MARIELEN DE SOUZA

STRATEGIES TO PROMOTE INTESTINAL HEALTH IN BROILER CHICKENS

Londrina
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Advisor at UEL: Prof. Dr. Ana Paula F.R.L. Bracarense.
Co-advisor at UEL: Prof. Dr. Ana Angelita S. Baptista
Advisor at UGent: Prof. Dr. Filip Van Immerseel

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Merelbeke, 24th April 2024.

To the most important person of my life, myself. It was a hard journey, but I was resilient enough to finish it. It seems selfish, but it's not.

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SOUZA, Marielen. **Estratégias para promover a saúde intestinal em frangos de corte**. 2024. 147p. Tese (Doutorado em Ciência Animal/ Ciências Veterinárias) – Universidade Estadual de Londrina/ Universidade de Gent, Londrina, 2024.

RESUMO

A saúde intestinal influencia significativamente o bem-estar animal, o desempenho e a lucratividade na avicultura. Este projeto de pesquisa teve como objetivo avaliar os efeitos deletérios dos desafios intestinais prevalentes em frangos de corte, incluindo doenças intestinais (enterite necrótica), micotoxinas (desoxinivalenol) e fatores antinutricionais (β-manano). O estudo abrangeu duas investigações principais: *i. Efeitos protetores de uma mistura de Lactobacillus spp. frente ao desafio com desoxinivalenol (DON) e Clostridium perfringens (CP)*. Este estudo investigou os impactos dos desafios simultâneos com DON e CP na saúde intestinal dos frangos de corte e avaliou o potencial de uma mistura de *Lactobacillus* spp. em aliviar os danos associados aos desafios. Frangos de corte (n = 252) foram divididos em sete grupos: Controle, DON, CP, CP+DON, VL (DON+CP + mistura de *Lactobacillus* spp. viáveis), HIL (DON+CP + mistura de *Lactobacillus* spp. inativados pelo calor) e LCS (DON+CP + sobrenadante de cultura da mistura de *Lactobacillus* spp.). Os resultados indicaram que os tratamentos com *Lactobacillus* spp., independentemente de suas formas de apresentação (viáveis, inativados pelo calor ou sobrenadante da cultura), reduziram parcialmente o dano intestinal induzido pelos desafios com DON e CP. Macroscopicamente o grupo CP+DON exibiu o maior escore de lesão, enquanto os grupos VL e HIL exibiram os menores. A análise microscópica demonstrou que todos os tratamentos com *Lactobacillus* spp. mitigaram as alterações morfológicas induzidas pelos desafios. Curiosamente, a defesa antioxidante contra o estresse oxidativo induzido por CP não melhorou com os tratamentos com *Lactobacillus* spp. *ii. Impacto da ingestão de β-mananas e suplementação com β-mananase na saúde intestinal de frangos de corte.* Este estudo investigou os efeitos da goma guar, uma fonte de galactomananas, em dietas para frangos de corte com ou sem suplementação de β-mananase, na composição da microbiota e seu potencial papel na saúde intestinal e desempenho zootécnico. Frangos de corte com um dia de idade (n = 756) foram distribuídos aleatoriamente em três tratamentos: dieta controle, dieta com goma guar (1,7%) ou dieta com goma guar suplementada com β-mananase (Hemicell® 330g/ton). Os resultados revelaram que a ingestão de goma guar impactou negativamente o desempenho zootécnico, enquanto a suplementação com β-mananase restaurou o desempenho para níveis do grupo controle. A dieta rica em mananas induziu disbiose da microbiota cecal, caracterizada por aumento de gêneros específicos e diminuição de outros. No entanto, a suplementação dietética com β-mananase restaurou eficazmente a composição da microbiota para níveis semelhantes ao grupo controle, indicando um efeito atenuante. Além disso, a suplementação com β-mananase reduziu as concentrações cecais de certos ácidos graxos de cadeia curta, sugerindo melhor digestão de proteínas e redução da fermentação de proteínas cecais. Concluindo, os três fatores avaliados – desafio de DON e *C. perfringens* e β-mananas – impactaram a saúde intestinal. No entanto, as soluções propostas, mistura de *Lactobacillus* spp. e a suplementação de β-mananase, demonstraram eficiência em mitigar ou reverter completamente os efeitos negativos associados a esses desafios.

Palavras-chave: DON. *Clostridium perfringens*. *Lactobacillus* spp. Polissacarídeos não amiláceos. β-mannanase.

SOUZA, Marielen. **Strategieën om de darmgezondheid bij vleeskuikens te bevorderen**. 2024. 147p. Proefschrift (Doctoraat in de Diergeneeskundige Wetenschappen) – Universidade Estadual de Londrina/ Universiteit Gent, Londrina, 2024.

ABSTRACT

De darmgezondheid heeft een grote invloed op het dierenwelzijn, de prestaties en de winstgevendheid in de pluimveehouderij. Dit onderzoeksproject was gericht op het beoordelen van de schadelijke effecten van veel voorkomende darmproblemen bij pluimvee, waaronder darmziekten (necrotische enteritis), mycotoxinen (deoxynivalenol) en antinutritionele factoren (β-mannan). Het onderzoek omvatte twee hoofddoelen i. Verzachtende effecten van *Lactobacillus* spp. mengsel op deoxynivalenol (DON) en Clostridium perfringens (CP) challenge. Dit segment verdiepte zich in de impact van gelijktijdige challenges met DON en CP op de darmgezondheid van vleeskuikens en onderzocht het potentieel van een *Lactobacillus* spp. mengsel bij het verlichten van de daarmee samenhangende schade. Vleeskuikens (n = 252) werden verdeeld in zeven groepen: Controle, DON, CP, CP+DON, VL (DON+CP + levensvatbaar *Lactobacillus* spp. mengsel), HIL (DON+CP + door hitte geïnactiveerd *Lactobacillus* spp. mengsel) en LCS (DON+CP + *Lactobacillus* spp. mengselkweeksupernatant). De resultaten gaven aan dat de *Lactobacillus* spp. behandelingen, ongeacht hun presentatievormen (levensvatbaar, door hitte geïnactiveerd of kweeksupernatant), gedeeltelijk beschermden tegen de darmschade veroorzaakt door DON- en CP-geïnduceerde schade. Terwijl macroscopische evaluaties de hoogste laesiescore in de CP+DON-groep aan het licht brachten, vertoonden de VL- en HIL-groepen de laagste scores. Microscopische analyse toonde aan dat alle *Lactobacillus* spp. behandelingen morfologische veranderingen verbeterden. Interessant is dat de antioxidantverdediging tegen CP-geïnduceerde oxidatieve stress niet werd verbeterd door *Lactobacillus* spp. behandelingen. ii. Impact van β-mannan- en β-mannanasupplementatie op de darmgezondheid van vleeskuikens. Deze fase onderzocht de effecten van guargom, een bron van galactomannans, aangevuld in vleeskuikendiëten met of zonder β-mannanasuppletie, op de samenstelling van de microbiota en de potentiële rol ervan in de darmgezondheid en -prestaties. Eén dag oude vleeskuikens (n = 756) werden willekeurig toegewezen aan drie behandelingen: controledieet, met guargom aangevuld dieet (1,7%), of met guargom aangevuld dieet + β-mannanase (Hemicell® 330 g/ton). Uit de resultaten bleek dat supplementatie met guargom een negatieve invloed had op de zoötechnische prestaties, terwijl suppletmentaie met β-mannanase de prestaties tot controleniveaus herstelde. Een mannanrijk dieet induceerde dysbiose in de caecale microbiota, gekenmerkt door een toename van specifieke genera en een afname van andere. Door supplementatie met β-mannanase via de voeding werd de samenstelling van de microbiota echter effectief teruggebracht tot controleniveaus, wat wijst op een voordelig effect. Bovendien verlaagde supplementatie met β-mannanase de caecale concentraties van bepaalde vertakte korte keten vetzuren, wat wijst op een verbeterde eiwitvertering en verminderde caecale eiwitfermentatie. Samenvattend hadden de drie geëvalueerde factoren – DON en C. perfringens challenges, en β-mannan – invloed op de darmgezondheid. De voorgestelde oplossingen, met name de *Lactobacillus* spp. mengsel- en β-mannanasupplementatie toonden efficiëntie aan bij het verbeteren of volledig ongedaan maken van de negatieve effecten die met deze uitdagingen gepaard gaan.

Kernwoorden: DON. *Clostridium perfringens.* *Lactobacillus* spp. Niet-zetmeelpolysachariden. β-mannanase.

SOUZA, Marielen. **Strategies to promote intestinal health in broiler chickens**. 2024. 147p. Thesis (Doctorate in Animal Science/ Veterinary Sciences) – Universidade Estadual de Londrina/ Gent University, Londrina, 2024.

ABSTRACT

Intestinal health significantly influences animal welfare, performance, and profitability in poultry farming. This research project aimed to assess the detrimental effects of prevalent intestinal challenges in poultry, including intestinal diseases (necrotic enteritis), mycotoxins (deoxynivalenol), and antinutritional factors (β-mannan). The study encompassed two main investigations: *i*. *Mitigating effects of Lactobacillus spp. mixture on deoxynivalenol (DON) and Clostridium perfringens (CP) challenge.* This segment delved into the impacts of concurrent challenges with DON and CP on broilers' intestinal health and explored the potential of a *Lactobacillus* spp. mixture in alleviating the associated damage. Broiler chickens (n = 252) were divided into seven groups: Control, DON, CP, CP+DON, VL (DON+CP + viable *Lactobacillus* spp. mixture), HIL (DON+CP + heat-inactivated *Lactobacillus* spp. mixture), and LCS (DON+CP + *Lactobacillus* spp. mixture culture supernatant). The results indicated that the *Lactobacillus* spp. treatments, irrespective of their presentation forms (viable, heat-inactivated, or culture supernatant), partially protected against the intestinal damage induced by DON and CP challenge. While macroscopic evaluations revealed the highest lesion score in the CP+DON group, the VL and HIL groups exhibited the lowest scores. Microscopic analysis demonstrated that all *Lactobacillus* spp. treatments mitigated morphological changes. Interestingly, the antioxidant defense against CP-induced oxidative stress was not improved by *Lactobacillus* spp. treatments. *ii. Impact of β-mannan and β-mannanase supplementation on broilers' intestinal health*. This phase investigated the effects of guar gum, a source of galactomannans, supplemented in broiler diets with or without β-mannanase supplementation, on microbiota composition and its potential role in gut health and zootechnical performance. One-day-old broiler chickens (n = 756) were randomly assigned to three treatments: control diet, guar gum supplemented diet (1.7%), or guar gum supplemented diet + β-mannanase (Hemicell® 330g/ton). The results revealed that guar gum supplementation negatively impacted zootechnical performance, while β-mannanase supplementation restored performance to control levels. A mannan-rich diet induced dysbiosis in the caecal microbiota, characterized by an increase in specific genera and a decrease in others. However, dietary β-mannanase supplementation effectively restored the microbiota composition to control levels, indicating a mitigating effect. Furthermore, β-mannanase supplementation reduced caecal concentrations of certain short-chain fatty acids, suggesting improved protein digestion and reduced caecal protein fermentation. In conclusion, the three factors evaluated—DON and *C. perfringens* challenge, and β-mannan—impacted intestinal health. However, the proposed solutions, particularly the *Lactobacillus* spp. mixture and β-mannanase supplementation, demonstrated efficiency in mitigating or completely reverting the negative effects associated with these challenges.

Keywords: DON. *Clostridium perfringens*. *Lactobacillus* spp. Non-starch polysaccharides. β-mannanase.

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ABBREVIATIONS AND ACRONYMS

ABPA	Brazilian Association of Animal Protein/ *Associação brasileira de proteína animal*
ABTS	2,20-azino-bis (3-ethylbenzothiazoline6-sulphonic acid)
AFLA	Aflatoxin
AGP	Antimicrobial Growth Promoter
ADG	Average daily gain
AX	Arabinoxylans
BMD	Bacitracin methylene disalicylate
BWG	Body weight gain
CFS	Cell-free supernatants
CP	*Clostridium perfringens*
DON	Deoxynivalenol
EU	European Union
FCR	Feed Conversion Ratio
FOS	Fructooligosaccharides
FRAP	Ferric-reducing antioxidant power
FUM	Fumonisin
GG	Guar gum
GOS	Glucooligosaccharides
GPDH	Glycerol-3-phosphate dehydrogenase
GSH	Reduced glutathione
GSH-PX/GPx/GPx4	Glutathione peroxidase
HMOX	Heme oxygenase
MDA	Malondialdehyde
MN	β-mannans

NE	Necrotic Enteritis
NSP	Non-starch polysaccharides
OR	Odds ratio
PP	Penicillin G potassium
ROS	Reactive species of oxygen
SA	Salinomycin
SCFAs	Short-chain fatty acids
T-AOC	Total antioxidant capacity
TBARS	Thiobarbituric acid reactive substances
T-ROS	Total reactive oxygen species
T-SOD	Total superoxide dismutase
UEL	Universidade Estadual de Londrina
UGENT	Gent University
XOR	Xanthine oxidoreductase
ZEA	Zearalenone

SUMMARY

1 INTRODUCTION

Poultry meat is the most affordable animal protein source (Oecd; Fao, 2021). The poultry industry generates millions of job opportunities and economic value worldwide and provides cheap protein to humans (Abpa, 2023). In the past few years this industry advanced a lot in productivity due to technological advances and breeder selection, however sanitary problems, mainly intestinal diseases, are still a challenge reducing the profitability of the chicken industry (Oviedo-Rondón, 2019; Abpa, 2023; Embrapa, 2023). Additionally, with global warming, some challenges in animal production tend to increase. There is a prediction that mycotoxin contamination will increase worldwide. In Europe, an increase in DON contamination of wheat and aflatoxin contamination of maize is expected (Medina; González-Jartín; Sainz, 2017).

New challenges are expected in the coming years in poultry production due to global warming. Among these challenges is the increase in contamination of cereals (the main nutrient base of feed) by mycotoxins. Furthermore, periods of prolonged drought make it necessary to obtain alternative sources of grains that are more adapted to drought and that are not intended for human consumption.

For several years, intestinal diseases were effectively controlled using antimicrobial growth promoters (AGP). Due to increasing rates of multi-resistant bacteria, especially those isolated from animal products and the transmission risk to humans through the food chain, in 2005, the European Union banned the use of AGP as a strategy to reduce the spread of antimicrobial resistance. After that, other important chicken meat producers also started to ban or restrain antimicrobial products (E.C., 2003; Mapa, 2020; Abd El-Hack et al., 2022). However, the consequence of this restriction was the increase in Necrotic Enteritis cases, an intestinal disease caused by *C. perfringens* that was previously controlled by the use of AGP, and economic losses began to accrue as a result of expenses related to treatment, mortality, and performance failures (Immerseel et al., 2009; Wade; Keyburn, 2015).

