produção de proteína (carne e ovos) é o objetivo elementar na cadeia avícola, no que diz respeito à oferta de alimento seguro e de qualidade. A utilização de probióticos na avicultura tem ganhado destaque em decorrência dos benefícios sobre a saúde dos animais, integridade do trato digestório e digestibilidade dos nutrientes da dieta, melhorando a eficiência alimentar das aves.

Jayaraman et al. (2017), ao avaliarem os efeitos da suplementação de *B. subtilis* PB6 (5×10^{11} UFC/kg) na dieta de frangos de corte de 1 a 35 dias, sobre a histomorfometria intestinal, verificaram melhora significativa na altura das vilosidades e profundidade de cripta do duodeno em comparação aos tratamentos contendo avilamicina e bacitracina metileno disalicilato. As medidas de altura das vilosidades e profundidade de cripta são geralmente usadas para avaliar a integridade intestinal. O mecanismo pelo qual a inclusão do probiótico melhora a morfologia intestinal, pode estar associado à sua capacidade de produzir substâncias semelhantes aos antibióticos, que podem proteger as vilosidades contra patógenos e toxinas e modificar bactérias intestinais (XING et al., 2015). O aumento da altura de vilosidade é sugestivo a uma maior área destinada à absorção dos nutrientes disponíveis no trato digestivo. No entanto, uma menor altura de vilosidade e criptas mais profundas podem ser responsáveis por uma má absorção de nutrientes, aumento da secreção de muco no trato gastrointestinal e desempenho reduzido dos animais (XU et al., 2003).

Hernandez-Patlan et al. (2019), observaram efeito positivo de *B. subtilis* sobre o ganho de peso e composição da população microbiana intestinal de frangos de corte aos 21 dias submetidos a um modelo de desafio, em relação ao grupo desafiado sem suplementação de probiótico na dieta. O grupo desafiado e com suplementação de *B. subtilis* apresentou redução de bactérias indesejáveis, enquanto os gêneros de *Lactobacillus* e *Bacillus* aumentaram.

Latorre et al. (2017), ao avaliarem a suplementação de *B. subtilis* (10^6 esporos/grama de ração) sobre a microbiota intestina de frangos de corte não desafiados, aos 28 dias de idade, constataram menor número de bactérias gram-negativas e aumento no número de bactérias lácticas em comparação com a microbiota do grupo não suplementado.

Darsi & Zhaghari (2021) verificaram que a suplementação de *B. subtilis* PB6 (2×10^7 UFC/kg) na dieta de matrizes pesadas com 57 semanas de idade, proporcionou melhora na

qualidade de ovos, unidade Haugh e espessura de casca, em comparação com aves alimentadas com dieta controle sem probiótico. A melhora da qualidade de casca de ovos provenientes de aves recebendo dieta com probióticos, pode estar associada à ação benéfica dos probióticos sobre o ambiente digestivo, permitindo uma condição favorável para maior absorção intestinal de cálcio, condição essa, decorrente da maior digestibilidade aparente do cálcio dietético, resultando em maiores níveis plasmáticos de cálcio durante a formação da casca (WANG et al., 2021).

Sobczak; Kozłowski, (2015) avaliaram os efeitos da suplementação de *B. subtilis* PB6 (1 x 10^8 UFC/kg) na dieta de poedeiras entre 18 a 42 semanas de idade, sobre o desempenho e qualidade de ovos. Os autores verificaram melhora significativa para qualidade de casca e unidade Haugh de ovos de aves recebendo dieta com probiótico em comparação às galinhas do tratamento controle.

Para Abdelqader; Al-Fataftah; Das (2013), os resultados para maior qualidade de ovos em aves suplementadas com *B. subitilis*, está relacionado à melhor absorção dos nutrientes da dieta. Ademais, Wang et al. (2021), reforçam que a melhor absorção é influenciada pela maior disponibilidade de nutrientes, visto que os microrganismos probióticos têm a capacidade de produzir enzimas digestivas endógenas ao longo do trato digestivo da ave, potencializando a digestão de nutrientes.

3.6 UTILIZAÇÃO DO CROMO ORGÂNICO NA NUTRIÇÃO

O cromo é considerado um mineral que necessita de maior investigação para que seja possível definir sua essencialidade na nutrição de aves. Sands & Smith, (1999) fornecem evidências de que o cromo exerce papel nutricional e fisiológico importante na nutrição de aves, tendo seus efeitos evidenciados sob condições de fatores estressores ambientais, nutricionais e hormonais. Ademais, a suplementação dietética de cromo pode afetar de maneira positiva a taxa de crescimento e a eficiência alimenta de aves em crescimento, particularmente em aves criadas sob condições de estresse por calor ou frio.

A Food and Drug Administration nos Estados Unidos aprovou a utilização do propionato de cromo como aditivo para frangos de corte em concentrações de até 0,20 mg Cr/kg (FDA, 2019). O cromo se caracteriza como um micro mineral e se apresenta na forma de complexos orgânicos (25 a 30% de disponibilidade) e inorgânicos (cerca de 13%) (PIVA et al., 2003). As formas orgânicas são cromo–L–metionina, complexo cromo-ácido-nicotínico, cromo picolinato, propionato de cromo e cromo levedura. A forma inorgânica é o cloreto de

cromo o qual apresenta menor taxa de absorção e menor atividade biológica quando comprado às formas orgânicas (SWAROOP et al., 2019).

As atribuições para a menor disponibilidade das fontes inorgânicas em relação as fontes orgânicas estão relacionadas à formação de óxido crômico insolúvel, à ligação com agentes quelantes naturais presentes nos alimentos (fitato, por exemplo) e à interferência de formas iônicas de outros elementos bem como baixa ou nenhuma conversão de cromo inorgânico para a forma bioativa (MOEINI et al., 2011).

De modo geral, o cromo está envolvido no metabolismo de carboidratos, lipídeos e ácidos nucleicos em diferentes espécies animais (ANDERSON, 1997). No metabolismo dos carboidratos, tem participação importante como componente integral da cromodulina, que é uma unidade oligopeptídica de ligação ao cromo com baixo peso molecular, que atua no processo de sinalização da insulina através de membranas celulares (VINCENT, 2000), o que possibilita o estímulo da captação de glicose pelas células do tecido alvo.

É conhecido que o cromo também tem participação na regulação do estresse, reduzindo os níveis dos glicocorticóides, refletindo em melhora sobre o desempenho, qualidade da carne e resistência a patógenos (OBA et al., 2012). De acordo Jackson et al. (2008), os resultados de pesquisa envolvendo a utilização de cromo na nutrição de aves são um tanto inconsistentes, ao que diz respeito ao não verificar efeito positivo sobre as mesmas características de desempenho estudadas em diferentes estudos. Para o autor isso se justifica pelo fato dos trabalhos anteriores utilizarem fontes de cromo com baixa disponibilidade.

4 ARTIGO I

Este capítulo é apresentado de acordo com as normas para publicação no Periódico **Animals.**

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Article

The Impact of *Bacillus subtilis* PB6 and Chromium Propionate on the Performance, Egg Quality and Nutrient Metabolizability of Layer Breeders

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Simple Summary: Environmental conditions can generate heat stress in poultry production. Feed additives have been supplemented in diets for layers and broilers to improve their health status, immune system and nutrition, as well as productive performance. This paper shows the effects of probiotic *Bacillus subtilis* PB6 and chromium propionate diet supplementation on productive parameters, egg and eggshell quality, excreta moisture, cortisol and serum biochemistry of laying breeder hens. Diets supplemented with *Bacillus subtilis* PB6, chromium propionate or a combination of the two resulted in improved egg production, feed conversion ratio, eggshell quality and nutrient metabolizability, without modifying the main serum biochemical parameters of hens from 55 to 70 weeks of age. Highlighted improvements in hen performance and eggshell quality were observed when hens were fed diets supplemented with a combination of the probiotic and chromium. This study expands our understanding concerning the combined supplementation of probiotics and organic chromium for poultry.

Abstract: The objective of this study was to evaluate the effects of *Bacillus subtilis* PB6, chromium propionate or a combination of the two on the performance, egg and eggshell quality, nutrient metabolizability and serum biochemistry of layer breeders. White Plymouth Rock and Red Rhodes Island breeder hens at 55 weeks of age were allocated in individual cages using a completely randomized block design with 16 replicates. Hens were fed control, control + probiotic (500 g/ton of *Bacillus subtilis* PB6), control + CrProp (50 g/ton of chromium propionate) and control + probiotic + CrProp diets from 55 to 70 weeks of age. Productive parameters and eggshell quality as well as cortisol and blood biochemistry were grouped each 28 d as well as for the overall period. The metabolizability of nutrients and energy was determined at 70 weeks of age. In the overall period, hens fed the control + probiotic or control + probiotic + CrProp diets had significantly higher egg production, egg mass, shell percentage, thickness and shell strength. The metabolizability of dry matter, nitrogen and energy increased in hens that were fed the control + probiotic + CrProp diet. In conclusion, diets supplemented with *Bacillus subtilis* PB6 and chromium propionate resulted in improved productive performance, eggshell quality and nutrient metabolizability of layer breeders, without modifying serum cortisol, albumin and triglycerides.

Keywords: blood biochemistry; breeder hen; eggshell quality; organic chromium; probiotic

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1. Introduction

Feed additives have been increasingly used in animal nutrition due to their remarkable benefits. The use of probiotics has aroused widespread interest in poultry farms,

being related to improvements in the immune system of hens, while beneficially affecting the hosts by modifying their intestinal microbiome as well as improving feed efficiency, digestion and production performance [1–3].

Probiotic effectiveness for poultry may depend on several factors, including microbial species composition, diets, supplemental dose, combination with other additives and environmental stress factors [4]. *Bacillus subtilis* PB6 is a natural strain isolated from healthy chicken gut that produces antimicrobial components with broad activity against microorganisms [5,6]. In laying hens, *Bacillus subtilis* PB6 increased feed efficiency and reduced excreta moisture as well as cholesterol content in egg yolk and serum [7,8]. Other beneficial effects of this probiotic for hens were improvements in egg production, egg quality, nutrient digestibility and return on investment [9–11].

Heat stress is considered as one of the most important environmental stressors that modifies the oxidant/antioxidant system and compromises the health status of laying hens, resulting in their poor performance worldwide [12]. It has been reported that dietary *Bacillus subtilis* supplementation could reduce heat stress in birds [13]. However, organic chromium has been the main additive that has demonstrated positive effects against stress conditions in birds; several researchers have started exploring the effect of chromium on laying hens, breeders and broilers, for different requirements [14–16]. The US Food and Drug Administration approved chromium propionate (CrProp) as an additive for broiler feeds at concentrations up to 0.20 mg Cr/kg [17].

Chromium has been supplemented from different sources and levels, and it has been shown to present antioxidant properties, which help to attenuate the negative effects of oxidative stress, while contributing to lipid, protein and nucleic acid metabolism [18]. Furthermore, chromium has also been related to improvements in cell preservation and immune responses, which contribute to animal homeostasis and thermoregulatory capacity under heat stress conditions [19]. In laying hens, chromium propionate supplementation resulted in increased egg production, feed efficiency and egg quality, whereas it reduced egg cholesterol levels and serum cortisol [16,20,21].

The effect of *Bacillus subtilis* or chromium propionate on performance improvements in broilers is well-documented; however, more data are needed on when these additives are supplemented in diets for layer or breeder hens as well as when both additives are combined. The objective of this study was to evaluate the effects of *Bacillus subtillis* PB6, chromium propionate or a combination of the two on the performance, egg and eggshell quality, nutrient metabolizability and serum biochemistry of laying breeder hens of 55 to 70 weeks of age.

2. Materials and Methods

All procedures involving live-bird management and healthcare were approved by the Ethics and Research Committee of the Federal University of Santa Maria, Santa Maria, Rio Grande do Sul, Brazil (approval number: 5404280717).

2.1. Birds and Treatments

A total of 32 White Plymouth Rock and 32 Red Rhodes Island laying breeder hens at 50 weeks of age, obtained from the Poultry Science Laboratory (LAVIC-UFSM, Santa Maria, RS, Brazil), were allocated in a conventional poultry house, weighed and placed in individual wire cages (0.33 m length × 0.46 m deep × 0.40 m height) until the end of the experiment at 70 weeks of age. Hens had free access to water and mash feeds. The lighting program was a 16L:8D cycle.

Hens were distributed by weight and egg production before starting the experiment in a completely randomized block design, where each treatment was composed of 8 White Plymouth Rock and 8 Red Rhodes Island breeder hens. Hens were subjected to a 4-week adjustment period to the experimental diets and then fed the experimental feeds from 55 to 70 weeks of age. Birds were fed 4 dietary treatments with 16 replicates, and the

experimental unit was the individually caged hen. Measurements were done in 28 d periods, defined as 55 to 58, 59 to 62, 63 to 66 and 67 to 70 weeks of age.

2.2. *Experimental Diets*

All ingredients utilized during the study were from the same batch. A common control diet (Table 1), formulated of corn, soybean meal and wheat bran, was mixed, and afterwards the additives were supplemented to obtain the other dietary treatments. Treatments consisted of control (control; without additives); control + probiotic (control diet supplemented with *Bacillus subtilis* PB6 probiotic); control + CrProp (control supplemented with chromium propionate); and control + probiotic + CrProp (supplemented with both the probiotic and chromium propionate) diets.

Table 1. Ingredient and nutrient composition of the control diet.

Item	Control Diet (55 to 70 Weeks of Age)
Ingredients, %	
Corn	58.52
Soybean meal, 46 % CP	19.64
Wheat bran	4.00
Soybean oil	2.36
Dicalcium phosphate	1.52
Limestone	12.84
Salt	0.43
DL-Methionine, 99%	0.30
L-Lysine-HCl, 78%	0.15
L-Threonine, 98.5%	0.09
Mineral and vitamin premix [1]	0.15
Nutrient and energy composition, % or as shown	
AME, kcal/kg	2750
Crude protein	14.50
Calcium	4.30
Available phosphorus	0.35
Total phosphorus	0.53
Sodium	0.18
Potassium	0.59
Chloride	0.35
Dig. Lys [2]	0.75
Dig. Met + Cys	0.70
Dig. Thr	0.58
Dig. Trp	0.15

[1] Composition per kilogram of feed: vitamin A, 8000 IU; vitamin D3, 2000 IU; vitamin E, 30 IU; vitamin K3, 2 mg; thiamine, 2 mg; riboflavin, 6 mg; pyridoxine, 2.5 mg; cyanocobalamin, 0.012 mg, pantothenic acid, 15 mg; niacin, 35 mg; folic acid, 1 mg; biotin, 0.08 mg; iron, 40 mg; zinc, 80 mg; manganese, 80 mg; copper, 10 mg; iodine, 0.7 mg; and selenium, 0.3 mg. [2] Digestible-amino-acids-to-digestible-Lys ratios were maintained at TSAA 0.94; Thr 0.77; Val 0.77; Trp 0.20; Arg 1.13; and Ile 0.67.

The probiotic was supplemented at 500 g/ton from a commercial *Bacillus subtillis* PB6, PTA-6737 + 98% calcium carbonate (CLOSTAT® Dry; Kemin Industries Inc., Des Moines, IA, USA) with 2.0×10^{11} CFU/g. The organic chromium was supplemented at 50 g/ton from a commercial product (KemTRACE® Chromium 0.4% Dry; Kemin Industries Inc., Des Moines, IA, USA), an organic-compliant form of chromium propionate.

2.3. *Hen Performance and Egg Quality*

Hens were fed twice a day and eggs were collected four times per day. Egg production by each hen was recorded daily. Feed intake (g/bird/day), feed conversion (kg feed/kg eggs and kg feed/dozen eggs), egg production (%), egg loss (%) and dirty eggs (%) were grouped per period of 28 d. The egg loss (%) was considered as eggs that were broken, cracked, porous or thin-shelled.

Daily egg mass was calculated by multiplying the laying rate (%) by the average weight of eggs (g) divided by 100. At the end of each 28 d period, average egg weight, specific weight, albumen height, percentage and shell thickness were measured for 4 consecutive days. All intact eggs from each hen were identified and individually weighed. The specific weight was determined using the flotation method in saline solution, where seven saline solutions were prepared, ranging from 1.070 to 1.094 g/cm^3 with a variation of 0.004 g/cm^3 for each solution. After the test, all eggs were used to determine the albumen height. Measurements in millimeters (mm) were related to egg weight to determine the Haugh unit: HU log $100(H + 7.57 - 1.7 W^{0.37})$, in which H = albumen height (mm) and W = egg weight (g).

Yolk and albumen weights were also obtained to determine yolk and albumen percentages of the entire egg. The yolk index (YI) was assessed by measuring yolk width (YW) and yolk height (YH), and was the ratio between these two parameters as YI = YH/YW. The percentage of moisture and total solids of yolks were determined using one egg per cage (method 950.46 [22]). The yolk samples were placed in individual plastic drying containers and taken to a forced-air oven with a temperature of 55 °C for 72 h (Marconi, MA035, Piracicaba, SP, Brazil). Then, total solids were evaluated in an oven at 105 °C for 24 h (method 934.01 [23]).

Shells were washed and dried at room temperature for 72 h, then weighed using a precision digital scale (0.001 g; Bioscale, São Paulo, SP, Brazil). Shell percentage was expressed relative to the egg weight. After weighing the shells, shell thickness was measured at 3 points in the central region of each shell without the external membrane using a digital micrometer (Mitutoyo Sul Americana, Sao Paulo, SP, Brazil).

All intact eggs produced on day 28 of each period were used for the measurement of shell strength (N) and yolk strength (N), which were obtained with a texturometer, a TA.XT2 Texture Analyzer with a cylindrical, stainless steel 6 mm probe (Texture Technologies Corp. and Stable Micro Systems Ltd., Hamilton, MA, USA) following the methodology described by Stefanello et al. [24]. After shell strength evaluation, the egg was broken to measure the yolk vitelline membrane strength, which was also obtained with a texturometer, a TA.XT2 Texture Analyzer with a cylindrical, stainless steel 2 mm probe.

2.4. *Blood Chemistry*

Blood was collected from 10 random hens before the start of the experimental period. At the end of each 28 d period, blood was collected from the wing vein of 8 hens per treatment and centrifuged (3400 RPM for 20 min), and the obtained serum was stored at −80 °C. The serum was used to determine glucose, total cholesterol, albumin and triglycerides using a BS-120 automatic biochemical analyzer (Mindray Headquarters, Nanshan, Shenzhen, China). Commercial kits (Bioclin®, Quibasa, Belo Horizonte, MG, Brazil) were used following the methodology proposed by the manufacturer for albumin, cholesterol, triglycerides and glucose. Serum cortisol (n = 32 per period) was determined using a chemiluminescence immunoassay kit. Samples were automatically diluted by the biochemical analyzer in the proportion of 1:10 (sample to distilled water), reducing the effects of lipemia, hemolysis and jaundice that any sample could present.

2.5. *Excreta Moisture*

At the end of each 28 d period, excreta were collected on trays covered with plastic placed under the cages. Excreta samples from each experimental unit were pooled by cage, mixed and stored at −20 °C until analysis. Subsequently, excreta were weighed and dried in a forced-air oven at 55 °C until reaching constant weight and ground to pass a 0.5 mm screen (Tecnal, R-TE-648, Sao Paulo, SP, Brazil). Moisture analysis of excreta was performed after oven-drying the samples at 105 °C for 16 h (method 934.01 [23]).

