MATHEUS DE PAULA REIS

SUPLEMENTAÇÃO DE UM PROBIÓTICO PARA FRANGOS DE CORTE SUBMETIDOS AO ESTRESSE TÉRMICO

LAVRAS – MG
2016

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Zootecnia, área de concentração em Produção e Nutrição de Não Ruminantes, para a obtenção do título de Doutor.

Orientador
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APROVADA em 07 de março de 2016

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LAVRAS - MG

2016

À minha mãe, um ser humano exemplar.

À Fabiana, minha eterna companheira.

Ao desenvolvimento da avicultura brasileira.

DEDICO

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RESUMO

Três experimentos independentes foram conduzidos com frangos de corte criados de 1 a 42 dias de idade, com o objetivo de avaliar o efeito da suplementação de um probiótico (*Bacillus subtilis* – DSM 17299) para frangos de corte. O experimento I foi conduzido em gaiolas de metabolismo e composto por dois tratamentos, com e sem a suplementação de probiótico. Foram avaliados o desempenho das aves semanalmente, o coeficiente de digestibilidade aparente ileal da proteína bruta (CDAPB) e da matéria seca (CDAMS) e os valores energéticos das dietas (EMA) referentes ao período inicial (18 a 21 dias) e final de criação (38 a 41dias). Avaliou-se ainda o pH dos conteúdos do trato gastro intestinal (TGI). Os experimento II e III foram conduzidos em casas coloniais composto de dois tratamentos até os 21 dias de idade (com e sem probiótico) e 4 tratamentos em esquema fatorial 2 x 2 (com e sem probiótico x com e sem estresse térmico (ET)) a partir de 22 dias de idade. As variáveis respostas estudas foram pH dos conteúdos do TGI, peso relativo de órgãos e comprimento relativo de segmentos do intestino delgado, o desempenho das aves e a morfologia do TGI. Houve uma influência positiva da suplementação do probiótico sobre os parâmetros de desempenho, no qual houve melhoras na conversão alimentar (CA), resultado de uma melhor eficiência alimentar das aves suplementadas com o probiótico. Foi possível observar diferença estatística para o pH dos conteúdos intestinais, no qual a suplementação de probiótico influenciou um aumento nesse parâmetro nas regiões do íleo aos 14 e 42 dias de idade. Aves suplementadas apresentaram redução no peso e comprimento do duodeno, com consequente aumento dos mesmos parâmetros no jejuno e íleo. Por meio do estudo da digestibilidade de nutrientes, foi possível observar uma melhora no aproveitamento da proteína bruta e da energia da dieta nas duas fases de criação. O estudo da morfologia intestinal demonstrou que as melhoras na absorção de nutrientes podem ter sido resultado de um aumento na superfície de absorção do TGI. Com exceção da profundidade de cripta aos 42 dias de idade, não houve interação entre os fatores estudados para nenhum parâmetro ou idade avaliada, demonstrando que o efeito do probiótico não foi alterado com o estresse das aves pelo aumento de temperatura. Os resultados do presente estudo demonstra que o probiótico composto por *B. subtilis* DSM 17299 possui um efeito benéfico, mesmo em aves submetidas a condições de temperatura elevada, contribuindo para o aumento da eficiência alimentar das aves, o que pode reduzir os custos de produção dos frangos de corte, trazendo maiores benefícios para a indústria avícola.

Palavras-chave: *Bacillus subtilis*. DSM 17299. Avicultura.

ABSTRACT

Three independent trials were conducted with broiler chickens raised from one to 42 days old, in order to evaluate the effect of a probiotic supplementation (*Bacillus subtilis* - DSM 17299) for broilers chickens. The first trial was conducted in metabolic cages and consisting of two treatments, with and without probiotic supplementation. We evaluated the performance of the birds weekly, the apparent ileal digestibility of crude protein (ADCP) and dry matter (ADDM) and energy content of diets (AME) for two periods, 18-21 days and 38 to 41days. It also evaluated the pH of the gastro intestinal tract (GIT) contents. Trial II was conducted in colonial houses consisting of two treatments until 21 days of age (with and without probiotic) and 4 treatments in a factorial design 2 x 2 (with and without probiotic x with and without heat stress (HS)) from day 22. The response variables evaluated were the pH of the GIT contents, relative organ weight (RW) and relative length (RL) of the segments from small intestine. The third trial followed the same distribution as the trial II, however, the measurement evaluated were performance and the morphology of the GIT. A positive influence of the probiotic supplementation on performance measurements, in which there were improvements in feed conversion ratio (FCR), a result of an improvement on feed efficiency of birds supplemented with the probiotic. It was observed a statistical difference in the pH of the intestinal contents, where the pH measurements were increased by probiotic supplementation, mainly, on ileum at days 14th and 42sd. Supplemented birds showed a reduction in the weight and length of the duodenum, with a consequent increase of the same measurement in the jejunum and ileum. The study of intestinal morphology demonstrated that improvements in nutrient uptake might have been a result of an increase in absorptive surface of the GIT. Except for the crypt depth at 42 days of age, there was no interaction between the factors studied to any measurement or assessed age, demonstrating that the effect of probiotic has not changed with the stress of the birds by an increase of the temperature. The results of this study demonstrated that probiotic consisting of *B. subtilis* DSM 17299 has a beneficial effect, even in birds subjected to high temperature conditions, contributing to the increase in feed efficiency of the poultry, which can reduce production costs of broilers, bringing greater benefits for the poultry industry.

Keywords: *Bacillus subtilis*. DSM 17299. Poultry.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

A indústria avícola destaca-se no cenário agropecuário por ser uma atividade dinâmica, na qual os seus dois principais produtos (carne de frango e ovos) tiveram grandes aumentos em produção e produtividade nas últimas décadas. Atualmente o setor desempenha um papel fundamental na produção de proteína animal no Brasil e no mundo, sendo responsável por 1,5% do PIB brasileiro (MENDES, 2014). No início dos anos 90, o consumo da carne de frango no Brasil era de 13 kg/habitante ano. Em pouco tempo, o Brasil passou por grandes mudanças em sua economia, concomitantemente e aliados à melhoria genética das aves, houve o aumento na eficiência de produção e adoção de novas tecnologias, o que proporcionou à atividade avícola altos índices de eficiência alimentar, reduzindo o preço do produto final. O resultado foi o aumento da oferta de proteína animal de alta qualidade a baixo preço de mercado gerando grande demanda por esses produtos. Em 2014, o consumo de carne de frango no Brasil foi de aproximadamente 42 kg/habitante ano (MAPA, 2015), registrando um aumento de mais de 300% em pouco mais de 20 anos.

Para suprir essa demanda, o Brasil tornou-se um dos maiores produtores de frango de corte garantindo quantidade e qualidade. Segundo a Associação Brasileira de Proteína Animal (ABPA, 2015), o Brasil, os Estados Unidos da América (EUA) e a China encerraram o ano de 2014 como os maiores produtores mundiais de carne de frango. O Brasil ocupa, atualmente, o segundo lugar no ranking dos produtores mundiais de frango de corte com aproximadamente 15% da produção mundial.

A produção avícola nacional vem se destacando também no mercado internacional, sendo que atualmente cerca de 40% da carne exportada no mundo

tem origem no Brasil. De acordo com o Mapa (2015), a taxa de crescimento da produção da carne de frango deve alcançar 4,22%, anualmente, nas exportações, com expansão prevista em 5,62% ao ano, o que deve manter o Brasil na liderança mundial em exportação de carne de frango.

Apesar dos números econômicos serem promissores, a competitividade no setor de frangos de corte preocupa, principalmente, os pequenos e médios produtores (MENDES, 2014). O cenário tende a se agravar perante as mudanças climáticas e elevação da temperatura no Brasil e no mundo que de acordo com registros, o ano de 2014 foi recorde em aumento de temperatura (HERRING, 2015; WMO, 2015a), e o ano de 2015 elevou novamente os registros de máxima nas temperaturas (WMO, 2015b, 2015c, 2015d, 2015e). De acordo com Etches et al. (2008) quando as aves são submetidas a ambientes com temperatura elevada observa-se mudanças comportamentais, fisiológicas, neuroendócrinas e molecular das aves, fatores que geralmente culminam em perda de desempenho das aves (AL-FATAFTAH; ABDELQADER, 2014; ETCHES et al., 2008; QUINTEIRO-FILHO et al., 2010; SOHAIL et al., 2012, 2013) e em casos mais extremos a morte dos animais (AZOULAY et al., 2011). Portanto, o sucesso e a manutenção no setor, seja de um produtor individual bem como de grandes companhias, depende do emprego de tecnologias em constante desenvolvimento em todos os setores de produção, dentre elas uma área que recebe muita atenção é a nutrição, justificado pela grande participação na composição do custo de produção dos frangos de corte, pois sabe-se que aproximadamente 70% do valor gasto para a criação desses animais é referente à alimentação.

As aves também enfrentam desafios de sanidade, como a presença de microrganismos patogênicos, que somados ao aumento da temperatura ambiente, pode afetar ainda mais a produção frangos de corte (CALEFI et al., 2014). A principal alternativa para o combate a microrganismos patogênicos é a suplementação subterapeutica de antimicrobianos, os melhoradores de

desempenho (AMD), entretanto essa prática vem sofrendo fortes críticas (CASTANON, 2007), e a sua total proibição realizada em 2006 na União Europeia, vem preocupando o setor de produção animal em todo o mundo.

Impulsionado pela questão apresentada, diversas alternativas aos melhoradores de desempenho passaram a ser pesquisadas e comercializadas, entre elas estão os modificadores da flora intestinal que vem se destacando devido aos recentes avanços nas pesquisas com microrganismos e seus benefícios a microbiota e ao hospedeiro, resultando em redução de patógenos e melhorias de desempenho favoráveis a criação animal (ALLOUI et al., 2014; ALLOUI MOHAMED et al., 2013; DE OLIVEIRA et al., 2014; KOLLANOOR-JOHNY et al., 2012; SPIVEY et al., 2014).

Aliados aos resultados positivos dos diversos produtos modificadores da flora intestinal, acreditasse que a suplementação dos probióticos possa proporcionar melhores resultados de desempenho para frangos de corte submetidos ao estresse por elevação da temperatura ambiente (AL-FATAFTAH e ABDELQADER, 2014), pois contribui para a manutenção da integridade epitelial (SONG et al., 2014), modulação do sistema imune (RAJPUT et al., 2013) e redução de patógenos (CALEFI et al., 2014).

Apesar dos muitos trabalhos desenvolvidos envolvendo a questão descrita, muito ainda precisa ser feito, pois além da grande diferença de resultado observada entre os diferentes microrganismos utilizados como probiótico, ainda restam dúvidas relacionadas aos mecanismos envolvidos na interação entre microbiota e hospedeiro. Objetivou-se com presente trabalho estudar os efeitos da suplementação de um probiótico via dieta, *Bacillus subtilis* sorotipo DSM 17299, sobre os parâmetros de digestibilidade de nutrientes e o desempenho das aves. Também foram avaliadas as variações na expressão genica de diferentes tecidos dos frangos de corte com e sem a suplementação do probiótico. Em um segundo momento, avaliou-se os resultados da suplementação do probiótico em aves

submetidas a temperaturas elevadas por tempo prolongado, contribuindo. Dessa forma, para o melhor conhecimento da suplementação de um microrganismo como ferramenta alternativa aos AMDs e seus efeitos positivos no contexto atual.

A fim de melhor elucidar os temas relacionados aos AMDs e alguns de seus produtos alternativos, foi elaborada uma revisão baseada nos principais estudos apresentados na literatura até a atualidade, abordando assuntos polêmicos no contexto dos antimicrobianos, bem como os ácidos orgânicos, prebióticos e probióticos, contudo, uma atenção maior foi dada ao último citado, pois está de acordo com os principais objetivos do presente trabalho.

2 REFERENCIAL TEÓRICO

2.1 Antimicrobianos melhoradores de desempenho (AMD)

Após a descoberta da penicilina em 1928 pelo pesquisador inglês Alexander Fleming, os antimicrobianos foram largamente empregados para a medicina humana, entretanto, na nutrição animal a sua utilização começou a ser pesquisada anos depois, quando Moore et al. (1946) suplementaram estreptomicina para frangos de corte e se surpreenderam com o aumento no peso vivo das aves. Após alguns anos surgiria o termo promotor de crescimento, termo este que foi substituído por antimicrobiano melhorador de desempenho (AMD) no Brasil, devido ao impacto negativo causado pelo nome promotor de crescimento e a relação que o nome fazia aos tão questionados hormônios anabolizantes, que no Brasil são proibidos na produção animal. Niewold (2007), cita que os promotores de crescimento também são definidos como "*growth permitting*", ou seja, permitindo o crescimento. Sob o ponto de vista legislativo, no Brasil, os AMDs se enquadram como aditivos zootécnicos, que por conceito, segundo o MAPA (IN 13, Anexo I, item 3.5.1.d), retrata aditivos zootécnicos

como “toda substância utilizada para influir positivamente na melhoria do desempenho dos animais”. Ainda dentro da classificação do MAPA os antimicrobianos quando utilizados em pequenas dosagens e não terapêuticas estão descritos como melhoradores de desempenho (IN 13, Anexo II, item 4. c) que são “substâncias definidas quimicamente que melhoram os parâmetros de produtividade”. Nesse grupo estão a bacitracina, sulfato de colistina, flavomicina, avilamicina, enramicina, entre outros.

