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USO DE ADITIVOS NA ALIMENTAÇÃO DE POEDEIRAS COMO FERRAMENTAS MELHORADORAS DE DESEMPENHO, SAÚDE INTESTINAL, BEM-ESTAR E QUALIDADE DE OVOS

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Dissertação apresentada como um dos requisitos à obtenção do Grau de Mestre em Zootecnia, na Universidade Federal do Rio Grande do Sul

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PRÓ-REITORIA DE PESQUISA

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CEUA UFRGS

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Comissão De Ética No Uso De Animais analisou o projeto:

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Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 07/12/2020 via Webconferência - Mconf UFRGS, em seus aspectos éticos e metodológicos, para a utilização de 600 aves, galinhas poedeiras, fêmeas provenientes de uma granja comercial, proprietária Ângela Petry Hardt (CPF 949617940.15) , de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa.

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ALEXANDRE TAVARES DUARTE DE OLIVEIRA
Coordenador da comissão de ética

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USO DE ADITIVOS NA ALIMENTAÇÃO DE POEDEIRAS COMO FERRAMENTAS MELHORADORAS DE DESEMPENHO, SAÚDE INTESTINAL, BEM-ESTAR E QUALIDADE DE OVOS[1].

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Orientadora: Ines Andretta; Raquel Melchior

O objetivo deste estudo foi avaliar se a suplementação de β-mannanase e probióticos podem influenciar o desempenho, bioquímica sérica, características morfométricas intestinais, qualidade de ovos frescos e armazenados e bem-estar animal de poedeiras. Poedeiras leves (36 semanas de idade) foram alojadas em 120 gaiolas (4 aves cada) atribuídas aleatoriamente a um dos quatro diferentes tratamentos, sendo eles: grupo controle, alimentados com dietas não suplementadas; dietas suplementadas com 300 g/ton de β-mannanase; dietas suplementadas com 50 g/ton de probiótico; ou dietas contendo 300 g/ton de β-mananase e 50 g/ton de probiótico. O desempenho e o bem-estar animal foram avaliados em todos os tratamentos, enquanto a qualidade dos ovos frescos e armazenados foi avaliada nos três primeiros tratamentos. O experimento teve duração de 182 dias, compreendendo três fases produtivas de 28 dias quando os tratamentos foram fornecidos aos animais e uma última fase sem suplementação. As médias foram comparadas por meio de análise de variância seguida do teste de Tukey considerando diferenças de 5 e 10%. A β-mannanase aumentou a taxa de postura em 11% ($P<0,05$), enquanto os probióticos aumentaram essa resposta em 7% ($P<0,05$), e os aditivos combinados aumentaram a taxa de postura em 11,5% quando comparados ao tratamento controle. O peso dos ovos frescos aumentou com o uso de todos os aditivos durante o período de suplementação ($P<0,05$). A bioquímica sérica, morfometria intestinal e massas de ovos das aves alimentadas com dietas contendo ambos os aditivos apresentaram diferenças significativas em relação ao grupo controle como ácido úrico, colesterol total e triglicerídeos. β-mannanase e probiótico melhoraram a qualidade de ovos frescos ($P<0,05$). Quanto à qualidade dos ovos armazenados, a β-mananase e o probiótico foram capazes de melhorar a qualidade dos ovos ($P<0,05$), principalmente quando relacionados à cor da gema, além de apresentarem menores níveis de TBARS e pH ($P<0,05$) quando comparados ao tratamento controle. Quanto ao bem-estar animal, a β-mannanase foi capaz de aumentar a frequência do comportamento alimentar em 49% ($P<0,05$) e os probióticos em 39% ($P<0,05$). O tempo gasto neste comportamento também foi maior nas aves suplementadas ($P<0,05$). Todos os tratamentos foram

capazes de reduzir a bicagem (P<0,05). Portanto, a adição de β-mannanase e probióticos às dietas de galinhas poedeiras é uma estratégia eficaz para melhorar o desempenho, a saúde, o bem-estar das aves, além de melhorar a qualidade em ovos frescos e armazenados.

Palavras chave: aditivos, alimento; saúde intestinal; comportamento animal; ovos.

1 Dissertação de mestrado em Zootecnia – Produção Animal, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil (151 páginas) Março, 2022

USE OF ADDITIVES IN LAYERS FEEDINGS AS A TOOL TO IMPROVE PERFORMANCE, INTESTINAL HEALTH, WELFARE AND EGG QUALITY[2].

Author: Camila Lopes Carvalho
Supervisor: Ines Andretta; Raquel Melchior

The objective of this study was to evaluate whether β-mannanase and probiotic supplementation can influence the performance, serum biochemistry, gut morphometric traits, quality of fresh eggs and stored eggs, and animal welfare in laying hens. The light-weight laying hens (36 weeks old) were housed in 120 cages (4 birds each) randomly attributed to one of four different treatments, namely: control group, fed non-supplemented diets; diets supplemented with 300 g/ton of β-mannanase; diets supplemented with 50 g/ton of probiotic; or diets containing both 300 g/ton of β-mannanase and 50 g/ton of probiotic. Performance and animal welfare was evaluated in all treatments, while quality of fresh and stored eggs were assessed in the three first treatments. The trial lasted for 182 days, comprising three productive phases of 28 days when the treatments were provided to the animals and a last phase without supplementation. Means were compared using variance analysis followed by Tukey test considering differences at 5 and 10%. β-mannanase was able to improve laying rate by 11% ($P<0.05$), while probiotics improved this response by 7% ($P<0.05$), and combined additives increased laying rate by 11.5% when compared to control treatment. The weight of fresh eggs was improved by all the additives during the supplementation period ($P<0.05$). The serum biochemistry, gut morphometry, and egg masses of birds fed diets containing both additives showed significant differences compared to the control group like uric acid, total cholesterol, and triglycerides. β-mannanase and probiotic improved quality of fresh eggs ($P<0.05$). As for quality of stored eggs, β-mannanase and probiotic were able to improve egg quality ($P<0.05$), specially when related to yolk color, besides showed lower TBARS levels and reduced pH ($P<0.05$) when compared to control treatment. And as for animal welfare, β-mannanase was able to increase the frequency of feeding behaviour by 49% ($P<0.05$) and probiotics also enhanced it by 39% ($P<0.05$). The time spend in this behavior was also higher in supplemented birds ($P<0.05$). All the treatments were able to reduce pecking ($P<0.05$). Therefore, the addition of β-mannanase and probiotics to laying hen diets is an effective strategy to improve the bird performance, health status, welfare, and increase quality traits in fresh and stored eggs.

Keywords: additives; feeding; gut health; animal behavior; eggs.

2 Master of Science dissertation in Animal Science, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. (151 pages) March, 2022.

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CAPÍTULO I

1. INTRODUÇÃO

O ovo é considerado como sendo ovo de galinha em casca, sendo os demais ovos acompanhados de designação de espécie (BRASIL, 1990). E é através da eficiente transformação biológica realizada pela ave de postura, a qual possui a capacidade de transformar recursos alimentares de menor valor biológico em produtos com alta qualidade nutricional para o consumo humano, que o ovo ganha cada dia mais espaço na mesa dos brasileiros (BERTECHINI, 2004). Ao possuir minerais como fósforo, ferro, selênio e zinco; vitaminas do complexo A, B, E, K; carotenoides como zeaxantina e luteína e gorduras; além de outros nutrientes benéficos à saúde, que agem na modulação do sistema imunológico com antivirais e antibacterianos, o ovo é considerado um representativo do modelo de proteína ideal (AMARAL et al., 2016; FIGUEIREDO, 2012).

Segundo a Organização das Nações Unidas para a Alimentação e Agricultura (FAO), o Brasil é o sexto maior produtor de ovos do mundo (FAO, 2017). Em 2020 cerca de 124 milhões de aves comerciais de postura foram alojadas e a produção superou 53 bilhões de ovos, com um aumento de cerca de 8% quando comparado ao ano anterior (ABPA, 2021). Sendo o estado de São Paulo o maior produtor e o estado do Rio Grande do Sul o maior exportador (EMBRAPA, 2019). Também vale ressaltar o aumento significativo do consumo per capta de ovos nos últimos anos, chegando em 2020 a 251 ovos consumidos por habitante ao ano no Brasil (ABPA, 2021).

Além da produtividade, um tópico amplamente discutido no setor de avicultura de postura é a segurança alimentar. Este é um tema estratégico para a humanidade, sendo incorporado as políticas agrícolas, as políticas socioeconômicas, à pesquisa, ao desenvolvimento agroindustrial, a vigilância sanitária, à saúde pública e em debates acerca dos direitos dos consumidores. A segurança alimentar possui dois conceitos, um deles é utilizado quando se diz respeito ao seu acesso, sendo este regular e permanente para cada indivíduo (CONSEA, 2004). Já o outro conceito se refere aos aspectos relacionados à sua qualidade, a qual garante ao consumidor a aquisição de alimentos com características nutricionais e sanitárias adequadas (ORTEGA & BORGES, 2012). Com ovos, a discussão permanece a mesma, visto que fatores podem influenciar a qualidade dos ovos. Entre eles, a alimentação das aves, que afeta as características internas e externas dos ovos, e pode gerar alterações físico-químicas do albúmen e da

gema, o que resulta em alterações na palatabilidade, no frescor e no sabor (OLIVEIRA; OLIVEIRA, 2013).

O uso de aditivos alimentares é uma das formas de modular a qualidade dos ovos, o desempenho e o bem-estar das aves. Probiótico é um desses aditivos, o qual é definido como suplemento alimentar constituído de micro-organismos (FULLER, 1989) que possui a capacidade de estabilizar e manter certas populações bacterianas no trato digestório sem interferir na sanidade de forma negativa (RAMOS et al., 2014). Segundo Ibrahim et al. (2018), além de melhorar o balanço da microbiota intestinal, o uso de suplementação com probióticos também pode melhorar o bem-estar das aves.

Outra forma de aumentar a produção de bactérias benéficas é através do uso de enzimas exógenas. A β-mannanase auxilia na liberação de açúcares como fonte de energia, melhora a imunidade, a digestão e a absorção de nutrientes, além de limitar o crescimento de bactérias patogênicas (MOHAYAYEE; RIMI, 2012; O'NEILL; SMITH; SAEED et al., 2019). Tais fatores ocorrem devido ao poder de hidrólise dos β-mananos frente aos mananos. Os mananos são encontrados na parede celular das plantas, como a soja, que é um ingrediente muito utilizado na ração animal (JACKSON, 2001; JANI et al., 2009), entretanto, possui efeitos antinutricionais (DELMASCHIO, 2018).

O uso de aditivos alimentares além de alterar a microbiota intestinal dos animais, também pode impactar no bem-estar das aves de postura. A ciência do bem-estar animal vem sendo muito discutida nos últimos anos e segundo Carvalho (2019), a evolução da avicultura de postura afetou negativamente aspectos ligados ao bem-estar animal, visto que nestes sistemas, no qual os animais são criados principalmente em gaiolas, as aves são impedidas de realizar a maior parte de seus comportamentos naturais. Tais fatores geram preocupação da sociedade acerca do bem-estar animal, gerando reflexos em diversos âmbitos, entre eles econômicos, culturais, científicos e legais. Assim, os consumidores se apresentam preocupados com a origem dos produtos que consomem e demandam mudanças da indústria para que estas melhorem seus padrões de bem-estar animal. Porém, alterações muito impactantes nos sistemas produtivos (como mudança nos sistemas de alojamento) são mais difíceis de serem promovidas e podem não ser amplamente implementadas em curto período de tempo. Nesse sentido, pequenas alterações que possam beneficiar a saúde e o bem-estar dos animais também merecem ser estudadas e validadas.

Como já exposto, o ovo se destaca atualmente como sendo uma das principais fontes de proteína na alimentação da população. E o uso de suplementação nas dietas com aditivos se mostra promissor em vários aspectos. Apesar destes aditivos já serem descritos em outros estudos, muito foram realizadas em outras espécies, como suínos e frangos de corte. Ademais, tais aditivos utilizados de forma sinérgica, ao nosso conhecimento, ainda não foram descritos na literatura. Assim, este trabalho foi desenvolvido a fim de avaliar se a utilização de β-mannanase e probióticos sozinhos, ou de forma conjunta, podem melhorar o desempenho, a qualidade dos ovos e o bem-estar de poedeiras. Neste contexto, uma revisão bibliográfica, quatro artigos científicos, considerações finais e conclusão serão apresentados nesta dissertação.

2. REVISÃO BIBLIOGRÁFICA

2.1 Qualidade de ovos

Um dos maiores atrativos observados pelos consumidores de ovos está na sua qualidade física, a qual engloba diferentes aspectos, e possui três componentes principais: a gema, que representa em torno de 30% do ovo; o albúmen, que representa cerca de 60% e a casca com aproximadamente 10%. Inúmeros fatores definem a qualidade do ovo, entre eles podemos citar: a espessura e a resistência da casca, peso, altura da câmara de ar, índice de gema, espessura do albúmen e unidade haugh (LAGHI *et al.*, 2005; OLIVEIRA; OLIVEIRA, 2013; QUEIROZ *et al.*, 2016; STADELMAN, 1977). Além da qualidade, outro fator que chama a atenção do consumidor é seu preço, quando este é comparado a outras fontes de proteína (OLIVEIRA; OLIVEIRA, 2013).

Fatores extrínsecos as aves influenciam nas características do ovo, sendo eles: temperatura, umidade relativa do ar, duração e condições de estocagem. Já em relação aos fatores intrínsecos as aves, podemos citar: linhagem, idade da poedeira, condição nutricional e sanitária do animal (LANA *et al*., 2008. OLIVEIRA; OLIVEIRA, 2013). Assim, a qualidade dos ovos é um motivo de preocupação para consumidores e produtores, pois além de perdas econômicas, os defeitos na qualidade podem ocasionar problemas para a saúde pública (KRAEMER *et al.,* 2003).

Um dos fatores que tem extrema influência na qualidade dos ovos é a nutrição das aves. Os nutrientes que mais influenciam são minerais como o cálcio, o fósforo, o zinco e o manganês, além das vitaminas D e C. O desequilíbrio desses nutrientes pode ocasionar problemas na qualidade da casca (GHERARDI; VIEIRA, 2018). A capacidade de transporte e utilização de nutrientes pelas aves, os quais podem ser alterados conforme a dieta, geram alterações na qualidade da gema e do albúmen, assim como particularidades na cor, no tamanho e na forma dos ovos (CARVALHO; FERNANDES, 2012). Assim, inúmeros estudos relacionam o uso de aditivos na ração das aves para tentar alterar a qualidade dos ovos (SANTOS *et al.*, 2020; GONG *et al.*, 2021; MACIT *et al.*, 2021; RAMIREZ *et al.*, 2021).

2.2 Probióticos

A Organização Mundial da Saúde (OMS) lançou no dia 18 de junho de 2019 uma campanha que convoca governos a adotarem medidas para conter a resistência

antimicrobiana. O uso inadequado de antibióticos tanto na medicina humana quanto na produção animal tornou-se um problema de saúde pública, o qual vem se agravando (IAGG, 2019). Na mesma esteira o Ministério da Agricultura, Pecuária e Abastecimento (MAPA), publica a Instrução Normativa Nº 1, de 13 de janeiro de 2020, a qual proíbe a importação, fabricação e comercialização de alguns antibióticos comumente utilizados como promotores de crescimento na alimentação animal (BRASIL, 2020). O uso de antibióticos é menos frequente na avicultura de postura em relação a outras atividades de produção animal, como avicultura de corte ou suinocultura. Essa diferença se deve principalmente a possibilidade de resíduos nos ovos. Em decorrência disto, alternativas têm sido estudadas para substituir o emprego de antibióticos através do uso de probióticos, prebióticos, ácidos orgânicos, entre outros. Estes aditivos podem ser uma ferramenta importante também para os produtores de ovos.

Probióticos foram definidos como um suplemento alimentar constituído de micro-organismos vivos que beneficiam o hospedeiro e melhoram o seu equilíbrio microbiano intestinal, sendo esses utilizados para prevenção e tratamento de desordens gastrointestinais (FULLER, 1989). Para serem considerados probióticos eficientes, eles devem ser capazes de: exercer benefícios ao animal hospedeiro; estar presentes como células viáveis; sobreviver e metabolizar no ambiente intestinal; serem estáveis e capazes de permanecer viáveis por períodos de armazenamento; serem produzidos em larga escala; serem espécie-específica com o hospedeiro; devem ser identificados genotípica e fenotipicamente e não podem ser tóxicos ou patogênicos (FAO/WHO, 2002; FULLER, 1992).

Os mecanismos de ação dos probióticos ocorrem através de diferentes processos (CALLAWAY *et al.,* 2008; DUGGAN *et al.*, 2002; STAHL *et al*., 2004; WU *et al*., 2008), podendo estar associados ou não, sendo eles:

-Efeito físico: ocorre através da exclusão competitiva ou competição por sítio de ligação, ao competir pelo mesmo sítio de ligação da mucosa intestinal, as bactérias benéficas contidas nos probióticos formam uma barreira física aos patógenos oportunistas, colonizam, assim, os segmentos intestinais e providenciam um melhor sistema imunológico ao trato intestinal.

-Efeito biológico: as bactérias anaeróbicas contidas no probiótico promovem um ambiente de baixa tensão de oxigênio e inibem assim o crescimento de patógenos.

-Efeito químico: produção de bacteriocinas.

-Efeito nutricional: as bactérias do probiótico competem com os patógenos por nutrientes e assim diminuem sua colonização no intestino.

Os probióticos mais comumente utilizados na alimentação animal possuem as seguintes bactérias: *Lactobacillus bulgaricus, Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus helveticus, Lactobacillus lactis, Lactobacillus salivarius, Lactobacillus casei, Lactobacillus subtilis, Enterococcus faecalis, Enterococcus faecium, Bifidobacterium spp., Streptococcus, Enterococcus, Lactococcus, Escherichia coli.* Alguns probióticos também constam em sua composição fungos e leveduras, como *Aspergillus oryzae e Saccharomyces cerevisiae* (HUANG *et al*., 2004).

Essas bactérias são utilizadas na alimentação animal na forma de aditivos e administradas de diversas formas, sendo essas adicionadas na ração, na água de beber, através da pulverização nas aves ou na cama das aves, através da inoculação em ovos embrionados ou introduzidos por via intra-esofagiana (PETRI, 2000).

Os benefícios dos probióticos ocorrem através do ganho de peso do animal, melhora na conversão alimentar, aumento da produtividade, eliminação de patógenos, melhores índices zootécnicos e econômicos, prevenção de infecções, e redução de mortalidade (ADHIKARI *et al*., 2017; DUARTE *et al*., 2014; SILVA, 2000).

2.3 β-mananase

É importante conhecer e identificar os fatores responsáveis pelos efeitos adversos que ocorrem na utilização de nutrientes na dieta de aves para que assim se obtenha uma boa produtividade. Os polissacarídeos não amiláceos, mais especificamente hemiceluloses, podem reduzir a digestibilidade dos nutrientes (SAEED, *et al*, 2019). Eles são componentes formadores da parede celular das plantas e estão presentes em muitos ingredientes encontrados na ração animal, como na soja. Dentre as principais hemiceluloses encontradas na parede celular das plantas está a manana. Tal manana pode ser subdividida em diversas formas, entre elas: galactomanana, glucomanana e glucogalactomanana (JACKSON, 2001; JANI *et al*, 2009).

Já os beta-mananos ocorrem nas formas de glucomanana e glucogalactomanana, as quais além de serem encontrados na parede celular das plantas, também são encontrados na superfície de bactérias, fungos e vírus. Deste modo, o sistema imune inato do animal é acionado quando são ingeridos alimentos que possuem beta-mananos, o qual responde com proliferação de monócitos, macrófagos, células dendríticas e

elevação na produção de citocinas. Tais fatores geram um gasto de energia ao animal, além do aumento da resposta inflamatória (HSIAO *et al*., 2006; KORVER, 2006).

Os efeitos negativos dos beta-mananos ocorrem principalmente pelo aumento da viscosidade intestinal, o que gera diminuição na velocidade da passagem dos alimentos pelo trato intestinal, interfere na difusão ou transporte de nutrientes, diminui o aproveitamento de gorduras e piora a conversão alimentar (CHOCT *et al*., 2004; KRABBE e LORANDI, 2014; MOHAYAYEE e KRIMI, 2012). Além disso, os beta-mananos podem alterar o perfil da microbiota intestinal, fornecendo substrato para a fermentação de bactérias potencialmente patogênicas como *Escherichia coli* e *Clostridium* spp. (HOPWOOD, PETHICK, HAMPSON, 2002). Segundo Dhawan e Kaur (2007), beta mananos estão presentes em altas concentrações no farelo de soja e nos grãos secos de destilaria.

O desenvolvimento de aditivos utilizando enzimas exógenas tem crescido devido ao elevado preço dos ingredientes base utilizados na ração de aves, a fim de aproveitar melhor os nutrientes encontrados nos alimentos vegetais e reduzir os efeitos antinutricionais (DELMASCHIO, 2018; OBA *et al.,* 2013). A β-mananase é uma enzima produzida através da fermentação do *Bacillus lentus,* responsável pela hidrólise dos beta-mananos. Sua ação pode favorecer diversos fatores como: a população de bactérias benéficas, a liberação de açucares como fonte de energia, a digestibilidade de mananos, a imunidade, a digestão e absorção de nutrientes e assim diminuir a poluição ambiental resultado dos excrementos das aves, além de limitar a proliferação de bactérias potencialmente patogênicas no intestino (MOHAYAYEE e KRIMI, 2012; O'NEILL; SMITH; BEDFORD, 2014; SAEED *et al.*, 2019).

Pesquisas recentes demonstram os malefícios de uma dieta com altos índices de β-mananos e os efeitos benéficos que a suplementação que a β-mananase pode trazer quando esta é adicionada na dieta de aves poedeiras. Zheng *et al.* (2020), demonstrou aumento na produção de ovos em dietas de baixa energia e aumento da massa de ovos em dietas de baixa e alta energia, além de diminuição na concentração de amônia. Já Calislar (2020) demonstrou que quanto maior a concentração de β-mannanos na dieta de aves, maiores são as perdas em diversos fatores na produção de ovos, como: diminuição do peso corporal final, piora na eficiência alimentar, diminuição na produção de ovos e menor peso dos ovos.

2.4 Bem-estar animal

A partir da escassez de alimentos sofrido pela Europa devido a segunda guerra mundial, sistemas intensivos de produção ganharam força (LUDKE et al., 2010). Entretanto, nestes sistemas, animais são abrigados em espaços menores e os questionamentos acerca do bem-estar animal começaram a ganhar espaço (HÖTZEL; NOGUEIRA; MACHADO FILHO, 2010).

A senciência dos animais ficou comprovada a partir de novas evidências científicas, o que significa que os animais têm a capacidade de sentir, não apenas sensações dolorosas, mas sentimentos também (DAWKINS, 1997, 1978; ABREU; MAZUCO; SILVA, 2017). As cinco liberdades do bem-estar animal passaram a ser amplamente disseminadas, e hoje são utilizadas como referência no meio cientifico pois são compostas de instrumentos de diagnóstico que abrangem os principais aspectos que influenciam na qualidade de vida dos animais. As cinco liberdades podem ser expressas como: liberdade nutricional (disponibilidade de alimento e água de qualidade, além de frequência e quantidade adequada), liberdade sanitária (saúde física como ausência de doenças e ferimentos), liberdade ambiental (instalações adequadas a raça e com abrigo das intempéries, além de conforto e temperatura adequada), liberdade comportamental (ambiente similar ao natural para semelhante comportamento da espécie) e a liberdade psicológica (ausência de medo e estresse; OIE, 2019; MOLENTO, 2006).