2.6. Total Tract Metabolizability

On the last day of the experiment (at 70 weeks of age), excreta were collected per cage to evaluate the apparent metabolizable energy (AME) and the apparent total tract metabolizability coefficients of dry matter (DM), nitrogen and energy. Celite at 1% was used in each experimental feed, and diets with markers were provided for 3 days before the excreta collection.

Excreta were collected twice daily for 2 days, immediately mixed and pooled by cage and stored at −20 °C until analysis. Prior to calorimetry, excreta were dried in a forced-air oven at 55 °C and ground to pass a 0.5 mm screen. Analysis of the concentration of dry matter of diets and excreta was performed after oven-drying the samples at 105 °C for 16 h. Diets and excreta were also analyzed for gross energy (GE) using an adiabatic bomb calorimeter (Parr Instrument Company, 6400 Calorimeter, Moline, IL, USA) with benzoic acid as a calibration standard. The nitrogen concentration of diets and excreta was determined via the dry combustion method using a CN analyzer (Thermo-Finnigan Flash EA 1112, Waltham, MA, USA). Acid insoluble ash concentrations in the diet and excreta samples were determined using the method described by Vogtmann et al. [25] and Choct and Annison [26].

Apparent total tract metabolizability coefficients of DM, nitrogen and energy were calculated using the following equation [27,28]:

$$\text{Metabolizability coefficient} = 1 - [(\text{Mi}/\text{Mo}) \times (\text{Eo}/\text{Ei})],$$

where Mi and Mo are the concentration of acid insoluble ash in diet and excreta (g/kg of DM) samples, respectively; Ei and Eo are the concentration of nitrogen or DM (g/kg of DM) or GE (kcal/kg of DM) in diet and excreta samples, respectively.

The AME (kcal/kg) of experimental feeds was calculated using the analyzed content of acid insoluble ash and GE as previously described by Kong and Adeola [27], Stefanello et al. [28] and Haetinger et al. [29].

2.7. Statistical Analysis

Data were submitted to the normality test and Levene's test for homogeneity of variance and were transformed using the arcsine square root percentage (z = arcsine (sqrt (y/100)) whenever not normally distributed [30]. Data were subjected to one-way analysis of variance using the MIXED procedure of SAS [31], and means were compared by the Tukey test at $p < 0.05$.

3. Results

Analysis of *Bacillus subtilis* PB6 in the experimental diets showed that the supplemental probiotic had in-feed concentration in agreement with the expected values. *Bacillus subtilis* PB6 was not detected in the control or control + CrProp feeds; however, the recovery of *Bacillus subtilis* in the control + probiotic feed was 6.8×10^7 CFU/g and the control + probiotic + CrProp feed had 7.0×10^7 CFU/g. Chromium was supplemented at 0.20 mg/kg of feed and it was not detected in water samples collected on the first and last day of the study.

No effect was observed between the two strains of layer breeders, previously blocked in the experimental design, and only treatments were considered as an independent variable. There were no effects ($p > 0.05$) of dietary treatments on body weight, feed intake, egg loss, dirty eggs and mortality evaluated per 28 d period or in the overall period.

Productive performance and excreta moisture of layer breeders are presented in Table 2 and demonstrate that the probiotic and organic chromium supplementation improved FCR, egg production and egg mass ($p < 0.05$). From 59 to 62 and 63 to 66 weeks of age, hens fed the control + probiotic + CrProp diet had lower excreta moisture compared to birds fed the control diet ($p = 0.047$ and $p = 0.052$, respectively); however, the excreta moisture was not different among treatments in the overall period from 55 to 70 weeks of age.

Table 2. Productive performance and excreta moisture of layer breeders fed diets supplemented with the probiotic and chromium propionate from 55 to 70 weeks of age.

Item	BW, g	EP [1], %	FI [2], g/hen/d	Egg Loss, %	Dirty Eggs, %	Egg Mass, g	FCR [3], kg/kg	FCR, kg/dz	Excreta Moisture, %
Period of 55 to 58 weeks of age									
Control	2163	69.9	130	11.2	6.7	40.3	3.25	2.22	79.9
Control + probiotic [4]	2161	72.6	130	9.1	6.4	43.3	3.03	2.20	79.6
Control + CrProp [5]	2095	71.9	128	9.4	6.4	41.6	3.09	2.15	79.2
Control + probiotic + CrProp	2112	73.2	129	3.9	6.4	42.4	3.04	2.07	79.0
SEM	22.8	0.88	1.7	1.6	0.9	0.52	0.05	0.04	0.19
p-value	0.648	0.574	0.918	0.394	0.974	0.216	0.464	0.620	0.355
Period of 59 to 62 weeks of age									
Control	2144	67.8	131	6.7	8.5	39.1	3.43	2.49	79.4^{a}
Control + probiotic	2159	72.6	129	3.7	5.3	42.9	3.07	2.19	77.8^{b}
Control + CrProp	2062	70.7	127	4.5	7.2	41.9	3.05	2.10	79.0^{ab}
Control + probiotic + CrProp	2076	72.8	130	2.8	3.7	42.4	3.09	2.08	77.5^{b}
SEM	23.0	1.19	1.6	2.0	1.3	0.70	0.06	0.06	0.29
p-value	0.345	0.429	0.858	0.517	0.621	0.237	0.102	0.068	0.047
Period of 63 to 66 weeks of age									
Control	2143	64.6^{b}	129	9.0	9.2	37.6^{b}	3.49^{a}	2.41	79.0^{a}
Control + probiotic	2177	68.9^{ab}	127	6.2	6.1	41.0^{a}	3.13^{b}	2.25	77.3^{ab}
Control + CrProp	2061	70.1^{a}	127	8.1	7.8	40.2^{ab}	3.19^{ab}	2.21	77.3^{ab}
Control + probiotic + CrProp	2078	72.3^{a}	123	2.8	7.1	41.9^{a}	2.94^{b}	2.17	76.2^{b}
SEM	24.7	0.99	1.90	1.4	1.3	0.61	0.07	0.05	0.40
p-value	0.301	0.042	0.718	0.392	0.862	0.059	0.025	0.341	0.052
Period of 67 to 70 weeks of age									
Control	2099	63.4^{b}	121	8.3	10.8	36.8^{b}	3.39^{a}	2.53^{a}	77.4
Control + probiotic	2170	69.2^{a}	123	6.2	7.3	41.0^{a}	3.05^{ab}	2.19^{ab}	76.9
Control + CrProp	2008	69.5^{a}	120	5.9	5.8	41.4^{a}	2.89^{b}	2.18^{ab}	76.2
Control + probiotic + CrProp	2059	70.8^{a}	122	5.9	5.5	42.4^{a}	2.95^{b}	2.10^{b}	75.5
SEM	25.8	1.04	2.13	1.1	1.5	0.65	0.07	0.08	0.43
p-value	0.150	0.049	0.933	0.859	0.573	0.009	0.059	0.190	0.422
Overall period (55 to 70 weeks of age)									
Control	2138	66.4^{b}	128	8.8	8.8	38.4^{b}	3.39^{a}	2.41^{a}	78.9
Control + probiotic	2167	70.8^{a}	127	6.3	6.3	42.1^{a}	3.07^{b}	2.21^{ab}	78.0
Control + CrProp	2056	70.5^{ab}	125	6.5	7.0	41.3^{a}	3.06^{b}	2.16^{b}	77.9
Control + probiotic + CrProp	2081	72.3^{a}	126	5.7	3.9	42.3^{a}	3.00^{b}	2.10^{b}	77.0
SEM	22.3	0.77	1.42	0.99	0.86	0.46	0.05	0.04	0.26
p-value	0.279	0.043	0.914	0.715	0.230	0.009	0.012	0.051	0.443

[1] EP = egg production. [2] FI = feed intake. [3] FCR = feed conversion ratio. [4] *Bacillus subtilis* PB6, PTA-6737 supplemented at 500 g/ton. [5] Chromium propionate supplemented at 50 g/ton. [a,b] Means with different superscript letters differ significantly ($p < 0.05$) based on the Tukey test.

Egg production increased when layer breeders were fed the control + CrProp or control + probiotic + CrProp diets from 63 to 66 weeks of age (p = 0.042), and hens fed control + probiotic or control + probiotic + CrProp diets presented higher egg mass (p = 0.059) and lower FCR (kg/kg) (p = 0.025) in this period. From 67 to 70 weeks of age, layer breeders fed diets supplemented with the probiotic, CrProp or both had higher egg production and egg mass than the non-supplemented hens ($p < 0.05$). The combination between the probiotic and chromium supplemented in hens' diets resulted in the lowest FCR in kg/kg and kg/dz from 67 to 70 weeks of age.

In the overall period, egg production increased when hens were fed the control + probiotic or control + probiotic + CrProp diets compared to the control (p = 0.043). Additionally, layer breeders fed diets supplemented with probiotic, CrProp or both additives had higher egg mass and lower FCR (kg/kg) than the non-supplemented hens ($p < 0.05$).

Egg quality and eggshell quality results are presented in Table 3. There were no effects ($p > 0.05$) of dietary treatments on the Haugh unit, albumen and yolk percentage, yolk strength, yolk index and total solids evaluated per 28 d period or in the overall period. However, shell thickness increased when hens were fed diets supplemented with the probiotic, CrProp or both additives compared to the control in all periods as well as from 55 to 70 weeks of age ($p < 0.05$). Shell strength also increased in hens fed the control + CrProp and control + probiotic + CrProp diets compared to the control and control + probiotic diets ($p < 0.05$) in all 28 d periods as well as in the overall period. From 63 to 66, 67 to 70 and 55 to 70 weeks of age, diets supplemented with the additives individually or in combination resulted in higher ($p < 0.05$) specific weight and shell percentage compared to the non-supplemented control diet.

The results of serum albumin, cholesterol, glucose and triglycerides at 50 weeks of age (n = 10), before the start of the experimental period, were 2.70 g/dL, 176 mg/dL, 210 mg/dL and 2012 mg/dL, respectively. Serum cortisol (n = 32 per period) was determined each 28 d period and there were no differences ($p > 0.05$) between cortisol concentrations for all treatments and periods, and the obtained mean values were 0.05 mcg/dL or lower. In the experimental period, dietary treatments did not affect ($p > 0.05$) albumin and triglycerides (Table 4); however, in the overall period, cholesterol and glucose serum concentration decreased (p = 0.009 and p = 0.037, respectively) when hens were fed the control + probiotic or control + probiotic + CrProp diets compared to the control diet.

The total tract metabolizability and AME of layer breeders at 70 weeks of age are shown in Table 5. The AME was not significantly different among treatments; however, metabolizability coefficients of dry matter and energy increased when hens were fed diets supplemented with the probiotic or the probiotic + chromium propionate compared to the control diet ($p < 0.05$). Hens fed diets supplemented with the probiotic + CrProp had the highest total tract metabolizability of nitrogen (p = 0.001).

Table 3. Egg and eggshell quality of layer breeders fed diets supplemented with the probiotic and chromium propionate from 55 to 70 weeks of age.

Item	Egg Weight, g	Specific Weight, g/cm^3	Haugh Unit	Albumen, %	Yolk, %	Yolk Strength, N	Yolk Index	Total Solids, %	Shell, %	Thickness, mm	Shell Strength, N
Period of 55 to 58 weeks of age											
Control	57.1	1084	89.1	62.2	28.8	7.9	0.47	56.3	8.5	0.352 ^{b}	26.3 ^{b}
Control + probiotic [1]	59.7	1086	89.6	62.6	29.0	7.9	0.49	55.8	8.9	0.372 ^{a}	26.4 ^{b}
Control + CrProp [2]	58.0	1085	90.4	64.5	28.3	7.8	0.49	55.7	8.8	0.373 ^{a}	29.6 ^{a}
Control + probiotic + CrProp	58.2	1084	89.7	62.6	28.5	7.8	0.49	55.9	9.0	0.376 ^{a}	29.7 ^{a}
SEM	0.49	0.56	0.57	0.47	0.27	0.02	0.003	0.23	0.09	0.003	0.24
p-value	0.294	0.419	0.885	0.330	0.828	0.428	0.479	0.781	0.238	0.016	0.001
Period of 59 to 62 weeks of age											
Control	57.8	1079 ^{b}	88.6	63.4	29.0	7.5	0.47	54.2	8.1	0.343 ^{b}	25.1 ^{b}
Control + probiotic	59.1	1084 ^{a}	89.0	63.4	29.1	7.6	0.48	54.0	8.5	0.366 ^{a}	25.3 ^{b}
Control + CrProp	57.9	1084 ^{a}	90.8	63.2	28.6	7.5	0.49	54.9	8.7	0.365 ^{a}	28.7 ^{a}
Control + probiotic + CrProp	59.0	1083 ^{a}	90.4	63.6	29.1	7.5	0.48	54.5	8.7	0.360 ^{a}	28.7 ^{a}
SEM	0.49	0.65	0.71	0.41	0.22	0.01	0.003	0.23	0.12	0.003	0.26
p-value	0.695	0.014	0.648	0.987	0.807	0.748	0.136	0.533	0.172	0.050	0.001
Period of 63 to 66 weeks of age											
Control	58.2	1079 ^{b}	88.9	61.6	29.9	7.4	0.44	53.7	8.1 ^{b}	0.347 ^{b}	24.1 ^{b}
Control + probiotic	59.6	1082 ^{ab}	89.3	61.8	29.4	7.4	0.45	54.5	8.7 ^{a}	0.364 ^{ab}	24.2 ^{b}
Control + CrProp	57.6	1084 ^{a}	91.0	62.6	28.9	7.4	0.46	52.7	8.9 ^{a}	0.365 ^{a}	28.0 ^{a}
Control + probiotic + CrProp	59.0	1081 ^{ab}	89.4	62.6	29.2	7.5	0.45	53.2	8.6 ^{a}	0.367 ^{a}	28.1 ^{a}
SEM	0.51	0.55	0.59	0.25	0.21	0.01	0.003	0.42	0.09	0.004	0.28
p-value	0.535	0.020	0.599	0.293	0.425	0.849	0.209	0.487	0.010	0.055	0.001
Period of 67 to 70 weeks of age											
Control	58.1	1079 ^{b}	88.1	62.4	29.9	7.5	0.46	53.1	8.2 ^{b}	0.345 ^{b}	24.6 ^{b}
Control + probiotic	60.5	1084 ^{a}	88.0	62.1	29.4	7.5	0.47	53.3	8.8 ^{a}	0.366 ^{a}	24.9 ^{b}
Control + CrProp	58.3	1083 ^{a}	90.2	63.4	28.9	7.6	0.47	53.2	8.7 ^{a}	0.369 ^{a}	28.6 ^{a}
Control + probiotic + CrProp	60.1	1083 ^{a}	88.6	62.8	29.2	7.5	0.46	53.5	8.7 ^{a}	0.370 ^{a}	28.6 ^{a}
SEM	0.49	0.53	0.57	0.26	0.20	0.01	0.003	0.14	0.09	0.003	0.29
p-value	0.198	0.008	0.488	0.350	0.321	0.112	0.545	0.713	0.045	0.003	0.001
Overall period (55 to 70 weeks of age)											
Control	57.8	1080 ^{b}	88.7	62.4	29.4	7.6	0.46	54.3	8.2 ^{b}	0.347 ^{b}	25.1 ^{c}
Control + probiotic	59.7	1084 ^{a}	88.9	62.5	29.2	7.6	0.47	54.4	8.7 ^{a}	0.367 ^{a}	25.2 ^{b}
Control + CrProp	57.9	1084 ^{a}	90.6	63.4	28.7	7.6	0.48	54.1	8.8 ^{a}	0.368 ^{a}	28.7 ^{a}
Control + probiotic + CrProp	59.1	1083 ^{a}	89.5	63.0	29.0	7.6	0.47	54.3	8.8 ^{a}	0.370 ^{a}	28.8 ^{a}
SEM	0.45	0.49	0.51	0.26	0.17	0.01	0.003	0.14	0.08	0.003	0.26
p-value	0.369	0.014	0.555	0.446	0.499	0.160	0.186	0.892	0.028	0.003	0.001

[1] *Bacillus subtilis* PB6, PTA-6737 supplemented at 500 g/ton. [2] Chromium propionate supplemented at 50 g/ton. $^{a-c}$ Means with different superscript letters differ significantly ($p < 0.05$) based on the Tukey test.

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Table 4. Blood biochemical results of layer breeders fed diets supplemented with the probiotic and chromium propionate from 55 to 70 weeks of age.

Item[1]	Albumin, g/dL	Cholesterol, mg/dL	Glucose, mg/dL	Triglycerides, mg/dL
		Period of 55 to 58 weeks of age		
Control	2.14	149	202	1887
Control + probiotic[2]	2.18	149	193	1774
Control + CrProp[3]	2.31	148	194	1902
Control + probiotic + CrProp	2.10	129	190	1498
SEM	0.04	6.63	2.33	132.9
p-value	0.211	0.622	0.324	0.704
		Period of 59 to 62 weeks of age		
Control	2.13	157 ^{a}	201 ^{a}	1917
Control + probiotic	2.16	157 ^{a}	186 ^{b}	1978
Control + CrProp	2.20	132 ^{ab}	181 ^{b}	1551
Control + probiotic + CrProp	2.08	119 ^{b}	188 ^{ab}	1391
SEM	0.03	5.44	2.54	110.1
p-value	0.386	0.020	0.034	0.172
		Period of 63 to 66 weeks of age		
Control	2.44	128	179	1626
Control + probiotic	2.30	115	173	1308
Control + CrProp	2.39	123	170	1404
Control + probiotic + CrProp	2.30	112	181	1251
SEM	0.03	5.55	1.71	96.9
p-value	0.367	0.143	0.080	0.556
		Period of 67 to 70 weeks of age		
Control	2.55	147	185	1648
Control + probiotic	2.59	134	186	1591
Control + CrProp	2.50	118	183	1196
Control + probiotic + CrProp	2.51	116	179	1478
SEM	0.04	5.44	2.33	101.2
p-value	0.900	0.157	0.760	0.414
		Overall period (55 to 70 weeks of age)		
Control	2.33	149 ^{a}	192 ^{a}	1770
Control + probiotic	2.33	139 ^{ab}	185 ^{ab}	1663
Control + CrProp	2.36	129 ^{bc}	182 ^{b}	1514
Control + probiotic + CrProp	2.25	119 ^{c}	183 ^{b}	1404
SEM	0.02	3.84	1.32	67.3
p-value	0.231	0.009	0.037	0.235

[1] Serum cortisol was determined each 28 d period and the obtained means were 0.05 mcg/dL or lower than the limit of detection for all experimental diets and periods ($p > 0.05$). The results of serum albumin, cholesterol, glucose and triglycerides at 50 weeks of age ($n = 10$) before the start of the experimental period were 2.70 g/dL, 176 mg/dL, 210 mg/dL and 2012 mg/dL, respectively. [2] *Bacillus subtilis* PB6, PTA-6737 supplemented at 500 g/ton. [3] Chromium propionate supplemented at 50 g/ton. $^{a–c}$ Means with different superscript letters differ significantly ($p < 0.05$) based on the Tukey test.