Os antimicrobianos que, futuramente, seriam conhecidos como melhoradores de desempenho somente começaram a ser utilizados em larga escala na produção animal a partir da década de 60, seguindo os avanços tanto dos compostos antimicrobianos como do conhecimento de seu mecanismo de ação, sendo que a partir da década de 50 vários pesquisadores documentaram o seu efeito benéfico para o desempenho das aves (BRENNAN et al., 2003; ENGBERG et al., 2000; GROSCHKE e EVANS, 1950; HUYGHEBAERT e DE GROOTE, 1997; MILES et al., 2006; MOTL et al., 2005; REIS et al., 2014; SANTOS et al., 2008; STOKSTAD e JUKES, 1950).

Com o avanço das pesquisas foram propostas diversas hipóteses para explicar os mecanismos de ação dos AMDs e de acordo com Reis et al. (2014), a atuação conjunta de vários mecanismos pode ocorrer e de fato melhorar o desempenho dos animais.

Posterior à confirmação de que os antibióticos orais não possuem efeito como melhorador de desempenho em animais isentos de germes (Coates et al., 1955, 1963) e aliado ao fato de que aves suplementadas com AMD na fase embrionária não apresentam melhora em seu desenvolvimento (VISEK, 1978), vários autores consideraram que a ação dos antimicrobianos se dá no lúmen intestinal, modificando seletivamente a microbiota intestinal (BAURHOO et al., 2009; BRENNAN et al., 2003; ENGBERG et al., 2000; HUYGHEBAERT e DE GROOTE, 1997; SIMS et al., 2004; WALDROUP et al., 1993), portanto, os

estudos que pretendiam elucidar o mecanismo de ação dos AMDs concentraram-se nas interações entre o antimicrobiano e a microbiota do intestino dos animais.

Argumentos interessantes, que ainda necessitam de maiores esclarecimentos, foram utilizados por Niewold (2007), no qual ele sugere que além dos efeitos sobre a microbiota os AMDs também devem ter ação direta sobre o organismo animal, alterando o seu sistema imune inato, pois diversos antimicrobianos podem se acumular, principalmente, dentro das células de defesa do animal (LÓPEZ e OLVERA, 2008), provocando um efeito anti-inflamatório. Um exemplo prático é a bacitracina de zinco que é amplamente utilizada como melhorador de desempenho na produção animal e sua concentração pode ser até 40 vezes maior no interior das células quando comparado ao meio extracelular. Entretanto isso só seria possível com a absorção dos AMDs pelo trato gastro intestinal (TGI), o que pode ocorrer em diferentes proporções entre os antibióticos, assim como a bacitracina de zinco que apresenta absorção de aproximadamente 5% (LÓPEZ e OLVERA, 2008) quando fornecidos na forma oral. A integridade da parede intestinal também pode afetar a absorção dos antimicrobianos no TGI. Entre os principais efeitos da adição dos AMDs tem-se: redução na concorrência por nutrientes entre hospedeiro e a microbiota; redução de metabólitos tóxicos liberados pela microbiota; redução da espessura da parede intestinal; exclusão competitiva; aumento na produção de nutrientes (ácidos graxos, aminoácidos e vitaminas) e da atividade enzimática (amilase e lipase pancreática e quimiotripsina) devido a mudança no perfil da microbiota; modulação seletiva da microbiota, aumento na retenção de nitrogênio; aumento na absorção de lipídeo e alteração do sistema imune (BAURHOO et al., 2009; BRENNAN et al., 2003; ENGBERG et al., 2000; MOORE et al., 1946; NIEWOLD, 2007; SIMS et al., 2004; VISEK, 1978; YEO e KIM, 1997).

De maneira geral, a maioria dos mecanismos de ação apresentados são provocados pelas variações na microbiota após a adição do AMD, portanto,

podemos concluir que os AMDs atuam, principalmente, no controle dos microrganismos do (TGI), seja pela redução em número da microbiota, possibilitando redução na competição por nutrientes (BAURHOO et al., 2009; BIGGS e PARSONS, 2008), ou até mesmo pela seleção e modulação em favor da microbiota benéfica (BAURHOO et al., 2009; ENGBERG et al., 2000; SIMS et al., 2004), o que pode gerar uma série de benefícios ao hospedeiro.

Apesar dos efeitos positivos dos aditivos antimicrobianos, um assunto que vem sendo constantemente discutido é a proibição dos AMDs na produção animal. De acordo com Castanon (2007), muitos antimicrobianos já foram utilizados para promover melhoras no desempenho dos animais e grande parte foi proibida para esse fim, pois seu uso, mesmo em baixas dosagens, ou tendem a selecionar bactérias resistentes, ou são tóxicos para os animais, ou podem gerar resíduos nos tecidos animais tornando o produto animal um risco para o consumo humano. A comunidade europeia, pioneira nas restrições ao uso dos antimicrobianos, relata proibições no uso de antibióticos a partir da década de 70, fazendo as últimas restrições em meados de 2006 (CASTANON, 2007).

Atualmente a busca por um alternativo aos antimicrobianos é alvo de muitos estudos, pois a proibição dos AMDs parece ser uma certeza que atinge toda a produção animal e somados a isso, a demanda por carne de frango de corte criados sem a suplementação dos AMDs vem aumentando em ritmo acelerado, sendo utilizado muitas vezes como estratégia de marketing por empresas do ramo.

2.2 Aditivos equilibradores da flora intestinal

Conforme citado anteriormente a busca por aditivos, alternativos aos AMDs, aumentou consideravelmente e diversos produtos já são comercializados para esse fim. Considerando os mecanismos de ação dos antimicrobianos descritos, os aditivos substitutos devem, primordialmente, ter ação direta ou

indireta sobre a microbiota intestinal, alterando a população de microrganismos do TGI, com o intuito de beneficiar o desempenho das aves frente aos desafios do ambiente em que são criados. Portanto, um grupo de aditivos, denominados equilibradores da flora intestinal pelo MAPA e caracterizados como "microrganismos que formam colônias ou outras substâncias definidas quimicamente que tem um efeito positivo sobre a flora do trato digestório" (MAPA, 2015), recebem destaque nesse cenário pois seu principal mecanismo de ação é justamente a modulação da microbiota do TGI em favor do organismo hospedeiro, nessa classificação podemos citar os ácidos orgânicos, prebióticos e os probióticos (FARIA FILHO et al., 2006; GIBSON et al., 2004; GIBSON e ROBERFROID, 1995; LEVENT OZDUVEN et al., 2009; LUTFUL KABIR, 2009; SONG et al., 2014; VAN IMMERSEEL et al., 2006).

2.2.1 Ácidos Orgânicos

Os ácidos orgânicos estão envolvidos na produção animal a mais de 40 anos, entretanto, inicialmente acreditava-se que seu principal efeito estava relacionado com seu potencial antifúngico (DIXON e HAMILTON, 1981; TABIB et al., 1984) sendo muito utilizado como aditivo de alimentos reduzindo a deterioração dos mesmos. Os principais constituintes deste grupo são ácidos saturados de cadeia linear monocarboxílica e seus derivados (Ricke, 2003). Contudo, a evolução nas pesquisas tem demonstrado os efeitos positivos dos ácidos orgânicos no controle de bactérias patogênicas, principalmente *Salmonela spp*, *Campilobacter spp*, *E. Coli*, *Clostridium perfringens*, entre outras (BYRD et al., 2001; CHAVEERACH et al., 2002, 2004; GRILLI et al., 2011; KOLLANOOR-JOHNY et al., 2012; STRINGFELLOW et al., 2009; VAN IMMERSEEL et al., 2006), afetando positivamente o desempenho, bem como, a redução de microrganismos patogênicos no produto final.

A variedade de ácidos orgânicos existentes para utilização na indústria animal, determina também os diversos mecanismos de ação. De uma forma geral a redução do pH no lúmem intestinal é um efeito observado em praticamente todos os compostos denominados ácidos orgânicos, o que pode causar equívocos quanto a sua principal forma de ação. A eficácia antimicrobiana dos ácidos orgânicos está relacionado a sua forma não dissociada, que por sua vez penetra na célula bacteriana e se dissocia, gerando uma instabilidade no pH intracelular e morte das bactérias (BRUL e COOTE, 1999).

2.2.2 Prebióticos

Outro composto que vem recebendo destaque na indústria avícola são os prebióticos. Segundo definição proposta por Gibson e Roberfroid (1995), um prebiótico é um ingrediente alimentar não digestível que afeta beneficamente o hospedeiro por meio do estímulo seletivo de um ou limitado número de bactérias, induzindo a saúde do hospedeiro. Para que o ingrediente alimentar seja classificado como prebiótico alguns pontos devem ser atendidos: 1) o ingrediente não deve ser hidrolisado ou absorvido na parte superior do TGI; 2) ser um substrato seletivo para uma ou um limitado número de bactérias comensais benéficas; 3) alterar favoravelmente a microbiota do hospedeiro; 4) induzir efeitos no lúmem intestinal ou sistêmico que proporcionem benefícios ao hospedeiro.

Segundo Baurhoo et al. (2009) para que as bactérias possam colonizar o TGI do hospedeiro, é necessário que o mesmo reconheça sítios de adesão na parede intestinal, de outra forma seriam carreados juntamente com a digesta para fora do organismo animal. Algumas bactérias gram negativas possuem o tipo de fímbria tipo I e reconhecem sítios D-manose no epitélio do TGI para a sua fixação. Nesse contexto, alguns prebióticos como os mananoligossacarídeos (MOS) possuem o sítio de ligação necessário para a adesão dessas bactérias, o resultado

é a liberação desses microrganismos juntamente com as fezes, reduzindo a sua concentração e seus efeitos danosos ao hospedeiro. Esses efeitos estão de acordo aos observados por Baurhoo et al. (2007) ao suplementar MOS para frangos de corte e observar redução na população de salmonela na microbiota do hospedeiro.

2.2.3 Probiótico

Dentro do grupo de substancias modificadoras da flora intestinal os probióticos tem recebido grande destaque, pois seus efeitos tanto na microbiota intestinal bem como no próprio animal descritos na literatura, podem trazer benefícios ao desempenho do animal hospedeiro, reduzindo perdas econômicas e maximizando a produção na indústria alimentícia.

2.2.3.1 Breve histórico dos probióticos e suas definições

Os primeiros registros documentados com probióticos datam do início do século passado em 1907, com o pesquisador Elie Metchnikoff, também considerado o pai dos probióticos modernos (FULLER, 1989). Esse pesquisador se baseou em observações de um biólogo búlgaro, que documentou os benefícios do consumo de iogurte para o TGI, isolando e identificando um organismo ativo nesse alimento denominado de *Lactobacillus bulgaricus* (HUME, 2011). Motivado por esses achados Metchnikoff se dedicou a promover a ideia de que o iogurte e as bactérias que o constituíam eram ingredientes essenciais a vida, atribuindo a maior longevidade observada em camponeses búlgaros ao consumo do iogurte que possuíam esses microrganismos. Entretanto, de acordo com (HUME, 2011), a produção, consumo e as qualidades do iogurte também eram conhecidas no oriente médio e Ásia, muito antes das observações feitas na Bulgária.

Apesar dos achados na área dos probióticos serem antigos, segundo Guarner et al. (2005), o termo probiótico foi introduzido na literatura científica somente em 1954, e de acordo com a etimologia, é uma palavra que teve origem na língua grega, que significa a favor da vida.

Um dos primeiros relatos disponíveis na literatura com relação a utilização dos probióticos foi feito por Lilly e Stillwell (1965), no qual os autores verificaram um efeito positivo de substancias secretadas por um microrganismo sobre o crescimento de outros. Desde então diversos conceitos foram propostos com o intuito de conferir a correta aplicação do termo probiótico e os avanços na ciência ao longo dos anos contribuíram para uma definição apoiada pela maioria dos pesquisadores da área. Os diversos conceitos de probiótico propostos estão apresentados, em ordem cronológica, abaixo:

1954 - substâncias ativas que são essenciais para o desenvolvimento da vida saudável (VERGIN, 1954);

1965 - substancias secretadas por um microrganismo que estimulam o crescimento de outro (LILLY e STILLWELL, 1965);

1974 - organismo e substancias que contribuem para o balanço da microbiota intestinal (PARKER, 1974);

1989 - suplemento alimentar de microrganismos vivos, que afeta beneficamente o hospedeiro animal por meio da melhoria do equilíbrio da microbiota (FULLER, 1989);

1992 - culturas de um ou mais tipos de microrganismos vivos que afetam beneficamente o hospedeiro por meio da melhoria das propriedades da microbiota do hospedeiro (FULLER, 1992);

1998 - microrganismos vivos, não patogênicos e não tóxicos, que quando administrado, in natura, por meio do TGI, são favoráveis a saúde do hospedeiro (GUILLOT, 1998);

2003 - microrganismos vivos, que quando administrados em quantidades adequadas conferem benefícios à saúde do hospedeiro (REID et al., 2003);

De acordo com a apresentação acima é possível notar claramente a evolução dos conceitos atribuídos aos probióticos, nos quais o avanço da ciência contribuiu diretamente.

É interessante ressaltar que os probióticos também estão sujeitos a definições determinadas por órgãos reguladores, a fim de regulamentar a sua comercialização. No Brasil, sob o ponto de vista da legislação do ministério da agricultura, os probióticos são classificados dentro de um grupo geral, (aditivos equilibradores da flora intestinal) sendo definido como "microrganismos que formam colônias ou outras substâncias definidas quimicamente que tem um efeito positivo sobre a flora do trato digestório" (MAPA, 2015). Nos Estados Unidos da América os probióticos são descritos como "uma fonte de microrganismos vivos (viável) de ocorrência natural". A definição proposta pela FAO/WHO, define os probióticos como "microrganismos vivos, que, quando administrados em quantidades adequadas conferem benefícios a saúde do hospedeiro", sendo esta definição a mesma apresentada por Reid et al. (2003) e a mais utilizada atualmente.