Mycotoxins are toxic metabolites produced by fungi that contaminate animals' feed. There are over 700 different molecules already described, but some are more important and well-described due to their prevalence and toxicity (Peltomaa; Benito-Peña; Moreno-Bondi, 2018; Stroka; Gonçalves, 2019). Deoxynivalenol (DON) is one of the most frequent mycotoxins detected on cereals, and climate change as consequence of the global warming has been increasing its prevalence (Biomin, 2023). Chickens are considered one of the most tolerant species to DON exposure; however, previous studies have described intestinal barrier dysfunction and a predisposition to intestinal diseases like Necrotic Enteritis after ingestion of

DON-contaminated diets (Maresca, 2013; Antonissen et al., 2014; Souza et al., 2020).

Probiotic strains are well known by their beneficial effects to the host. After AGP ban they became a common strategy to promote intestinal health in broilers flocks (Angelakis, 2017). Studies reported their positive effects on promoting intestinal health on animals challenged with DON or CP separately (Gong et al., 2020; Souza et al., 2020; Azizi et al., 2023). Recently the capacity of heat-inactivated cells and culture supernatants to adsorb or de-epoxide DON has been described (Franco et al., 2011; Qu et al., 2019; Maidana et al., 2021). In the current thesis, the first aim was to investigate the effects of concomitant exposure to DON and *C. perfringens* on broiler intestinal health and also to explore the effects of a mixture of *Lactobacillus* as a probiotic, paraprobiotic, or postbiotic in mitigating intestinal damage.

Another consequence of global warming are agricultural losses related to drought periods, that will be five times higher, in cereals drought periods are directly related to non-starch polysaccharides (NSP) content (Coles et al., 1997; Cammalleri et al., 2020). In this context alternatives are necessary to improve feed digestibility.

The production cost is an ongoing concern in the current scenario of modern poultry farming, and the industry is always looking for solutions to reduce costs (Alhotan, 2021). Animal feed is responsible for about 60% of all production costs. A poor choice in diet formulation will result in lower zootechnical performance and economic losses (Noblet; Wu; Choct, 2022).

Alternative feed ingredients might be an option to try to reduce feed prices, but, in general, they are rich in antinutritional factors. As an example, the leguminous Guar has seeds extremely rich in protein; however, it also has high levels of β-mannans, an antinutritional factor that increases digesta viscosity, reducing animal performance and may predispose to intestinal diseases like Necrotic Enteritis (Hussain et al., 2012; Jha et al., 2019; Al-Oubaidi; Fayyadh; Dakheel, 2020). Nowadays, there are enzymes available that are stable during the feed pelleting process and can be added to improve digestibility and, consequently, zootechnical performance (Bedford et al., 2022).

The second objective of this thesis was to examine how a diet rich in β-mannan, with or without β-mannanase supplementation, affects the intestinal health of broilers. This research project assessed the adverse effects and explored potential strategies for the predominant intestinal challenges encountered in poultry farming, including intestinal diseases (such as Necrotic Enteritis), mycotoxins (DON), and antinutritional factors (β-mannan).

2 THEORETICAL REFERENCE

2.1 Significance of Poultry Farming Globally and in Brazil: Exploring the Link between Intestinal Health and Profitability

Poultry meat has high nutritional value and serves as an affordable protein source without religious restrictions. It can be produced in a short period and requires fewer natural resources compared to other animal protein sources. It is estimated that by 2030, poultry meat consumption will constitute 41% of all meat protein sources worldwide (Oecd; Fao, 2021).

In Brazil, the poultry production chain generates more than 150 thousand job opportunities. The industrial poultry production includes about 21 thousand farms, primarily composed of small properties (less than 100 acres) operated by families. This sector is an essential economic activity, contributing significantly to wealth generation and sustaining rural communities (Embrapa, 2023). According to the latest report from the Brazilian Association of Animal Protein (ABPA) in 2022, the gross production value of Brazilian poultry production reached 112.145 billion reais. In the South region, which comprises Paraná, Santa Catarina, and Rio Grande do Sul, the main chicken slaughtering enterprises are concentrated, representing 65.42% of the national production. Concerning the destination of production, 66.80% is consumed by the domestic market, and 33.20% is exported, predominantly to Asian countries. Brazil holds the position as the leading poultry meat exporter and the second-largest producer in the global market (Abpa, 2023).

The European Union (EU) produced approximately 13.0 million tons of poultry meat in 2022, making it the major animal protein producer (Eurostat, 2023). In Belgium, poultry farmers generated 449.04 thousand tons of poultry meat, playing a significant role in the EU poultry market (Economics, 2023). In 2023, the EU ranked as the third-largest poultry meat exporter, highlighting the economic importance of its poultry farming sector (Abpa, 2023).

The profitability of poultry farming is directly linked to the gastrointestinal tract, which plays a crucial role in nutrient absorption and acts as the largest exposed body surface, serving as a barrier against ingested toxins and pathogens (Yegani; Korver, 2008). For many years, antimicrobial growth promoters (AGPs) were used to control opportunistic pathogenic microbiota and promote intestinal health. Since 2003, the use of AGPs has been globally banned or restricted, leading to the reemergence of several diseases that were previously controlled. Necrotic Enteritis (NE) is a classic example, and other intestinal challenges like mycotoxins have

increased in prevalence in feedstuffs worldwide due to climate changes (E.C., 2003; Van Immerseel et al., 2016; Biomin, 2023).

Another factor currently affecting the intestinal microbiome is nutrient excess in the gut. This occurs with inappropriate diet formulations, although uncommon today with advances in animal nutrition, or when alternative feed ingredients with lower digestibility are used without enzyme supplementation. The excess of non-digestible carbohydrates serves as a substrate for some microorganisms, leading to dysbiosis (Dey; Ray Chaudhuri, 2022; Ogunnusi; Toye; Akinwemoye, 2023).

In poultry farming, intestinal health significantly impacts animal welfare, flock production efficiency, food safety, systemic health, and the environment (resulting in higher water/feed demand and increased ammonia production rates) (Oviedo-Rondón, 2019). Therefore, this review focuses on three specific intestinal challenges: *C. perfringens*, deoxynivalenol, and β-mannans.

2.2 Avian Digestive System Anatomy and Histology

The avian digestive tract is composed of a mouth/beak, esophagus, crop, proventriculus, ventriculus or gizzard, intestine, caeca, rectum, and cloaca. As food progresses through these organs, a specific sequence of digestive events occurs, including grinding, acidifying, hydrolyzing, emulsifying, and transportation of the end products (Klasing, 1999).

The intestinal tract serves not only as a digestive organ but also as part of the immune system (Gao et al., 2022). Anatomically, the small intestine of birds is divided into three parts: duodenum, jejunum, and ileum, with its primary function being the absorption of nutrients, electrolytes, and water (Ito et al., 2009). The duodenum, the first segment, is U-shaped and surrounded by the pancreas, receiving the pancreatic and hepatic ducts. The jejunum extends from the ducts to Meckel's diverticulum (yolk-sac vestige), while the ileum extends from the diverticulum to the ileo-ceco-colic junction (Klasing, 1999; Yamauchi, 2007).

Histologically, a single layer of epithelial cells coats the villus, providing the interface between the body and intra-luminal chyme constituents. Covered with mucus, this layer must maintain a balance between nutrient absorption and preventing harmful substances from entering the body. Stem cells in the crypts proliferate and generate new cells. Cells migrating to the villous differentiate into enteroendocrine cells (producing serotonin, enteroglucagon, or gastrin), goblet cells (mucus-producing cells), or absorptive enterocytes (Wijtten; Langhout; Verstegen, 2012).

In broilers, the enterocyte migration process through the villus takes several days before these cells undergo apoptosis (Yamauchi, 2007).

Overall, villi become shorter and wider, and crypt depth decreases with the distance from the gizzard (Fletcher; Tahseen, 2016). The duodenal mucosa is covered by a thick mucus layer to protect the villi from the excessive acidity of the digesta leaving the gizzard. This segment exhibits a higher villus height compared to the jejunum and ileum (Klasing, 1999; Geyra; Uni; Sklan, 2001). The chickens' large intestine consists of a pair of caeca (finger-like shaped) and a short rectum located between the ileum and cloaca (Saleh; Altaey; Ahmed, 2022).

Cecal tonsils and Peyer's patches are gut-associated lymphoid tissues in chickens, containing dendritic cells, macrophages, plasma cells, $CD4^+$ and $CD8^+$ T cells, natural killer cells, and granulocytes (Broom; Kogut, 2018). Cecal tonsils are situated on the basal part of each caeca, showing diffuse lymphocyte infiltration 3-5 days post-hatch. Plasma cells appear at 14 days, with complete development observed within 35 days (Saleh; Altaey; Ahmed, 2022). Peyer's patches are located at the anti-mesenteric side of the chicken jejunum and in the ileum at the proximal ileocecal transition (Casteleyn et al., 2010). These patches are covered by follicle-associated epithelium containing M cells, which sample gut contents and deliver them to underlying immune cells, inducing antigen-specific immune responses (Zeinali Lathori, 2023).

Intestinal histology is closely tied to intestinal function, with damage causing villus adaptation, activating intestinal absorptive function not by increasing numbers but by fusing into larger villi (Yamauchi; Incharoen; Yamauchi, 2010). This underscores the importance of histological evaluation when assessing intestinal health.

However, in birds, histological evaluation can be challenging, especially under field conditions, as post-mortem autolysis occurs rapidly, beginning within 5-10 minutes in the intestine of broilers (Smyth, 2016). Changes such as epithelial cell loss and lamina propria detachment occur in this process, starting from the villus tip and later in the crypt, making diagnosis challenging due to the complexity of differentiating autolysis from mild lesions (Fletcher; Tahseen, 2016). This rapid autolysis observed in birds poses a challenge in obtaining high-quality histological cuts.

In an effort to standardize a technique ensuring high-quality histological cuts, the first step in this research project was to evaluate different fixatives and sampling methods for intestinal histological evaluation. It was concluded that the Swiss Roll sampling method and 10% buffered formalin fixative were the optimal choices (Souza et al., 2021).

2.3 MICROBIOME

The term “microbiome” refers to the characteristics of a microbial community in a well-defined habitat with distinct physio-chemical properties and activity. The term "microbiota" refers to all members, including bacteria, viruses, protozoa, and fungi, forming the microbiome (Berg et al., 2020). In this section, we will discuss the intestinal microbiome and the functions of relevant microbiota.

The gut microbiome of broilers plays a crucial role in carbohydrate fermentation and the production of short-chain fatty acids (SCFAs). The primary SCFAs include acetic acid, propionic acid, and butyric acid (Faden, 2022; Gao et al., 2022). In brief, acetate is absorbed and metabolized by peripheral tissues, propionate is mainly transported to the liver, and butyrate serves as the primary energy source for enterocytes, contributing to intestinal homeostasis through anti-inflammatory actions (Wong et al., 2006; Hamer et al., 2008; Parada Venegas et al., 2019).

Analysis of 16S rRNA gene sequences from the intestinal content of healthy broilers (at 42 days of age) revealed that Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Cyanobacteria were the most abundant phyla. There was a distinction in phyla abundance across different intestinal segments, with Firmicutes predominating in the duodenum, jejunum, ileum, and colon. However, Bacteroidetes was the dominant phylum in the cecum, Proteobacteria were most abundant in the duodenum, Actinobacteria predominated in the ileum, and Cyanobacteria were more abundant in the jejunum (Xiao et al., 2017).

Nevertheless, the microbial composition varies according to the intestinal segment, with a correlation observed between cloacal and cecal microbial composition (Andreani; Donaldson; Goddard, 2020).

The Firmicutes phylum constitutes the largest bacterial component of microbes in the gut microbiome. These organisms are involved in the digestion and fermentation of starch and play a crucial role in energy production (Oakley et al., 2014). Bacteroidetes are recognized for their ability to degrade complex carbohydrates that escape proximal gut digestion (Cheng et al., 2022). The Proteobacteria phylum is considered a biomarker for intestinal dysbiosis and poor zootechnical performance (Shin; Whon; Bae, 2015; Kollarcikova et al., 2019). Actinobacteria have a lower abundance in the intestinal microbiome but include beneficial microorganisms such as *Bifidobacterium*, a well-known probiotic strain with the capacity to ferment complex carbohydrates and produce short-chain fatty acids (SCFAs) (Jandhyala et al., 2015).

At the family level, *Enterobacteriaceae* can trigger intestinal inflammation by increasing

intestinal lipopolysaccharide abundance, leading to higher gut permeability (Bibbò et al., 2016). The abundance of *Escherichia-Shigella* is associated with poor zootechnical performance in broilers (Rubio et al., 2015).

On the other hand, the families *Lachnospiraceae* and *Ruminococcaceae* are considered beneficial microorganisms. They have the capability to degrade complex plant carbohydrates, such as cellulose and hemicellulose, fermenting them into acetate, propionate, or butyrate, which serve as energy sources for the host (Biddle et al., 2013).

At the family level, *Peptostreptococcaceae* is associated with gut homeostasis and short-chain fatty acid (SCFA) production (Leng et al., 2016; Kisuse et al., 2018). Supplementation with dried plum in heat-stressed birds demonstrated an increase in the ileal abundance of this family, correlating with higher SCFA levels found in the intestinal content of heat-stressed birds (Wasti et al., 2021).

At the genus level, *Lactobacillus* spp. is a well-studied and normal member of the broiler small intestinal microbiota, present in low levels in the caecum in healthy birds (Xiao et al., 2017). Recognized for its probiotic action, this microorganism can ferment complex carbohydrates, producing lactate (Takagi et al., 2016; Souza et al., 2020). However, under conditions of excessive nutrient availability and fermentation with elevated lactate production, this same beneficial microorganism can act as harmful microbiota, lowering the intestinal pH and creating adverse environmental conditions for other beneficial microorganisms, resulting in intestinal dysbiosis (Dey; Ray Chaudhuri, 2022; Souza et al., 2023).

The homeostasis of the gut microbiome can be disrupted by several factors. The rearing system impacts the microbial composition of laying hens, with lower α-diversity observed in cage range hens compared to free-range birds. A distinct difference was also observed in β-diversity analysis. However, birds raised in mixed systems (free-range/cage range or cage range/free-range) showed a similar gut microbial composition. Among the relative abundance of the top 10 microbes, cage range chickens had more *Lactobacillus* and *Akkernansia* at the genus level. Furthermore, free and cage range birds showed differences in 42 genera, with free-range hens having 31 more different microbes at the genus level. The authors discuss that the lower microbial diversity in the cage range system may result from stress and deprivation of natural behaviors (Chen et al., 2019).