Em relação ao bem-estar animal de galinhas poedeiras, suas bases científicas são fundamentadas através do conhecimento sobre a etologia, a saúde, e a fisiologia dos animais. Parâmetros zootécnicos podem ser utilizados para expressar índices de produtividade, que auxiliam na avaliação da influência do bem-estar animal sobre os métodos de manejo. Fatores relacionados diretamente às cinco liberdades dos animais, como alimento e água de qualidade, instalações, equipamentos, ambiência, biosseguridade e programas de luz adequados geram benefícios ao bem-estar das aves visto que respeitam a biologia animal e de tal modo o animal consegue manifestar suas potencialidades. Também, aconselha-se evitar práticas de manejo estressantes como a muda forçada e debicagem em poedeiras. (MENDEZ *et al*., 2008). Portanto, a avaliação do bem-estar animal é um processo multidisciplinar, no qual inúmeros parâmetros podem ser avaliados para se possa obter uma melhor compreensão sobre o bem-estar animal em qualquer sistema de criação.

Inúmeros estudos evidenciam o uso da nutrição na promoção do bem-estar animal. Sabe-se que o estresse possui efeitos negativos no balanço da microbiota

intestinal (SOHAIL *et al*., 2010; GUARDIA *et al.,* 2011). Segundo Ibrahim (2018) uma forma de diminuir o estresse das aves e melhorar seu bem-estar ocorre através da utilização de aditivos na água e na comida das aves. Entre esses aditivos podemos citar os probióticos, os quais melhoram o balanço da microbiota intestinal, melhoram performance e imunidade e consequentemente o bem-estar (CENGIZ et al., 2015; TEO & TAN, 2007; YU et al., 2008; ZHANG & KIM, 2013). Ainda não existem estudos descritos na literatura sobre a influência da β-mannanase no comportamento dos animais, nem sobre o efeito sinérgico dos aditivos propostos neste estudo sobre o bem-estar.

3. OBJETIVOS

Esta pesquisa foi desenvolvida a fim de avaliar os efeitos da suplementação de dietas com probióticos e β-mannanase sobre o desempenho, a qualidade dos ovos e o bem-estar de poedeiras comerciais. A hipótese principal é que os aditivos podem melhorar o desempenho e a qualidade dos ovos, além de alterar com o comportamento das aves de forma positiva.

CAPÍTULO II[1]

[1]Artigo escrito nas normas da revista Poultry Science

Dietary supplementation with *β*-mannanase and probiotics as a strategy to improve laying hen performance and egg quality

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ABSTRACT:

The objective of this study was to evaluate whether β-mannanase and probiotic supplementation can influence the performance, serum biochemistry, gut morphometric traits, and fresh egg quality in laying hens. The light-weight laying hens (36 weeks old) were housed in 120 cages (4 birds each) randomly attributed to one of four different treatments, namely: control group, fed non-supplemented diets; diets supplemented with 300 g/ton of beta-mannanase; diets supplemented with 50 g/ton of probiotic; or diets containing both 300 g/ton of β-mannanase and 50 g/ton of probiotic. The trial lasted for 182 days, comprising three productive phases of 28 days in which the diets were supplemented according to the proposed treatments followed by a period in which all animals received non-supplemented feed (from week 48 to 62). Performance, serum biochemistry, gut morphometric traits, and fresh egg quality were evaluated. Means were compared using variance analysis followed by Tukey test considering differences at 5 and 10%. β-mananase improved laying rate by 11% ($P < 0.05$) compared to control treatment. The use of probiotics also enhanced laying rate by 7% ($P < 0.05$), as well of the supplementation with combined additives (11.5%). Treatments showed an increase in egg masses, additive association improved by 13.9% ($P < 0.001$) compared to control treatment. The weight of fresh eggs was improved by all the additives during the supplementation period, and the benefits remained noticeable even after 14 weeks without supplementation ($P < 0.05$). The serum biochemistry and egg masses of birds

fed diets containing both additives showed significant differences compared to the control group like uric acid, total cholesterol, and triglycerides. The gut morphometry showed differences with combined additives. The quality of fresh eggs shows significant differences. β-mannanase improved specific gravity, yolk height, length and pH, and yolk color traits. The use of probiotic helped to improve yolk height, pH, and color. Besides, both additives improve yolk height, length, weight, pH, and better traits in yolk color. Therefore, the addition of β-mannanase and probiotics to laying hen diets is an effective strategy to improve the bird performance and health status, while increasing some quality traits in fresh eggs.
Keywords: additives; biochemical indicators; feeding; gut health; nutrition; poultry.

INTRODUCTION

In 2019, the World Health Organization (WHO) launched a campaign calling on governments to adopt measures to contain antimicrobial resistance. The inappropriate use of antibiotics both in human medicine and in animal production has become a public health problem, which has been worsening (World health organization, 2019). The use of antibiotics is less frequent in laying poultry due to the possibility of residues in the eggs. Still, the use of feed additives is a possible alternative to improve productivity, health status, and even egg quality.

Probiotics were defined by Fuller (1989) as a supplement consisting of live microorganisms that benefit the host and improve its intestinal microbial balance. The mechanisms of action of probiotics occur through different processes, which may or may not be associated. Physical effects (through competitive exclusion or competition for binding site, when competing for the same binding site of the intestinal mucosa, the beneficial bacteria contained in probiotics form a physical barrier against opportunistic pathogens), biological effects (the anaerobic bacteria contained in the probiotic promote a low oxygen tension environment and inhibit the growth of pathogens), and chemical effects (production of bacteriocins; nutritional effect: probiotic bacteria compete with pathogens for nutrients and decrease their colonization in the intestine) were already related to the probiotics (Callaway et al., 2008; Duggan et al., 2002; Stahl et al., 2004; Wu et al., 2008). The benefits of probiotics occur through, reduction of pathogens, better zootechnical and economic indices, prevention of infections, and reduction of

mortality (Adhikari et al., 2017). However, the impact of probiotics provided in diets on the performance of laying hens and on egg quality is still poorly studied.

Enzyme supplementation is another strategy that can benefit the gut health status by reducing the impacts of anti-nutritional components. The use of β-mannanase, for example, can help the nonruminant animals dealing with the non-starch polysaccharides, which can reduce nutrient digestibility (Saeed et al., 2019). Such components are found in plant cell walls and are present in many ingredients largely used in animal feeding, such as soybeans. Among the main hemicelluloses found in plant cell walls are β-mannans (Jackson et al., 2001), which can also be found on the surface of microorganisms. Thus, the animal's innate immune system is activated when foods that contain β-mannans are ingested, which responds with the proliferation of monocytes, macrophages, dendritic cells, and increased production of cytokines. Such factors generate an unnecessary energy expenditure, in addition to an increase in the inflammatory response (Hsiao et al., 2006). By hydrolyzing the β-mannans, this enzyme can improve the digestibility of mannans, increasing the population of beneficial bacteria, improving immunity, increasing digestion and absorption of nutrients, in addition to limiting the proliferation of potential pathogens in the intestine (Saeed et al., 2019).

Despite the benefits already described in previous studies, most of the available data was obtained in other poultry categories (i.e., broilers). In addition, both additives have complementary action modes, which can indicate the possibility of synergic effects when supplemented together in the feed. However, to our knowledge, the possible combined effects have not yet been described in the literature. Thus, the aim of this study was to evaluate whether β-mannanase and probiotic supplementation alone or combined can improve the performance and health status of commercial laying hens, as well as the quality of their fresh egg.

MATERIAL AND METHODS

Animals, Housing, and Experimental Design

This experimental protocol was approved by the Institutional Ethics Committee on the Use of Animals (CEUA/UFRGS) under protocol number 39783. The experimental units were randomly selected among the hens housed in a commercial farm (Salvador do Sul, Rio Grande do Sul, Brazil) with about 28 thousand light-weight

laying hens (Hyline W 36 lineage, 36 weeks old). From the population, 120 cages (4 birds each) were used in the trial. These replicates were assigned in a completely randomized design to the four treatments, that were: control (CON) treatment, which consisted of a basal diet, without supplementation with any other additive; β-mannanase (BMA), which was the control diet supplemented with 300 g/ton of β-mannanase; probiotic (PRO), that was the control diet supplemented with 50 g/ton of a multi-strain probiotic additive; and β-mannanase + probiotic (BMA + PRO) treatment, which was the control diet supplemented with 300 g/ton of β-mannanase and 50 g/ton of a multi-strain probiotic additive.

The β-mannanase (Hemicell™ HT, Elanco Animal Health, São Paulo, Brazil) used in this trial consists of an exogenous enzyme from the fermentation of the *Paenibacillus lentus* bacteria. The probiotic additive (Protexin™ Concentrate, Elanco Animal Health, São Paulo, Brazil) includes *Lactobacillus acidophilus* ($2.06x10^8$ UFC/g), *Lactobacillus bulgaricus* ($2.06x10^8$ UFC/g), *Lactobacillus plantarum* ($1.26x10^8$ UFC/g), *Lactobacillus rhamnosus* ($2.06x10^8$ UFC/g), *Bifidobacterium bifidum* ($2.0x10^8$ UFC/g), *Enterococcus faecium* ($6.46x10^8$ UFC/g) e *Streptococcus thermophilus* ($4.10x10^8$ UFC/g).

The experiment lasted 182 days. Birds were supplemented during the first 84 days of the project. For evaluating purposes, this period was divided into three different phases (phase 1, 36-40 weeks; phase 2, 41-44 weeks; and phase 3, 45-48 weeks). At the end of the supplementation period, all birds were fed the control diet for 14 weeks and a new evaluation was carried out (week 62).

The basal diet (Table 1) was a corn-soybean meal-based feed formulated according to the nutritional requirements of the genetic (Hyline, 2020). Inert material (kaolin) was included in the basal feed to replace β-mannanase and/or probiotic additives. Feed and water were both provided *ad libitum* throughout the experimental period using nipple drinkers and gutter feeders.

The birds were housed in conventional sheds, arranged in an east-west direction, with concrete floors and masonry walls complemented with wire mesh to the ceiling. The shed was equipped with side curtains, which were managed according to weather conditions to provide thermal comfort. The average minimum and maximum temperature and air relative humidity values recorded were 18 and 36 °C, and 35.8 and 94.7 %, respectively. The lighting regime was composed of 16 hours of light and eight hours of dark per day. The birds remained in galvanized-wire cages (100-cm long × 40-

cm wide × 45-cm high, resulting in a floor area of 500 cm^2/hen) throughout the experimental period.

Performance Analysis

Egg production was evaluated at weeks 4, 8, and 12 in 120 cages with 4 birds each, corresponding to 30 replicates per treatment. All eggs produced were individually weighed. Laying rate and egg mass were calculated considering all eggs (including non-marketable eggs) for each replicate (cage). The coefficient of variability was calculated for each cage considering the individual weight of all the eggs produced in each week. Same procedure was adopted for egg masses.

Due to management limitations related to the commercial system, feed intake measurement was not possible in this study. For that reason, feed conversion was also not evaluated.

Dirtiness Degree of the Eggshells

All eggs produced in each repetition at weeks 4, 8, and 12 were individually inspected for the presence of feces in the shells, which was classified by the same observer through visual analysis as clean eggs (absent; score 0), minor presence (score 1), and major presence (score 3 and 4). During data analysis, scores 3 and 4 were considered together due to the low casuistry of score 3.

Serum Biochemistry

Blood samples were collected from the ulnar vein of 8 birds randomly selected in each treatment at the end of week 12. This material was placed in a tube without anticoagulant to obtain serum. Subsequently, this material was centrifuged at 3500 rpm for 10 minutes and the serum separated, collected, and frozen (-20 °C) for biochemical analysis.

Samples were processed and analyzed (Bio-Plus 2000®, Biochemical Analyzer, Bioplus, São Paulo, Brazil) for total protein, albumin, uric acid, total cholesterol, triglycerides, glucose, alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase using commercial kits (Wiener Lab Group, São Paulo, Brazil).

Parasitology Tests

The excreta of three birds from 10 cages were collected at the end of the experimental period and were processed within two hours after collection using the centrifugal-flotation technique (Monteiro, 2010). A subsample of 1 g of excreta diluted in 15 ml of sucrose solution was centrifuged for 5 minutes and analyzed on a glass slide using an optical microscope (10x, 40x, and 100x) for counting oocysts.

Gut Morphometric Analyses

Six birds per treatment were slaughtered by cervical dislocation, in accordance with the animal welfare and euthanasia standards described in the euthanasia practice guidelines of the National Council for Control of Animal Experimentation (Brasil, 2013). Two-centimeter samples were collected from the duodenum, jejunum, and cecum of the euthanized birds, which were stored in flasks containing a 10% formaldehyde solution. Slides with histological cuts were made and stained with Archived Hematoxylin and Eosin (H&E). The crypt depth and the villus length were determined following the methodology of Caruso and Demonte (2005). Through a microchamber Digital Eyepiece Camera Video coupled to a biological trinocular microscope model TNB-41T-PL (40x), the histological images were captured. The crypt depth was determined by a line from the base of the crypt to the upper portion using ImageJ bundled with 64-bit Java 1.8.0_172. The villus length was obtained through a straight line from the tip of the villi to the upper portion of the crypts.

Quality of Fresh Eggs

On the last day of weeks 4, 8, and 12, fresh eggs (15 from each treatment in each phase) were randomly collected for quality evaluation. Cracked eggs were excluded from this evaluation. First, the value of specific gravity was based on Archimedes' principle, using the equation:

$$Specific\ gravity = \frac{Egg\ weight}{Egg\ weight\ in\ water\ x\ temperature\ corretion}$$

The albumen height was estimated by the average of three measurements taken at different points on the albumen at a distance of 10 mm from the yolk using a digital caliper (TMX PD – 150, China). Thus, the Haugh Unit (HU) was obtained through the equation proposed by Haugh (1937):

$$UH = 100\ log\ [H - \frac{\sqrt{(30W^{0,37} - 100)}}{100} + 1.19$$

Where: *H*= thickness of albumen (mm); *W*= mass of the entire egg (g).

Yolk width and height (mm) were measured with a digital caliper (TMX PD – 150, China). After, the yolk index was calculated as:

$$Yolk\ index = \frac{Yolk\ height}{Yolk\ width}$$

Yolk color was determined using the Roche colorimetric fan (DSM, São Paulo, Brazil), with scores ranging from 1 (light yellow) to 15 (reddish-orange). Complementarily, a spectrophotometer device (Delta Vista model 450G, Delta Color, São Leopoldo, Brazil) was also used for this evaluation, which determined colorimetric coordinates of luminosity (L*), red intensity (a*) and yellow intensity (b*). Chroma, which is the relation between a* and b* and demonstrates the real yolk color to be analyzed, was estimated considering the following equation:

$$C = (a*^2 + b*^2)^{1/2}$$

After yolk and albumen separation, both parts were weighted. Later, the dense and the fluid albumen were homogenized for 20 seconds and then the pH was determined using a digital pH meter (Kasvi model k39-2014B, Paraná, Brazil) previously calibrated with buffer solutions of pH 4, pH 7, and pH 10. The pH of the yolk was determined using the same pH meter.

The total solid content was determined separately in albumen and yolk. Five grams of albumen and yolk were weighed separately in previously dried porcelain crucibles. The albumen and yolk samples were kept in an oven at 60 ºC for 12h and weighed. After weighing, the samples were kept at 105 °C for 12 hours and weighed again. The shell weight was obtained after shell separation, washing, and drying.

Statistical Analyses

Data analyzes were performed using the SAS statistical program (v 9.3, SAS Institute Inc., Cary, NC). Experimental units varied among the responses, but briefly, it was the cage for performance, the bird for biochemical and gut responses, and each egg for quality assessment. Data were tested for normality and then submitted to variance analyses using PROC MIXED, except for the coefficient of variance of egg weight, which was analyzed using PROC GLIMMIX. Performance data were analyzed considering repeated measures over time. Egg quality was analyzed considering the effect of phase in the model, but only pooled means are presented here due to the lack of

interaction between treatment and phase. Eventual mean differences were compared by Tukey test at 5 and 10% probability.

RESULTS AND DISCUSSION

Animals performed according to the expected for the genotype throughout the entire trial. During the experimental period, no severe health problems were observed.

Performance and Dirtiness Degree of the Eggshells

In phase 1, the BMA and BMA + PRO groups presented a 12% higher posture rate compared to the CON (P<0.001, Table 2). In phase 2, all supplemented treatments had higher laying rates (21%) compared to CON (P<0.001). In phase 3, the group fed with BMA + PRO was 5% superior to CON (P<0.001), while the BMA and PRO groups were intermediate in relation to CON and BMA + PRO. In the overall period, all treatments had a higher (P<0.001) laying rate compared to CON, in which BMA + PRO had the highest laying rate followed by BMA and PRO.

The increase in the laying rate in birds fed with probiotics compared to the control was also observed by Zhan et al. (2019) when using 5×10^4 cfu/g of *Clostridium butiricum.* Ribeiro Jr et al. (2014) also observed an increase with the dose 8×10^5 cfu/g of *Bacillus subtilis,* as well as Saleh et al. (2016) when using 0.05% of *Aspergillus awamori*. The positive effect of probiotics on egg production is believed to be due to better nutrient absorption (Ribeiro Jr et al., 2014), promotion of intestinal health, improved immune function, and reduced stress in birds (Zhan et al., 2019).

Regarding β-mannanase, the present findings are in agreement with the results of Zheng et al. (2020), who observed an increase in egg production in laying hens fed β-mannanase on low-energy diets, with values similar to high energy diets without enzyme and higher than medium energy with and without enzyme. Similar data were also found by Wu et al. (2005). Such findings can be explained by the fact that β-mannanase, by avoiding the immune response in response to β-mannanase, directs energy and nutrients for the bird's performance (Klasing, 2007).

Regarding egg weight, the BMA + PRO group differed from the CON, with higher (P<0.001) egg weight in phase 1. In phase 2, all treatments differed (P<0.001) from CON, with BMA and BMA + PRO being similar to each other. In phase 3, the PRO and BMA + PRO treatments differed from CON (P<0.001); however, the PRO

group was similar to the control which also occurs in the overall period. At week 62, all treatments differed (P=0.013) from CON.

Ribeiro Jr et al. (2014), Song et al. (2019), and Xiang et al. (2019) did not observe an increase in egg weight with the use of probiotics. However, Khan et al. (2011), Alaquil et al. (2020), and Mikulski et al. (2020) observed an increase in egg weight in diets with probiotics, such disagreement may be associated with different factors such as probiotic strain type and dosages used, as well as environmental traits (challenge level). The present study also agrees with the findings of Ryu et al. (2017) that, when using 0.8 g β-mannanase/kg, observed an increase in egg weight compared to the CON.

All treatments differed (P<0.001) from CON in terms of the overall coefficient of variability of egg weight, with the lowest values observed in BMA + PRO group. A trend effect (P=0.072) was observed in phase 1, with lowest coefficient of variability attributed to PRO treatment. In phases 2 (P=0.007) and 3 (P=0.004) all treatments differed from CON, with lowest values observed in BMA group. However, at week 62 (after treatment removal), no significant differences were observed among the groups (P=0.564). Based on these results, we observed a stable and predictable production, which facilitates the processes and increases profitability by decreasing the number of declassified eggs.

Regarding egg masses (Table 3), in phases 1 and 3 (P<0.001), treatments BMA and BMA + PRO were different from the CON group, showing higher egg masses. In phase 2 and overall (P<0.001), all treatments increased egg masses compared to the CON. Ryu et al. (2017) observed higher egg masses using β-mannanase. As for probiotics, Saleh et al. (2016), Alaquil et al. (2020) and Ribeiro Jr et al. (2014) also observed higher egg masses when compared to control group.

The occurrence of clean eggs in treated birds differed from CON in all phases of the experiment (P<0.05, Figure 1). The BMA, PRO, and BMA + PRO groups were superior to CON in all phases. In the 62nd week of production, after 14 weeks without supplementation, the occurrence of clean eggs still differed (P<0.001) in the BMA and BMA + PRO groups compared to CON.

The changes in the occurrence of clean eggs with the use of β-mannanase can be explained by the probable decrease in feces viscosity. Soluble non-starch polysaccharides increase digesta viscosity by increasing water retention, impairing nutrient diffusion and transport. Daskiran et al. (2004) demonstrated that diets that used

β-mannanase significantly reduced the water of total fecal production in broilers. Likewise, Mehri et al. (2010) demonstrated that the viscosity of digesta from the jejunum of broiler chickens decreased in diets with the enzyme.

The results obtained with the use of probiotics can be explained by the greater stability of the intestinal microbiota and lower count of opportunistic bacteria. Higgins et al. (2011) and Deng et al. (2021) observed a decrease in *Salmonella* sp. colonies in birds supplemented by probiotics. Aalaei et al. (2018) found that the addition of multi-strain probiotics reduced the presence of *Escherichia coli* in broilers, and with that, it was possible to reduce diarrhea in the birds. Therefore, in the present study, the reduction in diarrhea could be the cause of the decrease in dirty eggs from the PRO treatment. To our knowledge, this is the first study that evaluates the occurrence of dirty eggs in laying hens fed β-mannanase alone or combined with probiotics.

Serum Biochemistry

Uric acid differed from CON ($P<0.001$) in all treatments, with the lowest values observed in BMA + PRO, BMA, and PRO, respectively (Table 4). Uric acid is the main product of nitrogen metabolism in birds, which is synthesized in the liver and kidneys. Disorders in renal function can increase the concentration of uric acid in the serum and plasma of birds (Campbell, 2014), as well as elevated temperatures (Qaid and Algaradi, 2021). Low uric acid levels also indicate lower protein turnover (Ran et al., 2014), that is, lower endogenous losses of nitrogen and ammonia. In the present study, which took place in summer, the birds faced high temperatures and even so the values of uric acid found in the blood were lower in all treatments compared to the control, which may indicate an improvement in the health of the birds and better efficiency in protein utilization due to additives. In addition, as it is related to protein metabolism, uric acid may explain the findings of greater albumen weight and egg mass observed in this study.

Total cholesterol and triglycerides differed from CON ($P<0.001$), with smaller values showed in supplemented treatments. Such results agree with other authors (Saleh et al., 2016; Yalcin et al., 2012; Song et al., 2019) in birds fed with probiotics. Previous results on β-mannanase are still contradictory. Shahbazi et al. (2012) did not observed significant differences in cholesterol, which contrasts with the findings of Karimi and Shokrollari (2013), who observed a decrease in LDL-cholesterol levels. In relation to triglycerides, Tang et al. (2017) did not observe significant differences. Serum

cholesterol and triglyceride levels reflect lipid metabolism. Saleh et al. (2013) reported that one of the possible mechanisms of cholesterol reduction by probiotics occurs through the production of HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA reductase), which reduces the deposition of abdominal fat by influencing the activity of the hormone-sensitive lipase and malate dehydrogenase enzyme in adipose tissues (Mersmann, 1998). Also, one of the supposed mechanisms of probiotics occurs through the reduction of hepatic bile acid synthesis (De Semet, 1998). Lactic acid bacteria such as those found in the tested product have the ability of reducing cholesterol in the bloodstream (Jin et al., 1998). The decrease in cholesterol by β-mannanase can be explained by the hypolipidemic effect of the enzyme, which reduces the absorption of lipids (Karimi, Shokrollari, 2013; Korolenko et al., 2020).

Serum glucose levels were higher in CON ($P<0.001$) birds in relation to the BMA and BMA + PRO treatments. The PRO treatment did not differ from the control, which is in agreement with the findings of Tang et al. (2017). The decrease in glucose by β-mannanase can be explained by the fact that this enzyme stimulates insulin secretion (Jackson, 1999), which may stimulate feed intake behavior and consequently be linked to increasing egg production.

Serum alkaline phosphatase was higher ($P= 0.007$) in PRO and BMA + PRO treatments than CON. This agrees with the findings of Yalcin et al. (2012). BMA was similar to CON and to other treatments in this study. Alanine aminotransferase was higher in CON ($P= 0.005$) than in PRO and BMA + PRO treatments. These findings are in agreement with Saleh et al. (2016) and Tang et al. (2017).

The serum concentration of liver enzymes such as alkaline phosphatase and alanine aminotransferase can provide information about tissue and organ damage (González and Silva, 2017). Alkaline phosphatase is also associated with calcium and phosphorus metabolism and with participation in osteoblastic and chondrogenic activities. Therefore, the increase in this enzyme is associated with bone growth, fracture consolidation and pre-ovulation and medullary calcification phase in chickens (Campbell, 2014). Furthermore, changes in alkaline phosphatase levels may indicate that the medullary bone promotes calcium during the formation of eggshells and stores calcium when there is no egg in the uterus (Etches, 1987). In relation to alanine aminotransferase in birds, it is believed that it may be elevated due to damage to multiple tissues, making its interpretation difficult (Harr, 2002).