2.2.3.2 Mecanismos de ação dos probióticos

Assim como os aditivos apresentados anteriormente, já é bem documentado na literatura que a maioria dos produtos probióticos possuem ação principal no TGI dos animais, alterando e regulando a microbiota (ALLOUI MOHAMED et al., 2013; AL-FATAFTAH e ABDELQADER, 2014; BIGGS e PARSONS, 2008; CHAUCHEYRAS-DURAND e DURAND, 2010; FULLER, 1989, 1992; GUILLOT, 1998; JEONG e KIM, 2014; KNAP et al., 2011; LUTFUL KABIR, 2009; WU et al., 2011). Entretanto, os mecanismo de ação

desse grupo de aditivos ainda não foi completamente elucidado e muitos trabalhos publicados recentemente tentam explicar melhor essa questão (BAI et al., 2013; CAO et al., 2012; DE OLIVEIRA et al., 2014; PARK e KIM, 2014; SONG et al., 2014).

Conformo discutido anteriormente, os probióticos vem sendo pesquisados a muitos anos, e são muitos os mecanismos de ação propostos na literatura, entre eles os principais relacionados com a avicultura são: a manutenção da microbiota intestinal normal por exclusão competitiva (EC) e antagonismo; alteração do metabolismo do hospedeiro por meio da melhoria da atividade enzimática bem como a redução da atividade de enzimas bacterianas e a produção de alguns metabólitos como a amônia; melhora no consumo de alimento e aumento na digestibilidade de nutrientes; e também o estímulo do sistema imune do hospedeiro.

Segundo Schneitz (2005), a EC foi descrita pela primeira vez por Nurmi e Rantala (1973), quando os autores observaram que lactobacilos não foram suficientes para controlar a salmonela em aves jovens, portanto, foi utilizada bactérias provenientes do TGI de aves adultas, resistentes a *Salmonela infantes*. Por meio da suplementação desse grupo indefinido de microrganismos, os autores obtiveram sucesso na resistência contra a salmonela. É possível notar que inicialmente o termo EC não foi criado para explicar a forma de ação dos probióticos e sim um procedimento adotado com o intuito de reduzir ou controlar microrganismos patogênicos, entretanto, o avanço das pesquisas e a melhor compreensão da EC ao longo dos anos (LUTFUL KABIR, 2009; NURMI et al., 1992; SCHNEITZ, 2005) demonstrou que esse conceito atende aos requisitos dos probióticos, sendo portanto, uma explicação plausível dos efeitos positivos observados nessa classe de aditivos, sendo várias as causas que explicam a EC.

A redução de pH intestinal é um dos fatores observados utilizado para explicar, em parte, a EC, pois já foi demonstrado que a suplementação de alguns

probióticos aumentam a produção de ácidos graxos de cadeia curta e média que por sua vez podem reduzir o pH intestinal (SERVIN, 2004) reduzindo o número de bactérias patogênicas no TGI (LUTFUL KABIR, 2009; SCHNEITZ, 2005; SERVIN, 2004).

Outro ponto importante observado na EC é a adesão de microrganismos ao epitélio intestinal, pois de acordo com Alloui Mohamed et al. (2013) é necessária a adesão dos patógenos em sítios específicos encontrados em todo o TGI do hospedeiro e a suplementação de probióticos que competem por esses sítios de adesão, pode ser uma estratégia eficiente pois reduz a população de bactérias patogênicas que, ao não se fixar no epitélio intestinal por falta dos sítios de adesão, são carreadas juntamente com o bolo alimentar para fora do TGI (AWAD et al., 2009; BAI et al., 2013; DE OLIVEIRA et al., 2014; KNAP et al., 2011; MOUNTZOURIS et al., 2010; NURMI et al., 1992; SERVIN, 2004) , reduzindo seus efeitos negativos.

Produtos utilizados como probióticos devem ser capazes de passar pelas condições extremas do TGI e chegar ao seu local de atuação, sofrendo pouca redução na sua concentração, e após se estabelecer, essa população de microrganismos utiliza substratos para seu crescimento, como aminoácidos, proteínas, oligossacarídeos, entre outros, reduzindo a disponibilidade de nutrientes no lúmem intestinal (MOUNTZOURIS et al., 2010; SCHNEITZ, 2005). Portanto a competição por nutrientes também favorece a EC reduzindo a concentração de bactérias patogênicas.

As bacteriocinas produzidas por uma grande variedade de microrganismos também estão relacionadas a EC. Também chamada de pequenos peptídeos antibióticos (PPA), variam de 30 a 100 sequencias de aminoácidos e possuem ação contra uma ou um grupo de bactérias. Já segundo Sangtanooa et al. (2014), os PPAs tem o papel de matar ou inibir uma grande variedade de organismos e células, incluindo bactérias gram positivas e negativas, vírus,

protozoários, parasitas, fungos e células do câncer. Portanto, o mecanismos de ação de alguns microrganismos probióticos tem sido atribuído à produção de PPAs (KLAENHAMMER, 1988; OSCÁRIZ e PISABARRO, 2001; SINGH et al., 2015).

O esgotamento dos sítios de adesão do epitélio intestinal proporcionado pela suplementação de microrganismos probióticos também podem reduzir os efeitos causados por toxinas produzidas pela microbiota, pois segundo Luciana Kazue et al. (2012) quando a adesão dessas toxinas ao epitélio não ocorre, seu efeito negativo reduz.

Existem outros mecanismos de ação atribuídos aos probióticos e descritos na literatura, como a prevenção da síntese de aminas tóxicas por meio da EC, produção de enzimas digestivas e/ou aumento da atividade enzimática do hospedeiro, bem como a síntese de alguns nutrientes que podem ser aproveitados pelo hospedeiro, melhorando a digestibilidade do alimento ingerido (MOUNTZOURIS et al., 2010), alteração das características morfológicas do TGI (AL-FATAFTAH e ABDELQADER, 2014; AWAD et al., 2009; SONG et al., 2014) e interação com o sistema imune do hospedeiro, podendo influenciar a resposta imunológica do animal por meio de um mecanismo complexo, resultando em melhora no desempenho animal frente ao desafio com microrganismos patogênicos (BAI et al., 2013; CAO et al., 2012; MOUNTZOURIS et al., 2010; SEIFERT et al., 2011).

Conforme apresentado, a lista dos mecanismos de ação dos aditivos probióticos é grande e a observação de múltiplos fatores para um mesmo produto pode ocorrer, dando suporte para a utilização de misturas de microrganismos em um produto probiótico. Entretanto, alguns microrganismos apresentam funções muito específicas que podem ser utilizados para corrigir problemas pontuais que os produtores e a indústria de carnes enfrentam. Foi demonstrado por Park e Kim (2014) que um sorotipo específico de *Bacillus subtilis* (B2A), proporcionou a

redução de salmonela no intestino de aves. Muitos resultados semelhantes foram demonstrados na literatura com diferentes microrganismos probióticos (CAO et al., 2012; DE OLIVEIRA et al., 2014).

2.2.3.3 Probióticos X stress térmico

Segundo Burkholder et al. (2008) animais submetidos a stress, podem se tornar mais susceptíveis a ação de microrganismos patogênicos, e nesse contexto, o stress causado por aumento da temperatura ambiente pode afetar negativamente a imunidade do animal, facilitando o aparecimento de doenças oportunistas (SOLEIMANI et al., 2012), bem como alterar a composição da microbiota (AL-FATAFTAH e ABDELQADER, 2014; SOHAIL et al., 2013; SONG et al., 2014). Somados a isso, o stress crônico causado pelo aumento de temperatura pode modificar a estabilidade do epitélio intestinal, alterando a expressão das proteínas de junções firmes, enfraquecendo a permeabilidade das membranas do epitélio, favorecendo a translocação bacteriana bem como de endotoxinas (TUOHY et al., 2014), entretanto, são poucos os trabalhos que tentam elucidar e minimizar essa questão (SONG et al., 2014). Segundo Cani et al. (2007), o aumento de endotoxina circulante no plasma, principalmente lipopolissacarídeo (LPS) proveniente de bactérias gran negativas, aumentou a resistência a insulina em ratos e também está relacionado a obesidade, descrevendo esse efeito como endotoxilemia metabólica. Somados a isso Tuohy et al. (2014), descrevem que o aumento do LPS sanguíneo pode estar relacionado ao aumento do risco de doenças cardiovasculares em humanos. Assim como demonstrado em ratos e humanos, as aves de produção também podem sofrer com os efeitos negativos da endotoxilemia metabólica, podendo ser uma das causas da queda no desempenho e em casos mais extremos a morte do animal documentada por diversos pesquisadores (AL-FATAFTAH E ABDELQADER, 2014; AZOULAY et al., 2011; AZZAM et al., 2011; DENG et

al., 2012; QUINTEIRO-FILHO et al., 2010; SOHAIL et al., 2013), entretanto, os trabalhos que abordam os efeitos da endotoxilemia metabólica em aves são escassos na literatura, com algumas recentes publicações (AL-FATAFTAH e ABDELQADER, 2014; SONG et al., 2014).

A situação acima descrita começou a ganhar foco nos últimos anos, pois os aumentos de temperatura em todo o planeta é uma realidade (WMO, 2015a; 2015e) sendo alvo de constantes debates e tratados entre as principais economias do mundo, como o encontro no ano de 2015 em Paris (COP21), que entre outras coisas teve o objetivo de firmar acordos a fim de definir metas para que os países minimizem seus efeitos perante o aquecimento global. Os desafios encontrados por produtores de aves e outros animais tem levado a mais pesquisas que contribuam para preparar a agricultura para esse cenário, favorecendo a oferta constante de carne.

Devido aos mecanismos de ação atribuídos aos probióticos, em especial, a redução de bactérias patogênicas (PARK e KIM, 2014; SONG et al., 2014) e consequentemente a redução de endotoxinas (AL-FATAFTAH e ABDELQADER, 2014), esses aditivos podem ser uma ferramenta a fim de minimizar os impactos do stress causado pelo aumento de temperatura, entretanto, poucos estudos demonstram os efeitos positivo dos probióticos frente a situações de stress térmico (AL-FATAFTAH e ABDELQADER, 2014; BURKHOLDER et al., 2008; SONG et al., 2014) e muito ainda precisa ser elucidado.

2.2.3.4 *Bacillus subtilis*

Atualmente, existe um grande número de microrganismos utilizados como probiótico na produção animal, pois muitos atendem aos requisitos dessa classe de aditivos (LUCIANA KAZUE et al., 2012), trazendo benefícios ao hospedeiro. Contudo, a forma de suplementação do probiótico, via dieta ou água,

e o ambiente extremo no interior do TGI é um desafio para a sobrevivência de qualquer microrganismos, principalmente devido ao processo de peletização da dieta, bem como pela rápida redução de pH intestinal que pode chegar próximo de 2 na moela (MORGAN et al., 2014).

Por outro lado, muitas bactérias patogênicas conseguem ultrapassar esse ambiente extremo e colonizar o TGI das aves e são vários os fatores envolvidos nessa questão como a idade do animal, estado fisiológico, temperatura ambiente, dieta, entre outros. Contudo, já é sabido que algumas bactérias tem a capacidade de formar esporos, sendo considerado como uma das formas de vida mais resistentes encontrada na natureza (LATORRE et al., 2014). Os esporos podem ser definidos como bactérias em seu estado dormente (HONG et al., 2005), entretanto, quando expostos a condições favoráveis, germinam para o seu estado vegetativo sendo metabolicamente ativo. Devido a essas características o *Bacillus subtilis* é uma boa opção de probiótico, pois tem a capacidade de formar esporos sendo de grande importância na nutrição animal e amplamente utilizado como probiótico a partir da suplementação de seus esporos, pois os mesmo são capazes de resistir a aumentos de temperatura e pressão bem como variações extremas de pH (HONG et al., 2005).

Os *B. subtilis* são bactérias comumente encontrada no solo, (HONG et al., 2005; LATORRE et al., 2014; MICHIKO e PETER, 1998), seu desenvolvimento se dá, principalmente, em presença de oxigênio (CARTMAN et al., 2008) o que poderia inviabilizar o seu uso como probiótico, contudo, é amplamente utilizado na suplementação de animais pois além de resistirem ao processo de peletização e suportarem o baixo pH intestinal (CARTMAN et al., 2008; LATORRE et al., 2014), em situações específicas, também são capazes de se desenvolver em ambientes com baixo ou nenhum oxigênio (MICHIKO e PETER, 1998). Quando fornecido via dieta ou água, seu processo de germinação se dá no próprio TGI do hospedeiro, devido a ação do baixo pH, a presença de

nutrientes entre outros fatores relevantes (SHIVARAMAIAH et al., 2011). Após a ingestão do *B. subtilis* pelo hospedeiro de acordo com Latorre et al. (2014) aproximadamente 90% dos esporos ingeridos pela ave atingem a germinação.

Entre os principais mecanismos de ação dos *B. subtilis* no TGI favoráveis ao hospedeiro tem-se: produção de enzimas que ajudam a hidrolisar os nutrientes da dieta (DURAN-PARAMO et al., 2000; SRINIVASAN e Rele, 1999); redução da concentração de amônia da digesta o que reduz danos ao epitélio intestinal (SAMANYA e YAMAUCHI, 2002); formação de colônias (biofilme) dificultando a adesão de outros microrganismos ao epitélio intestinal, incluindo bactérias patogênicas (PROBERT e GIBSON, 2002); modulação do sistema imune do hospedeiro (LA RAGIONE e WOODWARD, 2003; RAJPUT et al., 2013); aumento das bactérias produtoras de ácido lático (WU et al., 2011); produção de bacteriocinas que atuam na redução de microrganismos patogênicos (PINCHUK et al., 2001).