Table 5. Coefficient of total tract metabolizability and apparent metabolizable energy (AME) of layer breeders fed diets supplemented with the probiotic and chromium propionate at 70 weeks of age.

Item	Dry Matter	Nitrogen	Energy	AME, kcal/kg
Control	0.67 ^{b}	0.56 ^{c}	0.80 ^{b}	3155
Control + probiotic[1]	0.72 ^{a}	0.61 ^{b}	0.84 ^{a}	3215
Control + CrProp[2]	0.71 ^{a}	0.65 ^{ab}	0.82 ^{b}	3210
Control + probiotic + CrProp	0.72 ^{a}	0.68 ^{a}	0.85 ^{a}	3226
SEM	0.005	0.010	0.004	16.61
p-value	0.004	0.001	0.001	0.249

[1] *Bacillus subtilis* PB6, PTA-6737 supplemented at 500 g/ton. [2] Chromium propionate supplemented at 50 g/ton. $^{a–c}$ Means with different superscript letters differ ($p < 0.05$) based on Tukey test.

4. Discussion

Layer breeders fed diets supplemented with additives can provide useful results for commercial laying hens and broiler breeder hens. The present study had the objective of evaluating the effects that *Bacillus subtilis* PB6 and chromium propionate products would

have on serum biochemical parameters, excreta moisture, energy utilization, egg quality and productive performance of late-phase laying breeder hens.

Although probiotics and chromium present different main functions when supplemented in poultry feeds, these additives have some similar actions on stress control, improving the health status and immune system of poultry, which may result in enhanced bird performance. Marked benefits in performance and egg quality were observed when hens' diets were supplemented with both additives. Additionally, there are different genera of microorganisms commonly used as probiotics as well as variable sources of chromium, which may influence the obtained results when added in poultry feeds [32,33]. Therefore, *Bacillus subtilis* and organic chromium should be more explored for layers and breeder hens, because more data are needed considering the importance of these productions in the poultry industry.

The use *of Bacillus subtilis* PB6 (ATCC-PTA 6737) has been applied in the broiler feed industry, being well-documented in the literature [5,8]. For broilers, in addition to improving intestinal microflora, beneficial effects such as stimulated digestion, enhanced performance [8,34] and reduced mortality caused by disease [35] have been reported. There are also published data available on the effect of *Bacillus subtilis* on hen performance and egg quality [7,10,36]. However, little information is available on the role of *Bacillus subtilis* PB6 in layers' or breeders' production.

In the present study, *Bacillus subtilis* PB6 supplementation improved layer breeders' performance, egg and eggshell quality. These results corroborated the study conducted by Darsi and Zhaghari [11], where 63-week-old broiler breeder hens fed diets supplemented with *Bacillus subtilis* PB6 (2×10^7 CFU/g) presented higher egg production compared to hens fed non-supplemented diets. These authors observed similar egg weight, but increased Haugh unit and shell thickness from 60 to 63 weeks of age.

Darsi and Zhaghari [11] also observed that *Bacillus subtilis* PB6 supplemented in diets for broiler breeders from 56 to 63 weeks of age resulted in a lower percentage of cracked and dirty eggs, obtaining a positive effect on settable eggs and hatchling healthiness. Additionally, other studies reported decreased broken, cracked and shell-less eggs when probiotics were used [4,32,37]. In the present study, *Bacillus subtilis* PB6 supplementation decreased dirty and cracked eggs by 3% compared to non-supplemented hens in the overall period; however, the small number of layer breeders available for this research did not permit detection of small, statistically significant differences. The same reason explains the absence of hatchability results.

The increased eggshell thickness was already associated with the reduction in the number of cracked eggs, and it was suggested that the decreased damaged eggs could be caused by the increased calcium retention in layers [11,32]. In the current study, layer breeders fed diets supplemented with *Bacillus subtilis* PB6 probiotic presented higher specific weight and shell thickness as well as shell strength and shell percentage, mainly from 67 to 70 weeks of age or in the overall period.

In the study conducted by Sobczak and Kozłowski [10], increased percentage, thickness and strength of eggshells were obtained without modifying serum calcium, phosphorus, triglycerides and cholesterol in 42-week-old Lohmann Brown laying hens fed diets supplemented with *Bacillus subtilis* PB6 (1×10^8 CFU/kg feed) compared to the non-supplemented group. Fathi et al. [37] evaluated three different breeds of layers from 36 to 48 weeks of age fed 4×10^9 CFU/g of *Bacillus subtilis*, and reported increased eggshell percentage, thickness and strength with lower cholesterol compared to non-supplemented diets.

Some studies have shown that probiotic supplementation increased levels of calcium and decreased cholesterol levels in the serum of Brown laying hens [4,38,39]. Gilliland et al. [40] suggested that certain microorganisms present in probiotics might assimilate cholesterol from the gastrointestinal tract for their metabolism, thus reducing the amount of absorbed cholesterol. Probiotic bacterial strains are also able to inhibit the activity of hydroxymethyl-glutaryl-coenzyme A in the gastrointestinal tract [41] or modify the

enterohepatic cycle and reduce cholesterol through assimilating dietary cholesterol into bacterial cells [42].

Data on the digestibility and metabolizability of energy and nutrients using *Bacillus subtilis* PB6 supplemented in hens' feeds are scarce. In the present study, laying breeder hens fed diets supplemented with this probiotic from 55 to 70 weeks of age had improved egg production and eggshell quality, which were supported by increased energy, nitrogen and dry matter metabolizability. In broiler diets, probiotic supplementation has been related to improved performance along with increased AME and ileal digestibility of nutrients [43], because this additive may be able to enhance the maintenance and barrier function of the intestinal epithelium [1]. Dietary supplementation of *Bacillus subtilis* also allowed for a reduction in excreta moisture, thereby preventing problems caused by wet litter in poultry farms [7]. Excreta moisture was reduced in the current study, which was similar to findings by Ribeiro Jr. et al. [36], where lower excreta moisture was reported when 25- to 45-week-old layers were fed a *Bacillus subtilis* at 3 or 8 × 10^5 CFU/kg diet.

To further substantiate the use of probiotic and chromium as potential, more beneficial effects were obtained when hens were fed *Bacillus subtilis* PB6 + CrProp in the last period of age, where environmental temperatures were hotter. In the present study, a conventional poultry house was used, representing the climate and environmental conditions where hens have been raised in South and Southeastern regions of America, which is characterized by hot summers and mild winter temperatures. In these regions, hen producers do not prioritize investing in the environment of their farms. Additionally, the comparison of temperatures was not an objective of this study, and for this reason, it was not presented. Nevertheless, averaged maximum and minimum temperatures monitored on a daily basis inside the shed were 21.6 °C and 12.4 °C, 25.5 °C and 13.0 °C, 28.8 °C and 18.0 °C and 30.9 °C and 19.1 °C, respectively, from 55 to 58, 59 to 62, 63 to 66 and 67 to 70 weeks of age. The mean air humidity was 66.0%.

In the current study, CrProp supplementation improved the egg production, FCR (kg/kg) and egg mass of laying breeder hens. Ma et al. [21] supplemented increasing levels of CrProp in layers diets and observed enhanced egg production without affecting egg weight and FCR in late-phase brown laying hens. Egg production and FCR were also improved when CrProp was supplemented in diets for laying ducks under heat-stressed conditions without modifying FI, as reported by Chen et al. [16]. These authors also indicated that CrProp was probably effective in alleviating the negative effects of heat stress. Marked effects of CrProp were observed by the increased shell strength as well as shell thickness and percentage, with improved specific weight. Additionally, the majority of eggshell quality effects were observed after 67 weeks of age in the present study. Improved shell thickness was also reported by Ma et al. [21] in laying hens fed a 0 to 0.60 mg Cr/kg diet from 60 to 68 weeks of age.

Chromium supplementation was previously reported to decrease blood total cholesterol, low-density lipoprotein cholesterol and triglyceride [44,45]. In the present study, cholesterol and glucose decreased when hens were fed diets supplemented with CrProp from 55 to 70 weeks of age. However, albumin, triglycerides and cortisol were not affected. Bahrami et al. [46] observed reduced cortisol levels in the serum of broilers under heat stress conditions that were fed diets supplemented with high Cr supplementation (0, 0.80 or 1.20 mg). Since stress might have multiple origins, and cortisol could be a tool to evaluate stress tolerance, studies reported the association between chromium and stress metabolism through decreased sensitivity to stress and reduced concentration of cortisol in blood [47].

As mentioned above, along with stressful conditions and bird species, the results of probiotic supplementation in bird diets can be variable depending on factors related to microbial strains and supplemental doses. In the same context, contradictory results on performance, egg quality and blood biochemistry concentration can be expected due to chromium forms, sources and levels, as well as laying bird species, ages or production systems. The combination of *Bacillus subtilis* PB6 and chromium propionate seemed to beneficially affect the performance and eggshell quality of late-phase laying breeder hens.

5. Conclusions

In conclusion, diets supplemented with *Bacillus subtilis* PB6, chromium propionate or a combination of the two resulted in improved egg production, feed conversion ratio and eggshell quality as well as nutrient metabolizability, without modifying the main serum biochemical parameters in laying breeder hens from 55 to 70 weeks of age. Highlighted improvements in hen performance and eggshell quality were observed when hens were fed diets supplemented with a combination of probiotic *Bacillus subtilis* PB6 + chromium propionate. This study expands our understanding concerning combined probiotic and organic chromium supplementation for laying breeder hens.

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Institutional Review Board Statement: This study was conducted according to the guidelines of the Declaration of Helsinki, and was approved by the Ethics and Research Committee of the Federal University of Santa Maria, Santa Maria, RS, Brazil (protocol code: 5404280717, 15 August 2017).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data presented in this study are available on request from the corresponding author.

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References

1. Awad, W.A.; Bohm, J.; Razzazi-Fazeli, E.; Ghapeeb, K.; Zentek, J. Effect of addition of a probiotic microorganism to broiler diets contaminated with deoxynivalenol on performance and histological alterations of intestinal villi of broiler chickens. *Poult. Sci.* **2006**, *85*, 974–979. [CrossRef]
2. Mathivanan, R.; Kalaiarasi, K. *Panchagavya* and *Andrographis paniculata* as alternative to antibiotic growth promoters on haematological, serum biochemical parameters and immune status of broilers. *J. Poult. Sci.* **2007**, *44*, 198–204. [CrossRef]
3. Forte, C.; Acuti, G.; Manuali, E.; Proietti, P.C.; Pavonre, S.; Trabalza-Marinucci, M.; Moscati, L.; Onofri, A.; Lorenzetti, C.; Fraclosini, M. Effects of two different probiotics on microflora, morphology, and morphometry of gut in organic laying hens. *Poult. Sci.* **2016**, *95*, 2528–2535. [CrossRef] [PubMed]
4. Mikulski, D.; Jankowski, J.; Naczmanski, J.; Mikulska, M.; Demey, V. Effects of dietary probiotic (*Pediococcus acidilactici*) supplementation on performance, nutrient digestibility, egg traits, egg yolk cholesterol, and fatty acid profile in laying hens. *Poult. Sci.* **2012**, *91*, 2691–2700. [CrossRef]
5. Teo, A.Y.; Tan, H.M. Inhibition of *Clostridium perfringens* by a novel strain of *Bacillus subtilis* isolated from the gastrointestinal tracts of healthy chickens. *Appl. Environ. Microbiol.* **2005**, *71*, 4185–4190. [CrossRef] [PubMed]
6. Aljumaah, M.R.; Alkhulaifi, M.M.; Abudabos, A.M.; Aljumaah, R.S.; Alsaleh, A.N.; Stanley, D. *Bacillus subtilis* PB6 based probiotic supplementation plays a role in the recovery after the necrotic enteritis challenge. *PLoS ONE* **2020**, *15*, e0232781. [CrossRef]
7. Xu, C.L.; Ji, C.; Ma, Q.; Hao, K.; Jin, Z.Y.; Li, K. Effects of a dried *Bacillus subtilis* culture on egg quality. *Poult. Sci.* **2006**, *85*, 364–368. [CrossRef]
8. Jayaraman, S.; Thangavel, G.; Kurian, H.; Mani, R.; Mukkalil, R.; Chirakkal, H. *Bacillus subtilis* PB6 improves intestinal health of broiler chickens challenged with *Clostridium perfringens*-induced necrotic enteritis. *Poult. Sci.* **2013**, *92*, 370–374. [CrossRef] [PubMed]
9. Chung, S.H.; Lee, J.; Kong, C. Effects of multi strain probiotics on egg production and quality in laying hens fed diets containing food waste product. *Int. J. Poult. Sci.* **2015**, *14*, 19–22. [CrossRef]
10. Sobczak, A.; Kozlowski, K. The effect of a probiotic preparation containing *Bacillus subtilis* ATCC PTA-6737 on egg production and physiological parameters of laying hens. *Ann. Anim. Sci.* **2015**, *15*, 711–723. [CrossRef]
11. Darsi, E.; Zhaghari, M. Effects of *Bacillus subtilis* PB6 supplementation on productive performance, egg quality and hatchability in broiler breeder hens under commercial farm condition. *J. Appl. Anim. Res.* **2021**, *49*, 109–117. [CrossRef]

12. Lara, L.J.; Rostagno, M.H. Impact of heat stress on poultry production. *Animals* **2013**, *3*, 356–369. [CrossRef] [PubMed]
13. Zulkifli, I.; Abdulllah, N.; Azrin, N.M.; Ho, Y.W. Growth performance and immune response of two commercial broiler strains fed diets containing *Lactobacillus* cultures and oxytetracycline under heat stress conditions. *Br. Poult. Sci.* **2000**, *41*, 593–597. [CrossRef] [PubMed]
14. Rajalekshmi, M.; Sugumar, C.; Chirakkal, H.; Ramarao, S.V. Influence of chromium propionate on the carcass characteristics and immune response of commercial broiler birds under normal rearing conditions. *Poult. Sci.* **2014**, *93*, 574–580. [CrossRef] [PubMed]
15. Spears, J.W.; Lloyd, K.E.; Pickworth, C.A.; Huang, Y.L.; Krafka, K.; Hyda, J.; Grimes, J.L. Chromium propionate in broilers: Human food and broiler safety. *Poult. Sci.* **2019**, *98*, 6579–6585. [CrossRef] [PubMed]
16. Chen, X.L.; Zeng, Y.B.; Liu, L.X.; Song, Q.L.; Zou, Z.H.; Wei, Q.P.; Song, W.J. Effects of dietary chromium propionate on laying performance, egg quality, serum biochemical parameters and antioxidant status of laying ducks under heat stress. *Animal* **2021**, *15*, 100081. [CrossRef]
17. Food and Drug Administration (FDA). Food additives permitted in feed and drinking water of animals: Chromium propionate. *Fed. Regist.* **2020**, *85*, 48650–48651.
18. Farag, M.R.; Alagawany, M.; Abd El-Hack, M.E.; Arif, M.; Ayasan, T.; Dhama, K.; Patra, A.; Karthik, K. Role of chromium in poultry nutrition and health: Beneficial applications and toxic effects. *Int. J. Pharmacol.* **2017**, *13*, 907–915. [CrossRef]
19. Dalolio, S.; Albino, L.F.T.; Silva, J.N.; Campos, P.H.R.F.; Lima, H.J.D.; Moreira, J.; Ribeiro, V., Jr. Dietary chromium supplementation forheat-stressed broilers. *Worlds Poult. Sci. J.* **2018**, *74*, 101–116. [CrossRef]
20. Yildiz, A.O.; Parlat, S.S.; Yazgan, O. The effects of organic chromium supplementation on production traits and some serum parameters of laying quails. *Rev. Med. Vet.* **2004**, *155*, 642–646.
21. Ma, W.; Gu, Y.; Lu, J.; Yuan, L.; Zhao, R. Effects of chromium propionate on egg production, egg quality, plasma biochemical parameters, and egg chromium deposition in late-phase laying hens. *Biol. Trace Elem. Res.* **2014**, *157*, 113–119. [CrossRef]
22. Association of Official Analytical Chemistry. *Official Methods of Analysis*, 15th ed.; AOAC: Arlington, VA, USA, 1990.
23. Association of Official Analytical Chemistry. *Official Methods of Analysis*, 18th ed.; Analytical Chemists: Gaithersburg, MD, USA, 2006.
24. Stefanello, C.; Santos, T.C.; Murakami, A.E.; Martins, E.N.; Carneiro, T.C. Productive performance, eggshell quality, and eggshell ultrastructure of laying hens fed diets supplemented with organic trace minerals. *Poult. Sci.* **2014**, *93*, 104–113. [CrossRef] [PubMed]
25. Vogtmann, H.; Pfirter, H.P.; Prabucki, A.L. A new method of determining metabolisability of energy and digestibility of fatty acids in broiler diets. *Br. Poult. Sci.* **1975**, *16*, 531–534. [CrossRef]
26. Choct, M.; Annison, G. Anti-nutritive effect of wheat pentosans in broiler chickens: Roles of viscosity and gut microflora. *Br. Poult. Sci.* **1992**, *33*, 821–834. [CrossRef] [PubMed]
27. Kong, C.; Adeola, O. Invited review—Evaluation of amino acid and energy utilization in feedstuff for swine and poultry diets. *Asian Australas. J. Anim. Sci.* **2014**, *27*, 917–925. [CrossRef] [PubMed]
28. Stefanello, C.; Vieira, S.L.; Soster, P.; Santos, B.M.; Dalmoro, Y.K.; Favero, A.; Cowieson, A. Utilization of corn-based diets supplemented with an exogenous α-amylase for broilers. *Poult. Sci.* **2019**, *98*, 5862–5869. [CrossRef] [PubMed]
29. Haetinger, V.S.; Dalmoro, Y.K.; Godoy, G.L.; Lang, M.B.; de Souza, O.F.; Aristimunha, P.; Stefanello, C. Optimizing cost, growth performance, and nutrient absorption with a bio-emulsifier based on lysophospholipids for broiler chickens. *Poult. Sci.* **2021**, *100*, 101025. [CrossRef] [PubMed]
30. Ahrens, W.H.; Cox, D.J.; Budhwar, G. Use of the arcsine and square root transformations for subjectively determined percentage data. *Weed Sci.* **1990**, *38*, 452–458. [CrossRef]
31. SAS Institute. *SAS User's Guide: Statistics, Version 9.2*; SAS Institute, Inc.: Cary, NC, USA, 2009.
32. Khan, R.U.; Naz, S.; Dhama, K.; Saminathan, M.; Tiwari, R.; Jeon, G.J.; Laudadio, V.; Tufarelli, V. Modes of action and beneficial applications of chromium in poultry nutrition, production and health: A review. *Int. J. Pharmacol.* **2014**, *10*, 357–367. [CrossRef]
33. Sjofjan, O.; Adli, D.N.; Sholikin, M.M.; Jayanegara, A.; Irawan, A. The effects of probiotics on the performance, egg quality and blood parameters of laying hens: A meta-analysis. *J. Anim. Feed Sci.* **2021**, *30*, 11–18. [CrossRef]
34. Abudabos, A.M.; Alyemni, A.H.; Al Marshad, M.B.A. *Bacillus subtilis* PB6 based probiotic (CLOSTAT™) improves intestinal morphological and microbiological status of broiler chicken under *Clostridium perfringens* challenge. *Int. J. Agric. Biol.* **2013**, *15*, 978–982.
35. Park, J.H.; Kim, I.H. Supplemental effect of probiotic *Bacillus subtilis* B2A on productivity, organ weight, intestinal Salmonella microflora, and breast meat quality of growing broiler chicks. *Poult. Sci.* **2014**, *93*, 2054–2059. [CrossRef] [PubMed]
36. Ribeiro, V., Jr.; Albino, L.F.T.; Rostagno, H.S.; Barreto, S.L.T.; Hannas, M.I.; Harrington, D.; de Araujo, F.A.; Ferreira, H.C., Jr.; Ferreira, M.A. Effects of the dietary supplementation of *Bacillus subtilis* levels on performance, egg quality and excreta moisture of layers. *Anim. Feed Sci. Technol.* **2014**, *195*, 142–146. [CrossRef]
37. Fathi, M.; Al-Homidan, I.; Al-Dokhail, A.; Ebeid, T.; Abou-Emera, O.; Alsagan, A. Effects of dietary probiotic (*Bacillus subtilis*) supplementation on productive performance, immune response and egg quality characteristics in laying hens under high ambient temperature. *Ital. J. Anim. Sci.* **2018**, *17*, 804–814. [CrossRef]