In the present study, we can observe that birds fed with probiotics and probiotics with β-mannanase had higher serum alkaline phosphatase values, which indicates better health for these birds. The lower values of alanine aminotransferase observed in this study may indicate a more efficient metabolism of these birds due to less liver damage, which may explain the positive performance results.

No significant differences were observed in aspartate aminotransferase (P= 0.579) and total protein (P= 0.148), which corroborates with the findings of Tang et al. (2017) in relation to probiotics. There were also no significant differences in serum albumin (P= 0.237).

Gut Morphometry and Parasitological Analysis

No difference was observed for villus height, villus area, and crypt depth among the treatments. The villi width tended (P= 0.064) to be smaller in the BMA treatment compared to the control, whereas the BMA + CON treatment tended to be superior to CON and the PRO treatment was similar to CON. The relationship between the height of the villus and the depth of the crypt was significant (P= 0.007), with the highest relationship observed in the BMA + PRO treatment compared to the CON. The PRO treatment was similar to CON and BMA similar to CON and BMA + PRO.

Crypt height and depth measurements are often used to assess intestinal integrity. The height of the villi indicates a greater area for nutrient absorption and a deeper crypt indicates that there is greater tissue renewal. In the present study, the group treated with β-mannanase and probiotics at the same time showed a greater villus height and crypt depth ratio, demonstrating an improvement in intestinal health (Lei et al., 2013; Chen et al., 2020).

Previous studies have shown significant differences in the ratio between villus height and crypt depth (Song et al., 2019; Xiang et al., 2019) in the intestine of laying hens fed with probiotics. Even though there are no studies in relation to β-mannanase in laying hens, based on these results, it is believed that this additive can benefit the intestinal health of birds. The higher villus:crypt ratio in these groups, as it is associated with a greater surface area for nutrient absorption, may explain the better performance of these birds, especially in relation to egg weight and egg mass.

No parasites or oocysts were found in the fresh excrete samples, including the control treatment. This condition did not allow the evaluation of an eventual effect of treatments in parasite challenges.

Quality of Fresh Eggs

The BMA group showed higher ($P<0.001$) specific gravity when compared to the CON (Table 5), with higher values. In addition, higher ($P=0.009$) shell weights were observed in the BMA and BMA + PRO groups compared to CON. Eggshell is mainly composed of calcium carbonate, in addition to magnesium carbonate, calcium phosphate, among others. The balance between calcium and phosphorus ions is essential for the formation of the shell (Oliveira and Oliveira, 2013). Specific gravity indicates the amount of shell in relation to other components of the egg and is highly related to shell thickness and, consequently, to calcium carbonate deposition. Shell weight can also be used to confirm findings on specific gravity and assess calcium metabolism. The higher the specific gravity values, the higher will be the quality of the shell and the lower the probability of break during handling (Butcher and Miles, 2018; Gordon and Roland, 1998).

Yolk height showed significant differences in the BMA, PRO, and BMA + PRO groups compared to CON ($P=0.037$), all with higher values. Yolk width was also higher ($P=0.002$) in the BMA and BMA + PRO groups compared to CON. Yolk weight was higher in the BMA + PRO group ($P=0.004$) compared to the CON group. Yolk pH, on the other hand, differed from CON in all groups ($P=0.002$), with lower values. Egg freshness content can be assessed through parameters such as yolk pH, height, and width (Feddern et al., 2017; Huang et al., 2012).

Yolk and albumen weight have a positive relationship with egg weight. Their masses are higher in eggs with higher weight, when compared to those with lowest weight. Egg weight can be correlated with several factors, such as heritability, age and bird weight (Ledvinka et al., 2012). Egg weight also has a strong influence on dietary protein level (Shim et al., 2013). As already mentioned in this study, lower uric acid levels were observed in birds fed with β-mannanase when compared to control, which may indicate lower protein turnover. Furthermore, β-mannans are known to reduce viscosity and prevent the action of enzymes (Moreira and Filho, 2008). β-mannanase, by breaking down β-mannan, can facilitate the action of enzymes and increase the amount of absorbed protein, which may explain the higher yolk weight observed in this study. Another hypothesis is linked to the decrease in viscosity caused by β-mannanase (Lattimer and Haub, 2010), by the change in the form of micelles, (Anachkov et al.,

2018; Kamranfar and Jamialahmadi, 2014), which are lipid compounds of paramount importance because they are deposited in the yolk.

The BMA group showed a higher color score in the Roche colorimetric assessment compared to the CON (P=0.032). Regarding luminosity values (L color), the BMA + PRO group was superior to the CON group (P=0.002). Such findings indicate lower luminosity, that is, they were opaquer as they transmit less light. Higher red intensity (A color) and chroma values were observed in all supplemented groups than CON (P<0.001; Figure 2).

The more yellowish or reddish color of the yolk is, the more attractive it is to the consumer (Bessei, 2010). Pigmentation occurs through the absorption of carotenoid pigments present in the diet of birds (Garcia et al., 2002). In corn, the main carotenoids found are xanthophylls, luteins, and zeaxanthines (Perry and Rasmussen; Johnson, 2009). Such components are unsaturated and lipophilic (Cardoso, 1997), that is, they accumulate in the yolk that has the highest concentration of fat in the egg. One hypothesis for color change is that β-mannanase may improve nutrient absorption and/or increase the production of micelles, which transport carotenoids, accumulating these in the yolk. However, this hypothesis needs to be validated in future studies.

CONCLUSION

The present study indicates that β-mannanase, probiotics, and their association can increase the performance, which can be connected to some serum biochemical indicators and the highest ratio of villi height:crypt depth observed in this study. Supplementation also increase the occurrence of clean eggs and improve the quality of fresh eggs. Future studies are needed to elucidate the connection of the biochemical indicators with the better performance observed in this study and also to fully elucidate the mechanisms by which the additives improve the egg quality traits.

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DISCLOSURES

All authors approved the manuscript prior to its submission. We have no conflicts of interest to declare.

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Table 1. Composition of control diet.

Food	Control treatment
Corn	61.790
Soybean meal 45%	23.556
Limestone	9.283
Soybean oil	1.645
Dicalcium phosphate	1.549
Corn gluten 60%	1.024
Inert (washed sand)	0.262
Salt	0.497
DL-methionine	0.183
Vitamin premix[1]	0.100
Mineral Premix[2]	0.060
Choline chloride 70%	0.050
Calculated composition	
Metabolizable energy (kcal/kg)	2.800
Crude protein (%)	16.50
Calcium (%)	4.020
Available phosphorus (%)	0.380
Digestible methionine (%)	0.431
Digest. methionine+cystine (%)	0.668
Digestible lysine (%)	0.731
Digestible threonine (%)	0.559
Digestible tryptophan (%)	0.174
Digestible arginine (%)	0.984
Digestible valine (%)	0.690
Sodium (%)	0.220
Chlorine (%)	0.339
Potassium (%)	0.621

[1]Composition per kg of product: A vit. - 10,000,000 IU; D3 vit. - 2,500,000 IU; E vit. - 6,000 IU; K vit. - 1,600 mg; B12 vit. - 11,000 mg; Niacin - 25,000 mg; folic acid - 400 mg; pantothenic acid - 10,000 mg; Se - 300 mg.

[2]Composition per kg of product: MN - 150,000 mg; zinc - 100,000 mg; iron 100,000 mg; copper - 16,000 mg; iodine - 1,500 mg.

Table 2. Performance of laying hens fed diets supplemented with β-mannanase (BMA) and/or probiotics (PRO)

Responses	Treatments				SE[1]	*P*-value[2]
	CON	BMA	PRO	BMA+PRO		
Laying rate (%)						
Phase 1	85.31 ^{C}	93.71 ^{B}	83.25 ^{C}	97.66 ^{A}	0.11	<0.001
Phase 2	78.34 ^{B}	96.69 ^{A}	96.36 ^{A}	90.55 ^{A}	0.11	<0.001
Phase 3	91.05 ^{B}	92.59 ^{AB}	92.60 ^{AB}	95.90 ^{A}	0.09	<0.001
Overall	84.90 ^{C}	94.33 ^{A}	90.74 ^{B}	94.70 ^{A}	0.59	<0.001
Weight of fresh eggs (g)						
Phase 1	61.95 ^{B}	62.36 ^{B}	61.38 ^{B}	63.18 ^{A}	0.14	<0.001
Phase 2	61.28 ^{C}	63.09 ^{A}	61.98 ^{B}	62.79 ^{A}	0.15	<0.001
Phase 3	64.13 ^{B}	65.21 ^{A}	63.76 ^{B}	65.52 ^{A}	0.16	<0.001
Overall	62.47 ^{B}	63.55 ^{A}	62.37 ^{B}	63.83 ^{A}	0.09	<0.001
62 wk[3]	62.53 ^{B}	64.40 ^{A}	64.10 ^{A}	64.28 ^{A}	0.13	0.013
Coefficient of variability in egg weight (%)						
Phase 1	5.944 ^{b}	5.897 ^{b}	5.193 ^{a}	5.292 ^{ab}	0.014	0.072
Phase 2	7.152 ^{B}	5.625 ^{A}	5.750 ^{A}	5.734 ^{A}	0.018	0.007
Phase 3	7.088 ^{B}	5.397 ^{A}	5.608 ^{A}	5.405 ^{A}	0.019	0.004
Overall	6.728 ^{B}	5.640 ^{A}	5.517 ^{A}	5.477 ^{A}	0.01	<0.001
62 wk[3]	7.94	8.285	8.272	7.891	0.124	0.564
Total solids						
Albumen	10.78	10.59	10.66	11.59	0.21	0.383
Yolk	50.28	49.94	48.73	50.51	0.38	0.339

[1]Standart error.
[2] Probability of treatment effect. Means followed by different uppercase letters differ statistically at 5%, while lowercase letters were used to indicate differences at 10%.
[3]Treatments were not provided from week 48 to 62. Thus, the last evaluation was performed after 14 weeks without supplementation.

Table 3. Egg masses of laying hens fed diets supplemented with β-mannanase (BMA) and/or probiotics (PRO)

Responses	Treatments				SE[1]	*P*-value[2]
	CON	BMA	PRO	BMA+PRO		
Egg mass (g/hen/day)						
Phase 1	52.85 ^{B}	58.44 ^{A}	51.10 ^{B}	61.70 ^{A}	0.67	<0.001
Phase 2	48.01 ^{C}	61.00 ^{A}	59.72 ^{AB}	56.86 ^{B}	0.39	<0.001
Phase 3	58.30 ^{B}	60.38 ^{A}	59.04 ^{AB}	62.83 ^{A}	0.63	<0.001
Overall	53.08 ^{C}	59.94 ^{A}	56.62 ^{B}	60.46 ^{A}	0.39	<0.001

[1]Standart error.

[2] Probability of treatment effect. Means followed by different uppercase letters differ statistically at 5%, while lowercase letters were used to indicate differences at 10%.

Table 4. Serum biochemistry and intestinal morphometry of laying hens fed β-mannanase and/or probiotics

Responses	Treatments				SE[1]	*P*-value[2]
	CON	BMA	PRO	BMA+PRO		
Serum biochemistry						
Total protein (g/dL)	5.475	7.113	5.038	6.575	0.361	0.148
Albumin (g/dL)	1.850	2.100	1.875	2.043	0.052	0.237
Uric acid (mg/dL)	5.171 ^{A}	2.400 ^{BC}	3.171 ^{B}	2.062 ^{C}	0.247	<0.001
Total cholesterol (mg/dL)	301.3 ^{A}	149.5 ^{B}	204.1 ^{B}	161.9 ^{B}	14.1	<0.001
Triglycerides (mg/dL)	832.1 ^{A}	929.0 ^{A}	539.0 ^{B}	659.6 ^{B}	45.6	0.013
Glucose (mg/dL)	367.0 ^{A}	293.0 ^{B}	401.5 ^{A}	269.1 ^{B}	11.5	<0.001
FA (U/L)	378.1 ^{B}	624.0 ^{AB}	896.0 ^{A}	831.8 ^{A}	64.0	0.007
ALT (U/L)	8.207 ^{A}	7.020 ^{A}	3.201 ^{B}	1.667 ^{B}	0.811	0.005
AST (U/L)	144.3	149.7	149.0	140.4	2.7	0.579
Gut morphometry						
Villi height (µm)	1459	1294	1375	1561	30.6	0.428
Villi width (µm)	249.0 ^{ab}	226.7 ^{b}	246.4 ^{ab}	283.1 ^{a}	5.69	0.064
Villi area (µm$^{2)}$	367039	288838	343272	450579	1798	0.67
Crypt depth (µm)	224.9	186.4	227.6	202.1	4.96	0.397
Villi height: Crypt depth	6.839 ^{B}	7.245 ^{AB}	6.304 ^{B}	8.396 ^{A}	0.171	0.007

[1]Standart error.
[2] Probability of treatment effect. Means followed by different uppercase letters differ statistically at 5%, while lowercase letters were used to indicate differences at 10%.
[3]Treatments were not provided from week 48 to 62. Thus, the last evaluation was performed after 14 weeks without supplementation.

Table 5. Quality of fresh eggs from laying hens fed diets supplemented with β-mannanase (BMA) and/or probiotics (PRO)

Responses	Treatments[3]				SE[1]	*P*-value[2]
	CON	BMA	PRO	BMA+PRO		
General traits						
Spec. gravity (g/ml)	1.006 ^{B}	1.007 ^{A}	1.006 ^{B}	1.006 ^{B}	0.001	<0.001
Albumen traits						
Height (mm)	8.04	8.06	8.18	8.17	0.104	0.129
Weight (g)	36.82	37.39	36.30	36.59	0.239	0.424
pH	8.41	8.40	8.38	8.44	0.028	0.178
Yolk traits						
Height (mm)	17.98 ^{B}	18.15 ^{A}	18.27 ^{A}	18.18 ^{A}	0.063	0.037
Length (mm)	40.67 ^{B}	41.62 ^{A}	41.25 ^{AB}	41.82 ^{A}	0.118	0.002
Index	0.443	0.435	0.443	0.435	0.017	0.194
Weight (g)	15.33 ^{B}	15.70 ^{AB}	15.45 ^{B}	16.08 ^{A}	0.096	0.004
Haugh unit	89.40	90.10	89.88	89.55	0.558	0.132
pH	6.04 ^{B}	5.96 ^{A}	5.99 ^{A}	6.00 ^{A}	0.013	0.002
Yolk color						
Color score	5.60 ^{B}	5.98 ^{A}	5.77 ^{AB}	5.87 ^{AB}	0.052	0.032
Lightness (L*)	50.85 ^{B}	50.66 ^{B}	51.33 ^{AB}	52.16 ^{A}	0.161	0.002
Redness (a*)	7.12 ^{B}	7.66 ^{A}	7.67 ^{A}	7.66 ^{A}	0.100	<0.001
Yellowness (b*)	57.41	58.88	58.93	58.75	0.354	0.122
Chroma	57.85 ^{B}	59.67 ^{A}	59.67 ^{A}	59.25 ^{A}	0.357	0.003
Shell traits						
Weight (g)	5.81 ^{B}	6.15 ^{A}	5.96 ^{AB}	6.11 ^{A}	0.041	0.009

[1]Standart error.
[2] Probability of treatment effect. Means followed by different uppercase letters differ statistically at 5%, while lowercase letters were used to indicate differences at 10%.
[3]A subsample of 15 eggs from each treatment in each phase.

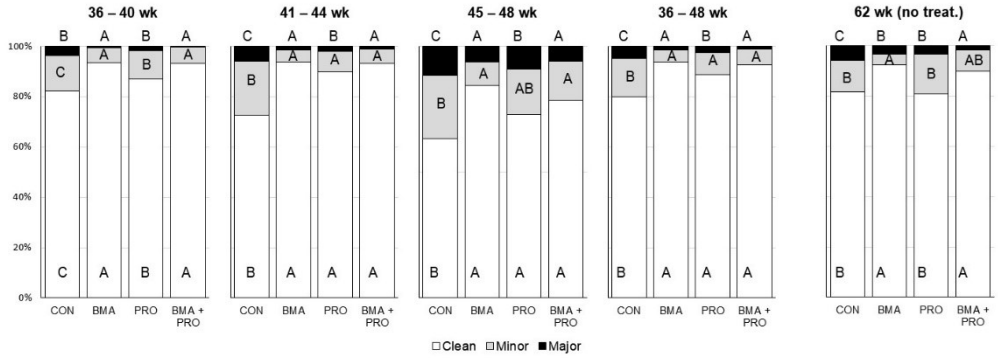

Figure 1. Occurrence of clean eggs or minor/major presence of feces (%) in eggs from laying hens fed β-mannanase and/or probiotics[1,2]

[1] Comparisons were performed among treatments in each period. Probability of treatment effect was P<0.001 for all responses, except for the period from 45 to 48 weeks in which all responses showed P<0.05. Different uppercase letters differ statistically at 5%.

[2] Treatments were not provided from week 48 to 62. Thus, the last evaluation was performed after 14 weeks without supplementation.

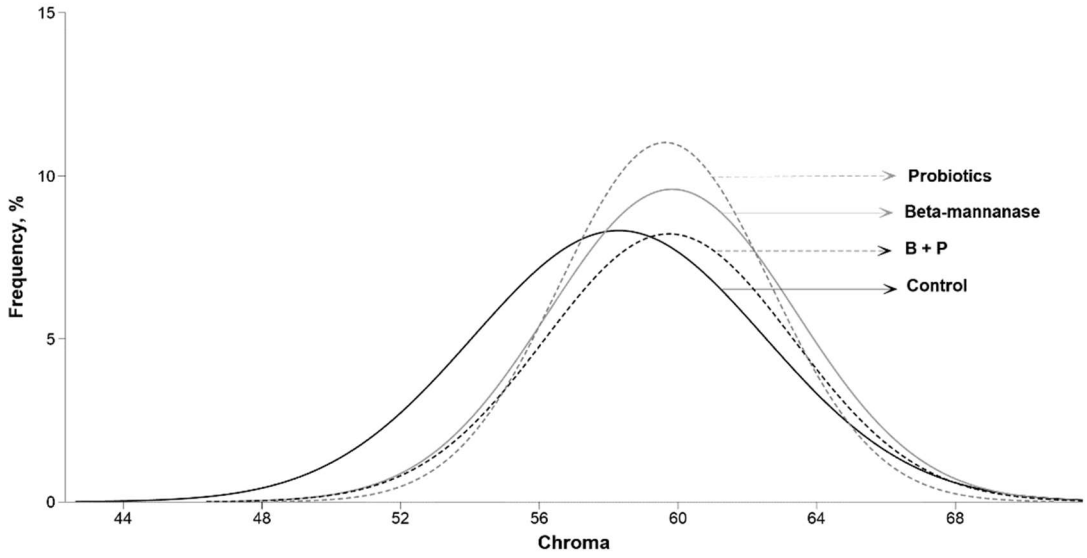

Figure 2. Frequency of different Chroma indexes in egg yolks from laying hens fed β-mannanase and/or probiotics

CAPÍTULO III[1]

[1]Artigo escrito nas normas da revista Livestock Science.

Dietary supplementation with *β*-mannanase and probiotics as a strategy to improve laying hen's welfare

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Abstract

A trend towards animal welfare improvement is observed in animal production, in addition to restrictions imposed on the use of antimicrobials. Thus, new approaches to maintain health, prevent disease, and improve the well-being of birds are gaining ground in research. Thereby, the objective of this study was to evaluate whether β-mannanase and probiotic supplementation can change the behaviour traits and guarantee an improvement of animal welfare. This trial was developed in a commercial farm, in which the light weight laying hens (36 weeks old) were housed in cages randomly attributed to one of four different treatments, namely: control group, fed non-supplemented diets; diets supplemented with 300 g/ton of beta-mannanase; diets supplemented with 50 g/ton of probiotic; or diets containing both 300 g/ton of β-mannanase and 50 g/ton of probiotic. The behaviour of 24 birds (randomly selected from a group of about 28 thousand animals) was recorded for a week using video cameras. The frequency and time of main behaviours (eating, walking, standing, sitting, drinking, and exploring) were analyzed in three periods per day (from 09:00 to 09:15; from 01:00 to 01:15, and from 04:00 to 04:15), as well as the time of other behaviours (stretching legs and wings, scratching, flapping wings and aggressive and non-aggressive pecks). The frequency of birds with lesions and lesion scores were also analyzed using a visual score of three body regions: neck, tail, and cloaca; as well as comb injuries. Means were compared using variance analysis followed by Tukey test considering differences at 5 and 10%. β-mannanase was able to increase the frequency

of feeding behaviour by 49% (P <0,05) and hens also spend 20% (P <0,05) more time in this behaviour compared to control treatment. The use of probiotics also enhanced by 39% (P <0,05) the frequency and 19% the time (P <0,05) and the supplementation with combined additives was able to increase by 29% (P <0,05) the frequency and 25% (P <0,05) the time in feeding behaviour. β-mannanase and probiotics also increased the frequency and time spent exploring behaviour (P <0,05) and promoted a higher frequency in standing behaviour (P <0,05). The additives also decreased the time spend in siting behaviours (P <0,05). The combined additives showed less frequency and time in siting behaviours (P <0,05), while increased flapping wings behaviour (P <0,05). All the treatments were able to reduce pecking (P <0,05). Therefore, the addition of β-mannanase and probiotics to laying hen diets is an effective strategy to improve bird welfare.

Keywords: additives, animal behaviour, image analysis, ethology, poultry.

1. Introduction

The poultry industry was intensified after the 1940s, generating eggs from caged birds (Singh et al. 2009). However, it is known that the rearing of birds in cages, mainly due to their reduced space, is not compatible with all the physiological needs of birds and ends up generating a greater susceptibility to stress (Castilho et al. 2015), which has negative effects in the intestinal microbiota balance (Guardia et al. 2011; Sohail et al. 2010). In broiler chickens, probiotics have already proven to be efficient by reducing heat stress and abnormal behaviour, in addition to improving their health. Such responses occur from the regulatory power of probiotics under the microbiota-gut-brain axis (Jiang et al. 2021). Thus, the modulation of gut microbiota has become a strategy for improving hosts' health and welfare under various conditions (Da Silva et al. 2021; Li et al. 2020). Probiotics also alleviate the stress response along the hypothalamic-pituitary-adrenal axis, reducing plasma or brain levels of corticotropin-releasing hormone, adrenocorticotropic hormone, and corticosterone (Ait-Belgnaouiet al. 2014; Sohailet al. 2010).

Enzyme supplementation is another strategy that can benefit the gut health status by reducing the impacts of anti-nutritional components. The use of β-mannanase can help the nonruminant animals dealing with the non-starch polysaccharides, which can reduce nutrient digestibility (Saeed et al. 2019). Such components are found in plant cell walls and are present in many ingredients largely used in animal feeding, such as

soybeans. Among the main hemicelluloses found in plant cell walls are β-mannans (Jackson et al. 2001), which can also be found on the surface of microorganisms. Thus, the animal's innate immune system is activated when foods that contain β-mannans are ingested, which responds with the proliferation of monocytes, macrophages, dendritic cells, and increased production of cytokines. Such factors generate an unnecessary energy expenditure, in addition to an increase in the inflammatory responses (Hsiao et al. 2006). By hydrolyzing the β-mannans, this enzyme can improve the digestibility of mannans, increasing the population of beneficial bacteria, improving immunity, increasing digestion and absorption of nutrients, in addition to limiting the proliferation of potential pathogens in the intestine (Saeed et al. 2019).

Improving animal health is one of the most important goals to achieve animal welfare and the feed additives can be helpful in this task. Despite the probiotic benefits already described in previous studies, most of the available data was obtained in other poultry categories (i.e., broilers). This is, to our knowledge, the first paper about the influence of β-mannanase in the behaviour and welfare of poultry hens. Besides, both additives have complementary action modes, which can indicate the possibility of synergic effects when supplemented together in the feed, and the possible combined effects have not yet been described in the literature. Thus, the aim of this study was to evaluate whether β-mannanase and probiotic supplementation combined or alone can change the behaviour and the welfare of commercial laying hens.