É importante ressaltar que os efeitos descritos acima estão relacionados ao gênero *Bacillus* da espécie *subtilis*, entretanto, o sorotipo pode variar entre as pesquisas relacionadas, gerando variação nos resultados, o que denota a importância em pesquisas constantes com os diversos tipos de probióticos e especificamente as bactérias que o compõe, definindo o sorotipo que está sendo avaliado.

Diversas pesquisas demonstram os efeitos positivos do B. subtilis (DSM 17299) ao desempenho (DENIZ et al., 2011; HOSSAIN et al., 2015; KNAP et al., 2011; KNARREBORG et al., 2008; LUND et al., 2005; MOKHTARI et al., 2010; MURSHED e ABUDABOS, 2015; OPALINSKI et al., 2007; ROSTAGNO et al., 2006; SZAKACS et al., 2015; ZAGHARI et al., 2015), a digestibilidade de nutrientes (HOSSAIN et al., 2015; KNAP 2011; ZAGHARI et al., 2015; ZHANG e KIM, 2014), a morfologia intestinal (HOSSAIN et al., 2015) e ao sistema imune (LOURENCO et al., 2012; SZAKACS et al., 2015), entretanto, a avaliação desse

sorotipo específico de bactéria necessita de mais estudos, como o acompanhamento semanal do desenvolvimento animal. Outro ponto que precisa ser melhor compreendido é a influência desse tipo específico de probiótico em aves submetidas a ambientes com temperatura elevada, pois esses dados são inexistentes na literatura atual.

Pesquisas que relacionem o uso do probiótico associados ao estresse por calor são escassos na literatura e muito ainda precisa ser elucidado. Nós acreditamos que a suplementação do *B. subtilis* (DSM17299), via dieta, pode melhorar a digestibilidade dos nutrientes e os parâmetros de desempenho de aves criadas em conforto térmico ou com elevadas temperaturas, contribuindo para o bem estar dos animais e, consequentemente, para a redução do custo de produção, aumentando a lucratividade do produtor.

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SEGUNDA PARTE

Artigo I - Normas da revista científica Journal of Applied Poultry Research

Effect of *Bacillus subtilis* (DSM 17299) on performance, digestibility, intestine morphology and pH in broiler chickens

Primary Audience: Nutritionists, Poultry Technicians, Veterinarians

SUMMARY

This study investigated the effect of supplementation of a *Bacillus subtilis* strain DSM 17299 on broilers, regarding performance, nutrient digestibility, intestinal morphology and content pH, comparing experimental birds receiving this probiotic and control birds without probiotic. The groups were also evaluated for economic value. Performance was improved for probiotic treated birds until week five post-hatch and economic evaluation observed at week six demonstrated that the addition of probiotic had no impact on production cost. Total tract digestibility of dry matter (DM), crude protein (CP) and energy metabolism were improved for probiotic supplemented broiler chickens. Furthermore, addition of probiotic increased the pH of the intestinal content, which was an unexpected outcome. The relative weight and relative length of intestine appear to be influenced by probiotic supplementation. Overall, an improvement on performance and a decrease in production costs is

expected when B. subtilis DSM 17299 is used on feed of broiler birds largely as a result of an improved nutrient digestibility.

Keywords: probiotic, grow promoter, microbiota, poultry.

DESCRIPTION OF PROBLEM

For many years antibiotics were one of the main forms of control of pathogens in poultry production and its use also functions as a growth promoter that provides good production rates [1-4]. However, rejection of the use of these additives increases every year, suffering a major milestone in 2006 when the European Community banned antibiotic use as a growth promoter (AGP) in animal production, due to the possibility of selection of resistant bacteria and also by strong consumer pressure [5].

Hence, several alternatives to growth promoters have been investigated and marketed, among them are probiotics. Probiotics are defined as live microorganisms which when administered in adequate amounts confer health benefits for the host [10]. Probiotics appear to have multiple benefits, including a reduction of pathogens along with performance improvements favorable to livestock [6-9].

Currently, there are a large number of microorganisms used as probiotics in animal production, as many additives meet the requirements of this class [11]. However, the form of supplementation of probiotic via the diet or water, and the extreme environment within the gastro-intestinal-tract (GIT) is a challenge for the survival of any microorganism [12]. Therefore, spore-forming bacteria have a great advantage when used as a probiotic as the spore provides a protected environment both prior to ingestion and with in the stomach. In this context *Bacillus subtilis* is a good choice of probiotics, as it is able to withstand a variety of insults including high temperature, pressure, and also extremes of pH [13, 14].

Currently, several strains of *B. subtilis* are commercially available for use as probiotic, however, despite belonging to the same species, the results between them vary [15]. This suggests a need for continuing research with the various types of *B. subtilis* strains to understand the relative merits of each strain.

In this context, the addition of *B. subtilis* (DSM 17299), via diet for broiler chickens, has been investigated since 2005 [16-20], reporting a reduction of pathogenic microorganisms and improvements in animal performance. The goal of this work was to study the effects of

supplementation with a probiotic diet-fed, *B. subtilis* strain DSM 17299 on performance measurements, nutrient digestibility, the gastrointestinal tract morphology and pH of the broiler chickens supplemented or not with probiotic from post-hatch days 1 to 42.

MATERIALS AND METHODS

Birds and Treatments

Male broiler chicks (Ross-708) were obtained from a Mountaire hatchery (Millford DE.) on day-of-hatch and randomly assigned to 24 cages separated in 2 treatment groups of 12 replications each. The experimental unit was the cage with 8 birds each. Diets in mash form and water were offered for *ad libitum* consumption. Lighting program was 23 h of light and 1 h of dark for the whole experiment. Electric room heaters maintained the temperature at 32 ºC for the first week, than decreased 2.5 ºC per week until 4th week when the temperature was set for 22 ºC for the remainder of the experiment. The commercial production required floor area per bird was followed. All of the experimental procedures were performed in compliance with protocols approved by University of Delaware Agricultural Animal Care and Use Committee.

Probiotic and Diet

The probiotic used was a *Bacillus subtilis*, strain DSM 17299, with a minimum declared concentration of 1,6 x 10^9 cfu/g of product. An amount of 500 g of product / ton of feed was used as per company recommendation. A day before the beginning of the experiment, the probiotic was weighed and then blended with basal diet. After blending the diets were transferred to the brooder room, which were weighed and stored in plastic buckets.

Corn and soybean meal based diet (starter from day 1 to 21 and finisher from day 22 to 42) were obtained from Southern States (Middletown DE.). Diets were representative of local commercial formulations, and calculated analyses met or exceeded NRC (1994) standards for the starter feed. The grower feed was formulated to have lower energy level than NRC standards. The diets did not contain any coccidiostat or other AGP (see Table 1). For the determination of ileal and total tract nutrient digestibility coefficients, titanium dioxide (as an indigestible marker) was added in each one of the experimental diets, at a final concentration of 3 g of TiO2 / kg of diet (0.3%).

Table 1. Ingredient composition of basal diet

Item	Diet[1]	
Ingredients (%)	Starter	Grower
Corn	58.20	65.62
Soybean meal	32.13	26.38
Corn DDGS[2]	3.32	3.70
Wheat bran	2.42	0.00
Phosphate dicalcium	1.81	1.80
Soy oil	0.00	0.56
Ground limestone	1.03	0.96
Salt	0.30	0.30
DL-Methionine 99%	0.12	0.03
Choline chloride 60%	0.10	0.10
Sodium bicarbonate	0.07	0.05
Trace mineral and Vitamin premix[3]	0.20	0.20
Titanium dioxide	0.30	0.30
Calculated analyses		
ME, kcal/kg	2,910	2,990
CP, %	21.50	19.00
Met + cys, %	0.85	0.70
Lys, %	1.22	1.04
Thr, %	0.83	0.74
Calcium, %	0.95	0.90
Available P, %	0.47	0.45
Sodium, %	0.20	0.20
Potassium, %	0.95	0.83
Chlorine, %	0.23	0.23
Electrolyte balance, mEq/Kg	264.90	232.60

[1]0.05% of probiotic was added on top of the diet to fed the supplemented birds

[2]DDGS = distillers dried grains with solubles.

[3]The starter premix supplied per kilogram of diet: trans-retinol, 3.60 mg; cholecalciferol, 0.062 mg; dl-α-tocopheryl acetate, 30 mg; thiamine, 2 mg; pyridoxine, 3 mg; calcium pantothenate, 10 mg; biotin, 0.07 mg; menadione, 3 mg; folic acid, 1 mg; nicotinic acid, 35 mg; cyanocobalamin, 0.015 mg; Se, 0.12 mg; antioxidant, 5 mg; Mn, 80 mg; Fe, 50 mg; Zn, 50 mg; Cu, 10 mg; Co, 1 mg; I, 1 mg.

Total tract and ileal nutrient digestibility of feed and energy metabolism

At days 18-20 and the days 38-40 excreta were collected, once per day in the morning. The waste from each pen was weighed, homogenized, and sampled for laboratory analysis of titanium (Ti), dry matter (DM), nitrogen (N) and gross energy (GE). The titanium dioxide was determined using [21] Short et al. (1996) method. DM was determined using AOC method 930.15 [22]. The N was determined using Kjedahl method. Gross energy was determined using adiabatic bomb calorimetry (Parr Instrument Company, Moline, IL).

At days 21 and 41, 2 birds per cage were sacrificed by cervical dislocation followed by necropsy. The ileum was defined as the segment of small intestine between the Meckel's diverticulum and the ileal-cecal junction. Five centimeter of both sides was discarded and then the ileum content was collected in plastic bags and immediately stored at -20 ^{0}C. After the end of the experiment the ileum content was pooled (2 birds per pool) oven-dried at 55 ^{0}C and ground by a coffee grinder. Dried samples were stored in air-tight containers at 4 ^{0}C. The samples were analyzed for Ti, DM, N and GE following the same steps cited above.

Total tract and ileal nutrient digestibility were calculated following Sakomura and Rostagno [23] methodology, using Ti as reference obtaining apparent digestibility coefficient (ADC) of DM and CP (CP - 6.25 x N of Kjedahl). Apparent metabolizable energy corrected by nitrogen (AMEn) was calculated for excreta content only.

Intestinal tract pH

At the days 14, 20 and 41, one bird from each pen was randomly selected and sacrificed by cervical dislocation. The crop, proventriculus, gizzard, duodenum, jejunum, anterior ileum, posterior ileum, cecum and large intestine contents were separated for pH measurements. pH measurements were taken with an Orion model 420a equipped with an electrode model 9157BN. The methodology used was described by [24] but sterilized physiological saline was replaced with deionized water and contents were diluted 1:10; all the content found inside the gut compartment was weighted and then an appropriate volume of deionized water was added. For example, when 10 g of content was found 90 mL of deionized water was added. The solution was homogenized in containers

then the electrode was inserted into the solution. The data was collect once the pH meter was stable.

Relative organs weights and small intestine length

At days 7, 21 and 42, one bird per cage was weighted and sacrificed by cervical dislocation. The liver, spleen, duodenum, jejunum, ileum and cecum was excised and weighed. The content of the gastrointestinal tract was removed the individual segments weighed and length determined. The relative organ weight was calculated by dividing the absolute organ weight by the live BW of birds multiplied by 100, following the methodology describe for [25]. The relative small intestine length was calculated by dividing the intestine segment by the length of the whole small intestine multiplied by 100.

Economic Evaluation

The application of the methodology for economic evaluation (EE) was adapted from [26], and it is described by the formula below:

$$\text{Economic evaluation (kg)} = \frac{\text{AFI (kg) x diet cost (US\$/Ton)}}{\text{ABG (kg)}}$$

Where:

AFI = average feed intake;

ABG = average body gain.

The values of total diet cost for control diet was 0.74 U$ per ton of diet. The prices were obtained from Southern States Cooperative, Inc. For the diet with probiotic, the same diet cost applied including the added cost of the probiotic, which was found as 2 U$ per ton of diet, recommended by CHR Hansen company. All the diets costs were expressed in kg.

Statistical Analysis

Descriptive statistics were used to analyze the normality assumption and if there were any outliers by a Proc Univariate and Proc Means procedures. One-way ANOVA was employed to determine the effects of a probiotic supplementation by a Proc GLM procedure. The least significant difference of Student's t-test was used. A correlation was applied to show the relation between relative weight and relative length of small intestine segments using a Proc Corr procedure. Differences were considered to be significant at a probability of 5%. The Statistical Analysis

System (SAS Institute Inc., Cary, NC) was used to perform all analyzes procedures.

RESULTS AND DISCUSSION

Performance

The mechanisms by which the probiotics act may be related to modulation of the host's microbiota [17, 27-35], changes in morphology of the intestine [36], or even to the immune system of the animal [37, 38], all of which could lead changes in digestibility of dietary nutrients [34, 35, 39, 40] and on broiler performance [16, 18-20, 34, 38, 40-44]. Many authors have demonstrated that *B. subtilis* spores are a good option as probiotic microorganism [14, 45, 46], as it can withstand elevated temperature and pressure of feed processing, along the adversities of intestinal lumen including a low pH and an anaerobic environment [47] and then germinate [13, 45]. After germination, the *B. subtilis* may influence the chickens' intestine microbiome improving their performance [33, 48] in a variety of ways. Different strains of *B. subtilis* are reported to have distinct effects on their host. Consequently, an important are of research is identifying the biological on the host of distinct strains [46, 49].