As an example of how diet influences the microbiome, complex glycans modulate microbiome metabolic pathways related to protein fermentation and putrefaction, resulting in a higher microbiome protein metabolism index and improved growth performance of broilers (Yan

et al., 2023). Furthermore, caecal microbiome modulation induced by galacto-oligosaccharides and xylo-oligosaccharides affected chicken's meat flavor (Yang et al., 2022).

Lundberg; Scharch;Sandvang (2021) found that high-performance chickens exhibit specific microbiome characteristics, such as higher microbial diversity and microbiome uniformity, increased levels of short-chain fatty acid (SCFA)-producing bacteria, and beneficial taxa like *Lachnospiraceae*, *Faecalibacterium*, *Butyricicoccus*, and *Christensenellales*.

The use of growth promoter antibiotics, such as bacitracin methylene disalicylate (BMD), penicillin G potassium (PP), and salinomycin (SA), disrupted chicken's cecal microbiome homeostasis. BMD, SA, and SA+PP decreased cecal microbiome α-diversity, and β diversity analyses revealed that SA+PP and BMD induced significant changes in the relative abundance of Gram-positive and Gram-negative bacteria (Kairmi et al., 2022).

After the restriction and prohibition of growth promoter antibiotics by certain markets, there was an increase in the incidence of necrotic enteritis (E.C., 2003; Mapa, 2020). Studies examining the effects of a *C. perfringens* challenge on the microbiome showed elevated ileal colonization by clostridia and *E. coli*, accompanied by a reduction in *Lactobacillus* counts (Daneshmand et al., 2022).

It can be concluded that microbiome composition is closely related to the broiler's intestinal health and performance. Although some knowledge on this topic is available, there is still much to be elucidated about microbiome composition and modulation and their relation to health and performance.

2.4 Intestinal Health Challenges

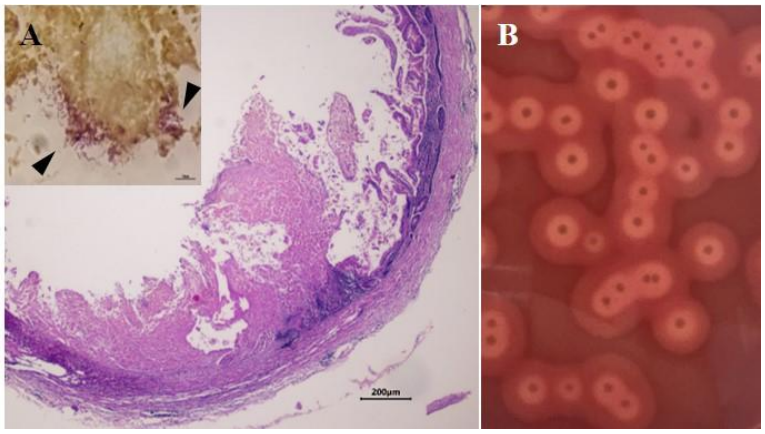
2.4.1 Necrotic Enteritis (NE)

Clostridium perfringens is an anaerobic, gram-positive, spore-former found in a wide range of natural environments, including the human and animal gastrointestinal tract. It can produce at least 20 different toxins (Figure 1) (Juneja; Taneja; Thakur, 2023). *C. perfringens* is the etiological agent of necrotic enteritis in chickens. This disease was controlled in the past with the use of AGPs and ionophore coccidiostats. After their prohibition in the European Union, and with the constant restriction of the molecules available for this purpose, this disease has become an emerging problem (E.C., 2003; Van Immerseel et al., 2016; Mapa, 2020).

It is believed that the economic losses induced by necrotic enteritis (NE) are around 6 billion dollars annually (Wade; Keyburn, 2015). The occurrence of the disease is dependent on

predisposing factors and can manifest as clinical cases, with signs such as dehydration, depression, orange-colored frothy diarrhea, and, in some cases, blood-tinged, along with ruffled feathers, resulting in high mortality. The characteristic gross lesion is intestinal mucosal necrosis coated in a yellow-brown pseudo-membrane filled with necrotic cells and bacterial colonies. At the microscopic level, destruction of intestinal villi is observed (Figure 1A); the subclinical cases are challenging to diagnose and result in performance failures, demonstrating a higher economic impact (Abd El-Hack et al., 2022).

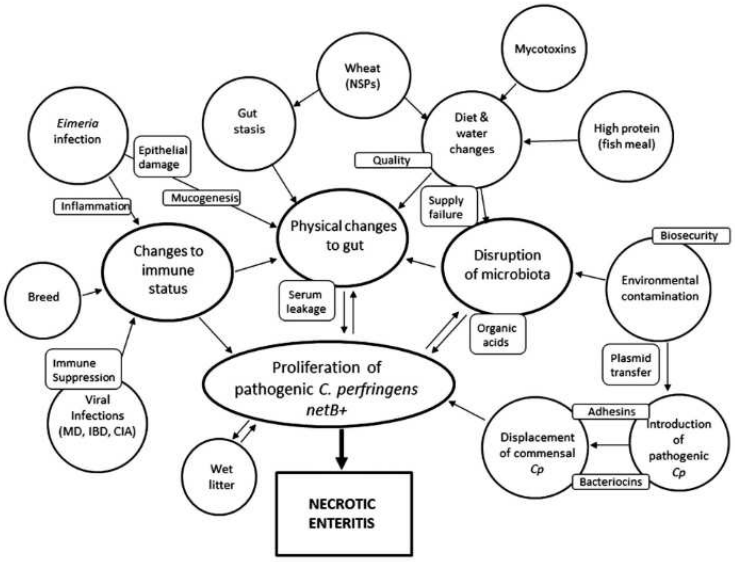
Figure 1. A- Small intestine, HE. Diffuse necrosis of villi, and inflammatory infiltrate in the submucosa. Scale bar 200 µm. Insert- Small intestine, Gram Goodpasture. Gram+ bacilli adhered to luminal cell debris. Scale bar 10 µm. B- Colonies of *Clostridium perfringens* on sheep blood agar reveal a double hemolysis zone, where the inner zone exhibits complete hemolysis, and the outer zone displays partial hemolysis.



Source: the author.

Predisposing factors for necrotic enteritis (NE) may include infectious agents, diet composition (non-starch polysaccharides [NSP], high protein and amino acid concentration), mycotoxins, bacterial toxins, environmental conditions, breed, high stocking density, or any other factor favoring an overgrowth of *C. perfringens* in the bird's intestine (Tsiouris, 2016; Tsiouris et al., 2018). Figure 2 cites the main predisposing factors (Moore, 2016). These factors lead to intestinal changes, such as intestinal stasis, resulting in delayed feed passage, increased digesta viscosity, higher nutrient availability for microbes, pH changes, and barrier failures, all of which promote the proliferation of *C. perfringens* (Paiva; Mcelroy, 2014). Due to the necessity of predisposing factors, experimental reproduction of the disease is challenging. A recent study concluded that obtaining satisfactory results requires an early, repeated *Eimeria* spp. challenge (10-fold the vaccination dose) 4 and 2 days before the start of the *C. perfringens* challenge, along with a fishmeal-supplemented diet as key factors (Dierick et al., 2021).

Figure 2. Necrotic enteritis predisposing factors. Predisposing factors are shown in circles and the major effects of these factors are shown in the ovals. Important factors that may drive the influence of the predisposing factors are shown in the small rectangular boxes. Cp = *C. perfringens*, IBD = infectious bursal disease, CIA = chicken infectious anemia, MD = Marek's disease, NSPs = non-starch polysaccharides



Source: Moore (2016).

In a survey conducted in the United Kingdom involving broiler practitioners and veterinarians, 32.8% of participants reported at least one flock affected by NE in 2001. The median age of affected flocks was 26 days, and wet litter (OR 2.39) and coccidiosis (OR 4.68) showed strong associations with the disease cases (Hermans; Morgan, 2007). In Belgian layer pullet flocks, 12.3% were affected by NE, with coccidiosis (OR 2.09) significantly associated with the diagnosis. Floor systems (OR 2.90) and enriched cage production systems (OR 1.86) were more prone to developing NE compared to aviaries, and the lowest frequency of cases occurred during the winter months (Goossens et al., 2020b).

Regarding strain characteristics, the genotype G, which produces α and net-B toxins, is identified as the primary cause for NE cases in a recent classification (Table 1) (Rood et al., 2018).

Table 1. New *C. perfringens* toxin-based typing scheme

Toxitype	α-toxin	β-toxin	ε-toxin	ι-toxin	CPE	NetB
A	+	-	-	-	-	-
B	+	+	+	-	-	-
C	+	+	-	-	+/-	-
D	+	-	+	-	+/-	-
E	+	-	-	+	+/-	-
F	+	-	-	-	+	-
G	+	-	-	-	-	+

Source: Rood et al. (2018). CPE- *C. perfringens* enterotoxin.

In terms of toxin characteristics, alpha-toxin acts as a phospholipase sphingomyelinase C. It has the ability to bind to intestinal cell membranes, hydrolyze phospholipids, and result in the formation of diacylglycerol. This process activates protein kinase C, triggering the arachidonic acid cascade responsible for synthesizing inflammatory mediators such as leukotrienes, thromboxane, platelet-aggregating factor, and prostacyclin. This cascade leads to blood vessel contraction, platelet aggregation, and myocardial dysfunction, ultimately causing acute death (Paiva; Mcelroy, 2014). Clinical signs induced by alpha-toxin include hemolysis, edema, fever, pain, gas gangrene, and lethality (Sakurai; Nagahama; Oda, 2004). The production of alpha-toxin can be easily assessed by strain plating on sheep blood agar, resulting in an inner complete zone of hemolysis caused by perfringolysin O and an outer incomplete hemolysis zone caused by the alpha toxin (Figure 1, B) (Goossens et al., 2016).

On the other hand, NetB forms discrete pores in the cellular membrane, leading to an influx of ions (e.g., Ca, Na, Cl), resulting in cell rounding and osmotic cell lysis. Another mechanism of cell death induced by NetB is programmed necrosis triggered by Ca influx, associated with calpain activation and release of cathepsins from lysosomes. Additionally, there is deregulation of mitochondrial activity leading to excessive ROS production and decreased ATP levels (Paiva; Mcelroy, 2014). Nowadays, NetB is considered a key factor for NE pathogenesis (Keyburn et al., 2008). Park et al. (2015) isolated NetB-positive strains in 47% of commercial chickens found dead with NE lesions."

The correlation between *C. perfringens* α-toxin and netB-toxin concentrations in litter and chick mortality was assessed in broiler farms undergoing anticoccidial control programs using either drugs or vaccines. Farms employing drugs for *Eimeria* spp. control exhibited the highest *C. perfringens* concentrations in litter at 2 weeks (∼105–106 spores/g). A strong correlation was observed between chick mortality in the 0-3 week period and the presence of

netB (OR 0.42–0.48) or α-toxin (OR 0.55–0.67) in the litter on the birds' first day of age. Conversely, in farms with vaccination programs, the highest concentration of *C. perfringens* (~105–106 spores/g) in litter was observed on the chicks' first day of age. A strong correlation was found between the presence of netB on the first day and chick mortality in the 0–1 week period (OR 0.67) (Jenkins et al., 2020). This data underscores the significance of netB in the occurrence of necrotic enteritis (NE).

Another emerging *C. perfringens* toxin is TpeL, first reported in 2007. It induces cytopathic effects in Vero cells, characterized by cell enlargement, the appearance of rounded cells, and the formation of cell aggregates that eventually detach from the well surface (Amimoto et al., 2007). TpeL enters target cells through self-mediated entry, mono-glycosylates Ras proteins, inhibiting cell growth signaling, and inducing apoptosis (Guttenberg et al., 2012). Evaluation of TpeL's contribution to inducing NE lesions in chickens revealed that animals inoculated with netB and TpeL-positive strains exhibited higher average macroscopic lesion scores than those inoculated with netB-positive and TpeL knockout strains. This suggests that TpeL potentiates the effect of other virulence factors from *C. perfringens* (Coursodon et al., 2012). Another study demonstrated that broilers challenged with different netB and TpeL positive strains displayed typical NE lesions, reduced growth rate, and highlighted TpeL as a key factor for lesion reproduction in an NE experimental model without *Eimeria* spp. challenge (Gu et al., 2019).

The ability of *C. perfringens* to penetrate the intestinal mucus layer is a crucial factor in disease pathogenesis. Intestinal mucus primarily consists of glycosylated proteins known as mucins. Anaerobic incubation of chicken mucin O-glycans with *C. perfringens* revealed that sialic acid was predominantly removed (Macmillan et al., 2019). Sialidases, including NanH, NanI, and NanJ, produced by *C. perfringens*, hydrolyze glycoproteins and glycolipids to release free sialic acid. These enzymes play roles in cell metabolism, adhesion, proliferation, immune function, and infectious processes under various conditions (Wang, 2020). Van Damme et al. (2022) demonstrated that *in vitro*, NanI may aid in the colonization of type G *C. perfringens* strains by providing nutrition for the bacteria and unmasking cryptic host ligands used for adherence. The presence of free sialic acids also increased toxin production, contributing to host tissue degradation and eventual tissue necrosis. NanI is considered another *C. perfringens* virulence factor that acts through two mechanisms: removing sialic acids from mucus and cell surface molecules, reducing charge repulsion effects, and exposing underlying *C. perfringens* cell adhesion sites that were otherwise masked (Li et al., 2021).

Chitinases, encoded by the plasmid NELoc-1, are additional mucus-degrading enzymes of *C. perfringens*. These enzymes likely act by hydrolyzing the chitobiose core of N-linked glycoproteins, contributing to mucus colonization and degradation (Prescott et al., 2016).

Adhesion is crucial for disease pathogenesis, and the ability to bind to collagen is strongly correlated with the induction of necrotic enteritis cases (Wade et al., 2015). The VR–10 B locus possesses an adhesin encoded by the gene cnaA, responsible for binding to collagen types IV and V, and gelatin. A cnaA mutant exhibited reduced adherence to collagen types IV and V and gelatin, and it was no longer capable of causing necrotic enteritis in chickens (Wade et al., 2016). The VR-10B locus also encodes a sortase-dependent pilus involved in collagen binding, specifically to types I, II, and IV (Lepp et al., 2021). *C. perfringens* autolysins have been reported to have a fibronectin-binding protein that acts as a fibronectin receptor (Aono et al., 2023).