2. Material and methods

This study was conducted at a commercial farm, in Salvador do Sul, state of Rio Grande do Sul, Southern Brazil. All procedures using animals were approved and followed guidelines recommended by the Institutional Ethics Committee on the Use of Animals (CEUA/UFRGS) under protocol number 39783.

2.1 Animals, Housing, and Experimental Design

The experimental units were randomly selected among the hens housed in a commercial farm with about 28 thousand light weight laying hens (Hyline W 36 lineage, 36 weeks old). The replicates were assigned in a completely randomized design to the four treatments, that were: control treatment, which consisted of a basal diet, without supplementation with any other additive; β-mannanase, which was the control diet supplemented with 300 g/ton of β-mannanase; probiotic, that was the control diet

supplemented with 50 g/ton of a multi-cepa probiotic additive; and β-mannanase + probiotic treatment, which was the control diet supplemented with 300 g/ton of β-mannanase and 50 g/ton of a multi-cepa probiotic additive.

The β-mannanase (Hemicell HT™, Elanco Animal Health, São Paulo, Brazil) consists of an exogenous enzyme from the fermentation of the *Paenibacillus lentus* bacteria. The probiotic additive (Protexin Concentrate™, Elanco Animal Health, São Paulo, Brazil) includes *Lactobacillus acidophilus* (2.06×10^8 UFC/g), *Lactobacillus bulgaricus* (2.06×10^8 UFC/g), *Lactobacillus plantarum* (1.26×10^8 UFC/g), *Lactobacillus rhamnosus* (2.06×10^8 UFC/g), *Bifidobacterium bifidum* (2.0×10^8 UFC/g), *Enterococcus faecium* (6.46×10^8 UFC/g) e *Streptococcus thermophilus* (4.10×10^8 UFC/g).

The basal diet (Table 1) was a corn-soybean meal-based feed formulated according to the nutritional requirements of the genetic (Hyline, 2020). Inert material (kaolin) was included in the basal feed to replace β-mannanase and/or probiotic additives. Feed and water were both provided *ad libitum* throughout the experimental period using nipple drinkers and gutter feeders.

The birds were housed in conventional sheds, arranged in an east-west direction, with concrete floors and masonry walls complemented with wire mesh to the ceiling. The shed was equipped with side curtains, which were managed according to weather conditions to provide thermal comfort. The average minimum and maximum temperature and air relative humidity values recorded were 18 and 36 ºC, and 35.8 and 94.7%, respectively. The lighting regime was composed of 16 hours of light and eight hours of dark per day.

The birds remained in galvanized-wire cages (100-cm long × 40-cm wide × 45-cm high, resulting in a floor area of 500 cm^2/hen) throughout the experimental period. XX birds were allocated in each cage. Birds were supplemented for 84 days and the assessments were performed in the last week of the trial.

2.2 Data Collection

Behavioural assessments were performed through image capture, combined with local feather scoring and comb abnormalities assessment. The evaluations of behavioural traits were carried out through images captured by four cameras, installed in front of the cages that were analyzed. The score of feathers and comb abnormalities was performed using the visual score at the end of the trial.

For the behaviour evaluation, six birds per treatment (one from each cage) were randomly selected for observation. The captures images were carried out for 7 consecutive days, in a period of 15 minutes in the morning (09:00 to 09:15); which is related to the highest peak of laying of the birds, plus 30 minutes in the afternoon, divided into two periods (from 1 pm to 1:15 pm and from 4 pm to 4:15 pm) referring to the hottest and cooler times of the day, respectively. Such methodology was adapted from Barbosa Filho et al. (2007), Garcia et al. (2015), and Pereira et al. (2013). The observation of behaviours for 15 continuous minutes was proposed by Bizeray et al. (2002).

Images were recorded and stored on media (pen drive) for further analysis by visual counting and frequency method. The images were analyzed by the same observer and, with the aid of a stopwatch, which counted the time of each behaviour expressed by the birds. An ethogram adapted from Pereira et al. (2013), Rudkin and Stewart (2003) was used for the behavioural analyses (Figure 1).

The lesion score was performed through a visual score attributed to three body regions: neck, tail, and vent from 25 birds per treatment (randomly selected). Possible injuries and different degrees of severity were analyzed using a scale from 0 to 5, with the best score being 0 (complete plumage, no damage) and the worst score being 5 (completely feathered areas with skin lesions). The methodology was adapted from Dennis et al. (2009) and Larsen et al. (2018). Comb abnormalities were observed in the same birds using the method proposed by Ali and Cheng (1985), Struthers (2019), and Welfare Quality (2009), which was adapted. In this test, the same 25 birds per treatment were analyzed using a scale from 0 to 3, with the best score being 0 (no evidence of comb abnormalities) and the worst score being 3 (3 or more comb areas with evidence of abnormalities).

All behavioural tests were carried out in the last week of the experiment, allowing the birds to remain exposed to the treatments for a longer period. The same animal was used only in one of the tests, thus preventing one test from interfering with the result of the other.

2.3 Statistical Analyses

Data analyzes were performed using the SAS statistical program. Behaviour data were submitted to variance analyses using PROC GLIMMIX considering the effects of treatment, time of day, and their interaction. Behaviour data were collected in the same

animal for seven days, their analysis was performed considering repeated measures over time. The PROC GLIMMIX was also used to evaluate the feather and comb abnormality scores. For these responses, only the treatment effect was considered. All residuals were tested for normality. Eventual differences were compared by Tukey test at 5 and 10% levels.

3. Results

Animals performed according to the expected for the genotype throughout the entire trial. During the experimental period, no severe health problems were observed.

Bird behaviour was evaluated in this trial in three different times of the day. Birds showed higher ($P<0.05$) eating and drinking frequencies and spent more time in these behaviours at 13:00 pm. Lower frequency of standing and exploring behaviours were found at 16:00 pm, while birds showed a higher frequency of siting at the same time of the day. Walking, scratching, flapping wings, stretching legs, stretching wings, as well as aggressive and non-aggressive peck behaviours were similar through the day.

3.1 Effect of Feeding Additives on the Main Behaviours

Birds supplemented with β-mannanase increased ($P<0.05$) the frequency of eating behaviour by 49% compared to control group, while probiotics enhanced this response by 39%, and combined additives by 29% (Table 2). The time spent in eating behaviour was increased ($P<0.05$) by β-mannanase in 20%, by probiotics in 19%, and by combined additives in 25% (Table 3). An interaction 'treatment vs time' ($P<0.05$) was observed for eating frequency and time spent in this behaviour. In this particular, the treatments were not able to modify the eating frequency during the laying pick (09:00 am), while no treatment effect was found the on time expended eating during the hottest time of the day (13:00 pm).

The walking behaviour (frequency and time) of birds supplemented with probiotics was similar to the control treatment. However, birds fed diets containing β-mannanase and combined additives presented lower ($P<0.05$) frequency and spent less time in this behaviour. The 'treatment vs time' interaction was found for the walking frequency ($P<0.05$) and tended to happen also for time spent walking ($P<0.10$), indicating that treatment effects are not constant throughout the day.

All supplemented treatments increased the frequency of the standing behaviour ($P<0.05$). β-mannanase increased it by 49%, probiotics by 72%, and combined additives

by 54%. The time spent standing was not affected by the treatments. Interaction 'treatment vs time' was observed for the time spent standing ($P<0.001$) and a tendency was also found for the behaviour frequency.

Supplemented treatments showed a lower frequency of siting behaviour ($P<0.05$), with β-mannanase decreasing this response by 45% and combined additives by 35%. The time spent in siting behaviour was also decreased ($P<0.05$) by β-mannanase (-62%), probiotics (-80%), and combined additives (-60%). Interactions 'treatment vs time' ($P<0.05$) were found for both responses, with treatment effects occurring at 16:00 pm.

Birds supplemented with β-mannanase increased ($P<0.05$) the frequency of drinking behaviour by 53% compared to control group, while other supplemented treatments showed intermediary results. Treatments were not able to modify the overall time spent drinking. However, interactions 'treatment vs time' were noticed ($P<0.05$) for both responses, with treatments influencing the drinking frequency at 13:00 and 16:00 pm, while time spent drinking was affected by the treatments at 9:00 am and 16:00 pm. All supplemented treatments increased ($P<0.05$) markedly both drinking frequency (around 6 times more) and time spent drinking (around 5 times more) at 16:00 pm compared to control group.

The β-mannanase and probiotic supplementation was able to increase the frequency of exploratory behaviour by 65 and 51%, respectively. The same was noticed for the time spent in this behaviour, which was increased in 61% by β-mannanase and in 63% by probiotics. Interaction 'treatment vs time' was noticed for both responses ($P<0.05$), which were not influenced by treatments at 16:00 pm.

3.2 Effect of Feeding Additives on the Other Behaviours

Birds supplemented with both additives combined increased the time flapping wings ($P<0.05$; Table 4), while the results observed in treatments with only β-mannanase or only probiotics were similar to control. All supplemented treatments were able to decrease ($P<0.05$) time spent pecking in both aggressive and non-aggressive forms. The β-mannanase decreased the time in aggressive and non-aggressive pecking by 73 and 94% compared to control group, respectively. In the same comparison, probiotics reduced the time in aggressive and non-aggressive pecking by 96 and 73%, while combined additives reduced them by 73 and 84%, respectively.

No differences among treatments were observed in the time spent scratching, scratching wings, and scratching legs (P >0.05). In addition, the interaction 'treatment by time' was not significant for any response presented in this section.

3.3 Effect of Feeding Additives on the Frequency and Score Lesions

All supplemented groups showed a tendency to present fewer birds with neck injuries than the control group (P<0.10; Table 5). In addition, the lesion score in the neck was also reduced (P<0.05; Table 6) by all supplemented treatments. Frequency and lesion score of neck injuries were reduced in birds fed diets containing β-mannanase by 39 and 38%, probiotics by 30 and 40%, and combined additives by 39 and 38%, respectively. However, no significant differences were observed among treatments for the frequency of lesions or for the lesion scores on the tail, cloaca, and crest.

4. Discussion

A factor that should be taken into account when analyzing the behaviour of birds is the time when they occur. Birds respond to luminous stimuli, in which the energy contained in the photons present in light is transformed into nerve stimuli that regulate the circadian rhythm, that is, the physiological responses of the animal are controlled by light. Maximum light sensitivity occurs between 10:00 am and 3:00 pm (Araújo et al., 2011).

The birds were fed *ad libitum* in this trial. Even so, the frequency of eating was higher at 13:00 pm, which agrees with the findings of Rodrigues et al. (2008), who observed a higher frequency of this behaviour between 13:00 pm and 14:00 pm in birds raised under thermal comfort. However, Giraldo et al. (2014) observed the higher frequency of eating behaviour between 9:00 am and 10:00 am.

Oviposition is negatively related to feed intake behaviour. Consumption decreases an hour or two before oviposition but increases soon after (Choi et al., 2004). This fact may also explain the exploratory behaviour observed in the birds on this study. Exploring behaviour is explained as dissatisfaction, as it occurs before oviposition, when the bird looks for a nest (Cooper and Albentosa, 2003; Olsson and Keeling, 2000). The birds fed with the additives had a higher egg production than the control group, which may explain the increase in exploratory behaviour, and, as the birds do not have

access to the nest, this behaviour was exacerbated by higher food intake and consequently higher posture.

In fact, it is believed that the positive effect of probiotics on egg production is due to a better absorption of nutrients (Ribeiro Jr et al., 2014), intestinal health promotion, improved immune function, and reduced stress in birds (Zhan et al., 2019). In relation to β-mannanase, by preventing the immune response that would be induced by β-mannans, this additive directs energy and nutrients to the bird performance (Klasing, 2007). In this study, feed consumption could not be quantified because the trial was conducted on a commercial farm. Despite the lack of this response, the most challenging environment (compared to research facilities) brings more reliable results (more applicable to production systems) in the variables of behaviour and animal welfare.

Social position is another factor considered important when analyzing the feeding frequency of birds (Cunningham and Tienhoven, 1983). Dominant birds tend to be more aggressive in feeders. In this study, the treatments were able to decrease the frequency of pecking, which may be another indication that the feed additives were able to reduce stress.

The bird hierarchy is based on the pecking of another individual of the same group, in which the hen's social position is determined by the number of individuals pecked (Izar et al. 2006). Bird pecking can be differentiated into non-aggressive and aggressive. Non-aggressive pecking is gentle and generally does not bother the receiving bird, unlike aggressive pecking which consists of plucking the feather from its receiver. Both patterns are defined as abnormal behaviours (Savory, 1995). Da Silva et al. (2006) found that, even after establishing a relationship of dominance and hierarchy, the birds continued to show pecking behaviour in cage systems. Feather pecking may also be associated with negative affective states such as fear (Rodenburg et al., 2013).

Cheon et al. (2020) observed that pecking peaks occurred close to feeding time. No effect of time was observed in the present study, but the feed additives were able to decrease the pecking behaviour, which indicates that the animals were less stressed.

Feather pecking is a stress-induced neuropsychological disorder in birds. Intestinal dysbiosis and inflammation are common features of these disorders. Thus, this behaviour may be linked to a set of consequence of dysregulated communication between the gut and the brain (Mindus et al., 2021; Van Staaveren et al., 2020). Mindus et al. (2021) demonstrated that the use of probiotics had an immunological effect by

increasing spleen T cells and cecal tonsils, in addition to limiting the dysbiosis of the cecal microbiota. Thus, with the results obtained in this study, it is possible to state that probiotics, by regulating the intestinal microbiota, were able to reduce stress. On the other hand, β-mannanase was able to improve the welfare of the animals by decreasing intestinal and systemic inflammation caused by β-mannans and beneficially modulating the microbiota.

Furthermore, negative emotions such as heat and oxidative stress negatively regulate the expression of "orexins" genes and oxerine gene receptors, which can inhibit feeding motivation (Greene et al., 2016; Nguyen et al., 2017). The treatments, by reducing the birds' stress, were able to increase the birds' feeding frequency and decrease the frequency of pecking, improving their welfare.

It is also worth mentioning the lesions on the crest when discussing the dominance effects. These lesions are also associated with dominance and aggression among birds, mainly due to crest pecking and neck aggression (Tauson et al., 2005; Savory, 1995). In this study, the control group had a greater frequency of neck injuries, in addition to having a greater number of pecks, as already shown, which may be an indication that the treatments reduced stress in the birds, by reducing fights.

It is also important to consider that drinking behaviour is related to eating behaviour. As already stated, birds normally have two peak moments of these behaviours, which are 2-3 hours after the light is turned on and 2-3 hours before the light is turned off (Li et al. 2019). In the present study, the birds showed a higher frequency of this behaviour at 4:00 pm, which agrees with the findings of Rodrigues et al. (2008) and Giraldo et al. (2014).

Another important factor to be taken into consideration is the lack of environmental enrichment in the facilities used for the project. In caged birds, the lack of attractants can be a stimulus for greater drinking behaviour, as it is performed as a distraction rather than thirst (Da Silva et al., 2006). However, in this study, all animals remained under the same conditions and showed a higher frequency of drinking behaviour with the use of treatments, which indicates that there was an influence on the animals, which led them to drink and eat more.

Regarding the sitting behaviour, it is known that this is linked to the nesting behaviour, which is manifested 1-2 hours before laying. When birds are prevented from displaying the behaviour, they become frustrated and sit down (Duncan, 1998). Birds supplemented with additives performed the standing behaviour more frequently, which

indicates greater exercise and is beneficial for leg muscles and bones, especially in caged birds (Li et al., 2015). Mohammed et al. (2021) observed that broilers supplemented by probiotics spent more time standing in the latency-to-lie test. They also observed higher tibial physical parameters (length, weight, and strength), in addition to higher concentrations of calcium and phosphorus in the blood. Thus, in this study, the birds performed the behaviour of standing for a longer time with a higher frequency, while the behaviour of sitting occurred for less time and less frequently, therefore, indicating that the treatments were effective in increasing animal welfare.

Regarding walking behaviour, De Hass et al. (2012) observed that the mean duration of walking in laying hens at six weeks of age was inversely proportional to the plasma corticosterone level. Thus, the higher the average walking distance of the animals, the lower the stress and the greater the welfare. Lei et al., 2013 and Sohail et al., 2012 also showed lower levels of corticosterone in animals supplemented with probiotics. In this study, probiotic treatment was similar to control, but an interaction between treatment and time was showed. In other periods, β-mannanase and combined additives decreased this behaviour. Thus, more studies should be carried out to understand further this relationship.

The behaviour of flapping the wings, on the other hand, is not fully clarified by the literature. Croney et al. 2007 showed that wing-flapping may be more present in dominant birds, because they learn more quickly. However, Zimmerman et al. (2011) conducted a study in which birds experienced three events, being positive, negative, and neutral. In positive events, the birds presented the flapping of wings, which was then recognized as a comfort movement. Thus, we believe that in this study, the birds fed with both additives, by demonstrating this behaviour more, had a beneficial effect on their welfare, due to the probable modulation of the microbiota.

Conclusion

The present study indicates that β-mannanase, probiotics, and their association can increase animal welfare. Probiotics, through the regulation of immunity modulated by the microbiota, alter the behaviour of animals and improve their welfare. The β-mannanase modulate the immune response and improve the animals' welfare. Future studies are needed to further elucidate the connection between those additives and the welfare biomarkers.

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Authors' Contributions

Camila Lopes Carvalho: Conceptualization, Methodology, Data acquisition, Formal analysis, Investigation, Writing – original draft. **Ines Andretta:** Supervision, Data curation. Conceptualization, Methodology, Writing – review & editing. **Gabriela Miotto Galli:** Data acquisition, Methodology, Visualization, Review. **Nathalia de Oliveira Telesca Camargo:** Translation, Data acquisition, Review. **Gabriel Bueno Martins:** Data acquisition, Review. **Raquel Melchior:** Supervision, Review**. Marcos Kipper da Silva:** Conceptualization, Review.

Declaration of conflict of interests

The authors declare no conflict of interest.

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Table 1. Composition of control diet.

Food	Control treatment
Corn	61.790
Soybean meal 45%	23.556
Limestone	9.283
Soybean oil	1.645
Dicalcium phosphate	1.549
Corn gluten 60%	1.024
Inert (washed sand)	0.262
Salt	0.497
DL-methionine	0.183
Vitamin premix[1]	0.100
Mineral Premix[2]	0.060
Choline chloride 70%	0.050
Calculated composition	
Metabolizable energy (kcal/kg)	2.800
Crude protein (%)	16.50
Calcium (%)	4.020
Available phosphorus (%)	0.380
Digestible methionine (%)	0.431
Digest. methionine+cystine (%)	0.668
Digestible lysine (%)	0.731
Digestible threonine (%)	0.559
Digestible tryptophan (%)	0.174
Digestible arginine (%)	0.984
Digestible valine (%)	0.690
Sodium (%)	0.220
Chlorine (%)	0.339
Potassium (%)	0.621

[1]Composition per kg of product: A vit. - 10,000,000 IU; D3 vit. - 2,500,000 IU; E vit. - 6,000 IU; K vit. - 1,600 mg; B12 vit. - 11,000 mg; Niacin - 25,000 mg; folic acid - 400 mg; pantothenic acid - 10,000 mg; Se - 300 mg.
[2]Composition per kg of product: MN - 150,000 mg; zinc - 100,000 mg; iron 100,000 mg; copper - 16,000 mg; iodine - 1,500 mg.

Table 2. Frequency of the main behaviours[1] observed in laying hens fed β-mannanase (βM) and/or probiotics (PB)[2]

Traits	Treatments				*Avg time*	*P*-value[3]		
	Control	βM	PB	βM+PB		Treat	Time	T×T
Eating								
9h	2.07	2.39	2.40	2.50	2.34^{X}	<0.001	0.034	0.001
13h	1.86^{C}	4.26^{A}	2.88^{B}	2.34^{B}	2.83^{Y}		SE[4]=0.16	
16h	1.07^{B}	3.18^{A}	2.98^{A}	2.25^{A}	2.37^{X}			
Avg treat	1.67^{B}	3.28^{A}	2.75^{A}	2.36^{A}				
Walking								
9h	0.90^{B}	0.33^{B}	1.14^{A}	0.62^{B}	0.75	<0.001	0.282	0.043
13h	0.88^{A}	0.37^{B}	0.93^{A}	0.42^{AB}	0.64		SE=0.12	
16h	0.60	0.55	0.81	0.40	0.58			
Avg treat	0.80^{A}	0.42^{B}	0.96^{A}	0.48^{B}				
Standing								
9h	1.71^{B}	2.61^{A}	2.83^{A}	2.19^{AB}	2.33^{X}	<0.001	0.003	0.071
13h	1.64^{B}	1.98^{AB}	2.48^{A}	2.05^{AB}	2.04^{XY}		SE=0.17	
16h	0.93^{B}	1.81^{A}	2.07^{A}	2.40^{A}	1.80^{Y}			
Avg treat	1.43^{B}	2.13^{A}	2.46^{A}	2.21^{A}				
Siting								
9h	0.48	0.48	0.50	0.16	0.40^{Y}	0.012	0.001	0.035
13h	0.59	0.26	0.59	0.55	0.50^{Y}		SE=0.10	
16h	1.19^{A}	0.49^{B}	0.62^{B}	0.75^{AB}	0.76^{X}			
Avg treat	0.75^{A}	0.41^{B}	0.57^{AB}	0.49^{B}				
Drinking								
9h	0.95	0.89	0.81	1.13	0.95^{Y}	<0.001	0.003	0.009
13h	1.05^{B}	2.35^{A}	1.67^{AB}	1.06^{B}	1.53^{X}		SE=0.14	
16h	0.21^{B}	1.54^{A}	1.12^{A}	1.56^{A}	1.01^{Y}			
Avg treat	0.74^{B}	1.59^{A}	1.20^{AB}	1.11^{AB}				
Exploring								
9h	0.57^{BC}	1.25^{A}	0.95^{AB}	0.35^{C}	0.78^{X}	<0.001	0.002	0.040
13h	0.28^{B}	0.99^{A}	0.55^{B}	0.49^{B}	0.58^{XY}		SE=0.09	
16h	0.07	0.44	0.43	0.55	0.37^{Y}			
Avg treat	0.31^{B}	0.89^{A}	0.64^{A}	0.46^{AB}				

[1] Times that each bird performed the behaviour during the observation window (15 minutes).
[2] Means followed by different uppercase letters differ statistically at 5%, while lowercase letters were used to indicate differences at 10%. Comparisons were performed among treatments - lines^{A,B,C,D} within each observation time and also for averages obtained when the three observation times were polled together (indicated as 'Avg treat'). The averages obtained when the four treatments were polled together in each observation time are also presented (indicated as 'Avg time') and compared within the column^{X,Y,Z}.
[3] Probability of treatment effect (treat), time of observation (time), and interaction (T × T).
[4] Standard error.