38. Panda, A.K.; Rama Rao, S.S.; Raju, M.V.; Sharma, S.S. Effect of probiotic (*Lactobacillus sporogenes*) feeding on egg production and quality, yolk cholesterol and humoral immune response of white leghorn layer breeders. *J. Sci. Food Agric.* **2008**, *88*, 43–47. [CrossRef]
39. Mahdavi, A.H.; Rahmani, H.R.; Pourreza, J. Effect of probiotic supplements on egg quality and laying hen's performance. *Int. J. Poult. Sci.* **2005**, *4*, 488–492. [CrossRef]
40. Gilliland, S.E.; Nelson, C.R.; Maxwell, C. Assimilation of cholesterol by *Lactobacillus acidophilus* bacteria. *Appl. Environ. Microbiol.* **1985**, *49*, 377–381. [CrossRef] [PubMed]
41. Fukushima, M.; Nakano, M. The effect of a probiotic on faecal and liver lipid classes in rats. *Br. J. Nutr.* **1995**, *73*, 701–710. [CrossRef]
42. Kalavathy, R.N.; Abdullah, N.; Jalaludin, S.; Ho, Y.W. Effects of *Lactobacillus* cultures on growth performance, abdominal fat deposition, serum lipids and weight of organs of broiler chickens. *Br. Poult. Sci.* **2003**, *44*, 139–144. [CrossRef] [PubMed]
43. Mountzouris, K.C.; Tsitrsikos, P.; Palamidi, I.; Arvaniti, A.; Mohnl, M.; Schatzmayr, G.; Fegeros, K. Effects of probiotic inclusion levels in broiler nutrition on growth performance, nutrient digestibility, plasma immunoglobulins, and cecal microflora composition. *Poult. Sci.* **2010**, *89*, 58–67. [CrossRef]
44. Anderson, R.A. Chromium, glucose tolerance, and lipid metabolism. *J. Adv. Med.* **1995**, *8*, 37–49.
45. Hanafy, M.M. Influence of adding organic chromium in diet on productive traits, serum constituents and immune status of Bandarah laying hens and semen physical properties for cocks in winter season. *Egypt. Poult. Sci. J.* **2011**, *31*, 203–216.
46. Bahrami, A.; Moeini, M.; Ghazi, S.H.; Targhibi, M.R. The effect of different levels of organic and inorganic chromium supplementation on immune function of broiler chicken under heat-stress conditions. *J. Appl. Poult. Res.* **2012**, *21*, 209–215. [CrossRef]
47. Chandrasekar, S.; Balakrishnan, U. Chromium nutrition in alleviating the effect of stressors in poultry production: A review. *Int. J. Anim. Sci.* **2019**, *3*, 1040.

5 ARTIGO II

Este capítulo é apresentado de acordo com as normas para publicação no Periódico **Animals.**

A presente publicação é composta por dados de dois ensaios experimentais distintos, sendo um deles realizado em outra instituição, o que foi necessário para complementar o artigo. Portanto, apenas o experimento 1 foi realizado na UFSM, instituição a qual o discente está vinculado e destaca-se que o primeiro autor e sua orientadora não tiveram participação no experimento 2 que foi realizado fora da UFSM.

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Article

Development and Evaluation of a Commercial Direct-Fed Microbial (Zymospore®) on the Fecal Microbiome and Growth Performance of Broiler Chickens under Experimental Challenge Conditions

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Simple Summary: Probiotics are recognized for their beneficial health-promoting properties, through competitive exclusion, promoting maintenance of intestinal epithelial integrity and host immune system homeostasis. The use of some spore-forming bacteria from the genus *Bacillus* has earned interest as a direct-fed microbial in recent years as a potential alternative to antibiotic growth promoters and growth enhancers. The present study evaluates the use of a *Bacillus subtilis* spore-based direct-fed microbial (Zymospore®, Vetanco, Villa Martelli, Argentina) compared to an antibiotic growth promoter on the performance of broiler chickens under experimental intestinal challenge conditions. The results suggest that Zymospore® increases the diversity of the broiler fecal microbiota and is an acceptable substitute for commonly used antibiotic growth promoters under defined and non-defined intestinal dysbiosis conditions.

Abstract: Direct-fed microbials (DFM) are added to broiler chicken diets in order to promote the proliferation of beneficial intestinal bacterial populations, which may lead to gains in performance efficiency and, potentially, reduce the level of enteric pathogens in the broiler chickens. The selection and laboratory evaluation of *Bacillus subtilis* strains as well as the experimental trial results of a novel *Bacillus*-based commercial DFM product are described. Fifteen wild-type *Bacillus subtilis* strains were characterized and assayed for their enzyme production capability, spore resistance to pH, salinity, and temperature, and ability to inhibit the growth of *E. coli* and *Salmonella* spp. The final DFM formulation was evaluated and compared to an antibiotic growth promoter (AGPs) in two experimental trials. In Experiment 1, broilers were given a defined challenge of *Eimeria* spp. and *Clostridium perfringens* to induce intestinal dysbiosis. The optimal dose of the DFM was determined to be 0.3 kg/ton of feed. At this dose, the broilers fed the DFM performed as well as the Flavomycin®-fed broilers. Further, intestinal microbiome analysis indicates that the use of the DFM enhances bacterial diversity of the gut flora by day 5 of age, increasing levels of lactic acid bacteria (LAB) and Clostridiales by 25 days of age, which may enhance the digestion of feed and promote growth of the birds. In Experiment 2, the broilers were raised on recycled litter and given an undefined challenge orally to mimic commercial growth conditions. In this trial, the DFM performed as well as the bacitracin methylene disalicylate (BMD)-11%-fed birds. The results of the present studies suggest that this novel DFM, Zymospore®, improves the performance of broiler chickens under experimental challenge conditions as effective as an AGP, providing a safe and effective substitute to the poultry industry.

Keywords: antibiotic growth promoter; broiler chicken; challenge conditions; direct-fed microbials; performance

1. Introduction

Intensive management practices in poultry production induce enteric microflora imbalances leading to diminishment of performance parameters [1,2]. To alleviate the effect of dysbiosis in the gastrointestinal tract, diets have been commonly supplemented with antibiotic growth promoters (AGPs), demonstrating an effective decrease in the presentation of digestive disorders [3], though the underlying mechanism of this AGP-driven enhancement is not well understood. Recent microbial genomics and metabolomic analysis of the broiler cecum indicates AGPs alter the bacterial community of the ceca, increasing the overall microbial gene content of the cecum, which enhances the bacterial community's ability to recycle host nitrogen compounds [4]. Further, it appears that AGP-modified bacterial communities promote increased levels of bile salt production, helping the host absorb fatty acids [4]. Both processes, in turn, drive performance of the broilers. Identification of novel non-antibiotic compounds and/or mixtures that have the same positive impact on performance is of great importance to the broiler as the concern for antimicrobial resistance (AMR) grows and bans on the use of AGPs spread around the world [5].

The indiscriminate and inappropriate use of antibiotics has led to the emergence of multidrug-resistant pathogens, resulting in a ban on many AGPs [6,7]. As an alternative to AGPs, probiotics have been under investigation as feed additives to modulate the intestinal microflora, which in turn support good productive responses in animals [8,9]. Among the species of microorganisms used as probiotics, some strains of the facultative anaerobic Gram-positive genus *Bacillus* are receiving important attention due to their augmentative properties on digestion, absorption of nutrients, and intestinal morphology [4,10,11]. Furthermore, the control of enteropathogens such as *Salmonella* spp., *Clostridium perfringens*, *Campylobacter* spp., and *Escherichia coli* in the gastrointestinal tract (GIT) has been associated with the use of *Bacillus*-based probiotics [12,13]. The genus *Bacillus* has the extraordinary capacity to produce endospores under stressful environmental conditions; some of these spores can resist high temperatures used during feed preparation (pelletization), extreme pH, dehydration, high pressures, and contact with caustic chemical substances [14]. These admirable features make selected *Bacillus* spores a direct-fed microbial (DFM) suitable for commercialization and distribution due to their AGP-like performance improvements, long shelf-life, and stability [15,16].

There is evidence supporting the theory that some *Bacillus* spores germinate in the GIT of chickens [17], mice [18], pigs, dogs, and humans [19]. Metabolically active cells are believed to produce antimicrobial substances, have immunomodulatory effects on the intestinal mucosa, and function as competitive exclusion agents interacting with host cells [20]. Furthermore, some *Bacillus* species can produce and export an array of extracellular enzymes, including protease, phytase, xylanase, keratinase, lipase, and cellulase [21,22]. These enzymes help to degrade complex feed molecules, improve absorption of nutrients, reduce intestinal viscosity in non-starch polysaccharide-rich diets (NSP), and decrease the amount of substrates available for the growth of pathogenic bacteria [23–25]. Additionally, it has been shown that the presence of *Bacillus* species, such as *Bacillus subtilis*, enhances the growth of other beneficial microorganisms, for example, *Lactobacillus*, by the production of subtilisin and catalase and also by decreasing intestinal pH [26].

All the benefits related to the utilization of *Bacillus*-DFMs in the diet make supplementation with *Bacillus* spores an accessible and applicable alternative to antibiotic growth promoters, while avoiding a concomitant increase in gastrointestinal diseases and maintaining or improving performance parameters in poultry production under commercial conditions. The purpose of the present study was to evaluate a recently developed com-

mercial direct-fed microbial (Zymospore®) relative to AGPs on the performance of broiler chickens under experimental intestinal challenge conditions. Further, the fecal microbiome of a subset of birds was evaluated by 16S DNA sequencing. The data revealed that the DFM-fed birds had a greater abundance and diversity of bacteria in their feces than the basal-diet-fed birds, a feature similar to AGP-fed birds [4]. The growth performance of broilers fed the DFM in these studies was better than the basal-diet-fed controls and similar to the performance of the AGP-fed birds.

2. Materials and Methods

2.1. Characterization of Bacillus subtilis Strains

Fifteen strains of *Bacillus* spp. previously isolated from soils collected from around the country of Argentina were initially screened on tryptic soy agar (TSA, Britania Labs, Caba, Argentina) and Spizizen potato agar (SPA, ATCC medium 423) plates for their ability to inhibit the growth of *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis, SE), *S.* Typhimurium (ST), *Escherichia coli* isolated from pigs afflicted with colibacillosis (EC-P), an avian pathogenic *E. coli* (APEC) isolate (EC-C), and an enteropathogenic *E. coli* (EPEC) isolate (EC-E) as previously described in [24] with the simple modification of using a sterile 1 µL loop to streak a straight line of each *Bacillus* isolate onto individual agar plates that did not touch the edge of the dish (2 agar plates per *Bacillus* isolate, 5 pathogen isolates). Each plate was overlaid the following day with the respective pathogen isolate of interest and incubated for 24 h at 37 °C. Zones of inhibition were measured and scored as follows: (−) no inhibition, (+) inhibition < 2.0 cm, (++) inhibition 2.1 to 4.5 cm, (+++) inhibition > 4.6 cm from edge of *Bacillus*, respectively. As growth and inhibition were best on SPA plates, *Bacillus* isolates were evaluated for each pathogen inhibition on SPA agar (Supplementary Table S1). An example (Figure S1) is provided in the Supplementary Data. Further, the *Bacillus* isolates (Table 1) that consistently ranked among the best for pathogen growth inhibition were selected for further characterization and were evaluated using enzyme-specific agar plates to detect extracellular enzyme production [24]. The spores of the strains were assayed for their resistance to temperature, salinity, and pH [14]. Based on a combination of pathogen growth inhibition and enzyme production, three strains were selected and the colony morphology and growth characteristics of the three strains on TSA plates were observed and recorded. Each strain was positively identified as *Bacillus subtilis* using a series of macroscopic, microscopic, and biochemical assays (bioMerieux API 50 CHB test kit, and bioMerieux, Lyon, France) (data not shown).

Table 1. Colony morphology description of selected *B. subtilis* strains on solid agar medium.

Strain	Form	Texture	Surface	Color	Elevation	Size	Margins
BS-009	Irregular	Rough	Dry	Yellowish white	Flat	0.5–1 cm	Curled
BS-020	Irregular	Rough	Dry	Yellowish white	Flat	0.5–1 cm	Curled
BS-024	Irregular	Rough	Mucoid	Yellowish white	Flat	0.5–1 cm	Curled/Lobate

Evaluated strains were assayed for the following extracellular enzymes: amylase (starch agar, Difco™ Starch Agar, BD, Franklin Lakes, NJ, USA), cellulase (carboxymethyl cellulose agar, ATCC medium: 1513), proteases (casein agar, Remel™, Lenexa, KS, USA) [27], and lipases (Difco™ Spirit Blue Agar, BD, Franklin Lakes, NJ, USA). The strains were cultured in tryptic soy broth (TSB, Britania Labs, Caba, Argentina) overnight at 37 °C, centrifuged and washed with 0.9% sterile saline the following day. The culture was quantified by serial dilution on TSA plates and stored at 4 °C overnight. The following day, each culture was diluted to 10^8 CFU/mL with sterile saline. Subsequently, 10 µL of each strain was placed at the center of each respective enzyme assay agar plate, allowed to absorb, and incubated at 37 °C overnight. To detect amylase and cellulase enzyme activity, the plates were flooded with Lugol solution (Sigma-Aldrich, St. Louis, MO, USA) for 2 min and 1 M NaCl (Sigma-Aldrich, St. Louis, MO, USA) solution for 15 min, respectively. Excess

solution was discarded, revealing potential zones of clearing in the agar indicative of the respective enzyme activity. A relative enzyme activity (REA) score was used to categorize the strains into Excellent, (REA > 5); Good, (REA 2 < 5); and Poor, (REA < 2) enzyme producers. The REA is calculated by dividing the diameter of the zone of clearing by the diameter of the colony in cm (REA = Ø of the zone of clearing (cm)/Ø colony (cm)) [24]. Each strain was analyzed by triplicate plating.

Spores of the selected *Bacillus subtilis* strains were assayed for their ability to withstand potential physiological environments found within birds for two and four hours. An isolated colony of each strain was inoculated into Difco Sporulation Medium (DSM, BD, Heidelberg, Germany) and incubated at 37 °C for 3 days. Sporulation was confirmed by microscopy and malachite green (Sigma-Aldrich, St. Louis, MO, USA) staining. The vegetive colony-forming unit capability of each spore preparation was determined using serial dilution on TSA plates. Subsequently, each spore preparation was diluted to 10^8 CFU/mL. Subsequently, 1 mL of the spore solution was added to 9 mL of sterile saline adjusted to the indicated salinity (NaCl, Sigma-Aldrich, St. Louis, MO, USA) or pH (1 M HCl, Sigma-Aldrich, St. Louis, MO, USA) in sterile borosilicate glass tubes and incubated at 37 °C. For the temperature resistance analysis, physiological saline was used. At 2 and 4 h of incubation, the tubes were vortexed, and 0.2 mL of the solution was serially diluted on TSA to determine the vegetative CFU/mL as an indirect measure of spore resistance to each treatment condition. These analyses were performed in triplicate.

2.2. *Direct-Fed Microbial (DFM) Product*

Zymospore® (Vetanco S.A., Buenos Aires, Argentina) is a *Bacillus subtilis* spore-based direct-fed microbial (DFM) containing at total of 5×10^{11} spores/gram from BS-009, BS-020, and BS-024.

2.3. *Experiment 1*

2.3.1. Housing Conditions

Experiment 1 was conducted at the Federal University of Santa Maria, Santa Maria, Brazil. Birds were reared in floor pens (1.5 m^2) with new wood shavings as litter in a clean experimental university poultry house. Each pen was equipped with one 18 kg feeder and one drinker. The average temperature was 32 °C at placement, being reduced by 1 °C every two days until reaching 23 °C to provide comfort throughout the study. A continuous lighting schedule was used until d 7 post-hatch, whereas an 18L: 6D cycle with constant intensity was used after that. Birds had ad libitum access to water and mash feeds. All procedures used in the current study were approved by the Federal University of Santa Maria, Santa Maria, Brazil (number 5404280717).

2.3.2. Animal Source and Diets

Seven hundred and fifty slow feathering male broiler day-old chicks (Cobb 500), vaccinated for Marek's and Avian Bronchitis diseases at the hatchery, were purchased from a local hatchery (Agrodanieli Group, Tapejara, RS, Brazil) and weighed (45 g ± 0.4 g). A four-phase corn–soybean-meal-based feeding program was used with pre-starter (1 to 7 d), starter (7 to 21 d), grower (21 to 35 d), and finisher (35 to 42 d) diets formulated according to Rostagno et al. [28] (Table 2) with or without additives. All chickens were weighed on days 1 and 42 to calculate body weight gain (BWG). Pen feed intake (FI) from days 1 to 42 was recorded to calculate the feed conversion ratio (FCR, d 42 Pen BW/accumulated (d1–42) Pen FI) at the end of the trial. Mortality was recorded daily.

Table 2. Ingredient and nutrient composition of the experimental diets fed to broilers in Experiment 1.