The performance of *B. subtilis* supplemented birds was positively affected between the first week and fifth week post-hatch (Table 2). Mainly, differences occurred at the FCR calculation, which was clearly affected first by the AFI and then by ABG. For the first 6 days of experiment, the supplemented birds were not different from the control birds ($P>0.05$). The average feed intake was 0.095 kg of feed, suggesting a low level of probiotic consumption during this period. Possibly increasing the amount of supplementation during the first week would improve performance during the first week. One-day-old birds have a low number and species of bacteria in the gastrointestinal tract [50, 51], although the supplementation of probiotic may help an establishment of a good microbiota [17, 27-30, 33, 34, 48, 52-54]. In this sense, an early probiotic supplementation could be a good choice although further study is needed to evaluate the impact of probiotic supplementation at different ages. Similar results were reported by Jeong and Kim [33], where the authors tested *B. subtilis* strain C-3102 with no statistical improvement were observed in contrast with no supplemented birds in the first week of birds life.

Table 2. Average feed intake (AFI), average body gain (ABG), feed conversion ratio (FCR) weekly and economic evaluation (EE) from the overall period of male broilers supplemented or not with probiotic[1]

Item	Week	Group		P-value[2]	SEM[3]
		Control	Probiotic		
AFI	First	98.25	94.42	0.17	1.39
ABG		92.92	91.75	0.42	0.71
FCR		1.06	1.03	0.26	0.01
AFI	Second	452.7	440.0	0.04	3.18
ABG		344.8	348.5	0.24	1.56
FCR		1.31	1.26	<0.01	0.01
AFI	Third	898.3	881.0	0.02	3.86
ABG		652.2	653.8	0.79	2.98
FCR		1.38	1.35	<0.01	0.01
AFI	Forth	1,916	1,883	0.21	12.78
ABG		1,188	1,204	0.41	8.88
FCR		1.61	1.56	<0.01	0.01
AFI	Fifth	2,916	2,914	0.24	21.74
ABG		1,674	1,717	0.07	23.75
FCR		1.75	1.70	0.04	0.02
AFI	Sixth	4,017	3,996	0.36	23.06
ABG		2,297	2,323	0.49	14.20
FCR		1.75	1.72	0.07	0.01
EE		1.14	1.12	0.42	0.01

[1]0.05% of a *B. subtilis* strain DSM 17299 (1,6 x 10^9 cfu/g) was used.
[2]Groups were consider statistically different when the probability were smaller than 0.05.
[3]Standard error of the mean.

The performance data obtained from the second and third weeks, showed an improvement of the AFI and FCR calculation (P<0.05) for

supplemented birds. Birds supplemented with *B. subtilis* strain DSM 17299 had a decrease of 2.7% and 1.9% of AFI values for each week, respectively ($P<0.05$), with no difference over the ABG, which also improved FCR in 3.8% and 2.2% ($P<0.05$). We hypothesize that the *B. subtilis* (DSM 17299), improved nutrient uptake because the supplemented birds have a reduced AFI but no differences were observed in ABG. This results is in accordance with [55], where the authors demonstrate an improvement of FCR of birds supplemented with a probiotic for 21 days. Probiotic supplemented birds also had an improvement for FCR calculation during weeks four and five ($P<0.05$), although, no AFI differences occurred ($P>0.05$). Similar results were reported by Park and Kim [48], where the authors used *B. subtilis* strain B2A and observed that the supplemented birds showed a low values of AFI and FCR ($P<0.05$) with no difference in ABG ($P>0.05$), according to authors this results may be induced by the effects of probiotic action, including the maintenance of beneficial microbial population, improved digestion, and increased activity of intestinal enzymes. No statistically significant improvements were observed for the overall period. However, using a p-value of 0.07 an improvement of 1.7% on FCR was observed. The performance results are in accordance with [33, 48, 55], where the

probiotic supplementation improved performance measurement in birds during the first 4 weeks post-hatch. However, some reports of positive FCR results have been reported for birds supplemented for 42 days [30]. The large variety of the microorganisms used as probiotic in these various studies may be the cause of the reported differences in effect. Given that bacteria from same species can present different results [30, 34], supporting the importance of research on each strain type.

The results for EE can also be observed in table 2. The statistical analyses shows no difference ($P>0.05$) for production cost between supplemented and non-supplemented birds, which mean that the probiotic inclusion, in a rate of 500g of probiotic per ton of feed, does not burdened the production cost, despite the probiotic cost (2 U$ per ton of feed).

Ileal and total digestibility

Considering that the feed is approximately 70% of broiler chickens production cost, the nutrient uptake (NU) of birds is an important component of feed cost. Many additives, such as exogenous enzymes, are used to improve digestibility in order to maximize the use of each feed ingredient. Furthermore, it is well known that many factors can influence

the status of GIT and microbiome composition leading to an alteration of digestibility. In this sense, probiotic could provide a good health status of the GIT [56], reducing pathogens, which may damage the GIT. Also, many species of probiotics can produce enzymes that could help feed digestibility [57, 58]. However, few studies have evaluated digestibility of feed from birds supplemented with *B. subtilis* DSM 17299 [34, 35, 39]. Hence, we evaluated both ileal and total digestibility supplemented or not with probiotic through the coefficient of digestibility of DM, CP and AMEn in two distinct stages (18-20 and 38-40 days).

The birds had no difference ($P>0.05$) for any ileal digestibility measures (Table 3). In this experiment only two birds per repetition was taken to collect digestion sample, which could influence the results accuracy. During the finish stage and using 11% of probability, DM digestibility was improved by 2.0%, which was sufficient to improve performance results and the reduced AFI and FCR from supplemented birds related in this study. Zhang and Kim [35] showed that the supplementation of *Lactobacillus acidophilus*, *B. subtilis* DSM 17299, and *Clostridium butyricum* did not influence digestibility of DM nor total nitrogen of birds; however, an improvement of amino acid digestibility

occurred. An improvement of digestibility of CP was demonstrated by [39], where the authors observed that *B. subtilis* DSM 17299 improved CP ileal digestibility by 3%.

Table 3. Ileal and total nutrient digestibility, dry matter basis, of broiler chickens supplemented or not with probiotic[1]

		Ileal digestibility[2]		Total digestibility[3]		
Item	Feed	DM	CP	DM	CP	AMEn
Control	Starter	72.70	80.36	76.20	68.04	3014
Probiotic		72.99	81.25	76.86	69.91	3035
P-value[4]		0.828	0.284	0.049	0.073	0.122
SEM[5]		0.584	0.381	0.170	0.519	6.618
Control	Finish	74.3	80.6	76.5	68.4	3155
Probiotic		75.9	82.8	77.9	70.5	3192
P-value		0.108	0.165	0.001	0.038	0.005
SEM		0.487	0.764	0.231	0.513	7.124

[1]0.05% of a *B. subtilis* strain DSM 17299 (1,6 x 10^9 cfu/g).
[2]Ileal apparent coefficients of digestibility of dry matter (DM) and crude protein (CP).
[3]Apparent coefficients of digestibility of dry matter (DM), crude protein (CP) and apparent metabolizable energy corrected by nitrogen (AMEn)
[4]Groups were consider statistically different when the probability were smaller than 0.05.
[5]Pooled Standard error of the mean.

Probiotic supplemented birds had improved DM digestibility ($P<0.05$), evidencing that the FCR improvement was a result of an improved nutrient uptake (table 3). No studies have yet demonstrated that the supplementation of *B. subtilis* DSM 17299 could influence DM digestibility of birds at the third week of age. For the finish stage, all

digestibility measurement, from excreta samples, were significantly influenced by probiotic supplementation ($P<0.05$). It was possible to observe an improvement of 1.8% for DM, 3.0% for CP digestibility ($P<0.05$) and 38 kcal/kg of AMEn ($P<0.01$) for birds fed with probiotic. These observations could be related to a variety of probiotic effects including release of enzymes [57, 58], reduction of pathogen load leading to improved GIT health [15, 17, 48, 59-61], and/or improved development of immune status [35]. In agreement with this study, Hossain, Begum and Kim [34], demonstrated that the inclusion of *B. subtilis* DSM 17299 improved nitrogen uptake in broiler supplemented on day 35, however, DM digestibility it was not influenced. Recently researchers have proposed a nutrient equivalence for DSM 17299 and interesting results have been demonstrated [40, 62-64], suggesting that this strain could be used in feed with reduced CP and energy levels without performance injury.

pH of the GIT content

The pH of chicken's GIT is influenced by feed, endogenous secretion of gastric acid, bile salts, pancreatic secretions and the gut's microbiome[24, 65]. Many pathogens are sensitive to low pH an additive

that contribute to the maintenance of low pH levels in the GIT may reduce pathogen load [65, 66] and improve broiler performance. For example, lactic acid bacteria have been shown to have a beneficial effect on GIT pH with a reduction in pathogenic bacterial load [67], along with an increase in production of small chain volatile fatty acid [65]. *B. subtilis* is not a lactic acid bacteria, but, Bacillus spp. can increase the acid lactic bacteria population [16] which is related to a pH alteration [8, 30]. Hence one objective of this study was to determine if *B. subtilis* DSM 17299 affects GIT pH.

The pH measurements are showed in table 4. At day 14 the pH differed ($P<0.05$) between groups in ileum segment. Supplemented birds, showed an increase pH value in anterior ileum and posterior ileum ($P<0.05$). The pH data from birds on 21st day show no differences between groups, in any segment ($P>0.05$). These results corroborate those of Chen et al. [68], when the authors supplemented a fermented feed with *B. subtilis* N21 + *Saccharomyces cerevisiea* Y10 and no pH difference where found throughout the intestinal tract. However, on day 41 (Table 4) the pH measurement of the intestinal content was different between groups in jejunum, anterior ileum, posterior ileum and large intestine ($P<0.05$). The

probiotic birds showed higher pH value in these compartments. To our knowledge, there is no direct evidence in the literature that chicken GIT pH is reduced by *B. subtilis*; the hypothesis that *B. subtilis* will reduce GIT pH was based on the observation that *B. subtilis* increases lactic acid bacterial content. Our data suggests that this hypothesis needs to be reevaluated.

Table 4. pH measurements from bird GUT compartments, supplemented or not with probiotic[1]

		Day 14			Day 21			Day 42		
Segment[2]	Group	pH	P-value[3]	SEM[4]	pH	P-value	SEM	pH	P-value	SEM
Crop	Control	4.997	0.723	0.117	6.088	0.691	0.093	5.117	0.154	0.084
	Probiotic	5.084			6.011			5.358		
Provent.	Control	4.228	0.766	0.104	4.314	0.853	0.084	4.701	0.159	0.081
	Probiotic	4.164			4.282			4.470		
Gizzard	Control	3.075	0.463	0.054	3.757	0.629	0.062	3.794	0.841	0.066
	Probiotic	3.157			3.695			3.766		
Duodenum	Control	6.191	0.980	0.048	6.680	0.182	0.051	6.441	0.866	0.036
	Probiotic	6.193			6.542			6.428		
Jejunum	Control	6.333	0.546	0.034	6.727	0.786	0.037	6.305	0.030	0.041
	Probiotic	6.374			6.748			6.482		
A. Ileum	Control	6.362	0.033	0.078	6.900	0.963	0.061	6.737	0.015	0.120
	Probiotic	6.691			6.894			7.300		
P. Ileum	Control	6.841	0.034	0.119	7.146	0.594	0.113	6.993	0.006	0.137
	Probiotic	7.337			7.020			7.711		
Cecum	Control	6.769	0.685	0.130	7.318	0.996	0.095	6.838	0.170	0.102
	Probiotic	6.668			7.317			6.553		
Colon	Control	6.552	0.269	0.138	6.591	0.191	0.154	6.328	0.004	0.177
	Probiotic	6.863			7.001			7.294		

[1]0.05% of a B. subtilis strain DSM 17299 (1,6 x 10^9 cfu/g) was used.
[2]Proventriculum, anterior ileum and posterior ileum.
[3]Groups were consider statistically different when the probability were smaller than 0.05.
[4]Pooled standard error of the mean.

Relative organs weight (RW) and small intestine relative length (RL)

Many probiotics function as immunomodulation agents [29, 34, 48, 50, 56, 69-72] potentially affecting immune organs (i.e. spleen) and altering intestine morphology [25], [32, 54, 73, 74]. In this context, one objective of this work was to investigate the impact of *B. subtilis* DSM 17299 on the RW of liver, spleen, and small intestine of birds. We collected measurements at two different ages (21 and 42) and compared them with the respective body weight. Also the RL of the compartments of small intestine was done in relation with the total length of the small intestine. Furthermore, a correlation between the RW and RL of the intestine compartments (duodenum, jejunum, and ileum) was applied to determine if the alteration of the intestinal RW is correlated with the alteration with it respective intestinal RL.

The RW of liver and spleen (Table 5) were not influenced by the probiotic supplementation ($P>0.05$), showing that in this experiment the probiotic has no influence on these measurements. Recently research have demonstrated that the *B. subtilis* strain B2A may not influence these measurement in broiler chickens [48]. However results were statistically different for the GIT between groups for both ages, specifically for

duodenum RW at days 21 and 42 and RL at day 42 for the same tissue, where supplementation of *B. subtilis* DSM 17299 decreased these values. A difference on jejunum relative length at day 21 also occurred ($P<0.05$), with an increase of this measurement for supplemented birds. Sohail at al. [36] have proposed that the alteration on intestine RW caused by probiotic supplementation may be a result of an alteration of histological changes on epithelial cells as a change on villi high and crypt depth, or an increase of thickness of mucus layer as reported by [73]. However, we have found a strong positive correlation ($P<0.05$) between the duodenal RW and RL (Figure 1). Based on that we hypothesize that the *B. subtilis* DSM 17299 could cause an alteration in duodenum through a decrease in RW resulting from decrease in duodenal RL. To our knowledge, this is the first work to suggest that the supplementation of *B. subtilis* DMS 17299 could reduce the RL of duodenum and consequently it RW in broiler chickens and more study should be done to better understand this results.