Table 3. Time expended (minutes/bird)[1] in each of the main behaviours by laying hens fed β-mannanase and/or probiotics[2]

Traits	Treatments				*Avg time*	*P*-value[3]		
	Control	βM	PB	βM+PB		Treat	Time	T×T
Eating								
9h	7.51^{B}	5.91^{B}	6.99^{B}	9.81^{A}	7.56^{XY}	0.004	0.016	<0.001
13h	7.39	8.47	7.94	9.25	8.26^{X}		SE[4]=0.42	
16h	3.85^{B}	8.98^{A}	8.24^{A}	5.87^{AB}	6.73^{Y}			
Avg treat	6.25^{B}	7.79^{A}	7.72^{A}	8.31^{A}				
Walking								
9h	0.84^{a}	0.37^{b}	0.88^{a}	0.43^{ab}	0.63	<0.001	0.330	0.065
13h	1.05^{A}	0.50^{AB}	0.89^{A}	0.25^{B}	0.67		SE=0.12	
16h	0.78^{a}	0.49^{ab}	0.53^{ab}	0.25^{b}	0.51			
Avg treat	0.90^{A}	0.45^{BC}	0.77^{AB}	0.31^{C}				
Standing								
9h	3.26^{AB}	4.78^{A}	4.13^{A}	2.52^{B}	3.67^{X}	0.259	0.028	<0.001
13h	3.03	2.77	2.94	2.38	2.78^{Y}		SE=0.38	
16h	2.25^{B}	2.19^{B}	3.64^{AB}	4.15^{A}	3.06^{XY}			
Avg treat	2.85	3.25	3.57	3.02				
Siting								
9h	1.07	1.99	0.89	0.62	1.14^{Y}	<0.001	<0.001	<0.001
13h	2.02	0.56	0.68	0.86	1.03^{Y}		SE=0.24	
16h	7.82^{A}	1.50^{BC}	0.65^{C}	2.77^{B}	3.19^{X}			
Avg treat	3.64^{A}	1.35^{B}	0.74^{B}	1.42^{B}				
Drinking								
9h	1.49^{A}	0.46^{B}	0.53^{B}	1.34^{A}	0.96^{Y}	0.352	0.002	1.49^{A}
13h	1.24	1.79	1.75	1.48	1.56^{X}		SE=0.14	
16h	0.25^{B}	1.31^{A}	1.24^{A}	1.27^{A}	1.02^{Y}			0.25^{B}
Avg treat	0.99	1.19	1.17	1.36				0.99
Exploring								
9h	0.70^{BC}	1.46^{AB}	1.69^{A}	0.29^{C}	1.04^{X}	0.008	0.005	0.012
13h	0.28	0.91	0.53	0.81	0.64^{XY}		SE=0.14	
16h	0.07	0.36	0.67	0.63	0.43^{Y}			
Avg treat	0.35^{B}	0.91^{A}	0.96^{A}	0.58^{AB}				

[1] Times that each bird performed the behaviour during the observation window (15 minutes).
[2] Means followed by different uppercase letters differ statistically at 5%, while lowercase letters were used to indicate differences at 10%. Comparisons were performed among treatments - lines^{A,B,C,D} within each observation time and also for averages obtained when the three observation times were polled together (indicated as 'Avg treat'). The averages obtained when the four treatments were polled together in each observation time are also presented (indicated as 'Avg time') and compared within the column^{X,Y,Z}.
[3] Probability of treatment effect (treat), time of observation (time), and interaction (T × T).
[4] Standard error.

Table 4. Time expended (minutes/bird)[1] in other behaviours by laying hens fed beta-mannanase and/or probiotics[2]

Traits	Treatments				*Avg time*	*P*-value[3]		
	Control	βM	PB	βM+PB		Treat	Time	T×T
Scratching								
9h	0.12	0.17	0.09	0.07	0.11	0.197	0.298	0.359
13h	0.02	0.17	0.00	0.11	0.07		SE=0.03	
16h	0.00	0.17	0.12	0.00	0.05			
Avg treat	0.05	0.15	0.07	0.06				
Flapping wings								
9h	0.02^{B}	0.03^{AB}	0.00^{B}	0.09^{A}	0.04	0.011	0.463	0.938
13h	0.00^{b}	0.05^{ab}	0.00^{b}	0.07^{a}	0.03		SE=0.02	
16h	0.00	0.002	0.00	0.04	0.01			
Avg treat	0.007^{B}	0.03^{AB}	0.00^{B}	0.07^{A}				
Stretching legs								
9h	0.00	0.02	0.00	0.00	0.005	0.113	0.609	0.808
13h	0.00	0.00	0.00	0.00	0.00		SE=0.005	
16h	0.00	0.02	0.00	0.00	0.005			
Avg treat	0.00	0.001	0.00	0.00				
Stretching wings								
9h	0.00	0.04	0.00	0.00	0.01	0.106	0.137	0.158
13h	0.00	0.00	0.00	0.00	0.00		SE=0.007	
16h	0.00	0.00	0.00	0.00	0.00			
Avg treat	0.00	0.01	0.00	0.00				
Non-aggressive peck								
9h	0.31^{A}	0.00^{B}	0.14^{B}	0.00^{B}	0.11	0.005	0.259	0.331
13h	0.12^{a}	0.05^{b}	0.00^{b}	0.09^{b}	0.06		SE=0.05	
16h	0.14^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.03			
Avg treat	0.19^{A}	0.01^{B}	0.05^{B}	0.03^{B}				
Aggressive peck								
9h	0.57^{A}	0.14^{B}	0.05^{B}	0.15^{B}	0.23	0.002	0.103	0.588
13h	0.24^{A}	0.04^{B}	0.00^{B}	0.13^{B}	0.10		SE=0.06	
16h	0.19^{a}	0.09^{b}	0.00^{b}	0.00^{b}	0.07			
Avg treat	0.33^{A}	0.09^{B}	0.01^{B}	0.09^{B}				

[1] Times that each bird performed the behaviour during the observation window (15 minutes).

[2] Means followed by different uppercase letters differ statistically at 5%, while lowercase letters were used to indicate differences at 10%. Comparisons were performed among treatments - lines^{A,B,C,D} within each observation time and also for averages obtained when the three observation times were polled together (indicated as 'Avg treat'). The averages obtained when the four treatments were polled together in each observation time are also presented (indicated as 'Avg time') and compared within the column^{X,Y,Z}.

[3] Probability of treatment effect (treat), time of observation (time), and interaction (T × T).

[4] Standard error.

Table 5. Frequency (%) of birds with lesions (disregarding the score) observed in groups of laying hens fed β-mannanase and/or probiotics[1]

Traits	Treatments				SE[2]	*P*-value[3]
	Control	βM	PB	βM+PB		
Tail	4	8	8	4	5	0.578
Cloaca	4	12	12	20	4	0.417
Neck	92[a]	64[b]	56[b]	56[b]	3	0.057
Comb	76	88	92	80	6	0.428

[1] Means (LSmeans) followed by different lowercase letters differ statistically at 10%.
[2] Standard error.
[3] Probability of treatment effect.

Table 6. Lesion score observed in laying hens fed β-mannanase and/or probiotics[1]

Traits	Treatments				SE[2]	*P*-value[3]
	Control	βM	PB	βM+PB		
Tail	0.04	0.16	0.08	0.12	0.09	0.952
Cloaca	0.04	0.12	0.36	0.32	0.11	0.282
Neck	1.48^{A}	0.92^{B}	0.92^{B}	0.88^{B}	0.16	0.039
Comb	1.04	0.96	1.24	1.12	0.14	0.556

[1] Means (LSmeans) followed by different lowercase letters differ statistically at 10%.
[2] Standard error.
[3] Probability of treatment effect.

ACTIVITY	BEHAVIOUR	DESCRIPTION	MEASUREMENT
STOPPED	1. Eating	Act of eating continuously	Frequency and time
	2. Walking	Take at least one step in any direction	Frequency and time
	3. Standing	Alert posture or standing in one place	Frequency and time
	4. Siting	Sitting with the head retracted and eyes open or closed	Frequency and time
	5. Drinking water	Continuous water intake	Frequency and time
MOVEMENT	6. Exploring feathers	Exploring feather with the beak, for maintenance or investigation	Frequency and time
	7. Scratching the head	Behavior in which the bird scratches its head with one of its paws	Frequency
	8. Flapping wings	Beating, stretching, shaking and ruffling the feathers	Frequency
	9. Stretching legs	Movement of stretching one leg and one wing, from the same hemisphere of the body	Frequency
	10. Stretching	Act of stretching one of the wings or legs	Frequency
	11. Non-aggressive peck	Light pecks aimed at other birds, usually in the head region or other parts of the body	Frequency
	12. Aggressive peck	Strong pecks from another bird causing tissue damage to the birds and/or damage to their combs	Frequency

Figure 1: Behavioural ethogram for laying hens in a cage system

CAPÍTULO IV[1]

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Effects of dietary β-mannanase supplementation on egg quality during storage

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ABSTRACT

The objective of this study was to evaluate whether dietary supplementation with β-mannanase can improve the quality of storage eggs from laying hens. The trial was developed in a commercial farm (14 thousand animals), in which light weight laying hens (36 weeks old) housed in cages (4 birds each) were randomly attributed to one of two different treatments: a control group fed non-supplemented diets or birds fed diets supplemented with 300 g/ton of β-mannanase. The trial lasted for 84 days, comprising three productive phases of 28 days each. One hundred and five eggs were collected randomly on the last day of each phase. The fresh egg quality was evaluated and then the other eggs were stored and randomly separated for quality assessment at each storage interval (7, 14, 21, 28, 35, and 42 days). Means were compared using variance analysis considering differences at 5 and 10%. β-mannanase was able to improve egg weight and albumen weight ($P<0.05$) during storage. Yolk color (pallete) was also improved by 2.5% ($P<0.001$), while an increase by 1.9% ($P<0.001$) in lightness, 7.7%

(P<0.001) in red intensity, and 4.10% (P<0.001) in yellow intensity was observed in comparison to control group. Besides, β-mannanase treatment was able to reduce by 2.4% (P<0.001) yolk pH, and TBARS levels when compared to control treatment. Therefore, the addition of β-mannanase to laying hen diets is an effective strategy to improve egg quality.

K**ey word:** dietary additives, egg characteristics, enzyme, laying hen, shelf life.

Introduction

Eggs are the result of the efficient biological transformation carried out by the laying hens, which have the ability to use and transform food resources of lower biological value into products with high nutritional quality for human consumption. However, eggs are perishable products and if they are not handled and stored correctly, they lose their quality in a short time.

The shelf life is defined as the storage period in which the eggs remain viable for consumption under certain conditions of temperature, light, relative humidity, and handling. The egg, being a nutrient-rich product, becomes an ideal medium for the growth of microorganisms, including pathogens (Nyholm, 2020). Therefore, establishing the shelf life of eggs is essential to ensure food quality and safety for the consumer. The diets provided for the laying hens are among many factors that can influence egg quality. Feeding can affect the internal and external characteristics of eggs, causing physicochemical changes in the albumen and yolk, which may result in changes in its palatability, freshness, and flavor (Oliveira and Oliveira., 2013). For that reason, some feeding practices can be used as alternatives to improve egg quality and its shelf life. In this context, the effect of many ingredients and dietary nutritional levels

were already studied. However, results on the antinutritive effects of some compounds on egg quality are still limited, as well as little scientific knowledge is available on the tools that can help producers to deal with this problem.

The β-mannans are non-starch polysaccharides that exhibit anti-nutritive activity when present in poultry diets (Saeed et al., 2019). Those components are found in plant cell walls, which are found in many ingredients largely used in animal feeding, such as soybeans and derived products (Jackson et al., 2001). β-mannans can also be found on the surface of microorganisms. For that reason, the animal's innate immune system is activated by feeds with β-mannans and responds with the proliferation of monocytes, macrophages, dendritic cells, and increased production of cytokines. Those factors generate an increase in the inflammatory responses and an unnecessary energy expenditure (Hsiao et al., 2006). By hydrolyzing the β-mannans, the β-mannanase enzyme can avoid the antinutritional effects, improving immunity, allowing a better digestion and nutrient absorption, in addition to limiting the growth of pathogenic bacteria (Saeed et al., 2019).

Previous studies report improvements in egg quality when birds are fed with β-mannanase (Çaliscar, 2020; Ryu et al., 2017). However, to our knowledge, there are no studies on its effects on egg quality during storage periods. In this study, the effects of β-mannanase supplementation in the diets of laying hens were tested to assess egg quality during different storage periods.

Material and Methods

2.1 Animals, Housing, and Experimental Design

This experimental protocol was approved by the Institutional Ethics Committee on the Use of Animals (CEUA/UFRGS) under protocol number 39783. One hundred cages were randomly selected in a commercial farm (Salvador do Sul, Rio Grande do Sul, Brazil) with about 14 thousand light-weight laying hens (Hyline W 36 lineage, 36 weeks old). These replicates were assigned in a completely randomized design to the two treatments, that were: control (CON) treatment, which consist of a basal diet, without supplementation with any other additive; and β-mannanase (BMA), which was the control diet supplemented with 300 g/ton of β-mannanase. The β-mannanase (Hemicell HT™, Elanco Animal Health, São Paulo, Brazil) consists of an exogenous enzyme from the fermentation of the *Paenibacillus lentus* bacteria.

The basal diet (Table 1) was a corn-soybean meal-based feed formulated according to the nutritional requirements of the genetic (Hyline, 2020). Inert material (kaolin) was included in the basal feed to replace β-mannanase. Feed and water were both provided *ad libitum* throughout the experimental period using nipple drinkers and gutter feeders.

The birds were housed in conventional sheds, arranged in an east-west direction, with concrete floors and masonry walls complemented with wire mesh to the ceiling. The shed was equipped with side curtains, which were managed according to weather conditions to provide thermal comfort. The average minimum and maximum temperature and air relative humidity values recorded were 18 and 36 °C, and 35.8 and 94.7 %, respectively. The lighting regime was composed of 16 hours of light and eight hours of dark per day. The birds remained in galvanized-wire cages (100-cm long × 40-cm wide × 45-cm high, 4 birds each, resulting in a floor area of 500 cm^2/hen) throughout the experimental period.

The experiment (supplementation) lasted 84 days. For evaluating purposes, this period was divided into three different phases (phase 1, 36-40 weeks; phase 2, 41-44 weeks; and phase 3, 45-48 weeks). Egg collection was performed on the last day of each phase, when 240 eggs were randomly collected (120 from each treatment).

2.2 Egg Quality Assessment

The fresh egg quality was evaluated in a subsample and then the other eggs were stored at room temperature (25 °C) and randomly separated for quality assessment at each storage interval (7, 14, 21, 28, 35, and 42 days). Fifteen eggs per treatment were evaluated weekly, except for the determination of substances that react to thiobarbituric acid (TBARS), total solids, and shell characteristics whose particularities are described later in this document.

Eggs were identified and weighed individually at weekly intervals during the storage period. The weight loss (%) of eggs during storage was calculated as described by Caner and Caniz (2008), using the following equation:

$$Weight\ loss\ \% = \frac{(Final\ weight)\ -(Initial\ weight)}{Initial\ weight} \times 100$$

The albumen height was estimated by the average of three measurements taken at different points on the albumen at a distance of 10 mm from the yolk using a digital caliper (TMX PD – 150, China). The Haugh Unit (HU) was obtained through the equation proposed by Haugh (1937):

$$UH\ =\ 100\ log\ [H\ -\ \frac{\sqrt{(30W^{0,37}\ -100)}}{100}\ +\ 1.19$$

where: h= thickness of albumen (mm); *W*= mass of the entire egg (g).

Yolk width and height (mm) were measured with a digital caliper (TMX PD – 150, China). The yolk index was calculated through the equation:

$$Yolk\ index = \frac{Yolk\ height}{Yolk\ width}$$

Yolk color was determined using the Roche colorimetric fan (DSM Animal Nutrition & Health, São Paulo, Brazil), with a score ranging from 1 (light yellow) to 15 (reddish-orange). A spectrophotometer (Delta Vista model 450G, Novo Hamburgo, Brazil) equipment was also used for this evaluation, which determined colorimetric coordinates of luminosity (L^*), red intensity (a^*), and yellow intensity (b^*).

After yolk and albumen separation, the dense and the fluid albumen were homogenized for 20 seconds, and then the pH was determined using a digital pHmeter (Kasvi model k39-2014B, Paraná, Brazil) previously calibrated with buffer solutions of pH 4, 7, and 10. The pH of the yolk was also determined using the same device.

Specific gravity was obtained according to Hempe et al. (1988). This method is based on Archimedes' principle, in which the value of specific gravity was obtained using the equation:

$$Specific\ gravity = \frac{Egg\ weight}{Egg\ weigh\ \ in\ water\ x\ temperature\ corretion}$$

The technique of Giampietro et al. (2008) was used for the determination of lipid oxidation. TBARS was assessed in a pool of three yolks per treatment for four storage periods (0, 21, and 42 days). The decomposition of lipid peroxides was measured using a spectrophotometer (532 nm). The 1,1,3,3 tetramethoxypropane (TMP) component was used as a TBARS standard, and the results were expressed in mg TMP/kg yolk.

Total solid content was determined in albumen and yolk. Five grams of albumen and yolk were weighed separately in previously dried porcelain crucibles. The albumen and yolk samples were kept in an oven at 60 °C for 12 hours and weighed. After, the

samples were kept at 105 °C for 12 hours and weighed again. Seven eggs from each treatment were evaluated at fortnightly intervals to determine the total solids.

Shell percentage was obtained after shell separation, washing, drying, and weighing on days 0, 21, and 42.

2.3 Statistical Analyses

A completely randomized design was used in the study. Each egg was considered an experimental unit. Statistical procedures were performed using SAS statistical software (9.4, SAS Inst. Inc., Cary, NC). The normality of the data was verified and, afterward, the data were submitted to analysis of variance using PROC MIXED. Statistical models considered the effects of treatment (control and β-mannanase), experimental phase (36-40, 41-44, and 45-48 weeks), days of storage (7, 14, 21, 28, 35, and 42 days), and interactions. To simplify the result presentation, the probability of treatment effect was obtained for each storage day of each experimental phase. Probabilities were then interpreted at 5 and 10% of significance.

To simplify the result presentation, a table will be presented with the overall means and probabilities for all responses evaluated in the study. Means will be further described (separately by phase and evaluation day) when any effect ($P<0.10$) relevant to the objective of the project (i.e., effect of treatment or its interaction with phase and/or day).

3. Results

3.1 General Traits

Eggs from laying hens fed diets containing β-mannanase were 2% heavier (P<0.05) than the control group (Table 2). No effect of treatment was found for weight loss during storage (Table S1). However, interactions (P<0.05) 'treatment by phase' and 'treatment by storage day' were noticed for this response. When individually assessing phases and days, it was possible to observe that the eggs from group fed with β-mannanase showed lower weight loss when compared to the control group on days 7 (P=0.004) and 21 (P<0.001) of storage in phase 1. The same was observed on day 42 of storage in phase 3 (P=0.020).

The supplementation of diets with β-mannanase did not affect the specific gravity in the overall trial. In addition, no interactions were found for this response.

3.2 Albumen Traits

The supplementation of diets with β-mannanase did not affect the albumen height (Table S2) in the overall database, however, interactions (P=0.050) 'treatment by storage day' were noticed for this response.

The β-mannanase group showed higher albumen weight than the control group in the overall database (P<0.001) and interaction (P=0.045) between 'treatment by phase'. On day 21 (P=0.063/ Table 3) of phase 1. The β-mannanase group also showed higher values on days 7 (P=0.016), 14 (P=0.023), and 35 (P<0.001) of storage in phase 2.

Albumen pH did not show significant differences or interactions.

3.3 Yolk Traits

Yolk height, length, index, weight were not affected by β-mannanase supplementation. Haugh unit was also similar for both treatments. In addition, no interactions treatment by period or by day were found for yolk height, yolk index, and Haught unit.

However, providing β-mannanase supplemented feed to laying hens reduced the yolk pH by 2% ($P<0.001$) when assessing the overall database. β-mannanase supplementation was able to minimize pH relative to the control group up to day 42 ($P=0.002$/ Table 4) in phase 1, up to day 7 ($P=0.002$) in phase 2, and up to day 35 ($P=0.047$) in phase 3, which indicated lower deterioration during storage in eggs from birds fed supplemented diets.

The supplementation of diets with β-mannanase did not affect the yolk length, however, interaction ($P=0.007$) 'treatment by storage day' was noticed for this response (Table S3). In phase 1, the group fed with β-mannanase showed lower yolk length when compared to the control group at day 21 ($P=0.005$) of storage. In phase 2, higher values were noticed in fresh eggs from supplemented birds compared to the control group ($P=0.021$). However, at days 7 ($P=0.008$) and 28 ($P=0.022$) of storage, values from supplemented treatment were lower than the control treatment. In phase 3, higher values were noticed at day 1 ($P=0.024$), while a tendency ($P=0.099$) with lower values than the control group was observed at day 14 of storage.

Interactions 'treatment by phase' ($P=0.014$) and 'treatment by storage day' ($P=0.005$) were observed for yolk weight (Table S4). Eggs from the group fed with β-mannanase showed higher yolk weight on day 42 ($P=0.003$) of phase 1. However, the β-mannanase group showed lower values than the control group on day 21 ($P<0.001$) in

phase 2. In addition, yolk weight was higher at day 1 of phase 3 (P=0.027) in the β-mannanase group when compared to the control.

3.4 Yolk Color

The supplementation of diets with β-mannanase improved all yolk color responses assessed in the database (P<0.01). Interactions treatment by phase and by day were also noticed for these responses.

Higher color scores were observed on days 28 (Table 5/ P<0.001) and 42 (P=0.002) of phase 1 when comparing the supplemented to the control group. In addition, higher values were noticed at days 1 (P=0.001) and 7 (P=0.024) of storage of phase 2.

The β-mannanase group showed higher yolk lightness (L* color) than the control group at days 21 (P=0.003), 28 (P<0.001), 35 (P<0.001), and 42 (P=0.029) of storage in phase 1 (Table 6). Higher values were also noticed when compared supplemented to the control group on days 7 (P<0.001), 14 (P=0.035), 21 (P=0.005), and 35 (P<0.001) of storage in phase 3.

Yolk redness (a* color) was improved by the β-mannanase on days 28 (P=0.007) and 35 (P=0.040) of storage (Table 7). Higher redness values were also observed on days 7 (P=0.025), 28 (P=0.007), and 35 (P=0.013) of storage in phase 2; and on day 42 (P=0.001) in phase 3 of storage.

β-mannanase improved yolk yellowness (b* color) on days 14 (P=0.030), 21 (P=0.005), and 28 (P<0.001) in phase 1 (Table 8). The same occurred in phase 2, on days 28 (P=0.012) and 35 (P=0.013) of storage. Besides, higher values were observed on days 1 (P=0.039), 28 (P=0.006), and 42 (P=0.009) of storage in phase 3.

Eggs from supplemented hens also presented lower heterogeneity (variability among eggs of the same treatment) for both redness and yellowness responses (Figure 1). This trait is not assessed in many studies but is certainly an important response for the poultry industry.

3.5 Shell Traits

Shell weight was improved by β-mannanase supplementation at 3% when considering the overall database. Treatment by phase and treatment by day interactions were found for this response ($P<0.05$; Table 9). When assessing the detailed data, it is possible to observe that most of the effect was observed in phase 3, when β-mannanase treatment showed higher shell weight than control group at days 1 ($P=0.001$), 7 ($P<0.001$), 14 ($P=0.001$), 28 ($P=0.015$), and 35 ($P<0.001$).

3.6 TBARS

Lower TBARS values were observed in β-mannanase group when compared to the control in fresh eggs from phase 2 (-17%; $P<0.05$) and 3 (-3%; $P=0.055$; Table 10). The same happened on day 1 from phase 2 ($P=0.018$). Eggs from supplemented laying hens also showed a 42% lower TBARS content after 42 ($P=0.035$) and 21 ($P=0.003$) days of storage in phase 3.

3.7 Total Solids

No supplementation effect was noticed for total solids of albumen (Table S5) and yolk when compared to the control group.

4. Discussion

The eggshell is mainly composed of calcium carbonate, in addition to magnesium carbonate, calcium phosphate, among others. The balance between calcium and phosphorus ions is essential for the formation of the shell (Oliveira and Oliveira, 2013). Specific gravity indicates the amount of shell in relation to other components of the egg and is highly related to shell thickness and, consequently, to deposition of calcium carbonate. Shell weight can also be used to confirm specific gravity findings and assess calcium metabolism. In this study, no significant differences in specific gravity was noticed, but higher values in terms of shell weight were found in eggs from supplemented birds. This is probably related to the greater preservation of albumen and yolk in eggs from β-mannanase treatment.

Still related to the shell is the albumen, which can suffer changes due to the porosity of the shell. Regarding albumen weight, higher values in birds fed with β-mannanase in relation to the control group were observed. The weight of the yolk and albumen has a positive relationship with the weight of the egg (Orhan et al., 2016), as their masses are greater in eggs of greater weight when compared to those of lower weight. Egg weight can be correlated with several factors, such as heritability, age, and bird weight. Egg weight also has a strong influence on the nutritional level of the diet (Shim et al., 2013). Furthermore, mannans are known to decrease viscosity and hinder the action of enzymes (Moreira and Filho, 2008). β-mannanase, by breaking down β-mannans, may facilitate the action of enzymes and increase the amount of protein

absorbed, which may explain the higher yolk and albumen weight observed in this study.