Item	Pre-Starter (1 to 7 d)	Starter (7 to 21 d)	Grower (21 to 35 d)	Finisher (35 to 42 d)
Ingredients, %				
Corn	48.41	50.63	57.05	63.43
Soybean meal	44.03	41.71	35.31	29.96
Soybean oil	3.74	4.35	4.70	4.22
Dicalcium phosphate	1.13	0.83	0.64	0.29
Limestone	1.38	1.23	1.14	0.97
Salt	0.55	0.52	0.50	0.47
DL-Met, 99%	0.35	0.33	0.28	0.24
L-Lys HCl, 78%	0.13	0.13	0.15	0.17
L-Thr, 98.5%	0.05	0.05	0.04	0.03
Choline chloride, 60%	0.03	0.04	0.05	0.07
Vitamin and mineral premix [1]	0.21	0.19	0.16	0.16
Nutrient and energy composition, % or as shown				
ME, Mcal/kg	2.97	3.05	3.15	3.20
Crude protein	24.16	23.27	20.86	18.92
Ca	1.01	0.88	0.79	0.63
Av. P	0.48	0.42	0.37	0.30
Na	0.23	0.22	0.21	0.20
Cl	0.42	0.41	0.40	0.40
Choline, mg/kg	1600	1600	1500	1500
Lys dig. [2]	1.31	1.26	1.12	1.01
Met + Cys dig.	0.66	0.64	0.56	0.50
Thr dig.	0.98	0.94	0.84	0.76
Trp dig.	0.86	0.83	0.74	0.67
Arg dig.	0.28	0.27	0.24	0.21
Val dig.	1.53	1.47	1.29	1.15
Ile dig.	1.01	0.97	0.87	0.78
Leu dig.	0.95	0.91	0.80	0.72

[1] Composition per kilogram of feed: vitamin A, 8000 UI; vitamin D_3, 2000 UI; vitamin E, 30 UI; vitamin K_3, 2 mg; thiamine, 2 mg; riboflavin, 6 mg; pyridoxine, 2.5 mg; cyanocobalamin, 0.012 mg; pantothenic acid, 15 mg; niacin, 35 mg; folic acid, 1 mg; biotin, 0.08 mg; iron, 40 mg; zinc, 80 mg; manganese, 80 mg; copper, 10 mg; iodine, 0.7 mg; selenium, 0.3 mg. Ronozyme HiPhos (GT) with 10,000 FYT/g (Novozymes A/S, Bagsvaerd, Denmark). [2] Ratios of digestible amino acids to digestible Lys were maintained at TSAA 0.75; Thr 0.65; Val 0.77; Trp 0.17; Arg 1.08; Ile 0.67 [28].

2.3.3. Experimental Design

Broiler chickens were distributed into 5 experimental groups with 6 replicates of 25 birds in a completely randomized design as shown below. Group 1 (basal diet, negative control) received commercial diets with no additives and no challenge. Group 2 (0.2 kg/ton) received the basal diet with the addition of 0.2 kg of DFM per metric ton of feed, Group 3 (0.3 kg/ton) received the basal diet with the addition of 0.3 kg of DFM per metric ton of feed, Group 4 (0.4 kg/ton) received the basal diet with the addition of 0.4 kg of DFM per metric ton of feed, and Group 5 (AGP, positive control) received the basal diet with the addition of 0.025 kg of flavomycin per metric ton of feed. Groups that received the DFM or AGP received it in all feed phases. All chickens were weighed on days 1 and 42 to calculate BWG. To calculate the FCR at the end of the trial, the Pen BW (d 42)/accumulated Pen FI (d1–d42) was utilized. Mortality was recorded daily.

2.3.4. Challenge Model to Induce Dysbiosis

All broilers were challenged on day 14 via individual oral gavage with 10× the regular dose of a commercially approved coccidian vaccine (Bio-Coccivet R® live vaccine, containing *Eimeria acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix*, *E. praecox*, *E. tenella*, and *E. mitis*; Biovet Vaxxinova, Vargem Grande Paulista, Brazil). At 19 days, all birds were individually orally gavaged with 1 mL/bird of *Clostridium perfringens* toxin Type A at an analyzed concentration of 3.1×10^9 CFU/mL (UFPR, Curitiba, Brazil). This intestinal

challenge model has been described to induce intestinal dysbiosis [29,30]. To obtain the *Clostridium perfringens* inoculum, 50 µL of the isolate from the bacterial stock was statically cultured in 3 mL of tryptic soy broth (TSB) (BD, Heidelberg, Germany) in a BD GasPak jar equipped with GasPak H2 + CO_2 generator envelopes and catalyst at 37 °C for 24 h. Then, the isolate was streaked across ten tryptic soy agar (TSA) plates (BD, Heidelberg, Germany) and cultured in a GasPak Jar as described above for 24 h in an incubator at 37 °C. The bacterial lawns were sterilely collected with cell scrapers and added to 25 mL of ice-cold sterile PBS. The inoculum was vortexed and centrifuged at 5400 × *g* for 20 min at 4 °C. The cells were washed once and diluted into 800 mL of PBS and kept on ice until use. The CFU of the inoculum was determined by serial dilutions on TSA plates and cultured as described above.

2.3.5. Sampling, DNA Extraction, Sequencing, and Bioinformatic Analysis

Fecal samples from the three different treatments (negative control, NC; 0.2 kg of DFM/ton of feed and 0.4 kg of DFM/ton of feed) at two different sampling points (5 and 25 days of life) were taken, and in total, 36 samples were analyzed. Each sample contained approximately 50 g of pooled, fresh feces from each replicate for each treatment. The d5 and d25 time points were chosen to evaluate potential changes in the fecal microbiome at an early and mid-life stage of the broilers at the minimum and maximum concentration of the DFM used in the experiment.

Fecal samples were processed (Imunova, Curitiba, Brazil) with the ZR Fecal DNA Miniprep kit (Zymo Research, Tustin, CA, USA) following the manufacturer's instructions. The extracted DNA was quantified by spectrophotometry at 260 nm with a NanoDrop® 2000 (ThermoScientific, Wilmington, DE, USA) spectrophotometer. To verify the integrity of the DNA, all the samples were evaluated with an agarose gel electrophoresis, stained with ethidium bromide (1% *w*/*v*, Glentham Life Sciences, Corsham, UK), and visualized with UV light.

A 250 base pair segment of the hypervariable V4 region of the ribosomal 16S rRNA gene was amplified using universal primers 515F and 806R with the following PCR conditions: 94 °C for 3 min; 18 cycles of 94 °C for 45 s, 50 °C for 30 s, and 68 °C for 60 s; followed by 72 °C for 10 min. From these, a metagenomic library was constructed using the commercial Nextera DNA Library Preparation Kit (Illumina, Hayward, CA, USA). The amplicons were pooled and sequenced using the Illumina MiSeq sequencing system [31]. To facilitate data visualization, the second sampling time (d25) was tagged as "b".

Sequencing data files were transferred, and analysis was conducted by the University of Minnesota Genomics Center. Sequence files were de-multiplexed with BBMap (https://sourceforge.net/projects/bbmap/; demuxbyname.sh, accessed on: 8 September 2021) and further processed in DADA2 (https://benjjneb.github.io/dada2/tutorial.html, accessed: 8 September 2021). The filter and trim parameters were as follows: maxN = 0, maxEE = 2, truncQ = 2, rm.phix = TRUE. The DADA2 algorithm was run with pseudo-pooling and chimeras were removed with the consensus method in 'remove BimeraDenovo' before assigning taxonomy using DADA2 'assignTaxonomy' and 'addSpecies' using the maintained databases of 'rdp_train_set_18.fa.gz' and 'rdp_species_assignment_18.fa.gz', respectively.

Further data analysis was performed in R. The beta diversity analysis used a customized CLR transform (https://github.com/trevorjgould/dada2_pipeline.git, accessed on: 8 September 2021) followed by PCA. The alpha diversity Simpson and Shannon indexes used the 'vegan package' (https://cran.r-project.org/web/packages/vegan/vegan.pdf, accessed on: 8 September 2021). The Chao1 index used the 'OTUtable' package. All plots used ggplot2, reshape2 (https://github.com/hadley/reshape, accessed on: 8 September 2021), and dlpyr (https://dplyr.tidyverse.org, accessed on: 8 September 2021) for processing.

For statistical analysis, samples were CLR transformed, and Analysis of Similarity (ANOSIM) was performed on Aitchinson distance. Indicator species analysis was performed using the multipatt function in the 'indicspecies' R library with 9999 permutations

while controlling for multiple test corrections using Benjamini–Hochberg correction. The 'adonis' function was performed on CLR transformed data with Euclidean distance. Betadisper function was tested and pairwise adonis function was performed on the three treatment groups for d5 and d25, separately, with multiple test correction using Benjamini–Hochberg formula. Data visualization utilized R and GraphPad Prism 9.

2.4. *Experiment 2*

2.4.1. Housing Conditions

This experiment was conducted at the experimental farm of Bioinnovo in Buenos Aires, Argentina. The broiler barn is an open-sided 600 square meter facility with a concrete floor housing 48 pens divided into 3 lines of 16 pens each. Each pen is 2.5 m^2 and equipped with individual feeders, individual in-line medicators, and fresh water. The heat is provided via air heaters, and the facility has six fans for heat relief. Each pen contained wood shavings 15 cm high, composed of 50% new wood shavings and 50% reused from previous experiments. The density, lighting program, and temperature were maintained within optimal parameters as outlined (www.cobb-vantress.com, accessed on 19 August 2019). Animal care was provided by an on-staff veterinarian.

2.4.2. Animal Source and Diets

Four hundred one-day-old male broiler chickens (Cobb 500) were obtained from a commercial hatchery and were vaccinated for Newcastle Disease and Marek's Disease at the hatchery. All birds received feed and water ad libitum. The study included two commercial diets in the form of micropellets, pre-starter from days 1 to 14, and finisher from day 15 to 42, the end of the trial (Table 3). Husbandry conditions such as environmental temperature and the light program were adjusted to the recommended guidelines of the genetic line. All animal handling procedures followed the guidelines of the Institutional Committee of use and care of experimental animals of the National Institute of Agronomic Technologies (INTA), protocol number 6/2021.

Table 3. Ingredient composition and nutrient content of the commercial feed diets used in Experiment 2 on as-is basis.

Item	Pre-Starter (1 to 14 d)	Finisher (15 to 42 d)
Ingredients (%)		
Corn	54.75	57.99
Soybean flour 46%	20.15	0.00
Deactivated soybean	0.00	16.00
Soybean expeller	20.00	18.14
Wheat	0.68	4.79
Grit	1.39	1.06
Salt	0.42	0.40
Mycotoxin binder	0.30	0.30
Dicalcium phosphate	1.16	0.54
Lysine	0.25	0.18
Methionine powder	0.37	0.25
Threonine	0.08	0.03
Choline chloride	0.10	0.08
Trace mineral premix [1]	0.10	0.10
Vitamin premix [2]	0.15	0.10
Nutrient and energy composition, % or as shown		
ME, Mcal/kg	2.95	3.10
Crude protein	21	18

Table 3. *Cont.*

Item	Pre-Starter (1 to 14 d)	Finisher (15 to 42 d)
Ca	1.02	0.82
Av. P	0.45	0.42
Lys dig.	1.2	1.0
Met dig.	0.48	0.40

[1] Mineral premix supplied the following per kilogram: manganese, 120 g; zinc, 100 g; iron, 120 g; copper, 10–15 g; iodine, 0.7 g; selenium, 0.4 g; and cobalt, 0.2 g. [2] Vitamin premix supplied the following per kilogram: vitamin A, 20,000,000 IU; vitamin D_3, 6,000,000 IU; vitamin E, 75,000 IU; vitamin K_3, 9 g; thiamine, 3 g; riboflavin, 8 g; pantothenic acid, 18 g; niacin, 60 g; pyridoxine, 5 g; folic acid, 2 g; biotin, 0.2 g; cyanocobalamin, 16 mg; and ascorbic acid, 200 g.

2.4.3. Experimental Design

In this experiment each treatment group contained 100 birds per group, divided into 5 repetitions with 20 birds/repetition. Group 1 (basal diet) received commercial diets with no additives and no challenge. Group 2 (BMD−) received commercial diets with the addition of 0.5 kg/metric ton of bacitracin methylene disalicylate (BMD) 11% in all the feed phases and no challenge. Group 3 (BMD+) received commercial diets with the addition of 0.5 kg/metric ton of BMD 11% in all the feed phases and was challenged with the litter filtrate. Group 4 (DFM+) received commercial diets with the addition of the DFM and Zymospore® at an inclusion rate of 0.3 kg/metric ton in all the feed phases and was challenged with the litter filtrate. All chickens were weighed on days 1 and 42 to calculate BWG. To calculate the FCR at the end of the trial, the Pen BW (d 42)/accumulated Pen FI (d1–d42) was utilized. Mortality was recorded daily.

2.4.4. Litter Filtrate to Recapitulate Commercial Farm Conditions

On days 7, 21, and 22 of life, chickens in specified groups received in the drinking water a liter filtrate as described by Sakomura and Rostagno [32]. Ten kilograms of reused litter (2 cycles minimum) from a commercial farm with a high historical prevalence of necrotic enteritis (75,000 oocysts per gram of litter) was mixed into fifty liters of distilled water at 22 °C for twenty-four hours. The solution was filtered through a stainless-steel metallic mesh with holes of 0.5 mm in diameter. The filtered solution was left untouched for one hour. One liter of this solution was then diluted with four liters of distilled water and administrated in the drinking water to each pen during a five-hour period on days 7, 21, and 22 of age. This procedure was repeated for each day of filtrate administration. The final dilution's average microbial count revealed that a liter of filtrate contained 3×10^9 CFU of total aerobic bacteria; 2×10^7 CFU of total anaerobic bacteria; and 7×10^6 CFU of total coliforms.

2.5. Statistical Analysis

All performance data were subjected to analysis of variance (ANOVA) as a completely randomized design using the general linear model (GLM) procedure of SAS [33]. For evaluation of growth performance parameters (body weight (BW), BWG, FI, and FCR), each of the replicate pens were considered as the experimental unit in each experiment, respectively. Treatment means were partitioned using Tukey's multiple range test with an alpha threshold set at ≤ 0.05, indicating statistical significance. Experiment 1: broiler chickens were distributed into 5 experimental groups with 6 replicates of 25 birds in a completely randomized design. Experiment 2: broiler chickens were distributed into 4 experimental groups with 5 replicates of 20 birds in a completely randomized design.

3. Results

3.1. Bacillus Species Characterization and Stain Selection

A selection of *B. subtilis* isolates were evaluated for their ability to inhibit the growth of pathogenic *Salmonella* and *Escherichia coli* isolates on TSA and SPA plates as an indirect

measure of their potential probiotic effect on broiler chickens and other animals (Supplementary Figure S1 and Table S1). Ultimately, three growth compatible strains, BS-009, BS-020, and BS-024 (data not shown) were selected for inclusion into the direct-fed microbial (DFM). Their colony morphology on solid agar media (Table 1) and vegetative cellular and spore characteristics and were observed microscopically (data not shown). All three strains presented as Gram-positive bacilli consistent with *B. subtilis*, which formed endospores as determined by malachite green staining (data not shown). Biochemical and fermentative characteristics of each strain were consistent with *B. subtilis* (data not shown). Colony morphology was similar among the three isolates, each exhibiting rough yellow-white colonies with irregular and lobate edges (Table 1). All three strains secreted various amounts of cellulase, amylase, lipase(s), and proteases (Table 4). The spores of the three strains exhibited resistance to temperature (Table 5), salinity (Table 6), and acid (Table 7). The spores of the three strains were highly resistant to all three stressors after two and four hours with each strain maintaining a vegetative CFU count consistent with the original spore inoculation of 10^7 spores/mL.

Table 4. Relative enzyme activity (REA) of the three *B. subtilis* strains in Zymospore® (mean ± SD).

Strain	Cellulase	Amylase	Lipases	Proteases
BS-009	3.55 ± 0.34	1.95 ± 0.08	3.16 ± 0.10	2.33 ± 0.09
BS-020	2.02 ± 0.08	1.71 ± 0.13	3.12 ± 0.40	1.96 ± 0.11
BS-024	2.30 ± 0.19	1.38 ± 0.05	2.32 ± 0.19	2.51 ± 0.16

Table 5. Temperature resistance of spores of the three *B. subtilis* strains in Zymospore®. Data represent the mean ± SD of vegetive cell counts ($\log_{10}$ CFU/mL) post-treatment.

Strain	15 °C		37 °C		45 °C	
	2 h	4 h	2 h	4 h	2 h	4 h
BS-009	7.03 ± 0.26	7.13 ± 0.32	7.42 ± 0.10	7.40 ± 0.30	7.20 ± 0.17	6.77 ± 0.68
BS-020	6.97 ± 0.06	7.20 ± 0.35	6.40 ± 0.17	6.30 ± 0.30	7.30 ± 0.0	7.55 ± 0.81
BS-024	7.26 ± 0.24	7.16 ± 0.15	6.95 ± 0.09	6.95 ± 0.09	7.10 ± 0.17	6.93 ± 0.13

Table 6. Salinity resistance of spores of the three *B. subtilis* strains in Zymospore®. Data represent the mean ± SD of vegetive cell counts ($\log_{10}$ CFU/mL) post-treatment.

Strain	NaCl 3.5%		NaCl 6.5%	
	2 h	4 h	2 h	4 h
BS-009	7.14 ± 0.15	7.33 ± 0.35	6.92 ± 0.08	6.77 ± 0.07
BS-020	7.28 ± 0.04	7.15 ± 0.32	7.36 ± 0.10	7.03 ± 0.05
BS-024	7.12 ± 0.21	7.15 ± 0.15	6.96 ± 0.34	6.73 ± 0.15

Table 7. Acid resistance of spores of the three *B. subtilis* strains in Zymospore®. Data represent the mean ± SD of vegetive cell counts ($\log_{10}$ CFU/mL) post-treatment.

Strain	pH2		pH3	
	2 h	4 h	2 h	4 h
BS-009	6.93 ± 0.08	7.33 ± 0.61	7.32 ± 0.28	6.87 ± 0.11
BS-020	7.16 ± 0.28	7.14 ± 0.29	7.01 ± 0.02	6.62 ± 0.54
BS-024	6.95 ± 0.09	6.67 ± 0.58	6.98 ± 0.03	6.95 ± 0.05

3.2. *Experiment 1*

3.2.1. Necrotic Enteritis Challenge and Performance Data

In addition to the food safety pathogens *Salmonella* and *E. coli*, broilers commonly face diseases such as coccidiosis and necrotic enteritis (NE) that cause intestinal dysbiosis, leading to reduced growth performance and increased production costs for farmers [34]. To evaluate potential disease mitigating effects of this newly developed DFM on broilers in the face of a performance reducing disease, a controlled NE challenge model was used in a pilot study to induce intestinal dysbiosis. While severe outbreaks of NE may cause up to 50% mortality in a flock, sub-clinical NE leads to diarrhea, dehydration, decreased feed consumption, and overall poorer performance of the broilers [35]. Further, the fecal microbiome of the broilers from a subset of the treatments was analyzed to evaluate changes in the bacterial community.

Table 8 shows the results of this pilot study, Experiment 1. There is a lack of statistical significance among any of the groups across the different parameters. Of note, throughout the study, particularly after the NE challenge was applied, the DFM and AGP groups had numerically better parameters than the basal diet. Particularly after day 21 of life, the NC + 0.3 kg DFM/metric ton of feed group had similar or better numerical total BW and BWG parameters compared to the AGP group, with both groups numerically better than the basal diet group. Further, the accumulated FCR and FI of the NC + 0.3 kg DFM/metric ton of feed group were numerically as good or better than the AGP group. Interestingly, the NC + 0.2 kg DFM/metric ton of feed and NC + 0.4 kg DFM/metric ton of feed groups numerically improved parameters over the basal diet, but the data suggest there is an optimal dosage of 0.3 kg Zymospore®/metric ton of feed. In this experiment, no significant differences were observed in mortality.

Table 8. Evaluation of different concentrations of the DFM (0.2 kg/t, 0.3 kg/t, or 0.3 kg/t) on body weight, body weight gain, feed intake, accumulated feed conversion ratio (FCR), and total mortality in broiler chickens given a defined challenge to induce dysbiosis at 42 days of age. Experiment 1.