Table 5. Relative weight and length of organs and small intestine of birds supplemented or not with probiotic[1]

		Relative weight (%)						Relative Length (%)		
Item	Day	Liver	Spleen	S. Intestine[2]	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum
Control	21	3.01	0.111	3.00	0.37	1.59	1.03	17.28	41.87	40.84
Probiotic		2.96	0.106	3.11	0.32	1.66	1.13	18.04	41.78	40.18
P-value[3]		0.677	0.601	0.435	0.024	0.406	0.396	0.196	0.916	0.520
SEM[4]		0.059	0.005	0.071	0.013	0.038	0.057	0.288	0.410	0.502
Control	42	2.04	0.122	1.94	0.20	0.97	0.78	19.53	37.61	42.86
Probiotic		2.20	0.124	2.04	0.14	1.07	0.84	17.44	39.05	43.51
P-value		0.101	0.884	0.170	0.002	0.040	0.296	0.003	0.212	0.541
SEM		0.047	0.007	0.036	0.010	0.025	0.028	0.376	0.566	0.514

[1]0.05% of a *B. subtilis* strain DSM 17299 (1,6 x 10^9 cfu/g) was used.
[2]Small intestine.
[3]Groups were consider statistically different when the probability were smaller than 0.05.
[4]Pooled Standard error of the mea

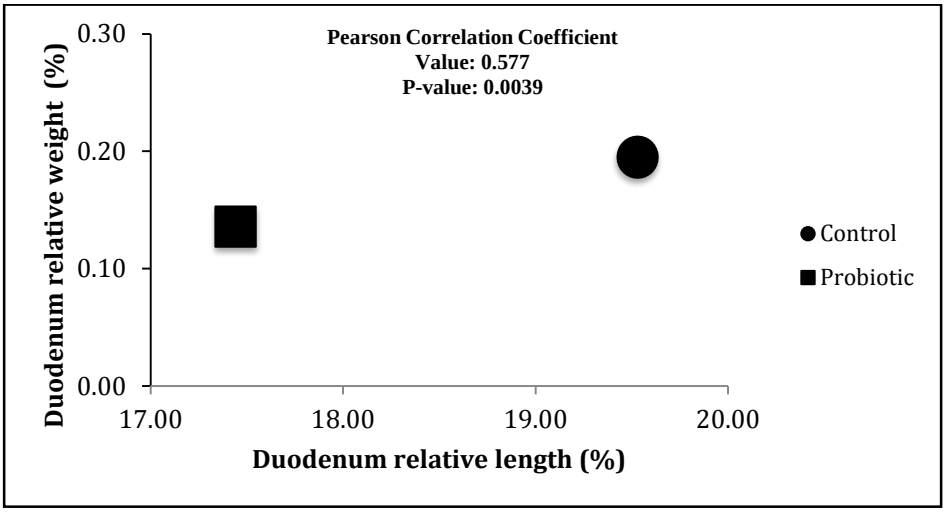

Figure 1. Correlation between duodenum RW and RL of bird supplemented from day one to 42 days of age with a *B. subtilis* strain DSM 17299.

CONCLUSION AND APPLICATIONS

1. The probiotic tested improved performance until fifth week. Also *B. subtilis* DSM 17299 should have no impact over the cost of broiler production, encouraging it uses as an alternative additive.

2. Ileal and total digestibility for probiotic supplemented birds were improved mainly at the finish state, suggesting it is useful on feed with reduced nutrient levels.

3. The alterations at the pH were not as expected, though new studies should be done to better understand this question, since our research

appears to be the first to evaluate the supplementation of *B. subtilis* DSM 17299 in broiler feed on pH of gastrointestinal tract content.

4. The relative weight and relative length of duodenum was reduced in supplemented birds, followed by an increase in the jejunum relative length, which may be related with an improvement of nutrient uptake observed digestibility measures.

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Artigo II - Normas da revista científica Animal Feed Science and Tecnology

Effect of *Bacillus subtilis* (DSM 17299) supplementation on performance, intestine morphology and pH, and relative organ measurements of broilers in heat stress challenge

Abstract

Two trials were conducted to evaluate the impact of supplementation with the probiotic *Bacillus subtilis* strain DSM 17299, to broiler chickens, under heat stress. For the first experiment the measurement were relative organ weight and pH of the gastrointestinal tract (GIT). At the second trial performance and intestinal morphology were evaluated. Four hundred and four hundred and forty male broiler chickens (Ross 708) were used for experiments I and II, respectively, in a total randomized design. Both experiments had the same distribution, where birds were assigned to 2 treatments from day 1 to 21 (with and without probiotic supplementation). At day 22 an additional factor was implemented (heat stress) until the end of the experiment, day 42, performing a factorial 2 x 2 (with and without probiotic supplementation x with and without heat stress). Despite the crypt depth for day 42, no interaction occurred between factors ($P>0.05$). An alteration on crypt depth occurred on day 21 and crypt depth and villus

height for day 42 ($P<0.05$), suggesting an increase on surface absorption. Probiotic supplementation improved feed conversion ratio (FCR) of supplemented birds ($P<0.05$) from day one to 21 until the end of the experiment. Average body weight (ABW) was also improved ($P<0.05$) on day one to 35 and 42 of supplemented birds. HS challenged birds showed a decrease performance for all measured measurements ($P<0.05$). Relative liver weight was reduced in HS broiler ($P<0.05$), despite probiotic supplementation. The influence on intestine morphological measurements by probiotic supplementation, may lead to an improvement of performance and a reduction on production cost, also for broiler challenge with high temperature.

Keywords: probiotic, *Bacillus subtilis*, DSM 17299.

1. Introduction

A main concern of broiler production during warm months is the environmental temperature. The issue is compounded in tropical regions where elevated temperatures are common. Heat stress (HS) reduces poultry performance, affects their overall welfare and reduces profitability.

Furthermore, accord to Burkholder et al. (2008), heat stress can negatively affect the immune system, increasing susceptibility to disease (Soleimani et al., 2012). Chronic HS could also modify the intestinal epithelium morphology and permeability (Deng et al., 2012; Al-Fataftah and Abdelqader, 2014), altering villus height and crypt depth along with changing the stability of tight junction proteins. Alterations in junctions can lead to increased epithelium permeability (Al-Fataftah and Abdelqader, 2014; Song et al., 2014), and elevated transport of bacteria and endotoxin from the intestinal lumen to the body (Tuohy et al., 2014). Elevated systemic levels of endotoxin, mainly lipopolysaccharide (LPS) from gram-negative bacteria have been implicated in a variety of adverse health effects. As cited before, broiler chickens are also affected by HS, which may cause reduced performance and death, documented in literature (Quinteiro-Filho et al., 2010; Azoulay et al., 2011; Azzam et al., 2011; Deng et al., 2012; Sohail et al., 2013; Al-Fataftah and Abdelqader, 2014). Climate change, which is resulting in a global rise in temperature (Herring, 2015; NOAA, 2015; WMO, 2015f; a; e; c; b; d) is also if increasing concern to the poultry industry.

Probiotics may reduce the impact of heat stress in broiler chickens (Deng et al., 2012; Sohail et al., 2012; Sohail et al., 2013; Al-Fataftah and Abdelqader, 2014; Song et al., 2014). Probiotics are defined as live microorganisms which when administered in adequate amounts confer health benefits for the host (Reid et al., 2003). The probiotic effect against pathogens has being described (Lilly and Stillwell, 1965; Nurmi and Rantala, 1973; Klaenhammer, 1988; Fuller, 1989; Fuller, 1992; Oscáriz and Pisabarro, 2001; Servin, 2004; Schneitz, 2005; Awad et al., 2009; Mountzouris et al., 2010; Knap et al., 2011; Alloui Mohamed et al., 2013; de Oliveira et al., 2014; Jeong and Kim, 2014; Wideman et al., 2015) along with an improvement of morphological measurement in intestinal epithelium (Awad et al., 2009; Deng et al., 2012; Tsirtsikos et al., 2012; Al-Fataftah and Abdelqader, 2014; Song et al., 2014). However, there is still a need to evaluate the use of probiotics to minimize the impact of HS (Deng et al., 2012; Sohail et al., 2012; Sohail et al., 2013; Al-Fataftah and Abdelqader, 2014; Song et al., 2014). Among the different species of bacteria used as probiotic (Luciana Kazue et al., 2012), *B. subtilis* has an important characteristic, in it forms spores. Sporulation allows *B. subtilis* to resist extremes encountered during feed processing, storage and with the

intestinal tract environment (Hong et al., 2005). However, different results are reported in the literature with variations observed even within the same bacteria species (de Oliveira et al., 2014), emphasizing the need of study on each strain. *Bacillus subtilis* strain DSM 17299 is a spore forming bacteria used as probiotic to improve poultry yield (Lund et al., 2005; Rostagno et al., 2006; Opalinski et al., 2007; Knarreborg et al., 2008; Mokhtari et al., 2010; Deniz et al., 2011; Knap et al., 2011; D. Harrington, 2014; Hossain et al., 2015; Zaghari et al., 2015). This strain was approved on European Commission (Commission Regulation (EC) No 1137/2007) as a direct fed microbial growth promoter for chickens for fattening (Knarreborg et al., 2008). Furthermore, to our knowledge, no research has being done to evaluate the supplementation of *B. subtilis* strain DSM 17299 to broiler chickens subjected to HS. The objectives of this experiment were to investigate if *B. subtilis* DSM 17299 could reduce the impact caused by HS in broiler chickens over performance, relative organs weight, pH from intestinal segments contents and intestinal morphology.

2. Material and methods

2.1. Birds and experimental design

All experimental procedures were performed in compliance with protocols approved by University of Delaware Agricultural Animal Care and Use Committee. The experiment was conducted at the Department of Food and Animal Science at University of Delaware. Two independent trials were conducted to evaluate probiotic supplementation for broiler chickens under heat stress condition. In both trials commercial 1-d-old straight run Ross 708 were raised from day one to 42 days of age.

The first trial was conducted to evaluate the pH of the gastrointestinal tract (GIT) content along with the relative organ weight and lengths. 400 birds were randomly distributed inside two identical colony houses with HS applied after day 21 in one house. Each house had 2 pen (with and without probiotic supplementation), with each bird as an experimental unit. A random block design was applied for the first three weeks, then, a second factor was set (HS) in one of two houses until the end of the trial, defining a factorial 2 x 2 (with and without probiotic x with and without heat stress).

In the second trial, performance and morphological analyses of jejunum were measured, in which birds were raised in three identical colonial houses, which each house containing eight pens, with each pen as an experimental unit. Birds were allotted in a fully randomized block design, with 2 treatments from day 1 to 21 (n = 20 birds per pen), and a factorial design 2 x 2 (with and without probiotic x with and without HS). The factor evaluated during the first three weeks was the probiotic, which totaled 12 repetitions with and without supplementation each. After day 22, the same distribution was maintained and a heat stress (HS) factor was applied in two of three colonial houses. This yielded an unbalanced factorial design with eight repetitions for HS and four repetitions for non-HS factor, for each supplemented and un-supplemented treatment (see Table 1).

For both trials a density of 12 birds per square meter was followed. The pens were provided with wood chips, hanging drinkers and tubular feeders. The food and water were offered for *ad libitum* consumption. Colony houses were equipped with automatic heathers and light, controlled by a central panel. The heaters maintained the temperature at 32 ºC for the first week, than decreased 2.5 ºC per week until 3th week when the

temperature was set for 22 ºC. Lighting program was 23 h of light and 1 h of dark for the whole experiment.

Table 1

Treatment distribution for trials 1 and 2.

Treatment[1]	Main factor		Pen / treatment	
	Probiotic	Heat stress	Trial I	Trial II
Probiotic	-	-	2	12
	+	-	2	12
Probiotic x Heat Stress	-	-	1	4
	+	-	1	4
	-	+	1	8
	+	+	1	8

[1]Treatment applied: probiotic from day 1 to 21; probiotic x heat stress from day 22 to 42.

2.2. Probiotic, Heat Stress and Diet

The probiotic used was *Bacillus subtilis*, strain DSM 17299, with a minimum concentration of 1,6 x 10^9 cfu/g of product. 500 g of product per ton of feed was used according to the company recommendation. One day before the beginning of the experiment, probiotic was weighed on a precision scale then blended with basal diet and then transported to the colony houses.

For HS, temperature was raised using automatic hanging heaters set inside the houses at the corner. The temperature inside the HS colony houses was kept at 36 ^{0}C for nine hours a day, from 07:40 am to 04:40 pm, and then reduced to 22 ^{0}C until the following morning. This cycle was maintained from day 21 through day 42. The temperature of the control colony house was maintained at 22 ^{0}C from day 21 until the end of the trial at day 42.

Corn and soybean meal based diet (starter from day 1 to 21 and grower feed from day 22 to 42) was obtained from Southern States (Middletown DE.). Diets were representative of local commercial formulations, and calculated analyses met or exceeded NRC (1994) standards for the starter feed, but the grower feed had a lower energy levels, which was replicated for all treatments. The diets were formulated without any coccidiostat or antibiotic growth promoter (see Table 2).

Table 2

Ingredient and nutrient composition of the basal diet (g/kg diet as fed basis).