During storage, changes in albumen and yolk are verified and the speed of these changes is affected by temperature (Oliveira and Oliveira, 2013) and other factors. Egg freshness can be evaluated through parameters such as pH (Huang et al., 2012). The changes that occur in the egg during storage affect the functional properties of the yolk. These changes include albumen thinning, pH increase, weakening and stretching of the yolk membrane (separates the albumen from the yolk), and increasing yolk water content (Jin et al., 2011; Karoui et al., 2005). In the present study, β-mannanase decreased yolk pH at all periods, improving its quality.

Regarding the yolk color, luminosity values (L* color) from β-mannanase group were higher than the control group. Such findings indicate lower luminosity, that is, they were opaquer because they transmit less light. Regarding the intensity of red (a* color) and the intensity of yellow (b* color), higher values were also noticed when compared to control group. Higher yolk color intensity increases egg acceptance by the consumers (Faitarone et al., 2014) and is seen as something positive. Pigmentation occurs through the absorption of carotenoid pigments present in hens diets (Garcia et al., 2002). In corn, the main carotenoids found are xanthophylls, lutein, and zeaxanthin (Perry et al., 2009). Such components are unsaturated and lipophilic (Cardoso, 1997), that is, they accumulate in the yolk that has the highest concentration of fat in the egg. Furthermore, carotenoids have many double bonds in their molecules and can be oxidized depending on storage time, lighting, and ambient temperature, which reduces yolk pigmentation (Oliver and Palou, 2000; Jin et al., 2011). Therefore, β-mannanase can decrease the effects of storage and, consequently, slow down the deleterious effects of yolk pigmentation. Furthermore, by improving the absorption of nutrients and/or

increasing the production of micelles, which transport carotenoids, β-mannanase can provide more carotenes to the yolk and generate a yellowish or reddish color.

In order to clearify the observed TBARS results, is important to understand that lipid oxidation (peroxidation) is one of the most relevant reactions in food chemistry, which consists of a series of chemical and biochemical reactions that cause changes in the type and concentration of molecules present in food, which can alter the taste and nutritional quality and produce toxic compounds. As for other lipid molecules, cholesterol is susceptible to oxidation, thus forming cholesterol oxidation products (COPs) or oxysterols. Oxysterols are present in many foods, especially foods rich in cholesterol, such as eggs. TBARS is the most used method for the quantification of malondialdehyde (MDA) in foods, which is one of the end products formed through the decomposition of certain lipid peroxidation products (Medina-Meza et al., 2014). Giampietro et al. (2014) observed that TBARS values of egg yolks increased over storage periods. In the present study, we observed that β-mannanase was able to decrease TBARS values, which may be related to a greater production of micelles and consequently a greater amount of carotenoids deposited in yolk, which act as antioxidants (Young and Lowe, 2018). Another factor that may be related is the lower viscosity generated by β-mannanase (Moreira and Filho, 2008). The viscosity impairs the absorption of nutrients and can lead to a greater amount of free radicals, the enzyme may reduce this production.

Few studies link intestinal health with egg quality. Regarding shelf life, our group did not find studies that relate the use of β-mannanase to this topic. The results found in this study can help and serve as an alternative in promoting the maintenance of intestinal health in laying hens, in addition to the possible decrease in the deterioration of egg quality by improving the use of nutrients by the bird.

5. Conclusion

The present study indicates that β-mannanase can increase the quality of eggs. Supplementation was able to improve the egg weight, albumen weight, yolk pH, and TBARS. Furthermore, β-mannanase showed to be efficient in improving yolk color, which is required by consumers. Future studies are needed to better elucidate the mechanisms by which this additive was able to improve some egg quality traits.

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CRediT authorship contribution statement

C. L. C did conceptualization, methodology, data acquisition, formal analysis, investigation and writing of the original draft; I. A. supervised the trial, did the data curation, conceptualization, methodology and review and edit original draft; G.M.G did data acquisition, methodology, visualization and review original draft; N. de. O.T.C did data acquisition and review original draft; T.B.S did data acquisition and review original draft; R.M supervised the trial and review original draft; M. K. da. S. did conceptualization, methodology, review and edit original draft

Declaration of Competing Interests

The authors declare no conflict of interest.

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Table 1. Composition of control diet.

Food	Control treatment
Corn	61.790
Soybean meal 45%	23.556
Limestone	9.283
Soybean oil	1.645
Dicalcium phosphate	1.549
Corn gluten 60%	1.024
Inert (washed sand)	0.262
Salt	0.497
DL-methionine	0.183
Vitamin premix[1]	0.100
Mineral Premix[2]	0.060
Choline chloride 70%	0.050
Calculated composition	
Metabolizable energy (kcal/kg)	2.800
Crude protein (%)	16.50
Calcium (%)	4.020
Available phosphorus (%)	0.380
Digestible methionine (%)	0.431
Digest. methionine+cystine (%)	0.668
Digestible lysine (%)	0.731
Digestible threonine (%)	0.559
Digestible tryptophan (%)	0.174
Digestible arginine (%)	0.984
Digestible valine (%)	0.690
Sodium (%)	0.220
Chlorine (%)	0.339
Potassium (%)	0.621

[1]Composition per kg of product: A vit. - 10,000,000 IU; D3 vit. - 2,500,000 IU; E vit. - 6,000 IU; K vit. - 1,600 mg; B12 vit. - 11,000 mg; Niacin - 25,000 mg; folic acid - 400 mg; pantothenic acid - 10,000 mg; Se - 300 mg.
[2]Composition per kg of product: MN - 150,000 mg; zinc - 100,000 mg; iron 100,000 mg; copper - 16,000 mg; iodine - 1,500 mg.

Table 2. Overall assessment of egg quality from laying hens fed diets supplemented with β-mannanase (BMA) during three phases and evaluated during a storage period of 42 days

Responses[2]	Treatments		*P*-values[1]						
	CON	BMA	T	D	P	T×D	T×P	P×D	T×P×D
General traits									
Weight (g)	61.76	62.80	0.002	0.405	<0.001	0.176	0.329	0.011	0.371
Weight loss (g)	1.87	1.82	0.201	<0.001	0.274	0.014	0.013	0.816	0.010
Spec. gravity (g/ml)	1.005	1.005	0.864	0.056	0.298	0.999	0.933	0.970	0.999
Albumen traits									
Height (mm)	4.278	4.089	0.238	<0.001	<0.001	0.050	0.249	<0.001	0.901
Weight (g)	33.91	35.04	<0.001	<0.001	<0.001	0.210	0.045	0.002	0.871
pH	9.16	9.16	0.819	<0.001	<0.001	0.993	0.936	<0.001	0.430
Yolk traits									
Height (mm)	13.12	12.99	0.169	<0.001	<0.001	0.772	0.771	<0.001	0.280
Length (mm)	46.41	46.25	0.360	<0.001	<0.001	0.007	0.611	<0.001	0.001
Index	0.290	0.288	0.290	<0.001	<0.001	0.585	0.207	<0.001	0.175
Weight (g)	16.87	16.91	0.759	<0.001	<0.001	0.005	0.014	<0.001	0.107
Haugh unit	56.97	55.89	0.131	<0.001	<0.001	0.179	0.842	<0.001	0.553
Ph	6.40	6.25	<0.001	<0.001	<0.001	0.173	0.028	<0.001	0.529
Yolk color									
Color score	5.65	5.79	0.004	<0.001	<0.001	0.001	0.071	<0.001	0.036
Lightness (L*)	56.09	57.15	<0.001	<0.001	<0.001	0.021	0.007	<0.001	0.009
Redness (a*)	6.33	6.82	<0.001	<0.001	<0.001	0.089	0.636	<0.001	0.485
Yellowness (b*)	56.54	58.86	<0.001	0.006	<0.001	0.049	0.106	<0.001	0.756
Shell traits									
Weight (g)	5.86	6.05	<0.001	0.019	<0.001	0.051	<0.001	0.042	0.048

[1] Probability of treatment effect.

[2] Means do not correspond only to fresh egg evaluation but represent an overall value comprising fresh and stored eggs. Quality assessment was performed at the last day of each phase (phase 1, 36-40 weeks; phase 2, 41-44 weeks; and phase 3, 45-48 weeks). Eggs were stored and fifteen eggs per treatment were evaluated weekly (7, 14, 21, 28, 35, and 42 days).

Table 3. Albumen weight (g) of eggs from laying hens fed β-mannanase (BMA) depending on the storage time

Treatments	Days of storage						
	1	**7**	**14**	**21**	**28**	**35**	**42**
	Phase 1 – 36 to 40 weeks						
Control	35.75	32.80	34.40	32.57	32.50	31.06	31.30
BMA	36.49	34.42	34.03	35.02	32.51	31.93	30.56
***P-value*[1]**	0.570	0.180	0.770	0.060	0.990	0.530	0.470
SE[2]	0.64	0.60	0.64	0.66	0.64	0.67	0.50
	Phase 2 – 41 to 44 weeks						
Control	36.80	34.10	32.60	31.38	31.94	29.96	30.71
BMA	37.63	37.98	35.06	32.92	33.33	34.03	31.28
P-value	0.430	0.010	0.020	0.170	0.340	<0.001	0.670
SE	0.51	0.82	0.55	0.56	0.72	0.59	0.66
	Phase 3 – 45 to 48 weeks						
Control	37.90	36.52	36.88	38.56	36.07	34.49	33.99
BMA	38.04	38.32	37.27	38.43	37.92	35.36	33.36
P-value	0.880	0.150	0.810	0.920	0.160	0.530	0.580
SE	0.47	0.62	0.78	0.66	0.65	0.68	0.57

[1] Probability of treatment effect.
[2] Standard error.

Table 4 – Yolk pH of eggs from Laying hens fed β-mannanase (BMA) depending on the storage time

Treatments	Days of storage						
	1	**7**	**14**	**21**	**28**	**35**	**42**
				Phase 1 – 36 to 40 weeks			
Control	6.22	6.62	6.47	6.82	6.50	6.65	6.75
BMA	6.18	6.30	6.18	6.60	6.40	6.39	6.42
***P-value*[1]**	0.451	0.039	0.089	0.456	0.449	0.014	0.002
SE[2]	0.028	0.092	0.085	0.144	0.066	0.054	0.059
				Phase 2 – 41 to 44 weeks			
Control	5.89	6.06	6.11	6.31	6.85	6.35	6.49
BMA	5.84	5.97	6.03	6.26	6.71	6.33	6.46
P-value	0.004	0.002	0.286	0.374	0.287	0.808	0.700
SE	0.010	0.015	0.033	0.028	0.062	0.032	0.035
				Phase 3 – 45 to 48 weeks			
Control	6.01	6.19	6.07	6.24	6.37	6.62	6.63
BMA	5.86	6.01	6.04	6.15	6.45	6.35	6.53
P-value	0.001	0.003	0.654	0.118	0.467	0.047	0.246
SE	0.023	0.076	0.026	0.029	0.052	0.067	0.043

[1] Probability of treatment effect.
[2] Standard error.

Table 5 – Yolk color score (palette) of eggs from laying hens fed β-mannanase (BMA) depending on the storage time

Treatments	Days of storage						
	1	**7**	**14**	**21**	**28**	**35**	**42**
	Phase 1 – 36 to 40 weeks						
Control	5.73	4.87	5.42	6.36	5.93	6.38	6.18
BMA	6.20	4.87	5.13	6.07	6.64	6.67	6.91
***P-value*[1]**	0.075	0.999	0.102	0.166	<0.001	0.202	0.002
SE[2]	0.131	0.104	0.0859	0.104	0.110	0.109	0.127
	Phase 2 – 41 to 44 weeks						
Control	5.33	4.57	4.67	6.07	6.80	6.46	6.36
BMA	6.00	5.07	4.73	5.85	6.79	6.69	6.33
P-value	0.001	0.024	0.739	0.452	0.928	0.371	0.886
SE	0.111	0.112	0.0977	0.146	0.0766	0.126	0.102
	Phase 3 – 45 to 48 weeks						
Control	5.73	5.20	5.00	5.15	5.33	5.75	5.50
BMA	5.73	5.07	4.87	5.00	5.64	5.69	5.77
P-value	0.999	0.299	0.168	0.478	0.561	0.820	0.101
SE	0.095	0.063	0.047	0.106	0.107	0.123	0.090

[1] Probability of treatment effect.
[2] Standard error.

Table 6 – Yolk lightness (L* color) of eggs from laying hens fed β-mannanase (BMA) depending on the storage time

Treatments	Days of storage						
	1	**7**	**14**	**21**	**28**	**35**	**42**
				Phase 1 – 36 to 40 weeks			
Control	51.30	58.18	57.60	57.13	55.27	55.38	57.77
BMA	50.33	58.72	58.70	58.45	58.72	58.60	59.14
***P-value*[1]**	0.068	0.336	0.548	0.003	<0.001	<0.001	0.029
SE[2]	0.266	0.274	0.894	0.235	0.505	0.497	0.321
				Phase 2 – 41 to 44 weeks			
Control	50.99	55.77	57.87	56.62	58.25	58.73	59.07
BMA	51.65	56.35	57.87	58.01	58.11	58.82	58.84
P-value	0.311	0.276	0.995	0.099	0.757	0.878	0.762
SE	0.321	0.265	0.292	0.418	0.215	0.287	0.371
				Phase 3 – 45 to 48 weeks			
Control	50.26	53.37	54.61	56.49	57.56	57.09	58.61
BMA	50.01	56.34	56.21	58.36	58.48	59.11	59.38
P-value	0.677	<0.001	0.035	0.005	0.219	<0.001	0.134
SE	0.292	0.455	0.385	0.352	0.369	0.275	0.255

[1] Probability of treatment effect.
[2] Standard error.

Table 7 – Yolk redness (a* color) of eggs from laying hens fed β-mannanase (BMA) depending on the storage time

Treatments	Days of storage						
	1	**7**	**14**	**21**	**28**	**35**	**42**
	Phase 1 – 36 to 40 weeks						
Control	7.58	6.03	7.21	7.02	6.57	5.80	7.01
BMA	8.26	6.72	7.62	7.41	7.46	6.79	6.95
***P-value*[1]**	0.058	0.081	0.265	0.266	0.007	0.040	0.862
SE[2]	0.181	0.199	0.179	0.173	0.171	0.240	0.169
	Phase 2 – 41 to 44 weeks						
Control	6.98	6.17	5.94	6.29	5.74	6.23	5.60
BMA	7.20	6.98	5.77	6.70	6.80	7.20	6.04
P-value	0.460	0.025	0.324	0.306	0.007	0.013	0.137
SE	0.143	0.184	0.0862	0.195	0.205	0.201	0.146
	Phase 3 – 45 to 48 weeks						
Control	6.81	7.03	6.32	6.09	5.49	5.58	5.39
BMA	7.52	6.82	6.28	6.04	6.05	6.33	6.38
P-value	0.169	0.543	0.920	0.937	0.233	0.069	0.001
SE	0.257	0.168	0.206	0.289	0.229	0.208	0.162

[1] Probability of treatment effect.
[2] Standard error.

Table 8 – Yolk yellowness (b* color) of eggs from laying hens fed β-mannanase (BMA) depending on the storage time

Treatments	Days of storage						
	1	**7**	**14**	**21**	**28**	**35**	**42**
	Phase 1 – 36 to 40 weeks						
Control	6.05	5.98	6.02	5.96	5.63	5.53	5.55
BMA	6.16	6.12	6.40	6.23	6.16	5.83	5.67
***P-value*[1]**	0.224	0.118	0.030	0.005	<0.001	0.089	0.507
SE[2]	0.433	0.453	0.894	0.504	0.786	0.902	0.885
	Phase 2 – 41 to 44 weeks						
Control	5.70	5.52	5.62	5.40	5.75	5.86	5.65
BMA	5.68	5.45	5.80	5.71	6.05	6.10	5.65
P-value	0.895	0.752	0.284	0.154	0.012	0.013	0.981
SE	0.865	1.03	0.829	1.06	0.614	0.500	0.667
	Phase 3 – 45 to 48 weeks						
Control	5.47	5.63	5.62	5.62	5.21	5.55	5.41
BMA	5.83	5.84	5.85	5.68	5.82	5.80	5.78
P-value	0.039	0.071	0.126	0.741	0.006	0.091	0.009
SE	0.878	0.561	0.748	0.824	1.160	0.744	0.731

[1] Probability of treatment effect.
[2] Standard error.

Table 9 – Shell weight (mm) of eggs from laying hens fed β-mannanase (BMA) depending on the storage time

Treatments	Days of storage						
	1	**7**	**14**	**21**	**28**	**35**	**42**
	Phase 1 – 36 to 40 weeks						
Control	5.78	6.17	6.01	5.81	5.88	6.01	6.20
BMA	6.07	6.10	6.02	6.08	5.94	6.01	5.93
***P-value*[1]**	0.262	0.635	0.931	0.129	0.660	0.984	0.103
SE[2]	0.129	0.070	0.067	0.087	0.074	0.100	0.081
	Phase 2 – 41 to 44 weeks						
Control	6.25	6.11	6.02	5.76	6.06	5.95	6.22
BMA	6.19	6.06	5.97	5.86	6.05	5.90	6.26
P-value	0.687	0.757	0.704	0.591	0.934	0.737	0.859
SE	0.072	0.075	0.062	0.084	0.058	0.064	0.098
	Phase 3 – 45 to 48 weeks						
Control	5.40	5.32	5.42	5.70	5.76	5.29	5.96
BMA	6.18	6.26	6.17	6.03	6.18	5.97	5.90
P-value	<0.001	<0.001	<0.001	0.051	0.015	<0.001	0.694
SE	0.125	0.119	0.117	0.086	0.089	0.084	0.074

[1] Probability of treatment effect.
[2] Standard error.

Table 10 – Thiobarbituric acid reactive substances in eggs from laying hens fed β-mannanase (BMA) depending on the storage time.

Treatments	Days of storage		
	1	**21**	**42**
	Phase 1 – 36 to 40 weeks		
Control	4.43	2.98	3.55
BMA	4.64	2.54	3.42
***P-value*[1]**	0.353	0.305	0.649
SE[2]	0.10	0.20	0.13
	Phase 2 – 41 to 44 weeks		
Control	4.77	3.91	2.96
BMA	3.97	3.41	3.23
P-value	0.018	0.103	0.480
SE	0.19	0.16	0.17
	Phase 3 – 45 to 48 weeks		
Control	4.14	3.18	3.16
BMA	4.02	2.37	1.87
P-value	0.055	0.003	0.035
SE	0.31	0.17	0.42

[1] Probability of treatment effect.
[2] Standard error.

Table S1 – Weight loss (g) of eggs from laying hens fed β-mannanase (BMA) depending on the storage time

Treatments	Days of storage						
	1	**7**	**14**	**21**	**28**	**35**	**42**
				Phase 1 – 36 to 40 weeks			
Control	1.017	1.673	1.748	2.691	3.251	3.750	1.017
BMA	0.744	1.168	1.221	2.591	3.223	3.653	0.744
***P-value*[1]**	0.004	0.099	<0.001	0.434	0.839	0.526	0.004
SE[2]	0.598	0.661	0.794	0.684	0.842	0.599	0.598
				Phase 2 – 41 to 44 weeks			
Control	0.736	1.242	2.056	2.387	2.923	3.807	0.736
BMA	0.756	1.315	2.024	2.515	3.098	3.717	0.756
P-value	0.537	0.160	0.612	0.130	0.244	0.607	0.537
SE	0.969	0.755	0.609	0.651	0.664	0.697	0.969
				Phase 3 – 45 to 48 weeks			
Control	0.615	1.348	1.523	2.137	2.439	3.675	0.615
BMA	0.541	1.221	1.562	2.300	2.121	3.263	0.541
P-value	0.131	0.152	0.708	0.241	0.291	0.020	0.131
SE	0.645	0.855	0.853	0.741	0.773	0.690	0.645

[1] Probability of treatment effect.
[2] Standard error.

Table S2 – Albumen height of eggs from laying hens fed β-mannanase (BMA) depending on the storage time

Treatments	Days of storage						
	1	**7**	**14**	**21**	**28**	**35**	**42**
				Phase 1 – 36 to 40 weeks			
Control	9.34	6.03	4.74	3.97	3.24	2.98	2.70
BMA	9.69	5.90	4.30	3.67	3.16	2.88	2.76
***P-value*[1]**	0.347	0.461	0.044	0.096	0.538	0.534	0.644
SE[2]	0.186	0.087	0.110	0.089	0.068	0.081	0.059
				Phase 2 – 41 to 44 weeks			
Control	6.83	5.33	3.92	3.23	2.74	2.75	2.62
BMA	6.65	4.91	3.58	2.89	2.58	2.57	2.67
P-value	0.504	0.007	0.058	0.016	0.242	0.173	0.531
SE	0.135	0.080	0.089	0.072	0.066	0.062	0.041
				Phase 3 – 45 to 48 weeks			
Control	7.97	5.49	4.74	3.67	2.96	2.66	2.68
BMA	7.83	5.28	4.30	3.13	2.82	2.48	2.47
P-value	0.576	0.294	0.051	0.016	0.245	0.057	0.106
SE	0.121	0.092	0.086	0.115	0.059	0.040	0.062

[1] Probability of treatment effect.
[2] Standard error.

Table S3 - Yolk lenght (mm) of eggs from laying hens fed β-mannanase (BMA) depending on the storage time

Treatments	Days of storage						
	1	**7**	**14**	**21**	**28**	**35**	**42**
				Phase 1 – 36 to 40 weeks			
Control	40.63	43.49	45.02	49.84	47.91	50.00	49.29
BMA	41.25	44.38	45.58	45.85	48.00	49.84	51.67
***P-value*[1]**	0.319	0.262	0.391	0.005	0.914	0.895	0.012
SE[2]	0.304	0.392	0.318	0.738	0.397	0.588	0.661
				Phase 2 – 41 to 44 weeks			
Control	41.43	44.73	45.89	47.80	50.39	47.90	51.59
BMA	42.52	42.80	45.67	47.42	48.15	48.50	51.91
P-value	0.021	0.008	0.713	0.564	0.022	0.416	0.739
SE	0.242	0.380	0.292	0.312	0.500	0.359	0.563
				Phase 3 – 45 to 48 weeks			
Control	39.94	42.03	46.63	46.08	46.08	48.06	50.00
BMA	41.10	42.63	45.40	46.10	45.47	47.96	49.04
P-value	0.024	0.301	0.099	0.977	0.328	0.928	0.418
SE	0.262	0.285	0.373	0.371	0.309	0.541	0.581

[1] Probability of treatment effect.
[2] Standard error.