Item	1 d	7 d	14 d	21 d	28 d	35 d	42 d	ADG [1]
Body weight (g)								
Negative control	45	200	526	990	1747	2449	3294	77.35
DFM, 0.2 kg/t	45	199	531	1008	1767	2482	3340	78.45
DFM, 0.3 kg/t	45	205	543	1026	1805	2537	3402	79.93
DFM, 0.4 kg/t	45	200	536	1014	1772	2497	3353	78.77
Positive control	45	196	537	1006	1753	2462	3328	78.15
SEM [2]	0.49	5.72	18.70	26.33	36.02	73.29	77.10	1.834
p-value	0.8416	0.1843	0.6004	0.2720	0.0766	0.3002	0.2116	0.2103
Body weight gain (g/d/b)	1–7 d	7–14 d	14–21 d	21–28 d	29–35 d	35–42 d		
Negative control	155	326	554	667	702	845		
DFM, 0.2 kg/t	154	331	568	668	715	858		
DFM, 0.3 kg/t	160	338	573	689	731	865		
DFM, 0.4 kg/t	155	335	568	668	725	856		
Positive control	151	341	559	656	710	865		
SEM	5.66	15.01	16.33	33.50	76.68	57.46		
p-value	0.1596	0.4425	0.2720	0.5449	0.9644	0.9702		
Feed intake (g/b)	1–7 d	7–14 d	14–21 d	21–28 d	29–35 d	35–42 d		
Negative control	182	426	738	989	1186	1298		
DFM, 0.2 kg/t	176	424	753	965	1165	1307		
DFM, 0.3 kg/t	182	432	757	965	1193	1300		
DFM, 0.4 kg/t	180	430	758	960	1186	1301		
Positive control	173	433	739	963	1184	1312		
SEM	7.14	15.95	25.12	74.31	142.10	87.72		
p-value	0.1240	0.8236	0.4647	0.9574	0.9979	0.9987		

Table 8. *Cont.*

Item	1 d	7 d	14 d	21 d	28 d	35 d	42 d	ADG [1]
FCR [3]	1–7 d	7–14 d	14–21 d	21–28 d	29–35 d	35–42 d		
Negative control	1.171	1.310	1.333	1.483	1.696	1.542		
DFM, 0.2 kg/t	1.142	1.282	1.325	1.443	1.637	1.530		
DFM, 0.3 kg/t	1.143	1.279	1.321	1.400	1.631	1.502		
DFM, 0.4 kg/t	1.156	1.283	1.335	1.437	1.640	1.519		
Positive control	1.142	1.271	1.320	1.464	1.664	1.516		
SEM	0.0235	0.0349	0.0237	0.0694	0.1368	0.0929		
p-value	0.1742	0.4011	0.7216	0.3314	0.9188	0.9589		
Mortality (%)	1–7 d	7–14 d	14–21 d	21–28 d	29–35 d	35–42 d	Total mortality	
Negative control	0.67	0.67	0.00	0.76	0.72	0.67	3.51	
DFM, 0.2 kg/t	0.00	2.17	0.67	0.00	0.76	0.67	4.29	
DFM, 0.3 kg/t	0.67	2.17	0.67	2.25	0.00	0.67	6.51	
DFM, 0.4 kg/t	0.00	0.67	0.00	0.72	0.76	0.00	2.18	
Positive control	0.67	2.17	0.00	0.00	0.79	0.00	3.70	
SEM	1.26	3.05	1.03	1.59	1.66	1.26	4.73	
p-value	0.7359	0.7826	0.5674	0.1272	0.9063	0.7359	0.6174	

Negative control—no antibiotic growth promoter. Positive control—feed supplemented with flavomycin at 25 g/ton. [1] ADG = average daily gain. [2] SEM—pooled standard error of the mean. [3] FCR—feed conversion rate, FI (d 1–42)/BW (d 42).

3.2.2. Fecal Microbiome Analysis

The fecal microbiome of a limited set of treatment groups was analyzed to help understand potential intestinal microbiome changes that may be occurring due to administration of the DFM. The groups analyzed were the basal diet (NC), NC + 0.2 kg/metric ton, and NC + 0.4 kg/metric ton groups. Fresh feces from each replicate within each treatment group was collected and pooled on days 5 and 25 of life. The samples were analyzed by 16S rDNA sequencing to evaluate bacterial changes that may be occurring within the broiler gastrointestinal tract.

The fecal bacterial community of all treatment groups was dominated by a few genera on d 5 (prior to challenge) of life with diversity increasing by varying degrees at d 25 (post-challenge) of life (Figure 1A–C). The level of alpha diversity as visualized by the Chao1, Shannon, and Simpson indices (Figure 1A–C), while statistically different, was relatively similar at d 5 in all treatment groups and was dominated by three genera, *Enterococcus*, *Lactobacillus*, and *Ligilactobacillus*. By d 25, the richness of taxa within each treatment group increased, as shown by the alpha diversity indices (Figure 1A–C). It is notable across all the indices at d 25, that the NC + 0.4 kg/metric ton DFM group results are roughly twice that of the NC and NC + 0.2 kg/metric ton groups, indicating greater species richness, but many of these genera are in low abundance (Figures 1A–C and 2A,B). Beta diversity analysis, displayed as a principal component analysis (PCA), indicates the d 5 samples cluster together, but the dominance of *Enterococcus* in NC + 0.2 kg/metric ton is highlighted by a small divergence from NC and NC + 0.4 kg/metric ton groups (Figure 1D). In the PCA, the NC + 0.4 kg/metric ton is significantly dissimilar to the NC and NC + 0.2 kg/metric ton groups, which group together, indicative of greater taxonomic diversity (Figure 1D).

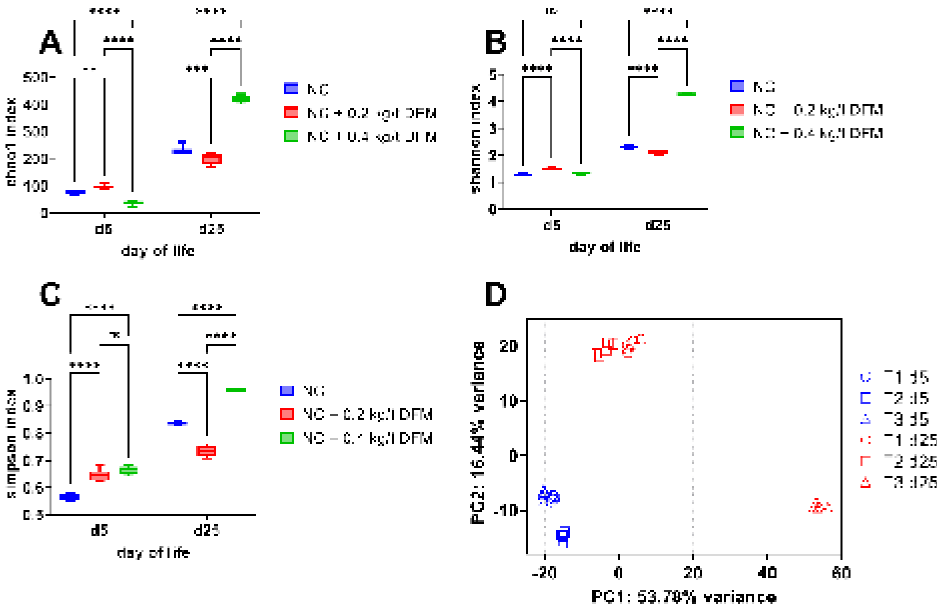

Figure 1. Alpha and beta diversity analysis. Levels of alpha diversity calculated as (**A**) Chao1, (**B**) Shannon, and (**C**) Simpson indices are depicted as boxplots by treatment group; d 5 and d 25 refer to day of life. (**D**) Bray-Curtis beta diversity shown as a principal component analysis (PCA). ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Taken together, the alpha and beta diversity indices indicate the DFM minimally impacted the diversity of the fecal microbiome early in life and prior to the necrotic enteritis challenge. The NC + 0.4 kg/metric ton group has greater species richness and diversity as compared to NC and NC + 0.2 kg/metric ton groups, with the treatment group explaining 57.78% of the variance between groups. The microbiota of NC and NC + 0.2 kg/metric ton groups were dominated by the Order Lactobacillale (Figure 2A,B), with *Lactobacillus* and *Enterococcus* contributing >80% of the total amplicon sequence variants (ASV) detected. To a lesser extent, the NC + 0.4 kg/metric ton also had a high proportion of Lactobacillale at d 5, but the distribution includes additional Lactobacillaceae, specifically *Ligilactobacillus* (Figure 2B), and to a lesser extent, *Enterococcus* compared to NC and NC + 0.2 kg/metric ton groups (Figure 2B). At d 25, the diversity of the NC + 0.4 kg/metric ton microbiota is underscored by the increased proportions of additional taxa as compared to NC and NC + 0.2 kg/metric ton groups (Figures 2B and S2). This greater diversity comes as the expense of *Lactobacillus* and *Enterococcus* genera, which make up a significantly smaller proportion of the microbiota in the NC + 0.4 kg/metric ton group by d 25 (Figures 2B and S2) but includes an increase in the proportion of other Families of the Order Lactobacillales (lactic acid bacteria) and Clostridiales, such as Faecalibacterium (Figures 2B and S2).

The results of Experiment 2 comparing an AGP (11% BMD) to the DFM on BW, FI, FCR, and total mortality in broiler chickens given a non-defined litter filtrate are summarized in Table 9. In this experiment, the use of (−) and (+) after the group acronym is to indicate if a specific group did not or did receive the litter filtrate via drinking water, respectively. The basal diet (NC−) group did not receive the litter filtrate or feed additive and established the basal growth performance parameters of the trial. The BMD-positive control group not receiving the litter filtrate (BMD−) showed a significant improvement ($p < 0.05$) in BW and FCR when compared to the NC− group, as expected. Notably, the BMD+ and DFM+

groups, which received the litter filtrate, were highly similar to each other across BW, FI, and FCR parameters and were statistically better than the NC− group even though BMD+ and DFM+ received the undefined litter challenge. No mortality differences were observed among the groups.

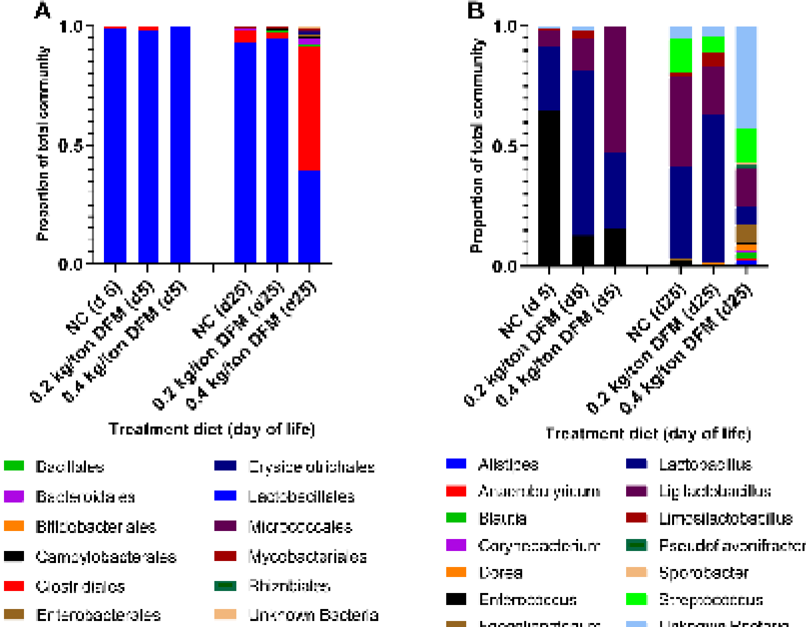

Figure 2. Biodiversity and proportionality of bacteria among treatment groups at d 5 and d 25 of life in broilers. Relative abundances of bacterial (**A**) Orders and (**B**) Genus among treatments within sampling days. When an ASV could not be resolved to a single taxon it was marked "Unknown" and resolved to the next highest taxa level. Unresolved taxa were grouped into "Unknown Bacteria". For genus-level data, only genera with ≥1% abundance are shown.

Table 9. Evaluation of Zymospore® on body weight (BW), feed intake (FI), accumulated feed conversion ratio (FCR), and total mortality in broiler chickens given a non-defined challenge. Experiment 2.

	BW g/broiler (d 42)	FI g/broiler (d 1–42)	FCR [1] (d 1–42)	Total Mortality (d 1–42)
Basal diet without filtrate (NC−)	2810 ^{b}	5302	1.97 ^{a}	5/100 (5.00%)
BMD without filtrate (BMD−)	3161 ^{a}	5319	1.76 ^{b}	3/100 (3.00%)
BMD with filtrate (BMD+)	3055 ^{a}	5402	1.82 ^{b}	3/100 (3.00%)
DFM with filtrate (DFM+)	3108 ^{a}	5456	1.83 ^{b}	3/100 (3.00%)
SEM [2]	110	3891	0.04	
p-value	0.0011	0.6784	0.0007	

NC−: Commercial feed formula with no additives and not challenged. BMD−: Commercial feed formula with the addition of BMD and no challenge. BMD+: Commercial feed formula with the addition of BMD and challenged. BMD 11% was included at a rate of 0.5 kg/metric ton in all the feed phases. DFM+: Commercial feed formula with the addition the DFM and challenged. Zymospore® DFM was included at a rate of 0.3 kg/metric ton in all the feed phases and challenged. [1] FCR—FI (d 1–42)/BW (d 42). [2] SEM—pooled standard error of the mean. [a,b] Values within columns with different superscripts differ significantly ($p < 0.05$); $n = 5$ replicates per treatment and $n = 20$ broilers/replicate.

3.3. *Experiment 2*

Intestinal Dysbiosis Challenge and Performance

It is a common practice to reuse litter on commercial farms, whereby fresh litter is laid over the top of used litter. This practice, while cost saving, perpetuates the cycling of potential pathogenic microorganisms through the new broiler flocks, thus the growth performance of new chicks may be impacted by sub-clinical diseases. To recapitulate this diverse and sub-clinical challenge of a commercial farm setting, an undefined litter filtrate was given via the drinking water in Experiment 2 (see Methods) to establish the cycling of microorganisms in the chicks' excreta and litter. Based on results from Experiment 1, a single concentration of the DFM at 0.3 kg/t of feed was used.

4. Discussion

The *Bacillus* genus is a phenotypically and genetically diverse endospore-forming taxa found within many ecosystems. Species of *Bacillus* produce a plethora of exo-proteins and -enzymes as well as bacterial antagonistic factors, including antibiotics [36,37]. The resistance of spores to environmental factors, cleaning agents, and sterilization methods as well as their diverse physiological properties make *Bacillus* of great interest to the food, animal, and biotechnology sectors. Specifically, the use of some spore-forming bacteria from the genus *Bacillus* have earned interest as direct-fed microbials in recent years as potential alternatives to AGPs. Inclusion of *Bacillus*-DFMs in broiler diets has been shown to have positive effects on the overall performance of boilers, the broiler immune system, and their resistance to disease [23,25,34,38–41]. The resilient capacity of spores to resist harsh environmental conditions, as well as their long shelf-life, make them feed-stable and suitable for commercialization in human and animal health and nutrition [42,43].

Nevertheless, it is essential to understand that not all *Bacillus* species can be used as DFMs. Each isolate has unique genetic and phenotypic characteristics, which in turn influence changes in the intestinal tract and the isolates' heat resistance capacity, rate of growth, sporulation rate, and persistence in the GIT [18,44]. Herein, we described the characterization and development of a novel *Bacillus*-based DFM that increased the diversity of the broiler fecal microbiota and performed as well or better than commonly used AGPs under defined and non-defined intestinal dysbiosis conditions.

A common feature of *Bacillus* species is their ability to inhibit the growth of other bacteria to varying degrees. Of interest to the poultry industry is the inhibition of colonization or growth of food-borne pathogens such as *Escherichia coli* and *Salmonella* species, among others, in or on the broiler. The novel strains isolated in this study, to varying degrees, displayed the ability to inhibit the growth of these pathogens in vitro (Figure S1 and Table S1), consistent with other *Bacillus* species isolated and characterized previously [45,46]. This antagonistic effect on the growth of other bacteria is associated with the production of a variety of natural antibiotics [36,47].

Bacillus subtilis produces a wide array of secreted enzymes known to promote and optimize the digestibility of non-starch polysaccharide (NSP)-rich diets such as xylanases, cellulases, and β-glucanases [23,24,48]. The inclusion of specific *Bacillus*-DFM candidates that produce exogenous enzymes, such as cellulases, amylases, and xylanase, in high NSP diets significantly reduced both viscosity and *C. perfringens* proliferation in an in vitro digestive model study simulating different compartments of the GIT [49]. The selected strains for this novel DFM produce extracellular enzymes, proteases, and lipases (Table 4) and were highly resistant to simulated GIT conditions (Tables 5–7) which may aid in transiting the GIT and, upon sporulation, facilitate the degradation of low-quality proteins and fats present in the diet that are used by the host for growth and prevention of detrimental enteric microflora changes. It was observed that the strains evaluated in this work had varying levels of enzyme production. Direct empirical comparisons are difficult because of the relative assay measurements, but recent work analyzing the relative enzyme activity of *B. subtilis* isolated from the broiler chick GIT identified a wide range of enzyme production

capabilities in the isolates [45]. This is not unsurprising, as the regulation and secretion of exoproteins in Bacilli is multi-factorial and complex.

The use of *Bacillus* species probiotics and DFMs as alternatives to AGPs to enhance growth performance metrics is well established. We tested this new *Bacillus* DFM formulation for broiler growth enhancement in two separate studies using different methods (1, necrotic enteritis model; 2, reused litter and oral challenge model) to disrupt intestinal homeostasis and stunt the growth of the chicks.

In Experiment 1, a previously defined NE challenge model [29,30] was utilized to examine the in vivo effects of this new DFM on performance parameters. Chickens supplemented with the *Bacillus*-DFM had similar growth performance parameters as those chickens supplemented with the AGP (flavomycin), which were greater than the basal control diets (Table 8). The lack of statistically significant differences in this trial is directly related to the limited replication and number of birds used in the trial. On an individual bird level, marginal gains in performance may seem nominal but accumulated across a large number of birds, and the impact can produce significant cost savings and economic returns to producers in the form of greater raw meat production and reduced feed costs. Based on these data of Experiment 1, the optimal dose of this new DFM formulation was determined to be 0.3 kg of DFM per metric ton (0.3 kg/ton).