In the past years, antimicrobial growth promoters (AGPs) were the tool used by practitioners to prevent necrotic enteritis cases, and antimicrobials remain the first choice for treating clinical disease cases. The most commonly used molecules include bacitracin, virginiamycin, amoxicillin, lincomycin, and tylosin (Brennan et al., 2001; Lanckriet et al., 2010; Agunos et al., 2019). Another strategy widely adopted in commercial production is the use of ionophore anticoccidials, as necrotic enteritis cases are often associated with *Eimeria* spp. infection (Lanckriet et al., 2010; Prescott et al., 2016).

Antimicrobial resistance has been reported in *C. perfringens* strains originating from necrotic enteritis (NE) cases. Strains (n=30) isolated from birds with or without clinical lesions exhibited complete resistance to gentamicin, neomycin, streptomycin, apramycin, and colistin. Additionally, they showed partial resistance to tetracycline, trimethoprim-sulfamethoxazole, sulfisoxazole, erythromycin, bacitracin, and clindamycin, indicating prevalent antimicrobial resistance in *C. perfringens*, irrespective of disease manifestation (Park et al., 2015). Another study found that 53% of isolates had a multidrug-resistant profile, including streptomycin, gentamicin, erythromycin, tetracycline, and bacitracin (Mwangi et al., 2019).

Due to high rates of antimicrobial resistance in bacteria from animal origin, the use of antimicrobials has been restricted in animal production for many markets over the years (E.C., 2003; Mapa, 2020). However, various alternatives have been investigated to replace antimicrobial use, including probiotic bacteria, prebiotics, enzymes, organic acids, essential oils, bacteriophages, vaccines, and oral supplementation with hyperimmune egg yolk antibodies (Johnson et al., 2019; Adhikari et al., 2020; Abd El-Ghany et al., 2022; Goo et al., 2023).

Necrotic enteritis is a complex and multifactorial disease. Recently, a clinical

manifestation named necro-hemorrhagic enteritis has been reported in the USA, characterized by the small intestine showing typical hemorrhagic features, with the mesentery often engorged with blood. Experts suggest renaming this manifestation as necro-hemorrhagic enteritis instead of necrotic enteritis (Goossens et al., 2020a).

2.4.2 Non-starch Polysaccharides (NSP)

Non-starch polysaccharides (NSP) are macromolecules consisting of monosaccharides linked by β glycosidic bonds present in the plant cell wall. These molecules resist degradation by endogenous enzymes in monogastric animals and thus reach the colon almost undigested (Căpriţă; Căpriţă; Julean, 2010). NSP can be categorized as cellulose, hemicellulose (including xylans, xyloglucans, mannans, glucomannans, and β-(1,3;1,4)-glucan), and pectic substances (Heredia; Jiménez; Guillén, 1995; Zhang et al., 2021). This discussion focuses on two water-soluble hemicellulose NSPs: arabinoxylans (AX) and β-mannans (MN). Table 2 provides the average concentration of both NSPs in various feed ingredients.

Arabinoxylans are the most prevalent NSP in cereals, with their content influenced by crop management and weather conditions (Knudsen, 2014). Higher temperatures during the grain-filling period and organic cropping systems increase AX content (Korge et al., 2023). Due to their viscosity-enhancing, gel-forming, water-absorbing, emulsion, and foam-stabilizing properties, arabinoxylans find many industrial applications (Izydorczyk, 2021).

Water-soluble NSPs, such as AX and MN, negatively impact nutrient utilization in monogastric animals. Their encapsulating effect on potentially available nutrients in the intestinal content, along with their viscous properties, hinders the digestion process, reducing passage rates and the digestibility of other nutrients (Knudsen, 2014; Jha et al., 2019; Gomez-Osorio; Oh; Lee, 2021). This lower digestibility induced by NSPs also results in morphological changes in the intestinal tract, such as increased length and weight (Jorgensen et al., 1996).

In broilers, the consumption of diets rich in arabinoxylans is associated with zootechnical performance failures, increased intestinal lesional scores, and heightened viscosity (Lei et al., 2016). Arabinoxylans also negatively impact nutrient digestibility, evidenced by reductions in total dry matter digestibility, retained nitrogen, total short-chain fatty acids (SCFAs), as well as concentrations of isovaleric, acetic, and butyric acids in the cecal content of broilers. This effect extends to the ileal and cecal microbiota, with lower microbiota counts and higher pH observed (Morgan et al., 2019). In broilers, the fermentative ability of arabinoxylans increases with age, being notably poor in the first two weeks and peaking at 35 days, the average slaughter age

(Bautil et al., 2019). An immunomodulatory effect of arabinoxylan ingestion has been reported, manifesting as higher humoral responses and lower *Eimeria* spp. oocysts per gram of droppings (Akhtar et al., 2012).

Another crucial and abundant type of non-starch polysaccharides (NSPs) is β-mannans, which include glucomannans, galactomannans, galactoglucomannans, and linear mannans, primarily found in leguminous seeds serving as carbohydrate storage forms (Shastak et al., 2015). Guar gum (GG) and palm kernel meal are the richest sources of β-mannans (Table 2).

Diets rich in guar gum are employed in experimental models to study the effects of β-mannans on monogastric animals. In terms of zootechnical performance, the ingestion of GG-rich diets leads to detrimental effects, including lower body weight gain, average daily gain, feed daily intake, and a higher feed conversion ratio (Owusu-Asiedu et al., 2006; Mishra et al., 2013; Saeed et al., 2019; Souza et al., 2023). These negative impacts are attributed to its high-water absorption capacity, resulting in enhanced digesta viscosity, lowered passage rate, and reduced digestibility (Maisonnier; Gomez; Carré, 2001; Latham et al., 2018).

The ingestion of β-mannan-rich diets, characterized by low digestibility and high nutrient availability in the hindgut, is associated with dysbiosis. This dysbiosis involves the proliferation of opportunistic microbiota (such as *E. coli*, *Shigella, Clostridium*) and carbohydrate fermenters (like *Roseburia* and *Lactobacillus*), coupled with a reduction in beneficial microbiota (including *Faecalibacterium* and the family *Ruminococcaceae*) (Souza et al., 2023).

A predisposing factor for NE is increased intestinal content viscosity. However, chickens fed diets containing either guar gum or pectin exhibited low intestinal lesional scores. This outcome is explained by the animals not fully consuming the diets, likely reducing the number of challenge organisms ingested (Branton et al., 1997).

Guar gum ingestion also elicited intestinal immune responses due to its structural similarity to cell wall components of certain pathogens (Saeed et al., 2019; Wang, W. et al., 2021). It is known that β-mannans may stimulate the immune system, inducing intestinal inflammation, however this effect could be mitigated by dietary enzymes supplementation (Li et al., 2010; Ferreira et al., 2016; Arsenault et al., 2017; Saeed et al., 2019). Conversely, Van Hung;Suzuki (2017) described that intact guar gum has an anti-inflammatory effect in the small intestine through toll-like receptor 2 and dectin-1 pathways. Partially hydrolyzed guar gum failed to activate these pathways, suggesting that the structure of intact guar gum is necessary to trigger the anti-inflammatory response.

Table 2. Average concentration of arabinoxylans and glucomannans on feed ingredients

Feed ingredient	Reported concentration		Reference
	Arabinoxylan	β-mannans	
Corn grains	4.7% DM	0.14% DM	(Knudsen, 2014; Hove et al., 2018)
Wheat grains	7.3% DM	0.27% DM	(Knudsen, 2014; Hove et al., 2018)
Oats grains	6.1% DM	0.31% DM	(Hove et al., 2018; Barjuan Grau et al., 2023)
Barley grains	8.4% DM	0.42% DM	(Knudsen, 2014; Hove et al., 2018)
Rice	9.5% DM	0.27% DM	(Knudsen, 2014; Hove et al., 2018)
Dehulled soybean meal 48% CD	1.1% DM	0.59% DM	(Hove et al., 2018; Ward, 2021)
Guar gum meal ≈40% CD	-	6.93% DM	(Hove et al., 2018)
Palm Kernel meal	3% of total NSP	78% of total NSP	(Düsterhöft; Posthumus; Voragen, 1992)

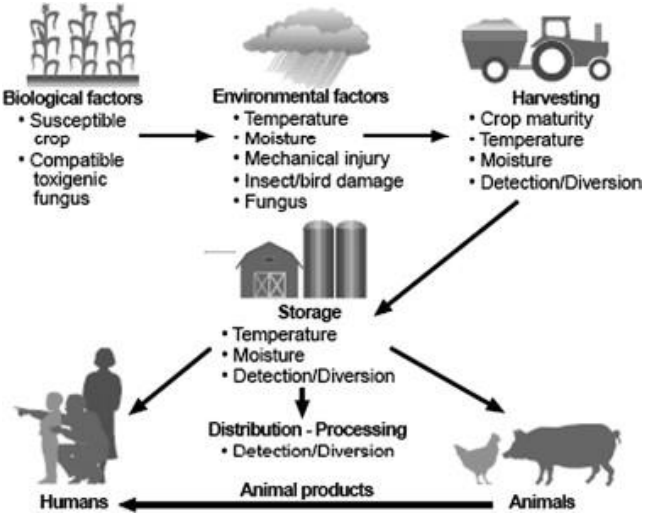
CD – crude protein; DM - dry matter

In this section, we have explored the adverse impacts of non-starch polysaccharides (NSP) on broiler performance and intestinal health. As we will investigate later, researchers are actively seeking solutions to mitigate these anti-nutritional effects and enhance nutrient utilization. This pursuit aims to enable the incorporation of alternative feed ingredients in poultry diets.

2.4.3 Mycotoxins – DON

The term "mycotoxin" translates to "fungus poison." With approximately 700 described molecules, these secondary metabolites of filamentous fungi have a low molecular weight (100-700 g/mol) and are too small to be recognized by the immune system (Peltomaa; Benito-Peña; Moreno-Bondi, 2018; Stroka; Gonçalves, 2019). Contamination by toxigenic fungi can occur at various stages of grain production, involving factors related to cultivation, harvesting, storage, and environmental conditions. Global warming, leading to increased temperature and humidity, favors fungal growth, exacerbating the issue of mycotoxin contamination (Figure 3) (Paterson; Lima, 2010).

Figure 3. Factors affecting mycotoxin occurrence in the food chain.



Source: Paterson;Lima (2010)

The most relevant mycotoxins for human and animal health include Aflatoxins (AFLA - B1, B2, G1, G2), T-2 toxins, Deoxynivalenol (DON), Zearalenone (ZEA), Fumonisin B1 (FB1), and Ochratoxin A (OTA) (Fao, 2001). In the first semester of 2023, *Fusarium* mycotoxins were the most prevalent in South America, with FUM present in 45% of all samples, followed by ZEA (44%) and DON (26%). In the different regions of Europe, the main mycotoxins were DON (68-44%), ZEA (77-42%) and FUM (78-14%) (Dsm, 2023). DON is considered the primary threat to farm animals (Maresca, 2013).

DON is globally present and is produced by *Fusarium graminearum* and *F. culmorum*, mainly contaminating maize, wheat, barley, and oats (Streit et al., 2013; Lee; Ryu, 2017). DON, a small sesquiterpenoid, possesses an epoxide group that allows it to bind to ribosomes, activating protein kinases, modulating gene expression, inhibiting protein synthesis, and inducing cell toxicity (Maresca, 2013). Although the full understanding of why fungi produce mycotoxins remains elusive, in the case of DON, its production is known to increase the pathogenicity of fungi, causing the disease called Fusarium Head Blight (Scab) (Stroka; Gonçalves, 2019).

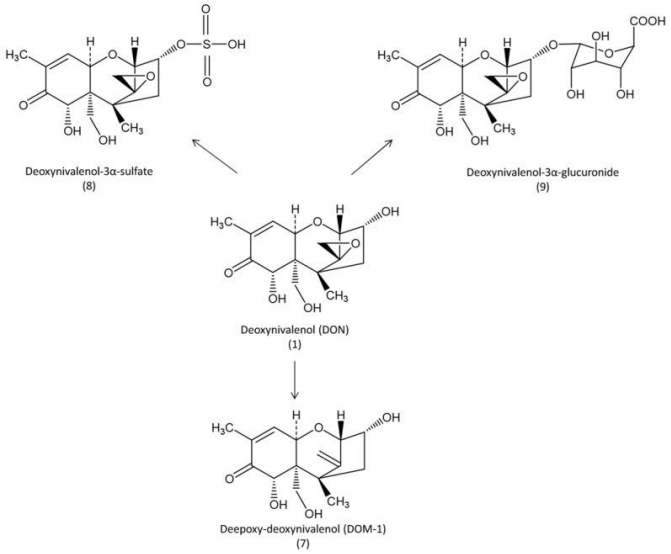
A survey of Brazil's rice harvest (2014-2015) revealed the presence of *F. graminearum* in 24.7% of the analyzed samples, with simultaneous contamination by at least three mycotoxins, most frequently zearalenone, beauvericin, and acetylated forms of deoxynivalenol (Moreira et al., 2020). Barley samples from São Paulo and Rio Grande do Sul (2017 harvest) showed contamination with at least one *Fusarium* toxin. In Rio Grande do Sul, levels of DON, ZEA, Enniatins B, and B1 were significantly higher, with over 20% of the samples exceeding European and Brazilian tolerated level legislations for DON and ZEA. Both regions exhibited co-

contamination with Beauvericin, DON, Enniatins, Nivalenol, and ZEA (Iwase et al., 2020).

In the European Union, the recommended maximum levels for broiler chicken feed are 5 mg kg^{-1} (E.C., 2006). Brazil's current recommended maximum levels for DON in chicken feed vary by category: 0.2 mg kg^{-1} in the initial phase, 0.5 mg kg^{-1} in growth, and 1 mg kg^{-1} for the final phase of breeder and layer feed (Lamic, 2023). However, Brazil only has legislation regulating aflatoxin levels in animal feed and feed ingredients, with a maximum tolerance level of 50 µg kg^{-1} (Mapa, 1988).

Birds are considered one of the most tolerant species to the toxic effects of DON, attributed to the low degree of absorption and systemic distribution of this mycotoxin in tissues. Metabolization mainly occurs in the intestine and liver, reducing systemic circulation DON levels. The interaction with microbiota in both the crop and intestine is a crucial factor in decreasing the available amount of DON for absorption (Maresca, 2013; Ghareeb et al., 2015). A toxicokinetic study revealed approximately 20% oral bioavailability in birds, with the highest concentrations of DON found in the small intestine, liver (mainly in bile), and kidneys (Figure 4) (Guerre, 2015).

Figure 4. Metabolic pathways of deoxynivalenol in birds



Source: Guerre (2015).

The primary metabolic route for birds is sulfonation in the liver, a process that enhances the solubility of toxic compounds, facilitating their excretion through bile and urine. Prolonged exposure to DON results in increased levels of liver enzymes responsible for metabolization (Guerre, 2015). In mammals, metabolism mainly occurs through de-epoxidation, yielding the

metabolite deepoxi-deoxynivalenol (Dänicke; Brezina, 2013; Guerre, 2015). The primary metabolite of DON in chickens is DON-3α-sulfate (Schwartz-Zimmermann et al., 2015).