Table S4 - Yolk weight (g) of eggs from laying hens fed β-mannanase (BMA) depending on the storage time

Treatments	Days of storage						
	1	**7**	**14**	**21**	**28**	**35**	**42**
				Phase 1 – 36 to 40 weeks			
Control	17.86	16.73	17.38	16.51	16.51	16.21	16.34
BMA	17.98	17.02	16.61	16.62	16.62	16.93	18.29
***P-value*[1]**	0.827	0.650	0.184	0.841	0.705	0.294	0.003
SE[2]	0.185	0.267	0.308	0.284	0.251	0.335	0.352
				Phase 2 – 41 to 44 weeks			
Control	16.75	17.73	17.10	17.47	17.00	16.16	16.18
BMA	16.51	16.87	17.36	16.09	16.29	16.46	16.51
P-value	0.464	0.104	0.066	<0.001	0.086	0.520	0.026
SE	0.159	0.265	0.290	0.205	0.208	0.225	0.271
				Phase 3 – 45 to 48 weeks			
Control	14.75	15.87	17.95	18.67	17.98	17.23	18.04
BMA	15.38	16.49	17.00	18.28	17.82	17.86	17.56
P-value	0.027	0.116	0.104	0.374	0.699	0.245	0.375
SE	0.147	0.221	0.294	0.216	0.204	0.264	0.268

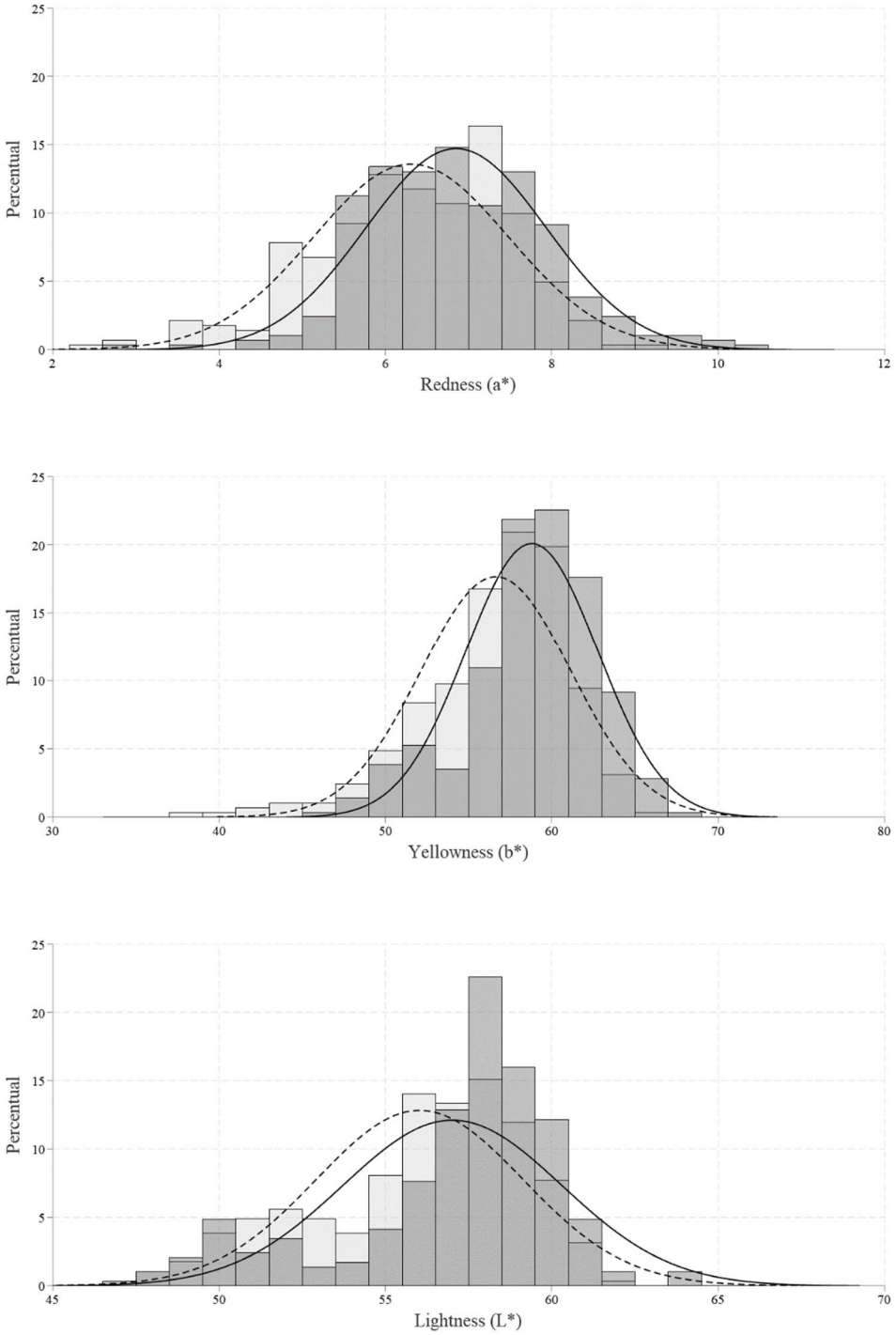
[1] Probability of treatment effect.
[2] Standard error.

Table S5 – Total solids of eggs from laying hens fed β-mannanase (BMA)

Treatments	Albumen	Yolk
Control	11.65	46.88
BMA	12.02	47.04
***P-value*[1]**	0.570	0.851
SE[2]	0.18	0.26

[1] Probability of treatment effect.
[2] Standard error.

Figure 1 - Yolk redness (a*), yellowness (*L), and lightness (L* color) of eggs from laying hens fed β-mannanase (dark gray bars) or control treatment (light gray bars)



CAPÍTULO V[1]

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Effects of dietary probiotic supplementation on egg quality during storage

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Abstract
The objective of this study was to evaluate whether probiotic supplementation to laying hens can improve the quality of eggs during storage. The trial was developed in a commercial farm (14 thousand animals), in which light weight laying hens (36 weeks old) housed in cages (4 birds each) were randomly attributed to one of two different treatments: a control group fed non-supplemented diets or birds fed with diets supplemented with 50 g/ton of probiotic. The trial lasted for 84 days, comprising three productive phases of 28 days each. One hundred and five eggs were collected randomly on the last day of each phase. The fresh egg quality was evaluated and then the eggs were stored and randomly separated for quality assessment at each storage interval (7, 14, 21, 28, 35, and 42 days). Means were compared using variance analysis considering differences at 5 and 10%. Probiotic was able to improve albumen weight, yolk length, yolk height, and yolk index ($P<0.05$) during storage. Yolk color (fan) was also improved by 3.9% ($P<0.001$), while an increase by 1.35% ($P<0.001$) in lightness, 8.05% ($P<0.001$) in red intensity, and 3.4% ($P<0.001$) in yellow intensity was observed in comparison to control group. Besides, probiotic treatment was able to reduce by 2.03% ($P<0.001$) yolk pH, and by 19.65% ($P<0.05$) TBARS levels when compared to control treatment. Therefore, the addition of probiotics to laying hen diets is an effective strategy to improve egg quality during storage.

Keyword: dietary additives, egg characteristics, enzyme, laying hen, shelf life.

1 Introduction

Eggs are an excellent protein source, in addition, have a huge amount of vitamins, minerals (such as iron, phosphorus, selenium, and zinc), carotenoids, and fats. Eggs may also have antibacterial and antivirals properties by immune system modulation (1). However, some problems came with all these nutrients: the egg quality deterioration begins to happen right after oviposition and keeps developing during storage, particularly in non-refrigerated environments. This deterioration is connected to several egg quality traits, such as albumen and yolk weight and pH. Losing eggs is a problem for food security worldwide and also represents an important problem in the poultry industry (2). And for that reason, it is important to establish the shelf life of eggs and ensure food quality and safety for the consumer.
Pathogens that affect the hens can also interfere on egg quality (3). Thus, the feeding practice applied to the birds is an effective way to modulate characteristics of eggs,

since changes in flavor, freshness, and palatability can be made this way (4). The use of food additives is also one of the ways to modulate egg quality, and probiotic is one of these available tools.

Probiotics are live microorganisms with a high potential to replace growth promoters, which have been restricted in several countries (5) due to the inappropriate use of antibiotics both in human medicine and in animal production. The use of antibiotics is less frequent in egg farming when compared to other animal production activities. However, the benefits attributed to probiotics are still very important to laying hens.

These additives can increase the protein digestibility and gross energy of diets, in addition to providing better animal performance, intestinal integrity, microbial profile (6), and immune system (7). Probiotics also have anti-inflammatory activity (8) and can increase short-chain volatile fatty acids, which are energy sources (6).

Different mechanisms of action were already attributed to probiotics, which may or may not be associated. Biological effects (anaerobic bacteria contained in probiotics promote an environment of low oxygen tension and thus inhibit the growth of pathogens), chemical effects (production of bacteriocins), nutritional effects (competition between the beneficial bacteria of the probiotic and the pathogens for nutrients), and physical effects (competitive exclusion or competition for a binding site were already described (9; 10; 11; 12).

Previous studies reported improvements in egg quality when birds are fed with probiotics (13; 14). However, to our knowledge, there are no studies on its effects on egg shelf-life. For that reason, the effects of probiotic supplementation in the diets of laying hens were tested to assess egg quality during different storage periods in this current study.

2 Material and Methods

2.1 Animals, Housing, and Experimental Design

This experimental protocol was approved by the Institutional Ethics Committee on the Use of Animals (CEUA/UFRGS) under protocol number 39783. One hundred cages were randomly selected in a commercial farm (Salvador do Sul, Rio Grande do Sul, Brazil) with about 14 thousand light-weight laying hens (Hyline W 36 lineage, 36 weeks old). These replicates were assigned in a completely randomized design to the two treatments, that were: control (CON) treatment, which consist of a basal diet, without supplementation with any other additive; and probiotic (PRO), that was the control diet supplemented with 50 g/ton of a multi-strain probiotic additive. The probiotic additive (Protexin Concentrate™, Elanco Animal Health, São Paulo, Brazil) includes *Lactobacillus acidophilus* (2.06×10^8 UFC/g), *Lactobacillus bulgaricus* (2.06×10^8 UFC/g), *Lactobacillus plantarum* ($1,26\times10^8$ UFC/g), *Lactobacillus rhamnosus* (2.06×10^8 UFC/g), *Bifidobacterium bifidum* (2.0×10^8 UFC/g), *Enterococcus faecium* (6.46×10^8 UFC/g) e *Streptococcus thermophilus* (4.10×10^8 UFC/g).

The basal diet (Table 1) was a corn-soybean meal-based feed formulated according to the nutritional requirements of the genetic (15). Inert material (kaolin) was included in

the basal feed to replace the probiotic additive. Feed and water were both provided *ad libitum* throughout the experimental period using nipple drinkers and gutter feeders.

The birds were housed in conventional sheds, arranged in an east-west direction, with concrete floors and masonry walls complemented with wire mesh to the ceiling. The shed was equipped with side curtains, which were managed according to weather conditions to provide thermal comfort. The average minimum and maximum temperature and air relative humidity values recorded were 18 and 36 °C, and 35.8 and 94.7 %, respectively. The lighting regime was composed of 16 hours of light and eight hours of dark per day. The birds remained in galvanized-wire cages (100-cm long × 40-cm wide × 45-cm high, 4 birds each, resulting in a floor area of 500 cm^2/hen) throughout the experimental period.

The experiment (supplementation) lasted 84 days. For evaluating purposes, this period was divided into three different phases (phase 1, 36-40 weeks; phase 2, 41-44 weeks; and phase 3, 45-48 weeks). Egg sampling was performed on the last day of each phase, when 240 eggs were randomly collected (120 from each treatment).

2.2 Egg Quality Assessment

The fresh egg quality was evaluated in a subsample and then the other eggs were stored at room temperature (25 °C) and randomly separated for quality assessment at each storage interval (7, 14, 21, 28, 35, and 42 days). Fifteen eggs per treatment were evaluated weekly, except for the determination of substances that react to thiobarbituric acid (TBARS), total solids, and shell characteristics whose particularities are described later in this document.

Eggs were identified and weighed individually at weekly intervals during the storage period. The weight loss (%) of eggs during storage was calculated as described by Caner and Cansiz (16), using the following equation:

$$Weight\ loss\ \% = \frac{(Final\ weight)\ -(Initial\ weight)}{Initial\ weight} \times 100$$

The albumen height was estimated by the average of three measurements taken at different points on the albumen at a distance of 10 mm from the yolk using a digital caliper (TMX PD – 150, China). The Haugh Unit (HU) was obtained through the equation proposed by Haugh (17):

$$UH\ =\ 100\ log\ [H\ -\ \frac{\sqrt{(30W^{0,37}\ -100)}}{100}\ +\ 1.19$$

where: h= thickness of albumen (mm); *W*= mass of the entire egg (g).
Yolk width and height (mm) were measured with a digital caliper (TMX PD – 150, China). The yolk index was calculated through the equation:

$$Yolk\ index = \frac{Yolk\ height}{Yolk\ width}$$

Yolk color was determined using the Roche colorimetric fan (DSM Animal Nutrition & Health, São Paulo, Brazil), with a score ranging from 1 (light yellow) to 15 (reddish-orange). A spectrophotometer (Delta Vista model 450G, Novo Hamburgo, Brazil) equipment was also used for this evaluation, which determined colorimetric coordinates of luminosity (L^*), red intensity (a^*), and yellow intensity (b^*).

After yolk and albumen separation, the dense and the fluid albumen were homogenized for 20 seconds, and then the pH was determined using a digital pHmeter (Kasvi model k39-2014B, Paraná, Brazil) previously calibrated with buffer solutions of pH 4, 7, and 10. The pH of the yolk was also determined using the same device.

Specific gravity was obtained according to Hempe et al. (18). This method is based on Archimedes' principle, in which the value of specific gravity was obtained using the equation:

$$Specific\ gravity = \frac{Egg\ weight}{Egg\ weight\ in\ water\ x\ temperature\ corretion}$$

The technique of Giampietro et al. (19) was used for the determination of lipid oxidation. TBARS was assessed in a pool of three yolks per treatment for four storage periods (0, 21, and 42 days). The decomposition of lipid peroxides was measured using a spectrophotometer (532 nm). The 1,1,3,3 tetramethoxypropane (TMP) component was used as a TBARS standard, and the results were expressed in mg TMP/kg yolk.

Total solid content was determined in albumen and yolk. Five grams of albumen and yolk were weighed separately in previously dried porcelain crucibles. The albumen and yolk samples were kept in an oven at 60 °C for 12 hours and weighed. After, the samples were kept at 105 °C for 12 hours and weighed again. Seven eggs from each treatment were evaluated at fortnightly intervals to determine the total solids.

Shell percentage was obtained after shell separation, washing, drying, and weighing on days 0, 21, and 42.

2.3 Statistical Analyses

A completely randomized design was used in the study. Each egg was considered an experimental unit. Statistical procedures were performed using SAS statistical software (9.4, SAS Inst. Inc., Cary, NC). The normality of the data was verified and, afterward, the data were submitted to analysis of variance using PROC MIXED. Statistical models considered the effects of treatment (control and probiotic), experimental phase (36-40, 41-44, and 45-48 weeks), days of storage (7, 14, 21, 28, 35, and 42 days), and interactions. To simplify the result presentation, a table will be presented with the overall means and probabilities for all responses evaluated in the study. Means will be further described (separately by phase and evaluation day) when any effect ($P<0.10$) relevant to the objective of the project (i.e., the effect of treatment or its interaction with phase and/or day). The probability of treatment effect was obtained for each storage day of each experimental phase. Probabilities were then interpreted at 5 and 10% of significance.

3 Results

3.1 General Traits

No difference was observed for egg weight, weight loss, and specific gravity between treatments (Table 2). However, an interaction ‘treatment by phase’ was observed for weight loss (Table S1, $P<0.001$). Despite no effects being attributed to the treatments in phase 2, birds fed diets with probiotics produced eggs that had lower weight loss in phases 1 and 3 after 42 days of storage. Eggs from supplemented birds showed a

cumulative weight loss 11% lower (P<0.01) than the control group in the phase 1, while a 15% lower egg loss was observed in phase 3 (P<0.001).

3.2 Albumen Traits

There were no differences between treatments for Haugh unit, height, and pH. However, an interaction 'treatment by day' was observed for Haugh unit (P=0.048; Table S2).

Probiotics increased on average 2.6% the albumen weight when compared to control treatment (P=0.002). An interaction 'treatment by phase' (P=0.001) was also observed for this response (Table 3) once a higher albumen weight was observed for the probiotic treatment on days 7 (P=0.036), 14 (P=0.020), 21 (P=0.009), 35 (P=0.003), and 42 (P=0.033) in phase 2 than the control group.

3.3 Yolk Traits

There was an increase of 1% in yolk height in the probiotic treatment when compared to the control (P=0.006), as well as an interaction between treatment by phase (P=0.001) and treatment by phase by day (P=0.042; Table 4). The treatment with probiotics showed higher yolk heights in phase 1, on days 28 (P=0.002) and 35 (P<0.001) when compared to the control group. The same was observed in phase 2, on days 7 (P=0.013), 35 (P=0.040), and 42 (P=0.007) of storage.

There was a decrease of 1.5% in yolk length in the probiotic treatment when compared to control (P<0.001), in addition, there was an interaction between all factors analyzed (P<0.05; Table 5). It is possible to observe lower values in relation to the yolk length of the group fed with probiotics compared to the control on days 21 (P=0.005) and 35 (P=0.017) of storage. The same occurred in phase 2, on days 7 (P=0.005), 14 (P=0.008), 21 (P=0.001), and 28 (P=0.003). However, on day 1 (P=0.032) in phase 3, we observed values higher than the control.

Consequently, there was an increase of 2% in yolk index in the probiotic treatment when compared to the control (P=0.002), as well as an interaction treatment by phase (P<0.001) and treatment by phase by day (P=0.033; Table 6). The probiotic treatment showed a higher yolk index compared to the control in phase 1, on days 21 (P=0.018), 28 (P<0.001), and 35 (P=0.002) of storage, the same occurred in phase 2, on days 7 (P<0.001) and 14 (P=0.020).

There was no difference between treatments for yolk weight. However, there was an interaction treatment by day (P<0.001; Table S3). A reduction of 2% in yolk pH was also observed in probiotic treatment when compared to the control (P<0.001), as well as an interaction between treatment by phase by day (P=0.047; Table 7). Lower pH values were observed in probiotic group compared to control in phase 1, on days 21 (P=0.040) and 42 (P=0.038) of storage; as well as in phase 2, on days 1 (P =0.038), 7 (P=0.012), 14 (P=0.030), and 28 (P=0.004); and in phase 3, on day 7 (P=0.007) of storage.

3.4 Yolk Color

There was a 4% increase in yolk fan color in probiotic treatment when compared to the control (P<0.001), and there was also an interaction treatment by phase by day (P=0.033). Higher values of yolk color in the probiotic treatment when compared to

control were observed in phase 1, on days 14 (P=0.031), 28 (P=0.001), and 42 (P=0.006) of storage (Table 8). The same occurred in phase 2, on days 1 (P=0.003) and 42 (P=0.006), and in phase 3, on days 21 (P=0.005) and 42 (P=0.045) of storage.

An increase of 1% in lightness was observed in yolks from probiotic treatment in relation to the control (P<0.001). There was an interaction treatment by phase by day (P=0.005) for this response, which is further described in Table 9. Higher lightness was observed in treatment with probiotics in relation to the control in phase 1 on days 28 (P=0.006) and 35 (P=0.010) of storage. The same occurred in phase 2 on day 21 (P=0.005) of storage and in phase 3 on days 14 (P=0.047) and 35 (P=0.015) of storage.

An increase in red intensity of 8.05% was observed in yolks from probiotic treatment when compared to control (P<0.001), as well as an interaction treatment by day (P=0.031) and treatment by phase (P= 0.023). A higher red intensity in yolks from probiotic treatment in relation to control was found in phase 1 on the day (P=0.003/ Table 10). The same occurred in phase 2 on day 1 (P=0.002), 7 (P=0.006), 21 (P=0.039), 28 (P=0.001), and 42 (P<0.001) of storage.

There was an increase in the yellow intensity of 3.4% in yolks from the treatment with probiotic compared to control (P<0.001), in the same way, interaction treatment by phase was obtained (P=0.006). Probiotic treatment had greater intensity of yellow when compared to the control group in phase 1 on day 28 (P=0.006) of storage (Table 11). The same occurs in phase 2 on days 1 (P=0.036), 7 (P=0.015), 21 (P=0.014), 28 (P=0.001), and 42 (P=0.016) of storage and in phase 3 on days 28 (P=0.026) and 42 (P=0.036) of storage.

3.5 Shell Traits

Probiotics showed a tendency to increase the eggshell weight by 1%, in contrast, to control (P=0.095). There was also an interaction for treatment by period (P<0.05; Table S4) with improvement on shell weight as a response to probiotic treatment in phase 2 (days 1, 7, 14, and 35 of storage; P<0.05) and tendency in phase 1 (fresh eggs).

3.6 Lipid Peroxidation and Total Solids

Lipid peroxidation data is presented in Table 12. Lower levels of TBARS were observed in fresh eggs compared to control treatment from phase 3 (P<0.001), while tendency of reductions was found for fresh eggs from phases 1 and 2 (P<0.10). In addition, it was observed that probiotic treatment tended to reduce TBARS levels in the first phase on days 21 and 42 (P<0.10) of storage.

There was no significant difference between treatments for total solids for both albumen and yolk (P>0.05; Table S5).

4 Discussion

Albumen is characterized as a clear colloidal solution which contains protein and is produced by epithelial cells in the magnum (20). Thereby, albumen quality is a parameter that reflects egg freshness (21) and protein quality. Thus, the increase in albumen weight observed in the probiotic treatment is probably due to higher protein

deposition in these eggs. This may have occurred due to a beneficial modulation of the intestinal microbiota, which provided better health and, consequently, better digestion and absorption of nutrients, regards amino acids.

The yolk index reflects the information of a fresh egg (22). This takes into account the height and width of the yolk, so the greater height and smaller width found in the probiotic treatment is an indication of a egg in which the effects of storage were minimized. Therefore, the increase in yolk index may be related to the ability and functionality of hepatocytes to synthesize vitellogenin (23). Vitellogenin is a protein that transports lipids from the liver to the growing oocytes that will give rise to the yolk. However, the exact mechanism used by the probiotic is not known, it may be linked to the synthesis of estradiol and, as a result, to an increase in hepatic estrogen receptors, which are responsible for the synthesis of this protein. Furthermore, the lower pH value in the yolk of probiotic-supplemented treatment is a beneficial effect and may be related to the higher deposition of antioxidants in the yolk that delayed lipid peroxidation (24). This hypothesis is supported by the increase in yolk color due to carotenoids and xanthophylls that have antioxidant properties and by the lower levels of TBARS.

The increase in yolk color is a desirable factor for consumers. Thus, the increase in the intensity of yellow and red is beneficial and depends on the carotenoid content present in the diet (25). Gul et al. (26) reported that yolk color is related to the amount of xanthophylls and the antioxidant activity of these pigments, such as carotene. Therefore, the greater amount of these pigments may explain the increase in yolk color and the decrease in lipid peroxidation observed in the probiotic treatment. In this context, it is known that lipid peroxidation is an undesirable factor, as it can cause rancid taste and reduce the nutritional and sensory quality of eggs. Our data are in agreement with Tang et al. (25) who also observed an increase in yolk color when layers were supplemented with *Bacillus subtilis.* The increase in luminosity (L* color) of yolk in the treatment supplemented with probiotic may have occurred due to the lower amount of solute present inside the yolk, which causes water to leave the intracellular medium to the extracellular medium, during this process an increase in humidity occurs on the gem surface due to greater reflection of incident light.

Few studies link the intestinal health of birds with egg quality. To our knowledge, this is the first study that evaluated the interaction of gut microbiota using probiotics with egg shelf life. Therefore, from this study, it is possible to observe that probiotics can delay and mitigate the negative effects of storage, such as the loss of pigmentation and yolk and albumen quality. Improvements were probably related to the effects of probiotics improving the intestinal health of laying hens.

5 Conclusion

The present study indicates that probiotics can increase egg quality. Supplementation was able to improve albumen weight, yolk pH, yolk length, yolk height, yolk index, and TBARS. Furthermore, probiotics showed to be efficient to improve yolk color, which is required by consumers. Future studies are needed to elucidate the better connection between this additive, microbiota, and egg quality.

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7 Authors' Contributions

Camila Lopes Carvalho: Conceptualization, Methodology, Data acquisition, Formal analysis, Investigation, Writing – original draft; Ines Andretta: Supervision, Data curation. Conceptualization, Methodology, Writing – review & editing; Gabriela Miotto Galli: Data acquisition, Methodology, Visualization, Review, Writing – original draft; Nathalia de Oliveira Telesca Camargo: Data acquisition, Review; Thais Bastos Stefanello: Data acquisition; Review; Raquel Melchior: Supervision, Review; Marcos Kipper da Silva: Conceptualization, Methodology, Writing – review & editing.

8 Declaration of conflict of interests

The authors declare no conflict of interest.

9 Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors without undue reservation.

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Table 1. Composition of control diet.