In Experiment 1, a resource-limited number of samples were analyzed to survey potential impacts of the DFM on the fecal microbiome consortium of the chickens at the minimum and maximum doses of the trial. Inclusion of the DFM into broiler diets altered the fecal microbiome of broilers as early as five days of age. At an inclusion rate of 0.4 kg/ton, the DFM suppressed the early dominance of *Lactobacillus* and *Enterococcus* and promoted greater diversity in species abundance by day 25. Within the greater context of the effect of *Bacillus* DFMs on the fecal microbiome and the relationship to body weight gain and feed conversion efficiency, a consistent profile of beneficial changes within taxa is lacking [10,11,50–52]. The lack of concurrence in these studies is likely a result of the varying methods of husbandry, feed, source and breed of chicks, tissue type and time of sample collection, and overall sequencing and analytical methods. The microbiome of the GIT is temporally dynamic and influenced by intra- and extra-host factors. The primary role of AGPs, and subsequently DFMs, is likely not to induce a defined beneficial microbiome per se, but rather to perpetuate the establishment and maintenance of a beneficial microbial genetic and metabolic profile in the host GIT. As metabolic genes are conserved across genera, the innate metabolic properties of the microbiome appear to be more important for host performance than the specific genera [4] that are present. The new DFM described here increased bacterial diversity, which in turn may establish a larger and more favorable bacterial metabolic profile, which helps the broiler efficiently utilize feed and overcome intestinal dysbiosis.

In Experiment 2, litter from a commercial farm was used and the broilers were orally given a non-defined challenge to induce intestinal dysbiosis with the goal of replicating potential stressors of a large-scale commercial farm operation. The results support the inclusion of this new DFM in feed at a rate of 0.3 kg/ton as an alternative to an AGP (BMD 11%) to enhance the growth performance of broilers and blunt the negative effects of on-farm microbial stressors.

The heterogeneity in the *Bacillus* genera, varying spore concentrations, and formulations of DFMs used makes individual comparisons between strains and studies difficult. Luise et al. recently analyzed the results from 131 studies utilizing *Bacillus* spp. DFMs and found "The benefits of *Bacillus* strains on these [growth] parameters [of broilers] showed results comparable to the benefit obtained by the use of antibiotics [5]". These benefits are received through four primary means: (1) direct effect on pathogenic bacteria, (2) favoring the colonization of the gut by beneficial bacteria, (3) host immunostimulatory effects, and (4) contributions to feed efficiency [53–55]. *Bacillus*-based DFMs are a safe and commensurate alternative to AGPs in broilers.

5. Conclusions

In summary, using both a defined (Exp 1, Table 8) and undefined dysbiosis-inducing challenge model (Exp 2, Table 9), chickens fed this new DFM had an observable growth advantage over basal-fed-diets, and the productive growth parameters of the DFM-fed chickens were at least equal to or numerically better than the AGP-fed groups with no observed negative side effects, indicating Zymospore® is a safe and effective AGP substitute for the poultry industry.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani12111436/s1, Table S1. Growth inhibition of *Salmonella* and *Escherichia coli* isolates by *Bacillus subtilis* on SPA agar plates; Figure S1: Growth inhibition of the select pathogen isolates by Bacillus subtilis isolate BS-009 on Spizizen potato agar (SPA) plate. SE, Salmonella Enteritidis; ST, Salmonella Typhimurium; EC-C, avian pathogenic Escherichia coli (APEC); EC-P, Escherichia coli isolated from a pig; EC-E, enteropathogenic Escherichia coli (EPEC); Figure S2: Order level proportions across individual samples for each treatment group and day of sampling. The height of each color-coded portion of the bar plot represents the percentage of that Order relative to the total number of 16S sequences classified to a taxonomic Order level identified in that sample. Individual replicates (Rn) for each respective treatment group and sampling timepoint are shown. Only Orders with ≥1% proportionality are shown.

Author Contributions: Conceptualization O.F.d.S., C.S. and S.L.; methodology, E.G. and F.M.; software, B.V.; validation, J.W.H. and S.L.; formal analysis, J.W.H.; investigation, O.F.d.S., B.V. and E.G.; resources, S.L.; data curation, O.F.d.S. and C.S.; writing—original draft preparation, O.F.d.S.; writing—review and editing, C.S., S.L., J.W.H. and X.H.-V.; visualization, E.G. and F.M.; supervision, B.V., C.S. and F.L.G.; project administration, B.V. and S.L.; funding acquisition, S.L. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All procedures used in the current study were approved by the Federal University of Santa Maria, Santa Maria, Brazil (number 5404280717) (Experiment 1), and followed the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the National Institute of Agronomic Technologies (INTA). The approved study protocol number is 6/2021 (Experiment 2).

Data Availability Statement: Not applicable.

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References

1. Teirlynck, E.; Gussem, M.; Dewulf, J.; Haesebrouck, F.; Ducatelle, R.; Van Immerseel, F. Morphometric evaluation of "dysbacteriosis" in broilers. *Avian Pathol.* **2011**, *40*, 139–144. [CrossRef]
2. Roberts, T.; Wilson, J.; Guthrie, A.; Cookson, K.; Vancraeynest, D.; Schaeffer, J.; Moody, R.; Clark, S. New issues and science in broiler chicken intestinal health: Intestinal microbial composition, shifts, and impacts. *Worlds Poult. Sci. J.* **2015**, *71*, 259–270. [CrossRef]
3. Parker, D.S. Manipulation of the functional activity of the gut by dietary and other means (antibiotics/probiotics) in ruminants. *J. Nutr.* **1990**, *120*, 639–648. [CrossRef] [PubMed]
4. Plata, G.; Baxter, N.T.; Susanti, D.; Volland-Munson, A.; Gangaiah, D.; Nagireddy, A.; Mane, S.P.; Balakuntla, J.; Hawkins, T.B.; Mahajan, M.A. Growth promotion and antibiotic induced metabolic shifts in the chicken gut microbiome. *Commun. Biol.* **2022**, *5*, 293. [CrossRef] [PubMed]
5. Luise, D.; Bosi, P.; Raff, L.; Amatucci, L.; Virdis, S.; Trevisi, P. *Bacillus* spp. probiotic strains as a potential tool for limiting the use of antibiotics, and improving the growth and health of pigs and chickens. *Front. Microbiol.* **2022**, *13*, 801827. [CrossRef]
6. The White House. National Action Plan for Combating Antibiotic-Resistant Bacteria. 2015. Available online: https://www.cdc.gov/drugresistance/pdf/national_action_plan_for_combating_antibotic-resistant_bacteria.pdf (accessed on 13 March 2022).
7. Tellez, G.; Latorre, J.D. *Alternatives to Antimicrobial Growth Promoters and Their Impact in Gut Microbiota, Health and Disease*; Frontiers Media: Lausanne, Switzerland, 2017; pp. 6–92. [CrossRef]
8. Bai, W.K.; Zhang, F.J.; He, T.J.; Su, P.W.; Ying, X.Z.; Zhang, L.L.; Wang, T. Dietary probiotic *Bacillus subtilis* strain fmbj increases antioxidant capacity and oxidative stability of chicken breast meat during storage. *PLoS ONE* **2016**, *11*, e0167339. [CrossRef]

9. Pandey, K.R.; Naik, S.R.; Vakil, B.V. Probiotics, prebiotics and synbiotics—A review. *J. Food Sci. Technol.* **2015**, *52*, 7577–7587. [CrossRef]
10. Wang, B.; Zhou, Y.; Tang, L.; Zeng, Z.; Gong, L.; Wu, Y.; Li, W.-F. Effects of *Bacillus amyloliquefaciens* instead of antibiotics on growth performance, intestinal health, and intestinal microbiota of broilers. *Front. Vet. Sci.* **2021**, *8*, 679368. [CrossRef]
11. Li, C.-L.; Wang, J.; Zhang, H.-J.; Wu, S.-G.; Hui, Q.-R.; Yang, C.-B.; Fang, R.-J.; Qi, G.-H. Intestinal morphologic and microbiota responses to dietary *Bacillus* spp. in a broiler chicken model. *Front. Physiol.* **2019**, *9*, 1968. [CrossRef]
12. Jadamus, A.; Vahjen, W.; Simon, O. Growth behaviour of a spore-forming probiotic strain in the gastrointestinal tract of broiler chicken and piglets. *Arch. Tierernahr.* **2001**, *54*, 1–17. [CrossRef]
13. Tellez, G.; Pixley, C.; Wolfenden, R.; Layton, S.; Hargis, B.M. Probiotics/direct-fed microbials for *Salmonella* control in poultry. *Food Res. Int.* **2012**, *45*, 628–633. [CrossRef]
14. Menconi, A.; Morgan, M.J.; Pumford, N.R.; Hargis, B.M.; Tellez, G. Physiological properties and *Salmonella* growth inhibition of probiotic *Bacillus* strains isolated from environmental and poultry sources. *Int. J. Bacteriol.* **2013**, *2013*, 958408. [CrossRef] [PubMed]
15. Vreeland, R.H.; Rosenzweig, W.D.; Powers, D.W. Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal. *Nature* **2000**, *407*, 897–900. [CrossRef] [PubMed]
16. Shivaramaiah, S.; Pumford, N.; Morgan, M.; Wolfenden, R.; Wolfenden, A.; Torres-Rodriguez, A.; Hargis, B.M.; Téllez, G. Evaluation of *Bacillus* species as potential candidates for direct-fed microbials in commercial poultry. *Poult. Sci.* **2011**, *90*, 1574–1580. [CrossRef] [PubMed]
17. Cartman, S.T.; La Ragione, R.M.; Woodward, M.J. *Bacillus subtilis* spores germinate in the chicken gastrointestinal tract. *Appl. Environ. Microbiol.* **2008**, *74*, 5254–5258. [CrossRef] [PubMed]
18. Casula, G.; Cutting, S.M. *Bacillus* probiotics: Spore germination in the gastrointestinal tract. *Appl. Environ. Microbiol.* **2002**, *68*, 2344–2352. [CrossRef] [PubMed]
19. Ghelardi, E.; Celandroni, F.; Salvetti, S.; Gueye, S.A.; Lupetti, A.; Senesi, S. Survival and persistence of Bacillus clausii in the human gastrointestinal tract following oral administration as spore-based probiotic formulation. *J. Appl. Microbiol.* **2015**, *119*, 552–559. [CrossRef]
20. Duc, L.H.; Hong, H.A.; Barbosa, T.M.; Henriques, A.O.; Cutting, S.M. Characterization of *Bacillus* probiotics available for human use. *Appl. Environ. Microbiol.* **2004**, *70*, 2161–2171. [CrossRef]
21. Hendricks, C.W.; Doyle, J.D.; Hugley, B. A new solid medium for enumerating cellulose-utilizing bacteria in soil. *Appl. Environ. Microbiol.* **1995**, *61*, 2016–2019. [CrossRef]
22. Mazotto, A.M.; Rodrigues, R.R.C.; Cedrola, S.M.L.; de Lima, M.F.; Couri, S.; Paraguai de Souza, E.; Vermelho, A.B. Keratinase production by three *Bacillus* spp. using feather meal and whole feather as substrate in a submerged fermentation. *Enzym. Res.* **2011**, *2011*, 523780. [CrossRef]
23. Latorre, J.D.; Hernandez-Velasco, X.; Kuttappan, V.A.; Wolfenden, R.E.; Vicente, J.L.; Wolfenden, A.D.; Bielke, L.R.; Prado-Rebolledo, O.F.; Morales, E.; Hargis, B.M.; et al. Selection of *Bacillus* spp. for cellulase and xylanase production as direct-fed microbials to reduce digesta viscosity and *Clostridium perfringens* proliferation using an in vitro digestive model in different poultry diets. *Front. Vet. Sci.* **2015**, *2*, 25. [CrossRef] [PubMed]
24. Latorre, J.D.; Hernandez-Velasco, X.; Wolfenden, R.E.; Vicente, J.L.; Wolfenden, A.D.; Menconi, A.; Bielke, L.R.; Hargis, B.M.; Tellez, G. Evaluation and selection of *Bacillus* species based on enzyme production, antimicrobial activity, and biofilm synthesis as direct-fed microbial candidates for poultry. *Front. Vet. Sci.* **2016**, *3*, 95. [CrossRef] [PubMed]
25. Latorre, J.; Hernandez-Velasco, X.; Vicente, J.; Wolfenden, R.; Hargis, B.; Tellez, G. Effects of the inclusion of a *Bacillus* direct-fed microbial on performance parameters, bone quality, recovered gut microflora, and intestinal morphology in broilers consuming a grower diet containing corn distillers dried grains with solubles. *Poult. Sci.* **2017**, *96*, 2728–2735. [CrossRef] [PubMed]
26. Hosoi, T.; Ametani, A.; Kiuchi, K.; Kaminogawa, S. Improved growth and viability of lactobacilli in the presence of *Bacillus subtilis* (natto), catalase, or subtilisin. *Can. J. Microbiol.* **2000**, *46*, 892–897. [CrossRef]
27. Jani, S.A.; Chudasama, C.J.; Patel, D.B.; Bhatt, P.S.; Patel, H.N. Optimization of extracellular protease production from alkali thermo tolerant actinomycetes: *Saccharomonospora viridis* SJ-21. *Bull. Environ. Pharmacol. Life Sci.* **2012**, *1*, 84–92.
28. Rostagno, H.S.; Albino, L.F.T.; Hannas, M.I.; Donzele, J.L.; Sakomura, N.K.; Perazzo, F.G.; Saraiva, A.; Texeira de Abreu, M.L.; Rodrigues, P.B.; Oliveira, R.F.; et al. *Tabelas Brasileiras para Aves e Suínos. Composição de Alimentos e Exigências Nutricionais*, 4th ed.; Universidade Federal de Viçosa: Viçosa, Brazil, 2017.
29. Belote, B.; Tujimoto-Silva, A.; Hümmelgen, P.; Sanches, A.; Wammes, J.; Hayashi, R.; Santin, E. Histological parameters to evaluate intestinal health on broilers challenged with *Eimeria* and *Clostridium perfringens* with or without enramycin as growth promoter. *Poult. Sci.* **2018**, *97*, 2287–2294. [CrossRef]
30. Stefanello, C.; Rosa, D.P.; Dalmoro, Y.K.; Segatto, A.L.; Vieira, M.S.; Moraes, M.L.; Santin, E. Protected blend of organic acids and essential oils improves growth performance, nutrient digestibility, and intestinal health of broiler chickens undergoing an intestinal challenge. *Front. Vet. Sci.* **2020**, *6*, 491. [CrossRef]
31. Degnan, P.H.; Ochman, H. Illumina-based analysis of microbial community diversity. *ISME J.* **2012**, *6*, 183–194. [CrossRef]
32. Sakomura, N.K.; Rostagno, H.S. *Métodos de Pesquisa em Nutrição de Monogástricos*; Fundação de Apoio a Pesquisa, Ensino e Extensão: Jaboticabal, Brazil, 2007; 262p.
33. SAS Institute Inc. *SAS User Guide. Version 9.1*; SAS Institute Inc.: Carry, NC, USA, 2002.

34. Hernandez-Patlan, D.; Solis-Cruz, B.; Pontin, K.P.; Hernandez-Velasco, X.; Merino-Guzman, R.; Adhikari, B.; López-Arellano, R.; Kwon, Y.M.; Hargis, B.M.; Arreguin-Nava, M.A.; et al. Impact of a *Bacillus* direct-fed microbial on growth performance, intestinal barrier integrity, necrotic enteritis lesions, and ileal microbiota in broiler chickens using a laboratory challenge model. *Front. Vet. Sci.* **2019**, *6*, 108. [CrossRef]
35. Palliyeguru, M.W.C.D.; Rose, S.P. Sub-clinical necrotic enteritis: Its aetiology and predisposing factors in commercial broiler production. *Worlds Poult. Sci. J.* **2014**, *70*, 803–816. [CrossRef]
36. Abriouel, H.; Franz, C.M.A.P.; Omar, N.B.; Gálvez, A. Diversity and applications of *Bacillus* bacteriocins. *FEMS Microbiol. Rev.* **2011**, *35*, 201–232. [CrossRef] [PubMed]
37. Priest, F.G. Extracellular enzyme synthesis in the genus *Bacillus*. *Bacteriol. Rev.* **1977**, *41*, 711–753. [CrossRef]
38. Lee, K.W.; Kim, D.K.; Lillehoj, H.S.; Jang, S.I.; Lee, S.H. Immune modulation by *Bacillus subtilis*-based direct-fed microbials in commercial broiler chickens. *Anim. Feed Sci. Technol.* **2015**, *200*, 76–85. [CrossRef]
39. Sandvang, D.; Skjoet-Rasmussen, L.; Cantor, M.D.; Mathis, G.F.; Lumpkins, B.S.; Blanch, A. Effects of feed supplementation with 3 different probiotic *Bacillus* strains and their combination on the performance of broiler chickens challenged with *Clostridium perfringens*. *Poult. Sci.* **2021**, *100*, 100982. [CrossRef] [PubMed]
40. Adhikari, B.; Hernandez-Patlan, D.; Solis-Cruz, B.; Kwon, Y.M.; Arreguin, M.A.; Latorre, J.D.; Hernandez-Velasco, X.; Hargis, B.M.; Tellez-Isaias, G. Evaluation of the antimicrobial and anti-inflammatory properties of Bacillus-DFM (Norum™) in broiler chickens infected with *Salmonella* Enteritidis. *Front. Vet. Sci.* **2019**, *6*, 282. [CrossRef]
41. Shehata, A.A.; Yalçın, S.; Latorre, J.D.; Basiouni, S.; Attia, Y.A.; Abd El-Wahab, A.; Visscher, C.; El-Seedi, H.R.; Huber, C.; Hafez, H.M.; et al. Probiotics, prebiotics, and phytogenic substances for optimizing gut health in poultry. *Microorganisms* **2022**, *10*, 395. [CrossRef]
42. Hong, H.A.; Duc, L.H.; Cutting, S.M. The use of bacterial spore formers as probiotics. *FEMS Microbiol. Rev.* **2005**, *29*, 813–835. [CrossRef]
43. Hong, H.; Huang, J.M.; Khaneja, R.; Hiep, L.; Urdaci, M.; Cutting, S. The safety of *Bacillus subtilis* and *Bacillus indicus* as food probiotics. *J. Appl. Microbiol.* **2008**, *105*, 510–520. [CrossRef]
44. Amuguni, H.; Tzipori, S. *Bacillus subtilis*: A temperature resistant and needle free delivery system of immunogens. *Hum. Vaccin. Immunother.* **2012**, *8*, 979–986. [CrossRef]
45. Penaloza-Vazquez, A.; Ma, L.M.; Rayas-Duarte, P. Isolation and characterization of *Bacillus* spp. strains as potential probiotics for poultry. *Can. J. Microbiol.* **2019**, *65*, 762–774. [CrossRef]
46. Nithya, V.; Halami, P.M. Evaluation of the probiotic characteristics of *Bacillus* species isolated from different food sources. *Ann. Microbiol.* **2013**, *63*, 129–137. [CrossRef]
47. Stein, T. *Bacillus subtilis* antibiotics: Structures, syntheses and specific functions. *Mol. Microbiol.* **2005**, *56*, 845–857. [CrossRef] [PubMed]
48. Monisha, R.; Uma, M.; Murthy, V.K. Partial purification and characterization of *Bacillus pumilus* xylanase from soil source. *KUSET* **2009**, *5*, 137–148.
49. Latorre, J.D.; Hernandez-Velasco, X.; Kogut, M.H.; Vicente, J.L.; Wolfenden, R.; Wolfenden, A.; Hargis, B.M.; Kuttappan, V.A.; Tellez, G. Role of a *Bacillus subtilis* direct-fed microbial on digesta viscosity, bacterial translocation, and bone mineralization in turkey poults fed with a rye-based diet. *Front. Vet. Sci.* **2014**, *1*, 26. [CrossRef] [PubMed]
50. Singh, K.M.; Shahm, T.M.; Reddy, B.; Deshpande, S.; Rank, D.N.; Joshi, C.G. Taxonomic and gene-centric metagenomics of the fecal microbiome of low and high feed conversion ratio (FCR) broilers. *J. Appl. Genet.* **2014**, *55*, 145–154. [CrossRef]
51. Singh, K.M.; Shah, T.; Deshpande, S.; Jakhesara, S.J.; Koringa, P.G.; Rank, D.N.; Joshi, C.G. High through put 16S rRNA gene-based pyrosequencing analysis of the fecal microbiota of high FCR and low FCR broiler growers. *Mol. Biol. Rep.* **2012**, *39*, 10595–10602. [CrossRef]
52. Diaz-Sánchez, S.; Perrotta, A.R.; Rockafellow, I.; Alm, E.J.; Okimoto, R.; Hawken, R.; Hanning, I. Using fecal microbiota as biomarkers for predictions of performance in the selective breeding process of pedigree broiler breeders. *PLoS ONE* **2019**, *14*, e0216080. [CrossRef]
53. La Ragione, R.M.; Woodward, M.J. Competitive exclusion by *Bacillus subtilis* spores of *Salmonella enterica* serotype Enteritidis and *Clostridium perfringens* in young chickens. *Vet. Microbiol.* **2003**, *94*, 245–256. [CrossRef]
54. Revolledo, L.; Ferreira, A.; Mead, G. Prospects in *Salmonella* control: Competitive exclusion, probiotics, and enhancement of avian intestinal immunity. *J. Appl. Poult. Res.* **2006**, *15*, 341–351. [CrossRef]
55. Khan, S.; Moore, R.J.; Stanley, D.; Chousalkar, K.K. Gut microbiota of laying hens and its manipulation with prebiotics and probiotics to enhance gut health and food safety. *Appl. Environ. Microbiol.* **2020**, *86*, e00600-20. [CrossRef]

6 CONSIDERAÇÕES FINAIS

A utilização de probiótico em dietas para matrizes de poedeiras se mostrou eficiente para melhorar a produção de ovos, conversão alimentar e qualidade de ovos. Já o uso combinado do probiótico com propionato de cromo, possibilitou otimizar esses resultados produtivos e de utilização dos nutrientes.