Despite birds' tolerance to DON, various adverse effects have been linked to its consumption in chickens. These effects include a reduction in daily weight gain (positively correlated with the supplied mycotoxin amount), an increase in feed conversion, mortality, liver weight, and a decrease in productive efficiency (Andretta et al., 2011).

Changes in intestinal morphology have been linked to the dietary contamination level of DON (Awad et al., 2011; Yunus et al., 2012). The primary small intestinal segments affected by the toxic effects of DON are the duodenum and jejunum (Awad et al., 2007). Birds exposed to DON at 5 mg kg^{-1} demonstrated a decrease in the absolute and relative weight of the small intestine, as well as a reduction in the height and width of the duodenal villi (Awad et al., 2006). Another study reported a decrease in villus height and an increase in crypt depth in the duodenum and jejunum of chickens after ingesting DON at 10 mg kg^{-1} (Wu et al., 2018).

Wang;Hogan (2019) assessed the effects of DON at 11.4 mg kg^{-1} on performance and intestinal morphometry at different growth stages in broiler chickens. Zootechnical parameters were reduced only when exposure occurred between 22-34 days. In the initial phase (15-21 days), there was a decrease in villi height in the jejunum and ileum; at 22-34 days, only the jejunum was affected, with lower villus height and a higher villus: crypt ratio. Continuous exposure throughout the production cycle (1-34 days) resulted in a reduction in villi height and crypt depth in the ileum. The authors concluded that chickens challenged with DON in the initial phase can recover losses in performance, with animals in the growth phase being more sensitive to deleterious effects on performance.

The ingestion of diets contaminated with DON (5-10 mg kg^{-1}) has been associated with a smaller intestinal absorption area, a decrease in the function and expression of glucose, fructose, and amino acid transporters, performance failures, and also allows higher nutrient availability in the small intestine, which may predispose to dysbiosis (Ghareeb et al., 2015). Bacteroidaceae, Ruminococcaceae, Lachnospiraceae, and Clostridiaceae are the main bacterial families present in the cecum of chickens (Aguzey et al., 2019). However, DON can cause a decrease in the genera *Oscillospira, Clostridium, Ruminococcaceae*, and an increase in an unassigned *Clostridiales* genus (Lucke et al., 2018).

Changes in the intestinal barrier have also been linked to DON ingestion, resulting in alterations in paracellular permeability (Awad et al., 2019) and lower expression of genes encoding claudin-1, occludin (Wu et al., 2018), and mucin (Antonissen et al., 2015). However,

an increase in the number of goblet cells has also been described (Wang; Hogan, 2019; Souza et al., 2020). Increased intestinal permeability facilitates the translocation of microorganisms, an event associated with increased expression of TRL 4 mRNA in the duodenum and jejunum after exposure to DON (7.54 mg kg^{-1} for three weeks) (Osselaere et al., 2013). Cytotoxic effects on intestinal lymphocytes (Awad et al., 2014), as well as modulation of the immune response and decreased vaccine response to infectious bursal disease and Newcastle virus, have been linked to increased susceptibility to disease in chickens exposed to DON (Awad et al., 2013). DON's intestinal toxicity is attributed to mitochondrial dysfunction, characterized by a failure in respiratory capacities and ROS overproduction, consequently initiating apoptosis early (Wang, S. et al., 2021).

Trichothecenes, including DON, can either stimulate or impair chickens' humoral immunity (Awad; Ghareeb; Böhm, 2012). Serum IgA mediates the transport of antigens from blood circulation into the bile, and hepatobiliary transport of IgA from blood serves to reinforce the intestinal supply of secretory IgA, protecting the mucosal surface against infection and preventing the penetration of antigens from the gut lumen (bacteria and viruses) (Awad; Ghareeb; Böhm, 2012). Broiler chickens fed a fusarium mycotoxin (DON, fusaric acid, ZEA, and 15-acetyldeoxynivalenol) contaminated diets linearly reduced peripheral blood monocytes and B-cell counts at the end of the experimental period (56 days), whereas the T-cell count on day 28 responded quadratically to the contaminated diets. The contaminated diets did not significantly alter serum or bile immunoglobulin (IgM, IgG, and IgA) concentrations, contact hypersensitivity to dinitrochlorobenzene, or antibody response to SRBC (Swamy et al., 2004). However, in another study similar to the previously cited, biliary IgA concentrations responded linearly and quadratically to the level of contaminated grains (Swamy et al., 2002).

The production of reactive oxygen species (ROS) is a physiological event; however, when there is an imbalance between ROS production and antioxidant capacity, oxidative stress is established. This imbalance can trigger various responses such as increased cell proliferation, apoptosis, or necrosis (Halliwell, 2007). Exposure to DON, in both in vitro and in vivo models, has been shown to increase ROS levels and induce oxidative stress in tissues (Ghareeb et al., 2015). Table 3 presents data related to the interaction between DON and the oxidative stress response in chickens.

Table 3. Deoxynivalenol (DON) affects reactive oxygen species (ROS) production and antioxidant defense.

Species	Mycotoxin; contamination level; exposure period	Organ	Effects	Reference
Broilers ***in vitro***	DON 0.001; 0.01; 0.1 e 1 µg/mL 24, 48 and 72 h	Lymphocytes	All DON concentrations increase MDA levels in 24 h 48-72 h only 1 µg/mL increase MDA	Lautert et al. (2014)
Broilers ***in vitro***	DON different concentrations 100, 500, 1000, e 2000 ng/mL 24 h	Chicken embryonic fibroblasts (DF-1 cells)	Increase MDA (500, 1000 and 2000 ng/mL) Decrease GSH (500, 1000 e 2000 ng/mL) Decrease SOD (100, 500, 1000 e 2000 ng/mL)	Li, D. et al. (2014)
Broilers ***in vitro***	DON 0-50 µg/mL 48 h	Splenic lymphocytes	Increase ROS	Ren et al. (2015)
Broilers	DON 4.6 mg kg^{-1} 2 weeks	Ileum	Increase mRNA HMOX	Antonissen et al. (2015)
Broilers	DON 10 mg kg^{-1} 5 weeks	Liver	No change in TBARS	Awad et al. (2012)
Broilers	DON 10 mg kg^{-1} 5 weeks	Jejunum	Increase TBARS	Awad et al. (2014)
Broilers	DON and ZEA 3.4 mg kg^{-1} 2 weeks	Liver	Increase MDA Decrease GPx	Borutova et al. (2008)
		Kidney	Increase MDA	
	DON and ZEA 8.2 and 8.3 mg kg^{-1} 2 weeks	Liver	Increase MDA Decrease GPx	
Broilers	AFLA 102.08 mg kg^{-1}; ZEA 281.92 mg kg^{-1}; FUMO 5,874.38 mg kg^{-1}; DON	Serum	Increase MDA Decrease T-SOD	Jiang et al.

	2.038,96 mg kg^{-1} 6 weeks			(2014)
Broilers	DON 7.54 mg kg^{-1} 3 weeks	Liver	Decrease mRNA HIF1, HMOX Increase mRNA XOR	Osselaere et al. (2013)
		Jejunum	Increase mRNA HMOX and XOR	
Broilers	*Low:* T-2 toxin 0.23 mg kg^{-1} and DON 4.96 mg kg^{-1} *Medium:* T-2 toxin 1.21 mg kg^{-1} and DON 12.38 mg kg^{-1} *High:* T-2 toxin 2.42 mg kg^{-1} and DON 24.86 mg kg^{-1} 1 week	Liver	At 3 and 7 days decrease MDA (low and high level) At 3 days increase mRNA GAPDH (medium and high level) At 3 days increase mRNA GPx4 (high level)	Pelyhe et al. (2018)
Broilers	DON 10 mg kg^{-1} 6 weeks	Serum	Decrease SOD No change in GSH-Px and MDA	Yang et al. (2017)
		Jejunum	No change in SOD Increase MDA	
Broilers	DON 19.3 mg kg^{-1} 7 days	Jejunum	Increase TBARS; decrease GSH, ABTS	Souza et al. (2020)
		Ileum	Decrease GSH	
		Liver	Increase NBT; decrease GSH, FRAP	
		Kidney	No change in relation to control group	
Broilers	DON 7 mg/kg 21 days	Jejunum	No change in ROS production Decrease CAT, GSH-PX and T-AOC	Wang et al. (2022)
		Liver	Increase 8-OHdG, MDA ans T-ROS Decrease T-AOC	

ROS: 8-OHdG- 8-hydroxy-2′-deoxyguanosine; TBARS- thiobarbituric acid reactive substances; MDA- malondialdehyde; T-SOD- total superoxide dismutase; XOR– xanthine oxidoreductase; Antioxidant defense: ABTS- 2,20-azino-bis (3-ethylbenzothiazoline6-sulphonic acid); FRAP- ferric-reducing antioxidant power; GSH- reduced glutathione; GSH-PX/GPx/GPx4– glutathione peroxidase; HMOX- heme oxygenase; GPDH- Glycerol-3-phosphate dehydrogenase; T-AOC- total antioxidant capacity; T-ROS- total reactive oxygen species.

2.5 ADDITIVES TO MODULATE THE INTESTINAL HEALTH

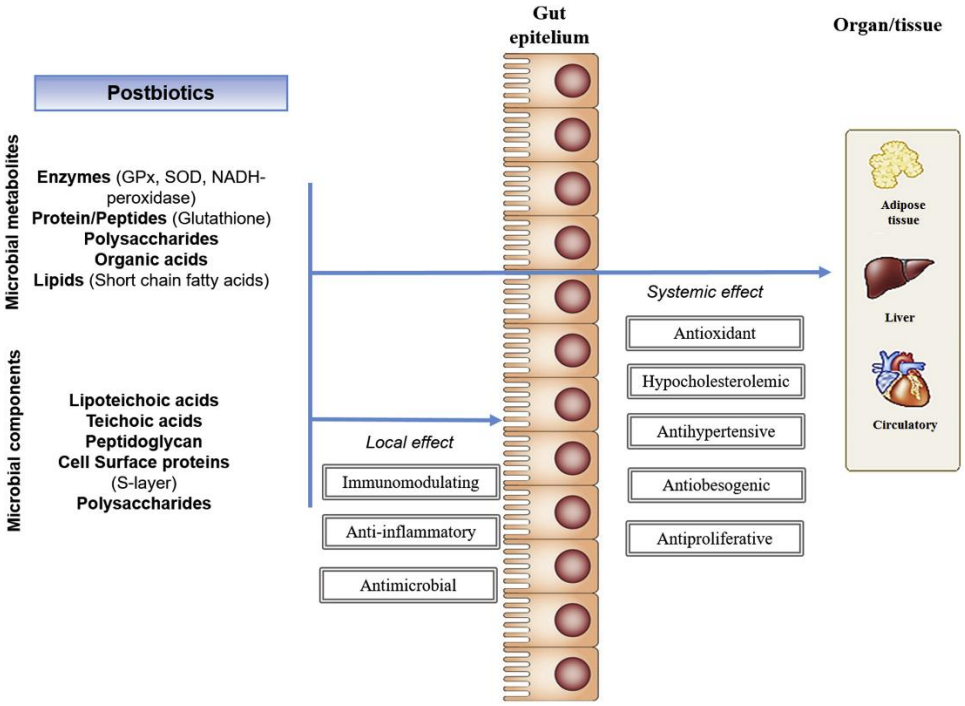
2.5.1 Microorganisms

The use of microorganisms for fermenting food and beverages to preserve them or treat diseases dates to ancient times, around 7,000 BC (Ozen; Dinleyici, 2015). Even Hippocrates recognized the significance of digestion, stating that "death sits in the bowels" and that "bad digestion is the root of all evil" (Gasbarrini; Bonvicini; Gramenzi, 2016). Throughout history, humanity has been utilizing fermented products and contemplating the effects of microorganisms on host health.

First, it is essential to briefly define some terms: prebiotics, probiotics, synbiotics, paraprobiotics, and postbiotics. Prebiotics are food ingredients that resist digestion by endogenous enzymes in the gastrointestinal tract, reaching the colon where they are fermented by the bacterial microbiota, stimulating the growth and activity of beneficial bacterial species. Examples include inulin, glucooligosaccharides (GOS), fructooligosaccharides (FOS), lactulose, and derivatives of galactose and β-glucans (Martyniak et al., 2021). Probiotic, meaning "for life," refers to microorganisms that, when administered in adequate doses, provide some benefit to the host (Hossain; Sadekuzzaman; Ha, 2017). Synbiotics combine probiotics and prebiotics, exhibiting a synergistic beneficial effect on host health. Paraprobiotics are dead probiotic microbial cells and cell constituents, considered a safe option for immunosuppressed hosts due to the absence of the risk of bacterial translocation (Martyniak et al., 2021). Postbiotics encompass bacterial metabolites and cell-free supernatants (CFS), soluble factors secreted by live bacteria or released after bacterial lysis. These byproducts offer physiological benefits to the host, including short-chain fatty acids, enzymes, peptides, teichoic acids, peptidoglycan-derived muropeptides, endo- and exo-polysaccharides, cell surface proteins, vitamins, plasmalogens, and organic acids (Figure 5) (Aguilar-Toalá et al., 2018).