Item	Diet[1]	
Ingredients	Starter (d 1-21)	Grower (d22-42)
Corn	582.0	656.2
Soybean meal	321.3	263.8
Corn DDGS[2]	33.2	40.0
Wheat bran	27.2	0.00
Phosphate dicalcium	18.1	18.0
Soy oil	0.00	5.6
Ground limestone	10.3	9.6
Salt	3.0	3.0
DL-Methionine 99%	1.2	0.3
Choline chloride 60%	1.0	1.0
Sodium bicarbonate	0.7	0.5
Trace mineral and Vitamin premix[3]	2.0	2.0
Calculated composition		
MEn (MJ/kg)	12.19	12.51
Crude protein	215.0	190.0
Methionine + cystine	8.5	7.0
Lysine	12.2	0.4
Threonine	8.3	7.4
Calcium	9.5	9.0
Available Phosphorus	4.7	4.5
Sodium	2.0	2.0
Potassium	9.5	8.3
Chloride	2.3	2.3
Electrolyte balance (mEq/Kg)	264.90	232.60

[1]0.5 g of probiotic per kg of feed was added on top to fed the supplemented birds

[2]DDGS = distillers dried grains with soluble.

[3]Supplied per kilogram of diet: trans-retinol, 3.60 mg; cholecalciferol, 0.062 mg; dl-α-tocopheryl acetate, 30 mg; thiamine, 2 mg; pyridoxine, 3 mg; calcium pantothenate, 10 mg; biotin, 0.07 mg; menadione, 3 mg; folic

acid, 1 mg; nicotinic acid, 35 mg; cyanocobalamin, 0.015 mg; Se, 0.12 mg; antioxidant, 5 mg; Mn, 80 mg; Fe, 50 mg; Zn, 50 mg; Cu, 10 mg; Co, 1 mg; I, 1 mg.

2.3. Analyses on trial I

For analysis of intestine segments, the duodenum was defined as that portion of the small intestine extending from the end of the gizzard through the pancreatic loop. The jejunum was defined as the segment extending from the end of the pancreatic to Meckel's diverticulum and the ileum was found from Meckel's diverticulum until the ileum-cecal junction. The double cecum was identified as fixed to the end of the ileum at the ileum-cecal junction. For pH measurements the ileum was split in half, and independent measurements obtained from the anterior and posterior portion.

2.3.1. Relative organs weights and small intestine length

At days 7, 21 and 42, eight birds per pen were weighed and sacrificed by cervical dislocation. The liver, spleen, duodenum, jejunum, ileum and cecum were identified. The content of the gastrointestinal tract was removed and weighed. The relative organ weight was calculated by dividing the absolute organ weight by the live body weight of birds

multiplied by 100 (Awad et al., 2009). The relative small intestine length was calculated by dividing the intestine segment by the length of the whole small intestine multiplied by 100.

2.3.2. pH of the GIT content

At the days 27 and 41, eight birds per pen was randomly selected and sacrificed by cervical dislocation. The duodenum, jejunum, anterior ileum, posterior ileum, cecum and large intestine contents were separated for pH measurements. The data measurements were done using an Orion model 420a pH meter equipped with a electrode model 9157BN using a methodology as described by Wu et al. (2013) but instead of sterilized physiological saline we used a deionized water in a dilution of 1:10. All the segment contents were weighed and then a volume of deionized water added. For example, when 10 g of content was found 90 mL of deionized water was added. The solution was homogenized in containers then the electrode was inserted into the solution. The data was collected once the pH meter was stable.

2.4. Analyses on trial II

2.4.1. Performance measurements

At days of 1, 7, 14, 21, 28, 35, and 42 each pen and feed waste was weighed and average feed intake (AFI), average body gain (ABG) and feed conversion ratio (FCR) was calculated for each period. A correction for mortality was applied for a true value of AFI and FCR.

2.4.2. Intestinal morphology

At days of 21 and 42, one bird from each pen was randomly selected and sacrificed by cervical dislocation. Five centimeters from the middle of duodenum, jejunum, ileum and cecum were separated for morphologic measurements. Samples were collected as described by (Al-Fataftah and Abdelqader, 2014), briefly: each tissue sample was washed with phosphate solution, and preserved in 10% buffered formalin. Then the tissue were trimmed, processed, embedded in paraffin, sectioned to 4 micrometer thickness, placed on glass slide, and stained with hematoxylin and eosin.

Histological sections were examined using ImageScope to measure villus height, and crypt depth. Villus height was measured from the tip of the villus to the villus-crypt junction, and crypt depth was defined as the

depth of the invagination between adjacent villi (Al-Fataftah and Abdelqader, 2014). A total of 40 reads per slide was applied, with 20 villus and 20 crypts reads.

2.4.3. Economic Evaluation

The application of the methodology for economic evaluation (EE) was adapted from (Gomes et al., 2012), and it is essentially described by the formula below:

$$\text{Economic evaluation (U\$/kg)} = \frac{\text{AFI (kg) x diet cost (U\$/kg)}}{\text{ABG (kg)}}$$

Where:

AFI = average feed intake;

ABG = average body gain.

The values of total diet cost for basal diet was 0.65 U$ per kg, obtained from our feed supplier (Southern States Cooperative, Inc), located in Delaware, USA. For the diet with probiotic, the same diet cost applied

was added the cost of the probiotic, which was 0.002 U.S. dollars per kg of diet, as recommended by the supplier (CHR Hansen company).

2.5. Statistical Analysis

Descriptive statistics were used to analyze the normality assumption and if there were any outliers by a Proc Univariate and Proc Means procedures. For the first three weeks, without HS factor, one-way ANOVA was employed to determine the effects of a probiotic supplementation by a Proc GLM procedure. The least significant difference of Student's t-test was used. To analyze a factorial design, two-way ANOVA was employed to determine the main effects (probiotic supplementation and heat stress) and their interaction by a Proc GLM. Tukey's student range test was used to show the difference of a factor inside another, when the interaction was significant and T test was applied to show the main difference effect for each factor separately. Differences were considered significant at probability of 5%. The Statistical Analysis System (SAS Institute Inc., Cary, NC) was used to perform mentioned analyzes procedures.

3. Results

3.1. Relative organ weight and small intestine length

The measurement of relative liver, spleen and intestine segments weight showed no difference ($P>0.05$) at day 7 and 21 for all main factors applied to birds as also the interaction between factors (see table 3). A similar result was found for relative small intestine length, with no statistical difference ($P>0.05$) for all ages and segments evaluated. However, a statistical difference ($P<0.05$) was found for supplemented birds on the relative weight of the ileum at day 42 (see table 4), despite the HS factor. Broilers fed with probiotic showed an increase of ileum relative weight of approximately 15%.

Table 3

Relative organ weight and relative small intestine length from supplemented and un-supplemented birds at days 7 and 21.

		Relative organ weigth (%)[1]						Relative length (%)		
Item	Day	Liver	Spleen	S. I.	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum
Control	7	4.08	0.08	4.63	0.71	2.22	1.24	21.40	39.72	38.88
Probiotic		4.09	0.08	4.60	0.76	2.37	1.43	19.23	41.47	39.30
P-value		NS	NS	NS	NS	NS	NS	NS	NS	NS
SEM[2]		0.195	0.008	0.189	0.055	0.087	0.124	0.887	0.601	0.559
Control	21	2.74	0.11	2.98	0.39	1.48	1.15	19.48	41.49	39.03
Probiotic		2.71	0.13	3.07	0.41	1.46	1.20	18.98	43.19	37.84
P-value		NS	NS	NS	NS	NS	NS	NS	NS	NS
SEM		0.06	0.006	0.078	0.013	0.039	0.039	0.262	0.609	0.618

[1]Relative organ weight measurements of liver, spleen, small intestine (S. I.), duodenum, jejunum, ileum and cecum of birds.
[2]Pooled standard error of the mean.

Table 4

Relative organ weight and relative small intestine length of birds receiving or not probiotic and heat stress factors at day 42.

Treatment[1]		Relative organ weight[2]							Relative length (%)		
HS	Probiotic	Liver	Spleen	S. I.	Duodenum	Jejunum	Ileum	Cecum	Duodenum	Jejunum	Ileum
no	-	2.06	0.13	1.92	0.22	0.92	0.76	0.15	20.90	37.64	41.46
	+	2.02	0.10	2.01	0.19	0.98	0.84	0.14	19.54	37.13	43.34
yes	-	2.42	0.10	1.94	0.23	1.01	0.70	0.15	19.49	36.88	43.62
	+	2.31	0.10	2.17	0.26	1.04	0.87	0.16	19.38	36.70	43.92
SEM[3]		0.087	0.006	0.072	0.012	0.037	0.028	0.005	0.468	0.407	0.494
Main Effect											
Probiotic	-	2.24	0.11	1.93	0.23	0.96	0.73	0.15	20.20	37.26	42.54
	+	2.17	0.10	2.09	0.22	1.01	0.85	0.15	19.46	36.91	43.63
HS	no	2.04	0.11	1.97	0.21	0.95	0.80	0.15	20.22	37.39	42.40
	yes	2.36	0.10	2.05	0.24	1.03	0.78	0.15	19.44	36.79	43.77
P-Value	Probiotic	NS	NS	NS	NS	NS	0.031	NS	NS	NS	NS
	HS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	Interaction	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[1]Treatments arranged in factorial design 2 x 2, with and without probiotic supplementation x with and without heat stress (HS).
[2]Relative organ weight measurements of liver, spleen, small intestine (S. I.), duodenum, jejunum, ileum and cecum of birds.
[3]Pooled standard error of the mean.

3.2. pH of GIT content

The pH measurement was not different (P>0.05) for all segments and ages evaluated. The interaction between factors was also not significant at 5% (see table 5 and 6).

Table 5

pH measurements of gastro intestinal tract contents of birds receiving or not probiotic and heat stress factors at day 27.

Treatment[1]		pH[2]					
HS	Probiotic	Duodenum	Jejunum	A. Ileum	P. Ileum	Cecum	L. Intestine
no	-	6.50	6.38	6.66	7.10	6.76	6.74
	+	6.33	6.22	6.54	7.19	6.47	6.56
yes	-	6.24	6.23	6.87	7.39	6.90	7.01
	+	6.27	6.45	7.05	7.47	6.99	6.71
SEM[3]		0.053	0.040	0.090	0.116	0.080	0.107
Main Effect							
Probiotic	-	6.37	6.30	6.76	7.25	6.83	6.88
	+	6.30	6.34	6.79	7.33	6.73	6.64
HS	no	6.41	6.30	6.60	7.15	6.61	6.65
	yes	6.26	6.34	6.96	7.43	6.94	6.86
P-Value	Probiotic	NS	NS	NS	NS	NS	NS
	HS	NS	NS	NS	NS	NS	NS
	Interaction	NS	NS	NS	NS	NS	NS

[1]Treatments arranged in factorial design 2 x 2, with and without probiotic supplementation x with and without heat stress (HS).
[2]Measurements of pH contents on duodenum, jejunum, anterior ileum (A. Ileum), posterior ileum (P. Ileum), cecum and large intestine (L. Intestine).
[3]Pooled standard error of the mean.

Table 6

pH measurements of gastrointestinal tract contents of birds receiving or not receiving probiotic and heat stress factors at day 41.

Treatment[1]		pH[2]					
HS	Probiotic	Duodenum	Jejunum	A. Ileum	P. Ileum	Cecum	Colon
no	-	6.55	6.58	6.98	7.38	6.72	6.53
	+	6.46	6.19	6.64	7.00	6.96	6.32
yes	-	6.64	6.26	6.56	7.34	6.55	6.48
	+	6.54	6.39	6.79	7.51	6.91	6.80
SEM[3]		0.061	0.062	0.094	0.079	0.082	0.090
Main Effect							
Probiotic	-	6.60	6.42	6.77	7.36	6.64	6.50
	+	6.50	6.29	6.71	7.25	6.94	6.56
HS	no	6.50	6.38	6.81	7.19	6.84	6.42
	yes	6.59	6.32	6.67	7.42	6.73	6.64
P-Value	Probiotic	NS	NS	NS	NS	NS	NS
	HS	NS	NS	NS	NS	NS	NS
	Interaction	NS	NS	NS	NS	NS	NS

[1]Treatments arranged in factorial design 2 x 2, with and without probiotic supplementation x with and without heat stress (HS).
[2]Measurements of pH contents on duodenum, jejunum, anterior ileum (A. Ileum), posterior ileum (P. Ileum), cecum and colon.
[3] Pooled standard error of the mean.

3.3. Performance measurement and economic evaluation

At days 7 and 14 no differences were observed, between supplemented and non-supplemented birds (see table 7), for all performance measurements ($P>0.05$). However, at day 21 the FCR was improved 4% ($P<0.05$) by probiotic supplementation of broiler chickens (table 7). With the addition of HS factor, no interaction was observed ($P>0.05$) in any measurement. Probiotic supplementation improved the FCR ($P<0.05$) of broiler chickens on days 28, 35 and 42 (see table 8). The ABG was also improved ($P<0.05$) for broiler chickens receiving probiotic for 35 and 42 days. Heat stress factor reduced AFI, ABG while increasing the FCR ($P<0.05$) for all evaluated ages. No interaction was observed for economic evaluation for day 42 ($P>0.05$), however, an increase in this measurement occurred ($P<0.05$) for both un-supplemented probiotic and heat stressed birds (see table 8).

Table 7

Performance measurements of birds supplemented or not with probiotic for 7, 14 and 21 days.