Food	Control treatment
Corn	61.790
Soybean meal 45%	23.556
Limestone	9.283
Soybean oil	1.645
Dicalcium phosphate	1.549
Corn gluten 60%	1.024
Inert (washed sand)	0.262
Salt	0.497
DL-methionine	0.183
Vitamin premix[1]	0.100
Mineral Premix[2]	0.060
Choline chloride 70%	0.050
Calculated composition	
Metabolizable energy (kcal/kg)	2.800
Crude protein (%)	16.50
Calcium (%)	4.020
Available phosphorus (%)	0.380
Digestible methionine (%)	0.431
Digest. methionine+cystine (%)	0.668
Digestible lysine (%)	0.731
Digestible threonine (%)	0.559
Digestible tryptophan (%)	0.174
Digestible arginine (%)	0.984
Digestible valine (%)	0.690
Sodium (%)	0.220
Chlorine (%)	0.339
Potassium (%)	0.621

[1]Composition per kg of product: A vit. - 10,000,000 IU; D3 vit. - 2,500,000 IU; E vit. - 6,000 IU; K vit. - 1,600 mg; B12 vit. - 11,000 mg; Niacin - 25,000 mg; folic acid - 400 mg; pantothenic acid - 10,000 mg; Se - 300 mg.

[2]Composition per kg of product: MN - 150,000 mg; zinc - 100,000 mg; iron 100,000 mg; copper - 16,000 mg; iodine - 1,500 mg.

Table 2. Quality of eggs from laying hens fed diets supplemented with probiotic (PRO).

Responses	Treatments[1]		*P-value*[2,3]						
	CON	PRO	T	D	P	T×D	T×P	P×D	T×P×D
General traits									
Weight (g)	61.76	61.96	0.532	0.294	<0.001	0.099	0.187	0.004	0.125
Weight loss (g)	1.87	1.88	0.933	<0.001	0.672	0.989	<0.001	0.014	0.156
Spec. gravity (g/ml)	1.005	1.005	0.959	0.070	0.342	1.000	0.897	0.901	1.000
Albumen traits									
Height (mm)	4.278	4.244	0.432	<0.001	<0.001	0.695	0.200	<0.001	0.556
Weight (g)	33.91	34.78	0.002	<0.001	<0.001	0.370	0.001	0.011	0.669
pH	9.16	9.15	0.323	<0.001	<0.001	0.980	0.206	<0.001	0.524
Haugh unit	56.97	56.48	0.355	<0.001	<0.001	0.048	0.871	<0.001	0.766
Yolk traits									
Height (mm)	13.12	13.28	0.006	<0.001	<0.001	0.131	0.001	<0.001	0.042
Length (mm)	46.41	45.73	<0.001	<0.001	0.002	0.006	0.001	<0.001	0.001
Index	0.290	0.295	0.002	<0.001	<0.001	0.801	<0.001	<0.001	0.003
Weight (g)	16.87	16.73	0.182	<0.001	<0.001	<0.001	0.381	<0.001	0.275
pH	6.40	6.27	<0.001	<0.001	<0.001	0.231	0.263	<0.001	0.047
Yolk color									
Color score	5.65	5.87	<0.001	<0.001	<0.001	0.088	0.173	<0.001	0.033
Lightness (L*)	56.09	56.85	<0.001	<0.001	<0.001	0.777	0.763	<0.001	0.005
Redness (a*)	6.33	6.84	<0.001	<0.001	<0.001	0.031	0.023	<0.001	0.272
Yellowness (b*)	56.54	58.46	<0.001	0.123	<0.001	0.103	0.006	<0.001	0.686
Shell traits									
Weight (g)	5.86	5.92	0.095	0.002	<0.001	0.289	<0.001	0.007	0.395

[1] Means do not correspond only to fresh eggs, but are representing the whole sample of fresh and stored eggs

[2] Means do not correspond only to fresh egg evaluation but represent an overall value comprising fresh and stored eggs. Quality assessment was performed on the last day of each phase (phase 1, 36-40 weeks; phase 2, 41-44 weeks; and phase 3, 45-48 weeks). Eggs were stored and fifteen eggs per treatment were evaluated weekly (7, 14, 21, 28, 35, and 42 days).

[3] Responses with treatment effect (P<0.10) are fully described in the next tables. Significant interactions with treatment (P<0.10) are described in the supplementary materials.

Table 3. Albumen weight (g) of eggs from laying hens fed with probiotics depending on storage time.

Treatments	Storage period (days)						
	1	**7**	**14**	**21**	**28**	**35**	**42**
				Phase 1 – 36 to 40 weeks			
Control	35.75	32.80	34.40	32.57	32.50	31.06	31.30
Probiotic	36.44	33.48	33.03	33.23	33.71	32.80	32.00
***P-value*[1]**	0.642	0.622	0.326	0.518	0.369	0.062	0.200
SE[2]	0.720	0.668	0.688	0.496	0.662	0.470	0.567
				Phase 2 – 41 to 44 weeks			
Control	36.80	34.10	32.60	31.38	31.94	29.96	30.71
Probiotic	36.16	37.13	35.41	34.17	33.88	33.01	33.22
P-value	0.613	0.036	0.020	0.009	0.227	0.003	0.033
SE	0.623	0.732	0.619	0.558	0.793	0.545	0.592
				Phase 3 – 45 to 48 weeks			
Control	37.90	36.52	36.88	38.56	36.07	34.49	33.99
Probiotic	36.30	36.58	37.29	36.77	37.18	34.86	33.12
P-value	0.173	0.960	0.739	0.132	0.445	0.775	0.518
SE	0.582	0.514	0.600	0.589	0.714	0.619	0.658

[1] Probability of treatment effect.
[2] Standard error.

Table 4. Yolk height (mm) of eggs from laying hens fed with probiotics depending on storage time.

Treatments	Storage period (days)						
	1	**7**	**14**	**21**	**28**	**35**	**42**
				Phase 1 – 36 to 40 weeks			
Control	18.66	15.77	13.15	11.82	10.51	10.07	9.96
Probiotic	19.13	15.36	13.10	11.94	11.21	11.01	10.07
***P-value*[1]**	0.147	0.184	0.873	0.633	0.002	<0.001	0.776
SE[2]	0.161	0.152	0.167	0.119	0.121	0.140	0.176
				Phase 2 – 41 to 44 weeks			
Control	17.68	15.50	13.47	12.33	10.86	10.88	9.64
Probiotic	17.94	16.27	13.78	12.23	10.83	11.39	10.49
P-value	0.215	0.013	0.306	0.610	0.900	0.040	0.007
SE	0.106	0.159	0.152	0.099	0.116	0.126	0.164
				Phase 3 – 45 to 48 weeks			
Control	17.59	15.97	14.05	13.07	12.39	11.48	10.74
Probiotic	17.75	15.81	13.65	12.67	12.38	11.23	10.79
P-value	0.636	0.564	0.103	0.131	0.961	0.315	0.872
SE	0.163	0.131	0.123	0.133	0.122	0.118	0.148

[1] Probability of treatment effect.
[2] Standard error.

Table 5. Yolk length of eggs (mm) from laying hens fed with probiotics depending on storage time.

Treatments	Storage period (days)						
	1	**7**	**14**	**21**	**28**	**35**	**42**
				Phase 1 – 36 to 40 weeks			
Control	40.63	43.49	45.02	49.84	47.91	50.00	49,29
Probiotic	41.32	43.50	44.48	45.93	46.71	47.15	50.56
***P-value*[1]**	0.261	0.986	0.328	0.005	0.098	0.017	0.175
SE[2]	0.299	0.277	0.272	0.730	0.361	0.613	0.463
				Phase 2 – 41 to 44 weeks			
Control	41.43	44.73	45.89	47.80	50.39	47.90	51.59
Probiotic	41.33	42.51	44.19	45.30	47.57	48.47	51.02
P-value	0.814	0.005	0.008	0.001	0.003	0.536	0.680
SE	0.196	0.410	0.332	0.420	0.504	0.450	0.665
				Phase 3 – 45 to 48 weeks			
Control	39.94	42.03	46.63	46.08	46.08	48.06	50.00
Probiotic	41.10	42.76	45.93	46.18	47.16	48.39	48.80
P-value	0.032	0.208	0.378	0.872	0.271	0.790	0.206
SE	0.273	0.289	0.393	0.297	0.431	0.600	0.470

[1] Probability of treatment effect.
[2] Standard error.

Table 6. Yolk index of eggs from laying hens fed with probiotics depending on storage time.

Treatments	Storage period (days)						
	1	**7**	**14**	**21**	**28**	**35**	**42**
				Phase 1 – 36 to 40 weeks			
Control	0.460	0.365	0.287	0.239	0.219	0.206	0.209
Probiotic	0.464	0.353	0.299	0.260	0.240	0.234	0.200
***P-value*[1]**	0.663	0.129	0.225	0.018	<0.001	0.002	0.412
SE[2]	0.004	0.003	0.005	0.004	0.003	0.004	0.005
				Phase 2 – 41 to 44 weeks			
Control	0.429	0.347	0.293	0.260	0.214	0.228	0.186
Probiotic	0.433	0.383	0.312	0.270	0.228	0.236	0.205
P-value	0.600	<0.00	0.020	0.190	0.074	0.240	0.064
SE	0.003	0.004	0.004	0.003	0.003	0.003	0.005
				Phase 3 – 45 to 48 weeks			
Control	0.441	0.380	0.314	0.284	0.271	0.239	0.220
Probiotic	0.432	0.366	0.301	0.275	0.263	0.229	0.222
P-value	0.340	0.155	0.147	0.197	0.399	0.271	0.848
SE	0.004	0.004	0.004	0.003	0.004	0.004	0.003

[1] Probability of treatment effect.
[2] Standard error.

Table 7. Yolk pH of eggs from laying hens fed with probiotics depending on storage time.

Treatments	Storage period (days)						
	1	**7**	**14**	**21**	**28**	**35**	**42**
				Phase 1 – 36 to 40 weeks			
Control	6.22	6.62	6.47	6.82	6.50	6.65	6.75
Probiotic	6.14	6.25	6.41	6.34	6.44	6.59	6.52
***P-value*[1]**	0.172	0.088	0.732	0.040	0.613	0.612	0.038
SE[2]	0.030	0.094	0.084	0.120	0.057	0.056	0.057
				Phase 2 – 41 to 44 weeks			
Control	5.89	6.06	6.11	6.31	6.85	6.35	6.49
Probiotic	5.85	5.97	5.96	6.29	6.52	6.26	6.44
P-value	0.038	0.012	0.030	0.776	0.004	0.257	0.512
SE	0.011	0.018	0.034	0.031	0.059	0.035	0.038
				Phase 3 – 45 to 48 weeks			
Control	6.01	6.19	6.07	6.24	6.37	6.62	6.63
Probiotic	5.99	6.05	6.08	6.22	6.38	6.49	6.49
P-value	0.735	0.007	0.846	0.691	0.951	0.322	0.090
SE	0.022	0.073	0.026	0.034	0.041	0.062	0.043

[1] Probability of treatment effect.
[2] Standard error.

Table 8. Yolk color score (palette) of eggs from laying hens fed with probiotics depending on storage time.

Treatments	Storage period (days)						
	1	**7**	**14**	**21**	**28**	**35**	**42**
				Phase 1 – 36 to 40 weeks			
Control	5.73	4.87	5.42	6.36	5.93	6.38	6.18
Probiotic	5.73	4.80	6.00	6.20	6.60	6.64	6.82
***P-value*[1]**	0.999	0.764	0.031	0.525	0.001	0.348	0.001
SE[2]	0.172	0.108	0.137	0.121	0.106	0.135	0.109
				Phase 2 – 41 to 44 weeks			
Control	5.33	4.57	4.67	6.07	6.80	6.46	6.36
Probiotic	5.93	4.87	4.80	6.00	7.00	6.79	6.87
P-value	0.003	0.135	0.493	0.826	0.094	0.139	0.006
SE	0.104	0.098	0.095	0.158	0.059	0.109	0.095
				Phase 3 – 45 to 48 weeks			
Control	5.73	5.20	5.00	5.15	5.33	5.75	5.50
Probiotic	5.64	5.00	4.93	5.67	5.46	5.57	5.86
P-value	0.614	0.120	0.591	0.005	0.194	0.482	0.045
SE	0.087	0.059	0.060	0.095	0.118	0.123	0.089

[1] Probability of treatment effect.
[2] Standard error.

Table 9. Yolk lightness (L* color) of eggs from laying hens fed with probiotics depending on storage time.

Treatments	Storage period (days)						
	1	**7**	**14**	**21**	**28**	**35**	**42**
				Phase 1 – 36 to 40 weeks			
Control	51.30	58.18	57.60	57.13	55.27	55.38	57.77
Probiotic	51.48	57.51	58.27	57.48	57.86	57.53	58.96
***P-value*[1]**	0.849	0.300	0.408	0.512	0.006	0.010	0.092
SE[2]	0.451	0.320	0.397	0.262	0.491	0.436	0.353
				Phase 2 – 41 to 44 weeks			
Control	50.99	55.77	57.87	56.62	58.25	58.73	59.07
Probiotic	52.06	56.45	58.54	58.93	58.31	58.89	59.10
P-value	0.079	0.343	0.284	0.005	0.870	0.750	0.948
SE	0.305	0.353	0.307	0.431	0.187	0.248	0.262
				Phase 3 – 45 to 48 weeks			
Control	50.26	53.37	54.61	56.49	57.56	57.09	58.61
Probiotic	50.45	54.60	56.57	56.69	57.34	58.03	58.97
P-value	0.768	0.167	0.047	0.733	0.760	0.015	0.384
SE	0.322	0.441	0.496	0.291	0.342	0.200	0.200

[1] Probability of treatment effect.
[2] Standard error.

Table 10. Yolk redness (a* color) of eggs from laying hens fed with probiotics depending on storage time.

Treatments	Storage period (days)						
	1	**7**	**14**	**21**	**28**	**35**	**42**
				Phase 1 – 36 to 40 weeks			
Control	7.58	6.03	7.21	7.02	6.57	5.80	7.01
Probiotic	7.63	6.28	7.28	6.71	7.15	7.28	7.45
***P-value*[1]**	0.837	0.572	0.860	0.534	0.102	0.003	0.203
SE[2]	0.137	0.212	0.182	0.241	0.177	0.264	0.170
				Phase 2 – 41 to 44 weeks			
Control	6.98	6.17	5.94	6.29	5.74	6.23	5.60
Probiotic	7.97	7.16	5.85	7.03	7.06	6.89	6.87
P-value	0.002	0.006	0.617	0.039	0.001	0.081	<0.001
SE	0.173	0.188	0.085	0.179	0.209	0.187	0.190
				Phase 3 – 45 to 48 weeks			
Control	6.81	7.03	6.32	6.09	5.49	5.58	5.39
Probiotic	7.40	6.54	6.48	6.38	6.11	6.22	5.89
P-value	0.211	0.195	0.751	0.512	0.235	0.125	0.097
SE	0.233	0.186	0.237	0.217	0.255	0.209	0.150

[1] Probability of treatment effect.
[2] Standard error.

Table 11. Yolk yellowness (b* color) of eggs from laying hens fed with probiotics depending on storage time.

Treatments	Storage period (days)						
	1	**7**	**14**	**21**	**28**	**35**	**42**
				Phase 1 – 36 to 40 weeks			
Control	6.05	5.98	6.02	5.96	5.63	5.53	5.55
Probiotic	5.96	6.00	5.98	5.96	5.98	5.61	5.77
***P-value*[1]**	0.178	0.880	0.799	0.997	0.006	0.688	0.252
SE[2]	0.338	0.533	0.642	0.551	0.656	0.977	0.929
				Phase 2 – 41 to 44 weeks			
Control	5.70	5.52	5.62	5.40	5.75	5.86	5.65
Probiotic	6.07	6.02	5.80	5.83	6.18	5.98	5.91
P-value	0.036	0.015	0.254	0.014	0.001	0.133	0.016
SE	0.900	1.070	0.778	0.885	0.693	0.411	0.552
				Phase 3 – 45 to 48 weeks			
Control	5.47	5.63	5.62	5.62	5.21	5.55	5.41
Probiotic	5.64	5.64	5.71	5.66	5.68	5.74	5.65
P-value	0.275	0.976	0.658	0.801	0.026	0.219	0.036
SE	0.793	0.579	0.905	0.656	1.08	0.748	0.595

[1] Probability of treatment effect.
[2] Standard error.

Table 12. Thiobarbituric acid reactive substances in eggs from laying hens fed probiotics.

Treatments	Storage period (days)		
	1	**21**	**42**
	Phase 1 – 36 to 40 weeks		
Control	4.43	2.98	3.55
Probiotic	4.09	2.53	0.22
***P-value*[1]**	0.056	0.088	0.051
SE[2]	0.21	0.22	0.16
	Phase 2 – 41 to 44 weeks		
Control	4.77	3.91	3.22
Probiotic	3.78	3.94	3.24
P-value	0.075	0.877	0.958
SE	0.28	0.13	0.16
	Phase 3 – 45 to 48 weeks		
Control	4.58	2.55	3.31
Probiotic	3.68	2.50	3.46
P-value	0.001	0.782	0.554
SE	0.17	0.08	0.11

[1] Probability of treatment effect.
[2] Standard error.

Table S1. Weight loss (g) of eggs from laying hens fed with probiotics depending on storage time.

Treatments	Storage period (days)						
	1	**7**	**14**	**21**	**28**	**35**	**42**
				Phase 1 – 36 to 40 weeks			
Control	-	1.017	1.673	1.748	2.691	3.251	3.750
Probiotic	-	0.743	1.407	1.422	2.132	2.797	3.351
***P-value*[1]**	-	0.003	0.196	<0.001	<0.001	0.003	0.011
SE[2]	-	0.685	0.700	0.619	0.719	0.639	0.635
				Phase 2 – 41 to 44 weeks			
Control	-	0.736	1.242	2.056	2.387	2.923	3.807
Probiotic	-	0.739	1.254	2.043	2.460	3.015	3.838
P-value	-	0.967	0.747	0.814	0.442	0.404	0.856
SE	-	0.893	0.654	0.555	0.772	0.746	0.760
				Phase 3 – 45 to 48 weeks			
Control	-	0.615	1.348	1.523	2.137	2.439	3.675
Probiotic	-	0.514	1.422	1.436	2.018	2.539	3.132
P-value	-	0.130	0.670	0.402	0.476	0.358	<0.001
SE	-	0.562	0.555	0.618	0.804	0.672	0.766

[1] Probability of treatment effect.
[2] Standard error.

Table S2. Haugh unit of eggs from laying hens fed with probiotics depending on storage time.

Treatments	Storage period (days)						
	1	**7**	**14**	**21**	**28**	**35**	**42**
				Phase 1 – 36 to 40 weeks			
Control	86.84	76.76	65.55	58.18	48.49	44.22	39.62
Probiotic	87.01	78.39	67.17	53.91	48.51	42.03	39.88
***P-value*[1]**	0.921	0.369	0.496	0.163	0.994	0.112	0.117
SE[2]	0.824	0.887	1.160	1.160	1.490	1.630	1.220
				Phase 2 – 41 to 44 weeks			
Control	84.42	70.58	58.24	47.09	41.03	43.40	38.10
Probiotic	85.31	69.00	57.42	46.60	41.68	39.60	39.96
P-value	0.566	0.433	0.746	0.863	0.759	0.163	0.497
SE	0.760	1.000	1.230	1.400	1.040	1.350	1.310
				Phase 3 – 45 to 48 weeks			
Control	86.93	71.65	51.62	49.65	42.56	41.32	38.10
Probiotic	87.33	67.55	52.39	46.87	38.53	42.19	38.35
P-value	0.770	0.577	0.030	0.911	0.572	0.139	0.379
SE	0.667	0.960	1.210	1.040	1.270	1.350	1.530

[1] Probability of treatment effect.
[2] Standard error.

Table S3.Yolk weight (g) of eggs from laying hens fed with probiotics depending on storage time.

Treatments	Storage period (days)						
	1	**7**	**14**	**21**	**28**	**35**	**42**
				Phase 1 – 36 to 40 weeks			
Control	17.86	16.73	17.38	16.51	16.51	16.21	16.34
Probiotic	16.90	16.27	16.58	16.71	16.71	16.44	16.39
***P-value*[1]**	0.118	0.454	0.146	0.705	0.701	0.689	0.881
SE[2]	0.184	0.276	0.299	0.273	0.253	0.283	0.179
				Phase 2 – 41 to 44 weeks			
Control	16.75	17.73	17.10	17.47	17.00	16.16	16.18
Probiotic	16.35	16.71	16.03	16.43	16.30	17.05	17.55
P-value	0.464	0.054	0.055	0.003	0.067	0.144	0.027
SE	0.154	0.267	0.280	0.184	0.190	0.301	0.314
				Phase 3 – 45 to 48 weeks			
Control	14.75	15.87	17.95	18.67	17.98	17.23	18.04
Probiotic	15.25	16.10	17.04	18.08	18.66	17.52	18.28
P-value	0.161	0.583	0.113	0.162	0.184	0.539	0.689
SE	0.177	0.207	0.288	0.209	0.255	0.228	0.287

[1] Probability of treatment effect.
[2] Standard error.

Table S4. Shell weight (g) of eggs from laying hens fed with probiotics depending on storage time.

Treatments	Storage period (days)						
	1	**7**	**14**	**21**	**28**	**35**	**42**
				Phase 1 – 36 to 40 weeks			
Control	5.78	6.17	6.01	5.81	5.88	6.01	6.20
Probiotic	6.05	6.15	6.18	5.93	5.98	6.04	6.18
***P-value*[1]**	0.071	0.901	0.300	0.539	0.492	0.887	0.920
SE[2]	0.076	0.068	0.083	0.094	0.076	0.106	0.104
				Phase 2 – 41 to 44 weeks			
Control	6.25	6.11	6.02	5.76	6.06	5.95	6.22
Probiotic	5.93	5.83	5.74	5.46	5.86	5.75	6.01
P-value	0.120	0.215	0.135	0.112	0.192	0.130	0.307
SE	0.092	0.111	0.091	0.094	0.073	0.063	0.101
				Phase 3 – 45 to 48 weeks			
Control	5.40	5.32	5.42	5.70	5.76	5.29	5.96
Probiotic	5.90	5.84	6.03	5.83	6.09	5.87	5.75
P-value	0.016	0.003	0.002	0.320	0.155	<0.001	0.216
SE	0.106	0.092	0.102	0.065	0.115	0.086	0.084

[1] Probability of treatment effect.
[2] Standard error.

Table S5. Total solids of eggs from laying hens fed probiotics depending on storage time.

Treatments	Albumen	Yolk
Control	11.65	46.88
Probiotic	11.84	46.97
***P-value*[1]**	0.241	0.698
SE[2]	0.16	0.26

[1] Probability of treatment effect.
[2] Standard error.

4. CONSIDERAÇÕES FINAIS

Ovos são produtos de alta qualidade nutricional, entretanto, perdem sua qualidade rapidamente. Umas das formas de modular sua vida de prateleira é através do uso de aditivos na alimentação das aves, o que beneficia não só consumidores, mas produtores. Neste estudo, ao utilizar probióticos e β-mannanase na alimentação de poedeiras, demonstramos que seu uso é positivo, sendo eficiente principalmente em relação à cor da gema, além de apresentarem menores níveis de TBARS e pH quando comparados ao tratamento controle.

Em relação a taxa de postura das aves, observamos aumentos significativos com o uso dos aditivos, além do peso dos ovos frescos também aumentarem sob seus efeitos. Bioquímica sérica, morfometria intestinal e massas de ovos das aves alimentadas com dietas contendo ambos os aditivos também apresentaram melhoras. Já em relação ao bem-estar animal, os aditivos foram capazes de modular o comportamento das aves, as quais apresentaram maiores frequências e tempo gasto no comportamento alimentar, além de redução de comportamento negativos como a bicagem. Assim, podemos concluir que alta produtividade pode estar alinhada ao bem-estar animal.

Portanto, a adição de β-mannanase e probióticos às dietas de galinhas poedeiras é uma estratégia eficaz para melhorar o desempenho, o bem-estar das aves, além de melhorar a qualidade em ovos frescos e armazenados. Futuros estudos, como microbiota intestinal e expressão gênica podem esclarecer os resultados encontrados neste estudo.

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