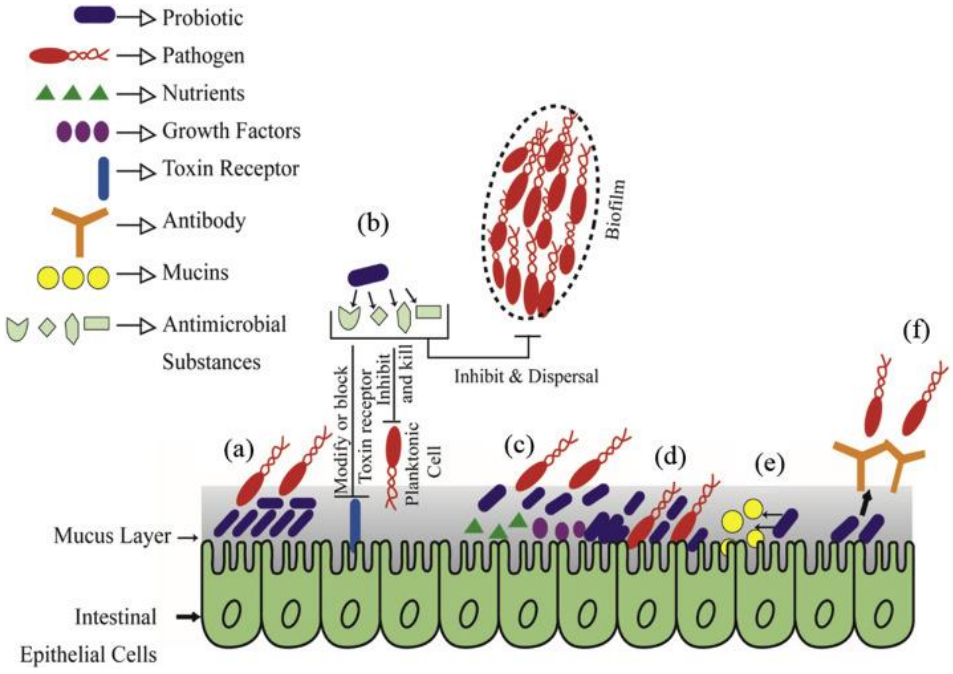
In this section, the primary focus will be on the effects of probiotics on poultry gut health. Genera commonly employed as probiotics include *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, *Carnobacterium*, *Enterococcus*, *Streptococcus, Pediococcus, Propionibacterium, Leuconostoc, Bacillus* species, *Saccharomyces* yeasts, and *Aspergillus* molds (Hossain; Sadekuzzaman; Ha, 2017). The ideal probiotic should be non-pathogenic, non-toxic, able to withstand gastric acidity, adhere to and persist in the intestinal epithelium, and produce substances with antimicrobial properties (Figure 6) (Angelakis, 2017).

Figure 5. Some postbiotics and their potential local and systemic positive effects in the host.



Source Aguilar-Toalá et al. (2018).

Figure 6. Mechanisms of probiotic action. a) Competitive exclusion of pathogenic microorganisms. b) Production of antimicrobial substances. c) Competition for nutrients and growth factors. d) Increase adhesion to intestinal mucosa. e) Enhanced epithelial barrier function. f) Enhanced Ig A secretion (immune stimulation).



Source: Hossain; Sadekuzzaman;Ha (2017).

Probiotics exert their regulatory influence on the microbiota, immune system, and gut nerve center primarily through effector molecules, including bacteriocins, peptidoglycan, teichoic acid, exopolysaccharides, secretory proteins, and short-chain fatty acids. Bacteriocins, small-molecule polypeptides or proteins secreted by probiotics, are natural antimicrobial substances that modulate host health by inhibiting harmful bacteria, thereby influencing the host immune system (Gao et al., 2022).

Lactobacillus spp. is known for their ability to produce bacteriocins. For instance, the *L. gasseri* strain KT7 produces an antimicrobial peptide known as gassericin KT7, found in the culture supernatant, which effectively inhibits the growth of *Clostridium*, *Listeria*, and *Enterococcus* bacteria (Zhu; Liu; Wu, 2000). Additionally, strains of *L. salivarius* and *L. plantarum*, when fermenting carbohydrates in poultry feed, create conditions with optimal pH and concentrations of lactic and acetic acids that can inhibit the growth of bacteria like *E. coli*, *S.* Typhimurium, and *C. perfringens* (Murry; Hinton; Morrison, 2004).

In vivo studies in broilers, where *Lactobacillus* spp. was ingested (mean log CFU/g feed: 1st week 5.85; 2nd week 5.96; 3rd week 5.84; 4th week 5.85; 5th week 5.88; 6th week 5.78), have demonstrated beneficial effects. These include an increase in small intestinal weight (jejunum and ileum) and villi:crypt ratio, along with a reduction in the number of enterobacteria in the ileum (Olnood et al., 2015). Another study reported enhanced food intake and weight gain in chickens supplemented with *L. plantarum* S27 (Benbara et al., 2020).

Supplementation with *Pediococcus acidilactici* has been shown to enhance jejunal villus height and crypt depth, increase the jejunal mRNA expressions of MUC2, claudin-1, and occludin, and elevate the relative abundance of *Bacteroides* in broilers fed a newly harvested corn diet. This supplementation has proven effective in ameliorating osmotic diarrhea induced by the experimental diet (Luo; Wang; Yuan, 2023). Moreover, broilers supplemented with *B. pullicaecorum* exhibited a lower abundance of *Campylobacter* spp. in the caecum, reduced levels of *Enterococcus* and *Escherichia*/*Shigella* spp. in the ileum and demonstrated an improved feed conversion ratio (Eeckhaut et al., 2016).

Probiotics have demonstrated the potential to enhance feed efficiency and productivity in laying hens while also improving egg quality. This improvement is evidenced by reduced yolk cholesterol levels, enhanced shell thickness, and increased egg weight (Angelakis, 2017).

2.5.1.1 Microorganisms and DON

Contamination of feedstuff by mycotoxins is a complex issue influenced by various factors, encompassing pre-harvest (such as environmental conditions, plant susceptibility, and crop management), harvest (grain moisture), and post-harvest factors (drying, fungal control in silos) (Cast, 2003). These metabolites pose a threat to both animal and human health (Grenier; Applegate, 2013; Ostry et al., 2017; Jia et al., 2023). Environmental conditions, beyond control, contribute to varying contamination levels within the same region and are exacerbated by climate changes linked to global warming, amplifying mycotoxin contamination challenges (Paterson; Lima, 2010).

Cereals and their by-products constitute the basis of monogastric animal diets, with dried distillers' grains with solubles (DDGS), a maize by-product, gaining global usage in animal feed. An analysis of more than 98% of samples from 2017 to 2020 revealed mycotoxin contamination, with fumonisin B1 (98.8%, mean 3,207 μg kg^{-1}) and B2 (97.6%, mean 1,243 μg kg^{-1}), aflatoxin B1 (32.3%, mean 1.47 μg kg^{-1}), ZEA (18.01%, mean 18.2 μg kg^{-1}), and DON (12.9%, mean 59.6 μg kg^{-1}) being the most prevalent (Mallmann et al., 2021). In maize samples from Paraná, southern Brazil (2012-2013), DON showed a prevalence of 58%, with a mean concentration of 184.4 μg kg^{-1} (Oliveira et al., 2017).

To address mycotoxin contamination in animal production, various strategies have been proposed to mitigate negative effects. The use of organic or inorganic adsorbents, such as yeast cell wall, bentonites, aluminum silicates, or synthetic cholestyramine polymers, is a prominent approach. These compounds bind to mycotoxins in the feed, allowing them to pass through the gastrointestinal tract without absorption. Additionally, the use of bio-transforming agents like bacteria, fungi, or enzymes capable of converting mycotoxins into non-toxic or less toxic metabolites has been explored (Oliveira et al., 2014; Vila-Donat et al., 2018).

Identification of microorganisms capable of detoxifying mycotoxins poses a challenge. Enhanced success in isolating microorganisms with the ability to detoxify deoxynivalenol (DON) from broiler chickens' intestinal content can be achieved through prior exposure to DON-contaminated diets (10 mg kg^{-1}), incubation in enriched medium (DON 100 μg/mL), and the application of the Polymerase Chain Reaction Denaturing Gradient Gel Electrophoresis (PCR-DGGE) technique. The predominant genera of isolated microorganisms were *Clostridiales, Anaerofilum, Collinsella*, and *Bacillus* (Yu et al., 2010).

Numerous studies conducted *in vitro*, *ex vivo*, and *in vivo* have highlighted the beneficial

effects of lactic acid bacteria on mycotoxin exposure. These effects are attributed to the interaction of lactic acid bacteria with mycotoxins, leading to a decrease in their absorption (Maidana; Souza; Bracarense, 2022). Gratz; Mykkänen;El-Nezami (2005) demonstrated *in vitro* that a combination of *L. rhamnosus* and *Propionibacterium* spp. neutralized 38-47% of aflatoxin B1 (5 μM) in solution and 25% of aflatoxin B1 (5 μM) in an *ex vivo* model. Another *in vitro* study, using *L. fermentum*, *L. plantarum*, and *L. casei*, achieved a reduction of 25-61% in the concentration of aflatoxin B1 (5 μg/mL) (Fazeli et al., 2009). El-Nezami et al. (2002) demonstrated *in vitro* the ability of viable and heat-inactivated strains of *L. rhamnosus* (two strains) and *Propionibacterium* (one strain) to neutralize mycotoxins produced by the *Fusarium* spp. genus, including DON (20 μg/mL), resulting in a reduction between 18-93%. Notably, only one strain of viable *L. rhamnosus* showed significant efficacy in reducing mycotoxins when compared to inactivated strains.

Moreover, *L. plantarum* strains isolated from wheat exhibited the capability to inhibit *F. graminearum* growth and remove DON *in vitro*. This effect was observed for both viable and heat-inactivated bacteria (Franco et al., 2011). An *ex vivo* study using the same strains of *L. plantarum* demonstrated a protective effect on jejunum villi integrity when exposed to DON (10 μM) in an explant model, utilizing the culture supernatant (Maidana et al., 2017).

In an *in vitro* study, it was identified that *L. paracasei* LHZ-1 cell walls could remove up to 40.7% of DON (media concentration of 50 μg/mL), culture supernatant up to 10.5%, and cellular lysate 8.9%. Laser scanning confocal microscopy revealed the formation of complexes as DON bound to the surface of bacterial cells (Zhai et al., 2019).

In broiler chickens, a diet contaminated with DON (10 mg kg^{-1}) induced a decrease in villi height and thickness in the duodenum and jejunum. However, dietary supplementation with *Eubacterium* spp. DSM 11798 (Biomin®) provided a protective effect on challenged animals (Awad et al., 2006). Another study found that a DON diet (10 mg kg^{-1}) induced liver hypertrophy in chickens, resulting in higher enzyme activity, and reduced total protein and albumin synthesis. Nonetheless, supplementation with *Lactobacillus* spp. ameliorated these adverse effects (Azizi et al., 2023).

Yang et al. (2017) evaluated the antioxidant capacity of a strain of *L. plantarum* isolated from the gastrointestinal tract of a healthy bird *in vitro*. Subsequently, they conducted an *in vivo* experiment where birds received a DON-diet (10 mg kg^{-1}), with or without *L. plantarum* supplementation. While no differences were observed in zootechnical parameters, the probiotic supplementation decreased oxidative stress in the jejunal mucosa and increased the expression

of the junction protein claudin-1 compared to the DON group. The study concluded that the probiotic was effective in antioxidant action and protecting the integrity of the intestinal barrier.

Supplementation with *L. plantarum* JM113 induced beneficial effects on DON (10 mg kg^{-1}) challenged birds. This supplementation increased duodenal expression of IL-10 and IL-12, ileal secretory IgA, and the expression of genes related to intestinal absorption and metabolism. The DON challenge disturbed the intestinal microbiome by decreasing *Proteobacteria, Escherichia, Lactobacillus*, and *Prevotella*. Nevertheless, probiotic supplementation increased the relative abundance of beneficial bacteria, such as *Bacteroidetes*, *Roseburia, Anaerofustis, Anaerostipe*, and *Ruminococcus bromii*. Consequently, there was a higher caecal content of propionic acid, butyric acid, and total short-chain fatty acids (Wu et al., 2018). Another study with the same strain concluded that *L. plantarum* JM113 supplementation ameliorates DON-induced apoptosis and intestinal inflammation by manipulating the bacterial community composition (Yang et al., 2020).

Beneficial effects of viable, heat-inactivated, and culture supernatant of *Lactobacillus* spp. (pool of 1 strain of *L. reuteri* and 2 strains of *L. plantarum*) supplementation were reported in chickens fed a DON-contaminated diet (19.3 mg kg^{-1}). DON increased the intestinal and liver lesion score, reduced villi height, increased crypt depths, raised the number of jejunal intraepithelial lymphocytes, induced an oxidative stress response, and reduced the antioxidant capacity in the small intestine and liver. In contrast, *Lactobacillus* spp. supplementation was able to revert these effects (Souza et al., 2020).

Biotransformation is one of the proposed mechanisms of action for probiotics against mycotoxins. *In vitro, L. rhamnosus* SHA113 efficiently degraded 60% of DON under optimal conditions. This transformation, observed only with live cells, converted DON into 3-epi-DON, a less toxic metabolite. *In vivo* studies demonstrated that *L. rhamnosus* SHA113 reduced the toxicity of DON to the immune system and organs, evidencing its potential as an effective strategy for mycotoxin detoxification (Qu et al., 2019).

Strategies aimed at mitigating the negative effects of DON have acquired increased interest, with probiotic bacteria emerging as a valuable alternative to alleviate DON toxicity in broiler chickens. These microorganisms have shown the ability to modulate intestinal health and oxidative responses, thereby reducing the toxic impact of DON. However, it is crucial to determine the optimal strains and presentation forms, whether viable or inactivated. In conclusion, while alternatives exist to diminish the toxic effects of DON in chickens, further research is essential to elucidate detoxification mechanisms, enhance detoxification efficiency,

and reduce associated costs. Additionally, consideration should be given to the fact that many adsorbents used come from non-renewable sources, such as clays and rocks.

2.5.1.2 Microorganisms and Necrotic Enteritis (NE)

Due to the escalating rates of antimicrobial multiresistance in bacteria originating from poultry, numerous markets have prohibited and/or restricted the use of antimicrobials as growth promoters (E.C., 2003; MAPA, 2020). Consequently, diseases like NE, previously controlled by growth promoters, have re-emerged (Immerseel et al., 2009).

C. perfringens is a natural inhabitant of the chicken's intestinal microbiome, and the disease can manifest as clinical cases characterized by high mortality and clinical signs, or as subclinical cases causing decreased nutrient absorption and digestion, impaired feed conversion ratio, and reduced body weight gain, with low or absent mortality. Subclinical cases are more challenging to diagnose and have a higher economic impact (Abd El-Hack et al., 2022). Due to antimicrobial resistance, there is growing pressure on animal production, especially in poultry farming, to eliminate the use of antimicrobials and seek eco-friendly alternatives. Probiotics, paraprobiotics, and postbiotics emerge as promising solutions in this scenario. Probiotic strains can enhance the antioxidant system, thereby neutralizing oxidative stress induced by NE, and effectively suppress apoptosis by increasing the levels of Bcl-2 family proteins (Obianwuna et al., 2023).

In the context of NE clinical disease, mortality rate, and intestinal lesion scores, supplementation through drinking water with a postbiotic (*L. acidophilus* fermentation product) or probiotic (*B. subtilis* and *B. lischeniformis*) in combination with amoxicillin, and postbiotic without antibiotics, showed positive effects. Changes in the microbial population were also observed, with a reduction in intestinal *C. perfringens* and total coliform CFU/mL. All treatments (probiotic, postbiotic either alone or in combination with amoxicillin, administered through water or feed) were effective in maintaining counts lower than the positive control (Abd El-Ghany et al., 2022).