Item[1]	Day	Treatment		P-value	SEM[2]
		Control	Probiotic		
AFI		0.127	0.127	NS	0.002
ABG	7	0.120	0.120	NS	0.002
FCR		1.057	1.054	NS	0.009
AFI		0.439	0.426	NS	0.008
ABG	14	0.344	0.352	NS	0.004
FCR		1.277	1.214	NS	0.029
AFI		1.000	0.982	NS	0.009
ABG	21	0.685	0.705	NS	0.006
FCR		1.460	1.396	0.039	0.016

[1]Performance measured: average feed intake (AFI), average body gain (ABG) and feed conversion ratio (FCR). All measurement was calculated in kg and FCR in kg/kg.

[2] Pooled standard error of the mean.

Table 8

Performance and economic evaluation (EE) measurements of birds receiving or not probiotic and heat stress factors at days 28, 35 and 42.

Treatment[1]		Performance[2]									
HS	Probiotic	AFI28	ABG28	FCR28	AFI35	ABG35	FCR35	AFI42	ABG42	FCR42	EE
no	-	1.926	1.212	1.589	3.123	1.854	1.686	4.396	2.508	1.753	1.140
	+	1.858	1.235	1.504	3.086	1.928	1.601	4.453	2.658	1.675	1.092
yes	-	1.744	1.058	1.649	2.642	1.545	1.711	3.496	1.963	1.782	1.158
	+	1.737	1.075	1.616	2.673	1.595	1.676	3.557	2.038	1.747	1.139
SEM[3]		0.020	0.017	0.013	0.047	0.035	0.011	0.093	0.062	0.010	0.007
Main Effect											
Probiotic	-	1.805	1.110	1.629	2.803	1.648	1.703	3.796	2.144	1.773	1.152
	+	1.778	1.129	1.578	2.810	1.706	1.651	3.855	2.245	1.723	1.123
HS	no	1.892	1.224	1.547	3.105	1.891	1.643	4.424	2.583	1.714	1.116
	yes	1.741	1.067	1.633	2.657	1.570	1.694	3.526	2.000	1.764	1.149
P-Value	Probiotic	NS	NS	0.011	NS	0.046	0.006	NS	0.025	0.004	0.010
	HS	<.0001	<.0001	<.0001	<.0001	<.0001	0.010	<.0001	<.0001	0.006	0.006
	Interaction	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[1]Treatments arranged in factorial design 2 x 2, with and without probiotic supplementation x with and without heat stress (HS).

[2]Performance measurements measured: average feed intake (AFI), average body gain (ABG) and feed conversion ratio (FCR) at days 28, 35 and 42. Economic evaluation (EE) for day 42. All measurements were calculated in kg and FCR in kg/kg

[3] Pooled standard error of the mean.

3.4. Intestinal morphology

The results of histological measurement for day 21 (table 9) showed a difference ($P<0.05$) in crypt depth and the relation villus height / crypt depth between groups. The probiotic supplemented group had a reduced crypt depth and an increased ratio of the villus height / crypt depth (see Figure 1). For day 42 an interaction occurred ($P<0.05$) only for crypt depth, where probiotic supplemented birds had an increased crypt depth when heat stress was applied (see table 10). Probiotic supplemented birds had a villus height increase ($P<0.05$) of 7% at day 42. Epithelium injury it was also observed in HS birds (see Table 10 and Figure 1).

Table 9

Morphology measurement of jejunum tissue from birds supplemented or not with probiotic at day 21.

Treatment	Villus height	Crypt depth	Villus/Crypt
Control	994.38	342.83	3.06
Probiotic	995.60	321.28	3.26
P-value	NS	<.001	0.03
SEM[1]	7.83	3.72	0.05

[1] Pooled standard error of the mean.

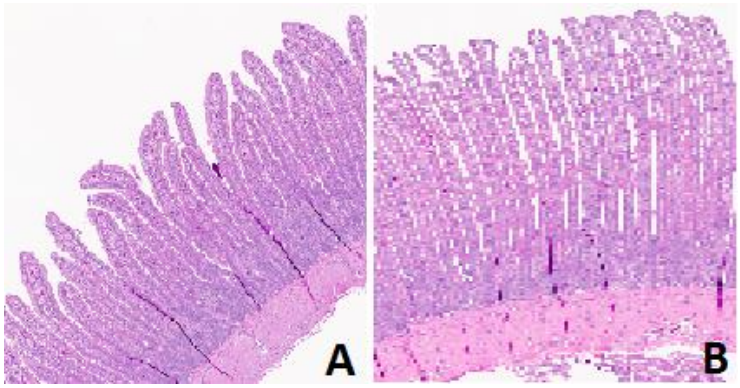

Figure 1 Villi of Jejunum epithelium from 21 days old broiler chickens un-supplemented (A) or supplemented with probiotic (B).

Table 10

Morphology measurement of jejunum tissue from birds receiving or not probiotic and heat stress factors at day 42.

Treatment[1]		Histology measurement		
HS	Probiotic	Villus height	Crypt depth	Villus/Crypt
no	-	1114.83	277.68 a	4.22
	+	1163.07	266.68 ab	4.63
yes	-	939.99	230.21 c	4.53
	+	1051.52	246.46 bc	4.59
SEM[2]		10.55	3.05	0.07
Main Effect				
Probiotic	-	1027.41	253.94	4.38
	+	1107.30	256.57	4.61
HS	no	1138.95	272.18	4.43
	yes	995.75	238.33	4.56
P-Value	Probiotic	<.0001	NS	NS
	Heat Stress	<.0001	<.0001	NS
	Interaction	NS	0.042	NS

[1]Treatments arranged in factorial design 2 x 2, with and without probiotic supplementation x with and without heat stress (HS).
[2]Pooled standard error of the mean.

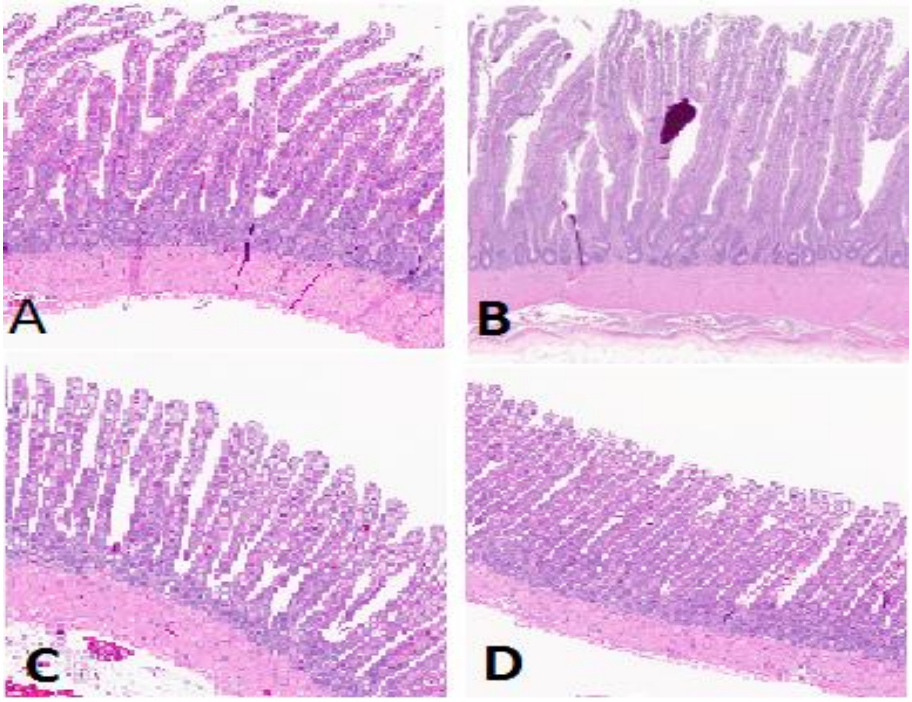

Figure 2 Villi of Jejunum epithelium from 42 days old broiler chickens. A: with HS and without probiotic; B: with HS and with probiotic; C: without HS and without probiotic; D: without HS and with probiotic.

4. Discussion

At the present experiment, we found no influence of *B. subtilis* DSM 17299 on broiler relative organ weight on 7 and 21 days of supplementation. Similar results were reported by Park and Kim (2014) evaluating broiler chickens supplemented with *B. subtilis* strain B2A. In a

previous study (first paper from *Parte 2*) we observed that the probiotic had no influence on liver and spleen RW, but a difference was noted for duodenum RW and RL, which was not observed in this work. However, for day 21 a statistical difference was found for RW of ileum. This result could indicate that the probiotic could influence the epithelial cells of the GIT, causing changes in villi high and crypt depth, or increase the thickness of mucus layer as reported by (Tsirtsikos et al., 2012). In our previous work (first paper from *Parte 2*), we observed a correlation between the RW and RL of duodenum, suggesting that the probiotic supplementation could affect the morphology of the TGI.

The histological analyses of the jejunum at 21 days showed that birds without probiotic supplementation had an increase on crypt depth without any difference on villus height. A deeper crypt is suggests an increase in epithelial metabolism (Awad et al., 2009) and when this is not accompanied by an increase in villus height, an excessive cell turnover may be occurring. This would leading to higher energy cost to produce this class of birds. The results of the present study for performance, before heat stress, showed that the probiotic supplementation did not affect birds until day 21, when the FCR was improved by probiotic. Since broiler chickens

acquire and stabilize its microbiota from the environment after hatching (Cartman et al., 2008), precocious probiotic supplementation may be important for a microbiota stabilization. An improvement of FCR at day 21 reported in this study could be related with histological measurements evaluated at the same age. Birds without probiotic supplementation had deeper crypt depth, increasing energy demand, although, no AFI difference was observed. We hypothesize that probiotic supplementation may influence the histology of the small intestine of birds, reflecting a better performance. Results that support the influence of probiotic on histologic epithelium measurement are described by many authors using different bacteria as probiotic additive (Awad et al., 2009; Deng et al., 2012; Tsirtsikos et al., 2012; Rajput et al., 2013; Al-Fataftah and Abdelqader, 2014; Song et al., 2014).

Broiler chickens subjected to heat stress could have an alteration in microbiota composition (Burkholder et al., 2008; Al-Fataftah and Abdelqader, 2014), with a destabilized microbiome allowing an increase of pathogenic bacteria, thereby increasing the competition for nutrient, (Al-Fataftah and Abdelqader, 2014) and causing damage to the GIT (Calefi et al., 2014). The pH of segment contents is influenced by microbiota and

some pathogens are pH sensitive, furthermore, researchers have demonstrated the impact of lactic acid bacteria on pH, influencing the pathogen bacteria population (Alakomi et al., 2000). In this sense, *Bacillus spp.* may increase lactic acid bacteria (Knarreborg et al., 2008) leading to pH reduction. However no difference was observed on digest pH for any GIT segment, independent of the factor evaluated. The environment status where birds are raised can influence the acquired microbiota by broilers, which may explain the absence of difference on digest pH even when a heat stress factor was applied. Sohail et al. (2013) reported no difference in microbiota composition of broiler chickens subject to heat stress, suggesting that the same result could be happened in this experiment, leading to no pH measurement alteration.

Modern broiler chickens are vulnerable to high temperature (Etches et al., 2008; Azoulay et al., 2011; Abdelqader and Al-Fataftah, 2014; Al-Fataftah and Abdelqader, 2014; Calefi et al., 2014) and in the present study heat stressed broilers had a decreased performance, which is in agreement with literature research (Quinteiro-Filho et al., 2010; Sohail et al., 2012; Sohail et al., 2013; Abdelqader and Al-Fataftah, 2014; Al-Fataftah and Abdelqader, 2014). A reduction on AFI clearly occurred for HS broilers,

which reduced the ABG and increased the FCR measurements. Probiotic supplementation reduced the negative effect of heat stress. Positive results on performance of broiler chickens supplemented with *B. subtilis* DSM 17299 are described by many authors (Lund et al., 2005; Rostagno et al., 2006; Opalinski et al., 2007; Knarreborg et al., 2008; Mokhtari et al., 2010; Deniz et al., 2011; Knap, 2011; Kehlet et al., 2014; Murshed and Abudabos, 2015; Szakacs et al., 2015; Zaghari et al., 2015), but the physiologic changes caused by this specific strain are not completely understood and no prior literature has evaluated the effect of DSM 17299 supplementation on heat stressed birds.

At day 42, probiotic supplemented birds showed an increase in villus height, despite the heat stress, suggesting that the probiotic supplementation improved the surface absorption area on jejunal segment. This could play an important role in the improved performance observed with probiotic treated birds. An interaction occurred for crypt depth, where under HS supplemented birds had deeper crypts, followed by an increase on villi height, suggesting that the supplementation of probiotic reduced the injury caused by heat stress. Reduction in heat stress induced epithelial injury could impact the immune status of the bird. (Etches et al., 2008;

Quinteiro-Filho et al., 2010). An increase in epithelium permeability (Song et al., 2014), which may permit a translocation of bacteria and endotoxin to the blood, increasing the requirement of energy by immune system, reducing performance. Heat stressed broiler could also divert systemic blood flows from internal organs to peripheral circulation, in order to dissipate heat (Al-Fataftah and Abdelqader, 2014), which could also affect physiology of the GIT, changing its characteristics as villi height and crypt depth, decreasing performance. In agreement with the present study, Al-Fataftah and Abdelqader (2014) have documented that *B. subtilis* strain PB6 supplementation improved the epithelium histology measurements of heat stressed bird, reducing the negative effects of high temperature, which may be an important nutritional strategy on poultry production in tropical regions.

5. Conclusion

Supplementation of *B. subtilis* DSM 17299 improve performance through an improvement of morphological measurements of jejunum on birds heat stressed or not, leading to an equal economic evaluation, even

considering the cost of the addition of probiotic on feed, suggesting its use on poultry production.

Conflict of interest

The authors declare that they have no conflict of interest.

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