A utilização de aditivos probióticos comerciais na ração representa uma importante alternativa ao uso dos antibióticos promotores de crescimento, uma vez que foi constatado efeito positivo na manutenção da microbiota benéfica do trato digestório e no desempenho produtivo de frangos de corte, sob condições de desafio, semelhantes as encontradas à campo.

REFERÊNCIAS

ABDELQADER, A.; AL-FATAFTAH, A.-R.; DAŞ, G. Effects of dietary Bacillus subtilis and inulin supplementation on performance, eggshell quality, intestinal morphology and microflora composition of laying hens in the late phase of production. **Animal Feed Science and Technology**, v. 179, n. 1–4, p. 103–111, jan. 2013.

AKINYEMI, F. T. et al. Dynamic distribution of gut microbiota during embryonic development in chicken. **Poultry Science**, v. 99, n. 10, p. 5079–5090, 2020.

AL-KHALAIFAH, H. S. Benefits of probiotics and/or prebiotics for antibiotic-reduced poultry. **Poultry Science**, v. 97, n. 11, p. 3807–3815, nov. 2018.

ALAGAWANY, M. et al. The use of probiotics as eco-friendly alternatives for antibiotics in poultry nutrition. **Environmental Science and Pollution Research**, v. 25, n. 11, p. 10611–10618, 2018.

ANDERSON, R. A. Nutritional factors influencing the glucose/insulin system: Chromium. **Journal of the American College of Nutrition**, v. 16, n. 5, p. 404–410, 1997.

APAJALAHTI, J.; KETTUNEN, A.; GRAHAM, H. Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. **World's Poultry Science Journal**, v. 60, n. 2, p. 223–232, 2004.

BAJAJ, B. K.; CLAES, I. J. J.; LEBEER, S. Functional mechanisms of probiotics. **Journal of Microbiology, Biotechnology and Food Sciences**, v. 04, n. 04, p. 321–327, 2015.

BERMUDEZ-BRITO, M. et al. Probiotic mechanisms of action. **Annals of Nutrition and Metabolism**, v. 61, n. 2, p. 160–174, 2012.

BEZERRA;, W. G. A. et al. Antibióticos no setor avícola : uma revisão sobre a resistência microbiana. **Archivos de Zootecnia**, v. 66, n. 254, p. 301–307, 2017.

BRASIL. **Ministério da Agricultura Pecuária e AbastecimentoInstrução normativa n° 1, 13 de janeiro de 2020**Brasília, DFDiário Oficial da União, Seção 1, p. 6, , 2020. Disponível em: <https://www.gov.br/agricultura/pt-br/assuntos/insumos-agropecuarios/insumos-pecuarios/alimentacao-animal/arquivos-alimentacao-animal/legislacao/INM000000012020.pdf>

BRENES, A.; ROURA, E. Essential oils in poultry nutrition: Main effects and modes of action. **Animal Feed Science and Technology**, v. 158, n. 1–2, p. 1–14, jun. 2010.

BRON, P. A.; VAN BAARLEN, P.; KLEEREBEZEM, M. Emerging molecular insights into the interaction between probiotics and the host intestinal mucosa. **Nature Reviews Microbiology**, v. 10, n. 1, p. 66–78, 2012.

CARRASCO, J. M. D.; CASANOVA, N. A.; MIYAKAWA, M. E. F. Microbiota, gut health and chicken productivity: What is the connection? **Microorganisms**, v. 7, n. 10, p. 1–15, 2019.

CASTANON, J. I. R. History of the Use of Antibiotic as Growth Promoters. **Poultry Science**, v. 86, n. 11, p. 2466–2471, 2007.

CHEN, J.; YING, G. G.; DENG, W. J. Antibiotic Residues in Food: Extraction, Analysis, and Human Health Concerns. **Journal of Agricultural and Food Chemistry**, v. 67, n. 27, p. 7569–7586, 10 jul. 2019.

CHICHLOWSKI, M. et al. Metabolic and physiological impact of probiotics or direct-fed-microbials on poultry: A brief review of current knowledge. **International Journal of Poultry Science**, v. 6, n. 10, p. 694–704, 2007.

CLAVIJO, V.; FLÓREZ, M. J. V. The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: A review. **Poultry Science**, v. 97, n. 3, p. 1006–1021, 2018.

CUTTING, S. M. Bacillus probiotics. **Food Microbiology**, v. 28, n. 2, p. 214–220, abr. 2011.

DARSI, E.; ZHAGHARI, M. Effects of Bacillus subtilis PB6 supplementation on productive performance, egg quality and hatchability in broiler breeder hens under commercial farm condition. **Journal of Applied Animal Research**, v. 49, n. 1, p. 109–117, 1 jan. 2021.

DAVIS, M. F. et al. An ecological perspective on U.S. industrial poultry production: the role of anthropogenic ecosystems on the emergence of drug-resistant bacteria from agricultural environments. **Current Opinion in Microbiology**, v. 14, n. 3, p. 244–250, jun. 2011.

DIARRA, M. S.; MALOUIN, F. Antibiotics in Canadian poultry productions and anticipated alternatives. **Frontiers in Microbiology**, v. 5, 17 jun. 2014.

DING, J. et al. Inheritance and Establishment of Gut Microbiota in Chickens. **Frontiers in Microbiology**, v. 8, n. OCT, p. 1–11, 10 out. 2017.

DONALDSON, E. E. et al. The time-course of broiler intestinal microbiota development after administration of cecal contents to incubating eggs. **PeerJ**, v. 5, n. 7, p. e3587, 20 jul. 2017.

DOWARAH, R.; VERMA, A. K.; AGARWAL, N. The use of Lactobacillus as an alternative of antibiotic growth promoters in pigs: A review. **Animal Nutrition**, v. 3, n. 1, p. 1–6, mar. 2017.

FDA. Antimicrobials sold or distributed for use in food producing animals. **US Food and Drug Administration: Silver Spring, MD, USA**, 2019.

FENG, Y. et al. Identification of changes in the composition of ileal bacterial microbiota of broiler chickens infected with Clostridium perfringens. **Veterinary Microbiology**, v. 140, n. 1–2, p. 116–121, jan. 2010.

FULLER, R. Probiotics in man and animals. **Journal of Applied Bacteriology**, v. 66, n. 5, p. 365–378, 1989.

GADDE, U. et al. Alternatives to antibiotics for maximizing growth performance and feed efficiency in poultry: a review. **Animal Health Research Reviews**, v. 18, n. 1, p. 26–45, 9 jun. 2017.

GOETTING, V.; LEE, K. A.; TELL, L. A. Pharmacokinetics of veterinary drugs in laying hens and residues in eggs: a review of the literature. **Journal of Veterinary Pharmacology and Therapeutics**, v. 34, n. 6, p. 521–556, dez. 2011.

HEMARAJATA, P.; VERSALOVIC, J. Effects of probiotics on gut microbiota: Mechanisms of intestinal immunomodulation and neuromodulation. **Therapeutic Advances in Gastroenterology**, v. 6, n. 1, p. 39–51, 2013.

HERNANDEZ-PATLAN, D. et al. Impact of a Bacillus Direct-Fed Microbial on Growth Performance, Intestinal Barrier Integrity, Necrotic Enteritis Lesions, and Ileal Microbiota in Broiler Chickens Using a Laboratory Challenge Model. **Frontiers in Veterinary Science**, v. 6, n. April, p. 1–11, 24 abr. 2019.

HOSPENTHAL, M. K.; COSTA, T. R. D.; WAKSMAN, G. A comprehensive guide to pilus biogenesis in Gram-negative bacteria. **Nature Reviews Microbiology**, v. 15, n. 6, p. 365–379, 2017.

JACKSON, A. R. et al. The effect of chromium propionate on growth performance and carcass traits in broilers. **Journal of Applied Poultry Research**, v. 17, n. 4, p. 476–481, 2008.

JAYARAMAN, S. et al. Bacillus subtilis PB6 improves intestinal health of broiler chickens challenged with Clostridium perfringens-induced necrotic enteritis. **Poultry Science**, v. 92, n. 2, p. 370–374, fev. 2013.

JAYARAMAN, S. et al. Use of Bacillus Subtilis PB6 as a potential antibiotic growth promoter replacement in improving performance of broiler birds. **Poultry Science**, v. 96, n. 8, p. 2614–2622, ago. 2017.

KALMENDAL, R. et al. Sunflower Meal and Exogenous Enzymes in Initial Diets for. **Rev Caatinga**, v. 44, n. 4, p. 234–243, 2016.

KHAN, R. U.; NAZ, S. The applications of probiotics in poultry production. **World's Poultry Science Journal**, v. 69, n. 3, p. 621–632, 1 set. 2013.

KIRCHHELLE, C. Pharming animals: a global history of antibiotics in food production (1935–2017). **Palgrave Communications**, v. 4, n. 1, p. 96, 7 dez. 2018.

LATORRE, J. D. et al. Effects of the inclusion of a Bacillus direct-fed microbial on performance parameters, bone quality, recovered gut microflora, and intestinal morphology in broilers consuming a grower diet containing corn distillers dried grains with solubles. **Poultry Science**, v. 96, n. 8, p. 2728–2735, ago. 2017.

LAXMINARAYAN, R.; HEYMANN, D. Challenges of drug resistance in the developing world Causes of resistance Increasing use of antibiotics. 2012.

MINGMONGKOLCHAI, S.; PANBANGRED, W. Bacillus probiotics: an alternative to antibiotics for livestock production. **Journal of Applied Microbiology**, v. 124, n. 6, p. 1334–1346, jun. 2018.

MOEINI, M. M. et al. The Effect of Different Levels of Organic and Inorganic Chromium

Supplementation on Production Performance, Carcass Traits and Some Blood Parameters of Broiler Chicken Under Heat Stress Condition. **Biological Trace Element Research**, v. 144, n. 1–3, p. 715–724, 21 dez. 2011.

MOHD SHAUFI, M. A. et al. Deciphering chicken gut microbial dynamics based on high-throughput 16S rRNA metagenomics analyses. **Gut Pathogens**, v. 7, n. 1, p. 1–12, 2015.

MONTEAGUDO-MERA, A. et al. Adhesion mechanisms mediated by probiotics and prebiotics and their potential impact on human health. **Applied Microbiology and Biotechnology**, v. 103, n. 16, p. 6463–6472, 2 ago. 2019.

MROZ, Z. et al. Carboxylic acids as bioregulators and gut growth promoters in non-ruminants. In: **Biology of Growing Animals**. [s.l: s.n.]. p. 81–133.

MUND, M. D. et al. **Antimicrobial drug residues in poultry products and implications on public health: A reviewInternational Journal of Food Properties**Taylor and Francis Inc., , 3 jul. 2017.

NAVA, G. M. et al. Probiotic alternatives to reduce gastrointestinal infections: the poultry experience. **Animal Health Research Reviews**, v. 6, n. 1, p. 105–118, 2005.

NEIJAT, M. et al. Bacillus subtilis Strain DSM 29784 Modulates the Cecal Microbiome, Concentration of Short-Chain Fatty Acids, and Apparent Retention of Dietary Components in Shaver White Chickens during Grower, Developer, and Laying Phases. **Applied and Environmental Microbiology**, v. 85, n. 14, p. 1–20, 15 jul. 2019.

OBA, A. et al. Características produtivas e imunológicas de frangos de corte submetidos a dietas suplementadas com cromo, criados sob diferentes condições de ambiente. **Revista Brasileira de Zootecnia**, v. 41, n. 5, p. 1186–1192, maio 2012.

PALAMIDI, I. et al. Probiotic form effects on growth performance, digestive function, and immune related biomarkers in broilers. **Poultry Science**, v. 95, n. 7, p. 1598–1608, 2016.

PAN, D.; YU, Z. Intestinal microbiome of poultry and its interaction with host and diet. **Gut Microbes**, v. 5, n. 1, p. 108–119, 31 jan. 2014.

PARK, J. W. et al. Management and production: Effect of dietary supplementation with a probiotic (Enterococcus faecium) on production performance, excreta microflora, ammonia emission, and nutrient utilization in ISA brown laying hens. **Poultry Science**, v. 95, n. 12, p. 2829–2835, 1 dez. 2016.

PIVA, A. et al. The effect of dietary supplementation with trivalent chromium on production performance of laying hens and the chromium content in the yolk. **Animal Feed Science and Technology**, v. 106, n. 1–4, p. 149–163, abr. 2003.

POURABEDIN, M.; ZHAO, X. Prebiotics and gut microbiota in chickens. **FEMS Microbiology Letters**, v. 362, n. 15, p. 1–8, 2015.

REHMAN, H. U. et al. Indigenous bacteria and bacterial metabolic products in the gastrointestinal tract

of broiler chickens. **Archives of Animal Nutrition**, v. 61, n. 5, p. 319–335, 2007.

REVOLLEDO, L.; FERREIRA, A. J. P.; MEAD, G. C. Prospects in Salmonella Control : Competitive Exclusion , Probiotics , and Enhancement of Avian Intestinal Immunity. **Journal of Applied Poultry Research**, v. 15, n. 2, p. 341–351, 2006.

SAKOMURA, N. K. et al. **Nutrição de não ruminantes**. 1. ed. Jaboticabal: Funep, 2014.

SANDS, J. S.; SMITH, M. O. Broilers in Heat Stress Conditions: Effects of Dietary Manganese Proteinate or Chromium Picolinate Supplementation. **Journal of Applied Poultry Research**, v. 8, n. 3, p. 280–287, out. 1999.

SEKELJA, M. et al. Abrupt temporal fluctuations in the chicken fecal microbiota are explained by its gastrointestinal origin. **Applied and Environmental Microbiology**, v. 78, n. 8, p. 2941–2948, 2012.

SNEERINGER, S. et al. Economics of Antibiotic Use in U.S. Livestock Production United States Department of Agriculture. **USDA**, n. 200, 2015.

SOBCZAK, A.; KOZŁOWSKI, K. The effect of a probiotic preparation containing Bacillus subtilis ATCC PTA-6737 on egg production and physiological parameters of laying hens. **Annals of Animal Science**, v. 15, n. 3, p. 711–723, 1 jul. 2015.

STANLEY, D.; HUGHES, R. J.; MOORE, R. J. Microbiota of the chicken gastrointestinal tract: Influence on health, productivity and disease. **Applied Microbiology and Biotechnology**, v. 98, n. 10, p. 4301–4310, 2014.

SWAROOP, A. et al. Benefits of chromium(III) complexes in animal and human health. In: **The Nutritional Biochemistry of Chromium (III)**. Second Edi ed. [s.l.] Elsevier, 2019. p. 251–278.

TEO, A. Y. L.; TAN, H.-M. Effect of Bacillus subtilis PB6 (CloSTAT) on Broilers Infected with a Pathogenic Strain of Escherichia coli. **Journal of Applied Poultry Research**, v. 15, n. 2, p. 229–235, jul. 2006.

TEO, A. Y.; TAN, H.-M. Evaluation of the Performance and Intestinal Gut Microflora of Broilers Fed on Corn-Soy Diets Supplemented With Bacillus subtilis PB6 (CloSTAT). **Journal of Applied Poultry Research**, v. 16, n. 3, p. 296–303, out. 2007.

THOMAS, C. M.; VERSALOVIC, J. Probiotics-host communication. **Gut Microbes**, v. 1, n. 3, p. 148–163, 27 maio 2010.

TORTORA, CHRISTINE L. C. B. R. F. G. J. **Microbiologia**. 12. ed. [s.l.] Artmed Editora, 2017.

VAN DER WIELEN, P. W. J. J. et al. Spatial and temporal variation of the intestinal bacterial community in commercially raised broiler chickens during growth. **Microbial Ecology**, v. 44, n. 3, p. 286–293, 2002.

VINCENT, J. B. The Biochemistry of Chromium. **The Journal of Nutrition**, v. 130, n. 4, p. 715–718, abr. 2000.

VOHRA, A.; SYAL, P.; MADAN, A. Probiotic yeasts in livestock sector. **Animal Feed Science and Technology**, v. 219, p. 31–47, set. 2016.

WANG, J. et al. Effects of dietary Bacillus subtilis supplementation and calcium levels on performance and eggshell quality of laying hens in the late phase of production. **Poultry Science**, v. 1, p. 1–10, 2021.

XING, Y. et al. Effects of dietary supplementation with lysine-yielding Bacillus subtilis on gut morphology, cecal microflora, and intestinal immune response of Linwu ducks. **Journal of Animal Science**, v. 93, n. 7, p. 3449–3457, 2015.

XU, Z. R. et al. Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora and morphology of male broilers. **Poultry Science**, v. 82, n. 6, p. 1030–1036, 2003.

YADAV, S.; JHA, R. Strategies to modulate the intestinal microbiota and their effects on nutrient utilization, performance, and health of poultry. **Journal of Animal Science and Biotechnology**, v. 10, n. 1, p. 1–11, 2019.

YOU, Y.; SILBERGELD, K. Learning from agriculture: understanding low-dose antimicrobials as drivers of resistome expansion. **Frontiers in Microbiology**, v. 5, p. 284, 2014.