Johnson et al. (2019) observed improvements in zootechnical performance, a reduction in mortality, lesion scores, and *C. perfringens* counts in chickens supplemented with a postbiotic (fermented product containing organic acids produced from a cocktail containing *Pediococcus acidilactici, L. reuteri, Enterococcus faecium*, and *L. acidophilus*). The study concluded that the postbiotic had an immunomodulatory effect, reducing the proinflammatory

response induced by the infection.

Supplementation with *B. pullicaecorum* resulted in fewer birds with NE lesions (Eeckhaut et al., 2016). NE-challenged birds supplemented with *B. subtilis* exhibited increased weight gain, upregulation of genes coding for tight junction proteins (CLDN1, JAM2, TJP1), cytokines (IL12, interferon gamma, TGFβ), and Toll-like receptors (TLR5, TLR21), suggesting enhanced immunity and intestinal integrity (Keerqin et al., 2021). The effects of *B. subtilis* supplementation after NE challenge were also evaluated. Supplemented chickens displayed improved zootechnical performance, lower intestinal lesion scores, and higher cecal acetic and butyric acid concentrations. However, supplementation with *B. subtilis* was unable to reverse the changes in the gut microbiome induced by *C. perfringens*. (Aljumaah et al., 2020).

NE-challenged broilers supplemented with *L. plantarum* 1.2567 showed increased average daily gain, reduced small intestine gross lesion scores, improved ileal morphology, reduced intestinal inflammation (nitric oxide concentration and myeloperoxidase activity, chTNF-α, and IL-1β), and oxidative stress (MDA levels). Additionally, it enhanced antioxidant system activity (SOD and CAT activity), reducing NE effects (Cao et al., 2019).

In subclinical NE, chickens supplemented with *L. johnsonii* BS15 exhibited better average daily gain, improved liver function, and a positive effect on beneficial gut microbiota. Another study with the same strain revealed that *L. johnsonii* BS15 supplementation positively influenced antioxidant capacity, improved intestinal and serum immune responses, which may be one of the mechanisms contributing to its beneficial effects in healthy and SNE-afflicted broilers (Qing et al., 2017; Wang et al., 2017; Wang, H. et al., 2018).

L. johnsonii, L. salivarius, L. reuteri, and *L. crispatus* were individually supplemented to NE-challenged birds. Overall, all strains induced modulation of the immune response and intestinal microbiota composition, characterized by an increase in phyla *Actinobacteria* and *Firmicutes,* resulting in a reduction in NE severity (Shojadoost et al., 2022).

In vitro, L. plantarum inhibited the growth of *C. perfringens* by reducing pH levels. Furthermore, it significantly increased the mRNA expression levels of host defense peptides, suppressed the gene expression of proinflammatory cytokines and pattern recognition receptors, and decreased the adhesion of *C. perfringens* to IPEC-J2 cells (Zhou et al., 2021).

Broiler chickens challenged with *C. perfringens* exhibited an intestinal apoptotic status, as evidenced by the upregulation of Bax and p53, and downregulation of Bcl-2 expression. Inflammation was observed in the ileum mucosa with higher levels of IFN-γ, IL-6, IL-1β, iNOS, and IL-10. *C. perfringens* infection also altered the microbiota composition and metabolic

pathways, including enhancing B-vitamin biosynthesis, peptidoglycan biosynthesis, amino acid biosynthesis, and ribonucleotide biosynthesis, while decreasing pyruvate fermentation to acetate and lactate II pathway. *Lactobacillus plantarum* 16 effectively reversed these effects (Gong et al., 2020).

Currently, studies have associated the ingestion of diets contaminated by *Fusarium* toxins (DON and FUM) with more severe NE cases, impaired gut immune response, failure in the intestinal barrier, and changes in caecal microbial composition (Antonissen et al., 2014; Shanmugasundaram et al., 2022; Shanmugasundaram et al., 2023). However, there is no data about a single solution to address both issues.

2.5.2 Enzymes

Feed enzymes are proteins capable of accelerating chemical reactions. An ideal enzyme exhibits high specific activity at body temperature, an optimal pH profile, pepsin resistance, and high thermostability (Velázquez-De Lucio et al., 2021; Bedford et al., 2022). The use of feed enzymes began in the 1980s. Xylanases and mannanase, classified as fiber-degrading enzymes within the hydrolases class, are widely employed in the poultry industry (Bedford et al., 2022).

Xylanases, enzymes that degrade arabinoxylan, are typically derived from microorganisms such as *Bacillus* spp. and *Trichoderma reesei* (Velázquez-De Lucio et al., 2021). Supplementation of diets with xylanases results in improvements in zootechnical performance (average daily gain, feed conversion ratio), reduced digesta viscosity in the jejunum and ileum, lower intestinal lesion scores, and improved carcass, leg, and liver weights in broilers (Lei et al., 2016; Van Hoeck, Veerle et al., 2021). Additionally, a modulation of the intestinal wall barrier has been reported, with enhanced expression of claudin-1, occludin, and MUC2 (Luo; Wang; Yuan, 2023). The effects on the gut microbiome are attributed to a prebiotic effect of xylan-oligosaccharides, resulting in the stimulation of *Lactobacillus* spp. and *Bacillus* spp. growth (Lei et al., 2016; Van Hoeck, V. et al., 2021).

β-mannanases are enzymes synthesized by strains of *Aspergillus niger, Paenibacillus lentus, Bacillus subtilis,* or *Trichoderma longibrachiatum*, capable of hydrolyzing the β-1,4-glycosidic linkage of β-mannans (Li, Y.-F. et al., 2014; Saeed et al., 2019).

β-mannanase is well-known for its positive impact on zootechnical performance metrics such as feed conversion ratio (FCR), body weight gain (BWG), and average daily gain (ADG), as well as for reducing intestinal viscosity, enhancing digestibility of alimentary

content, influencing gut morphometry, and promoting short-chain fatty acid (SCFA) production. These benefits stem from the release of mannan-oligosaccharides and improved nutrient utilization (Hussain; Rehman; Khalid, 2012; Caldas et al., 2018; Latham et al., 2018; White et al., 2021; Souza et al., 2023). Recently, the positive effects of β-mannanase supplementation were observed in the intestinal microbiome of broilers fed a guar gum diet. The enzyme restored most of the microbiota shifts induced by the experimental diet, contributing to the preservation of gut homeostasis (Souza et al., 2023).

A diet rich in β-galactomannan led to enhanced activation of jejunal signaling pathways related to immune responses, that leads to intestinal inflammation. However, β-mannanase significantly attenuated the majority of these immune-related signaling pathways. Changes in specific metabolic and gut function pathways, including insulin signaling, ErbB pathway, adipocytokine signaling, mTOR pathway, tight junctions, and adherens junctions, were also observed in animals supplemented with β-mannanase. These alterations were suggested as potential action mechanisms by which the enzyme improved performance and feed conversion (Arsenault et al., 2017).

Studies associating β-mannanase with mannans and infectious agents have reported several advantages. For instance, broilers challenged with *C. perfringens* exhibited improved performance and reduced intestinal lesional scores when receiving a β-mannanase-supplemented diet (Jackson et al., 2003). In a coccidiosis challenge, the protozoa reduced intestinal microbiota diversity, and β-mannanase mitigated these effects by preserving beneficial microbiota members such as *Lactobacillus*, *Ruminococcaceae*, and *Akkermansia*, while reducing non-beneficial *Bacteroides* (Bortoluzzi et al., 2019).

Additional benefits attributed to the use of β-mannanase include the improvement of intestinal tonus and the intestinal integrity index, a reduction in excessive cellular sloughing, prevention of litter eating habits, protection against gizzard erosion, and a decrease in pododermatitis lesion scores (Poulsen; Mathlouthi; Bargen, 2023). In conclusion, fiber-degrading enzymes, such as β-mannanase, offer viable alternatives to reduce production costs by enhancing digestive processes and supporting gut homeostasis, particularly during challenges with infectious agents.

3 HYPOTHESES

I. Ingestion of a diet contaminated with Deoxynivalenol (19.3 mg kg^{-1}) induces toxic effects on broiler chickens' intestinal health and exacerbates necrotic enteritis cases. The use of a *Lactobacillus* spp. pool in three different presentation forms (viable, heat-inactivated and culture supernatant) can mitigate the negative effects caused by the mycotoxin and *Clostridium perfringens* challenge.

II. Ingestion of a *β*-mannan rich diet can impair broilers chickens' zootechnical performance and gut homeostasis; supplementation with a dietary *β*-mannanase can reduces these effects.

4 GOALS

4.1 MAIN GOAL

I. Evaluate the impact of nutritional and sanitary challenges on the intestinal health of broiler chickens.

II. Investigate the effects of a *Lactobacillus* spp. pool on the immune response and intestinal health of broiler chickens fed a DON-contaminated diet and challenged with *Clostridium perfringens.*

III. Examine the effects of a β-mannan-rich diet and the supplementation with a commercial β-mannanase on the intestinal health of broiler chickens.

4.2 SPECIFIC GOALS

I. Screen strains of *Lactobacillus* spp. exhibiting inhibitory activity against *C. perfringens* and assess their potential to mitigate toxic effects of DON.

II. Examine the impact of a mixture of *Lactobacillus* spp. presented as viable cells, heat-inactivated, and their culture supernatant on the macroscopic characteristics of the intestine in broiler chickens.

III. Investigate the effects of treating broiler chickens challenged with DON and *C. perfringens* with the *Lactobacillus* spp. mixture (viable cells, heat-inactivated, and culture supernatant) on intestinal morphology and morphometry.

IV. Assess the impact of DON and *C. perfringens* challenge on the humoral immune response in broilers, considering the treatment with the *Lactobacillus* spp. pool in viable, heat-inactivated, and culture supernatant forms.

V. Examine the influence of a β-mannan-rich diet (inclusion level 1.7 %) and the supplementation with a commercial β-mannanase on the zootechnical performance and intestinal morphometry of broiler chickens.

VI. Analyze the impact of a β-mannan-rich diet and the supplementation with a commercial β-mannanase on the ileal and cecal microbiome of broiler chickens.

VII. Evaluate the effects of a β-mannan-rich diet and the supplementation with a commercial β-mannanase on cecal concentrations of short and branched-chain fatty acids in broiler chickens.

5 MANUSCRIPT A – MODULATION OF BROILER INTESTINAL CHANGES INDUCED BY *CLOSTRIDIUM PERFRINGENS* AND DEOXYNIVALENOL THROUGH PROBIOTIC, PARAPROBIOTIC, AND POSTBIOTIC SUPPLEMENTATION [1]

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ABSTRACT

Deoxynivalenol (DON) is a predisposing factor for necrotic enteritis. This study aimed to investigate the effects of a DON and *Clostridium perfringens* (CP) challenge on the intestinal morphology, morphometry, oxidative stress, and immune response of broilers. Additionally, we evaluated the potential of a *Lactobacillus* spp. mixture as an approach to mitigate the damage induced by the challenge. One-day-old broiler chickens (n = 252) were divided into seven treatment groups: Control, DON, CP, CP + DON, VL (DON + CP + viable *Lactobacillus* spp. mixture), HIL (DON + CP + heat-inactivated *Lactobacillus* spp. mixture), and LCS (DON + CP + *Lactobacillus* spp. mixture culture supernatant). Macroscopic evaluation of the intestines revealed that the CP + DON group exhibited the highest lesion score, while the VL and HIL groups showed the lowest scores. Microscopically, all *Lactobacillus* spp. treatments

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mitigated the morphological changes induced by the challenge. DON increased levels of reactive oxygen species (ROS) in the jejunum, and CP increased ROS levels in the jejunum and ileum. Notably, the *Lactobacillus* spp. treatments did not improve the antioxidant defense against CP-induced oxidative stress. In summary, a *Lactobacillus* spp. mixture, whether used as a probiotic, paraprobiotic, or postbiotic, exerted a partially protective effect in mitigating most of the intestinal damage induced by DON and CP challenges.

Keywords: intestinal health; mycotoxins; detoxification; immunity; necrotic enteritis

Key Contribution: The combined challenge of deoxynivalenol (DON) and *Clostridium perfringens* exacerbates necrotic enteritis lesions in broilers, yet supplementation with a *Lactobacillus* spp. mixture effectively mitigates the resulting intestinal changes, highlighting its potential as a protective measure.

INTRODUCTION

Intestinal health plays a key role in broiler performance and productivity (Yegani; Korver, 2008). The growing concern regarding the emergence of multi-resistant bacteria from poultry has led many markets to prohibit or restrict the use of antimicrobials as growth promoters (E.C., 2003; Mapa, 2020). Consequently, maintaining intestinal health in poultry flocks has become increasingly challenging, resulting in the reemergence of intestinal diseases such as necrotic enteritis (NE) (Van Immerseel et al., 2016).

Necrotic enteritis (NE) is a bacterial disease caused by strains of *Clostridium perfringens* (CP) (Kulkarni et al., 2022). CP is a Gram-positive, anaerobic, spore-forming bacterium that is a natural component of the poultry gut microbiota (Abd El-Hack et al., 2022). This disease occurs when there is an abnormal increase in the population of *C. perfringens* in the gastrointestinal tract (GIT), combined with predisposing factors such as coccidia infection, diets rich in non-starch polysaccharide grains, and exposure to mycotoxins, among others. Virulent strains produce the plasmid-encoded NetB toxin (Antonissen et al., 2014; Dierick et al., 2019; Mwangi et al., 2019; Abd El-Hack et al., 2022). NE can be manifested as either clinical (resulting in high mortality rates) or subclinical (leading to growth performance failures), with an estimated annual cost of approximately USD 6 billion, equivalent to USD 0.05 per chick (Wade; Keyburn, 2015).

Contamination with mycotoxins is a growing concern in feedstock due to climate change (Paterson; Lima, 2010). Mycotoxin exposure significantly contributes to the occurrence of NE, and poultry are frequently exposed to deoxynivalenol (DON) (Antonissen et al., 2014; Guo et al., 2021; Shanmugasundaram et al., 2022). DON is one of the most prevalent mycotoxins contaminating finished feed and raw commodities worldwide (Biomin, 2023). In poultry, DON exposure has been linked to villus atrophy, failure in intestinal barrier function, increased intraepithelial lymphocyte infiltration, heightened goblet cell abundance, intestinal oxidative stress, and disruption of gut microbiome diversity (Osselaere et al., 2013; Awad; Zentek, 2015; Souza et al., 2020; Von Buchholz et al., 2022; Jia et al., 2023). Consumption of DON has additionally been linked to increased susceptibility to coccidiosis and necrotic enteritis, particularly in more severe cases (Antonissen et al., 2014; Grenier et al., 2016; Shanmugasundaram et al., 2022).

Probiotics consist of beneficial microorganisms that can enhance host health through various mechanisms (Cremon et al., 2018). Paraprobiotics, on the other hand, refers to dead probiotic microbial cells and their constituents, making them a preferred choice for immunosuppressed hosts due to the absence of the risk of bacterial translocation (Martyniak et al., 2021). Postbiotics encompass bacterial metabolites, cell-free supernatant (CFS), and soluble factors (products or metabolic byproducts) produced by live bacteria or released following bacterial lysis (Aguilar-Toalá et al., 2018). *Lactobacillus* spp. strains, in different forms—be it live cells, heat-inactivated cells, or culture supernatants—have demonstrated protective effects in chickens individually challenged with either DON or CP (Cao et al., 2019; Gong et al., 2020; Abd El-Ghany et al., 2022; Kulkarni et al., 2022; Maidana; Souza; Bracarense, 2022).

This study aimed to investigate the impact of a dual challenge involving DON and CP on intestinal morphology, morphometry, immune response, and oxidative stress in broilers. Additionally, we have evaluated the efficacy of a *Lactobacillus* spp. mixture, administered as a probiotic (live cells), paraprobiotic (heat-inactivated cells), and postbiotic (culture supernatant), as a potential alternative to mitigate the damage induced by both of these factors.

MATERIALS AND METHODS

1. Study Location and Ethical Approval

This study was conducted at the avian medicine experimental facilities at Universidade Estadual de Londrina, Londrina, Paraná, Brazil, and received approval from the institutional

ethics committee for the use of animals (*Comitê de Ética no Uso de Animais* CEUA-UEL, protocol number 12433.2018.03, approval date 24 September 2018).

2. Animals and Treatments

One-day-old broiler chickens ($n = 252$), Ross 308 lineage, were housed in cages with water, feed, and heating provided, following lineage guidelines (Aviagen, 2018). The animals were divided into seven treatment groups ($n = 36$ each), as follows: Control—uncontaminated diet; DON—diet containing DON at 19.3 mg kg^{-1}; CP—uncontaminated diet + *Clostridium perfringens* challenge; CP + DON—diet containing DON at 19.3 mg kg^{-1} + *C. perfringens* challenge; VL—diet containing DON at 19.3 mg kg^{-1} + *C. perfringens* challenge + viable *Lactobacillus* spp. mixture; HIL—diet containing DON at 19.3 mg kg^{-1} + *C. perfringens* challenge + heat-inactivated *Lactobacillus* spp. mixture; LCS—diet containing DON at 19.3 mg kg^{-1} + *C. perfringens* challenge + *Lactobacillus* spp. mixture culture supernatant.

3. Diets

The experimental diets were formulated to meet the nutritional requirements of the animals (Table 3), consisting of three different diets used during the trial period: Diet 1 (0–6 days)—primarily composed of corn (48.16%) and soybean meal (43.70%), free of DON; Diet 2 (7–14 days)—composed of corn (15%), soybean meal (35.31%), and wheat (40.13%); Diet 3 (15–23 days)—composed of corn (15%), wheat (40.43%), and fish meal (35.31%). Fish meal was added to diet 3 to elevate the crude protein level and create an intestinal environment favorable for the experimental induction of necrotic enteritis (Drew et al., 2004).

Table 3. Composition of the experimental diets.

Ingredients (g/kg)	Diet 1 0–6 Days	Diet 2 7–14 Days	Diet 3 15–23 Days
Corn	481.6	150	150
Soya bean meal (46% CP)	437	353.14	-
Soybean oil	32	54	54
Wheat	-	404.33	404.33
Fishmeal	-	-	353.14
Dicalcium phosphate	25	9.28	9.29
Limestone	1.5	13.17	13.16
Sodium chloride	6	5.03	5.04
Premix	5	5	5
L-lysine HCL	0.968	2.1	2.08
DL-methionine	3.36	3	2.99
L-threonine	0.38	0.98	0.98
Nutritional Levels			
Energy mcal/kg	2975	3050	3010
Protein (%)	24.27	23.31	25.61
Linoleic acid (%)	3.355	-	-
Calcium (%)	0.971	0.878	2.910
Phosphorus available (%)	0.463	0.310	1.563
Lysine dig (%)	1.307	1.256	1.418
Methionine dig (%)	0.646	0.600	0.869
Methionine + cistine dig (%)	0.967	0.929	1.176
Threonine dig (%)	0.863	0.829	0.931
Tryptophan dig (%)	0.277	0.271	0.190
Sodium (%)	0.225	0.218	0.451

Premix composition per kg of product: Iron 8,400.00 mg; Copper 3,200.00 mg; Manganese 13.60 g; Zinc 10.80 g; Iodine 146.00 mg; Selenium 52.00 mg; Vitamin A 2,500,000.00 UI; Vitamin D3 420,000.00 UI; Vitamin E 6,000.00 UI; Vitamin K3 500.00 mg; Vitamin B1 500.00 mg; Vitamin B2 1,600.00 mg; Niacin 7,000.00 mg; Vitamin B6 900.00 mg; Folic acid 200.00 mg; Biotin 36.00 mg; Vitamin B12 16,000.00 mcg; Choline 80.00 g; Methionine 178.20 g.

On the 7th day, DON-challenged groups (excluding the control) began receiving a DON-contaminated diet containing 19.3 mg kg^{-1} (Figure S1). The crude DON extract used to contaminate the diets was provided by the Laboratory of Mycology, Luiz de Queiroz College of Agriculture, University of São Paulo. A blend (standard diet + DON) was prepared at the Universidade Estadual de Londrina facilities using a commercial feed mixer. The diets were sent to Lamic laboratory (Santa Maria—RS/Brazil), where the mycotoxins levels were assessed using the HPLC/MS-MS method. Three diet samples were collected throughout the experimental period: first, 0–6 days (diet 1); second, 7–23 uncontaminated diet (diets 2 and 3); and third, 7–23 DON-contaminated diet (diets 2 and 3). The results of the mycotoxin (deoxynivalenol, aflatoxins, fumonisins, and zearalenone) analysis are shown on Table 4.

Table 4. Mycotoxin contamination levels of the experimental diets used in the trial, as determined by HPLC/MS-MS

Mycotoxin	Contamination Level [µg kg^{-1}]		
	1–6 Days Uncontaminated Diet	7–23 Days Uncontaminated Diet	7–23 Days Contaminated Diet
DON	<LOQ	200	19,309.4
AFB1	<LOQ	<LOQ	<LOQ
AFB2	<LOQ	<LOQ	<LOQ
AFG1	<LOQ	<LOQ	<LOQ
AFG2	<LOQ	<LOQ	<LOQ
FB1	252.9	<LOQ	<LOQ
FB2	<LOQ	<LOQ	<LOQ
ZEA	31.4	<LOQ	4878.7

LOQ: limit of quantification. DON: deoxynivalenol; AFB1: aflatoxin B1; AFB2: aflatoxin B2; AFG1: aflatoxin G1; AFG2: aflatoxin G2; FB1: fumonisin B1; FB2: fumonisin B2; ZEA: zearalenole. LOQ: DON, 200 µg kg^{-1}; AFB1, AFB2, AFG1, AFG2, 1 µg kg^{-1}; FB1, FB2, 125 µg kg^{-1}; ZEA, 20 µg kg^{-1}.

4. Necrotic Enteritis Induction

Animals in the CP, CP + DON, VL + DON, HIL + DON, and LCS + DON groups underwent necrotic enteritis induction. They were orally challenged with 4000 oocysts of *Eimeria* spp. from a commercial vaccine (Livaccox® Paulínia, Brazil) and a 10-fold dose of a commercial Gumboro disease vaccine (Bursa F® Campinas, Brazil) (Shojadoost; Vince; Prescott, 2012; Sivaseelan et al., 2013) on the 14th day. The non-challenged groups received 1 mL of sterile PBS to replicate the same stress.

A *C. perfringens* type G, netB positive strain from the Avian Medicine UEL collection was used to challenge the birds. The strain was grown in BHI (Brain Heart Infusion, HiMedia Sumaré, Brazil) broth at 37 °C for 18 h under anaerobic conditions using a commercial kit (GasPak® Becton Dickinson Osasco, Brazil). From the 16th to the 22nd day, animals received 1 mL of fresh CP culture (approximately 4×10^8 CFU/mL) via oral gavage twice daily (Figure 1). On each challenge day, an inoculum sample was 10-fold diluted and plated on SFP agar® (Becton Dickinson Osasco, Brazil), followed by incubation under anaerobic conditions to determine the CFU count. The non-challenged groups received 1 mL of BHI broth to simulate the same stress.

5. Lactobacillus spp. Mixture Administration

Animals in the VL, HIL, and LCS groups received 1 mL of a *Lactobacillus* spp. mixture (approximately 2.2×10^9 CFU/mL) via oral gavage every other day throughout the experimental period (Figure 1). Groups not supplemented with the *Lactobacillus* spp. pool received 1 mL of sterile MRS (De Man, Rogosa, and Sharpe medium, HiMedia Sumaré, Brazil) broth.

The *Lactobacillus* spp. mixture comprised an equal quantity of three strains: two isolated from broiler chickens (*L. reuteri* and *L. plantarum*, not deposited in GenBank) and one from wheat (*L. plantarum*—accession number CP053912) in previous studies (Franco et al., 2011; Rocha et al., 2014; Maidana et al., 2021). The strains were grown separately in MRS broth and incubated at 37 °C for 24 h under microaerophilic conditions. Samples were provided in three different forms: (i) a fresh culture of viable *Lactobacillus* spp. mixture; (ii) a heat-inactivated culture of *Lactobacillus* spp. mixture, and (iii) a supernatant culture from a heat-inactivated *Lactobacillus* spp. mixture. The inactivation and mixture preparation followed previously described methods (Franco et al., 2011; Souza et al., 2020). Cell density was assessed daily through 10-fold dilution and plating.

6. Sample Collection

Throughout the experimental period, four samplings were conducted. On days 7, 14, and 20, ten animals per treatment group were euthanized, and on day 23, 6 animals per treatment were used for biological sample collection. The intestinal samples underwent macroscopic lesion scoring, histological examination, ELISA (enzyme-linked immunosorbent assay), and oxidative stress response assessments.

- *Macroscopic intestinal lesion score*

On the 23rd day, 6 animals per treatment were euthanized, and an intestinal lesion score was determined following previously described criteria, ranging from 0 to 5 (Cravens et al., 2013).

- *ELISA*

Intestinal fluid was collected from 10 animals per treatment at 7, 14, and 20 days. For this purpose, 2 mL of a wash buffer (PBS pH 7.2, thimerosal 0.01%, 1% BSA, 1 mM phenylmethylsulfonyl fluoride, and 5 mM EDTA) was injected into the proximal duodenum and collected at the distal ileum. The collected samples were then centrifuged at 1200 × *g* for 15 min at 4 °C, and the resulting supernatant was collected and stored at −20 °C. The levels of IgA were determined using the chicken IgA ELISA quantitation kit (Bethyl® Laboratories, Montgomery, TX, USA). The assay was performed in triplicate following the manufacturer's instructions, with the plates read at 450 nm.

- *Histology and scanning electron microscopy*

Morphological and morphometric evaluations were carried out on the intestines (duodenum, jejunum, and ileum) of 6 animals per treatment on the 23rd day. The Swiss roll

technique (Souza et al., 2021) was used to collect and prepare the samples. The tissues were fixed in a 10% buffered formalin solution and subsequently subjected to routine histological processing. Sections of 5 µm thickness were obtained and stained with hematoxylin-eosin (HE) and Alcian Blue (AB). AB staining was utilized to determine goblet cell density.

Morphometric analysis was performed on 30 randomly selected villi and crypts per slide (180 villi and crypts per treatment) using image analysis software (Motic Image Plus 2.0, Motic Instruments, Richmond, BC, Canada). Measurements of villus height, crypt depth, and villus: crypt ratio were conducted in the duodenum, jejunum, and ileum. The morphological evaluation of the intestines followed the scoring system described by Terciolo et al. (2019), with minor modifications (including additional lesions in the score: inflammatory infiltrate, congestion, bacteria adhered to the villi, presence of *Eimeria* spp., and cell debris). The count of intraepithelial lymphocytes (IELs) was performed on 12 randomly chosen villi per slide (72 villi per treatment), considering IELs positioned above the enterocyte nucleus. Goblet cell density was determined exclusively in the ileum by evaluating 15 random villi per slide (90 villi per treatment).

Scanning electron microscopy was conducted specifically in the jejunum. Samples were collected on the 23rd day, fixed in a 2.5% glutaraldehyde buffered solution (sodium cacodylate solution 0.1 M, pH 7.2) for 24 h, and subsequently washed with sodium cacodylate buffer (0.1 M, pH 7.2). Treatment involved exposure to 1% osmium tetroxide in sodium cacodylate buffer (0.1 M, pH 7.2) for 1 h. Subsequent steps included gradual dehydration in different ethanol concentrations (70, 80, 90, 100%) and drying to the critical point using a CPD 030 critical point dryer (Bal-Tec Union Ltd., Vaduz, Liechtenstein). Following this, tissues were coated with gold (Sputter Coater SDC 050, Bal-Tec Union Ltd., Vaduz, Liechtenstein), and the morphology of the intestinal villi was examined using a scanning electron microscope (FEI Quanta 200, Field Electron and Ion Company, Hillsboro, USA).

- *Oxidative stress response*

The oxidative stress response was evaluated in both the jejunum and ileum. On the 20th day, 4 animals per treatment were euthanized, and samples from these tissues were collected in microtubes and preserved at −80 °C until processing. The antioxidant capacity was assessed by quantification of reduced glutathione (GSH) following the method of Sedlak;Lindsay (1968), ferric reducing ability (FRAP), and reduction of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), as described by Katalinic et al. (2005).

The oxidative response was evaluated using the nitroblue tetrazolium assay (NBT) (Fattori et al., 2015) and the quantification of thiobarbituric acid reactive substances (TBARS) (Sattler; Malle; Kostner, 1998; Manchope et al., 2018). Tissue homogenization was carried out in buffer using a Tissue-Tearor (Bjospec, São Paulo, SP, Brazil). For the FRAP, ABTS, NBT, and TBARS protocols, the buffer consisted of KCL (1.15%) and EDTA (0.02 M) for GSH analysis.

7. Statistical Analysis

The experimental design was entirely randomized, except for the ELISA analysis, which followed a factorial 3 × 7 design (3 time points and 7 treatments). Each animal was considered one experimental unit. Data analysis was carried out using the free software R® version 3.4.4, and ANOVA was performed using the AgroR package with a significance level of 5%. If the means exhibited statistical significance, the data were submitted to a Scott–Knott multiple comparison test at a 5% significance level. Data that did not meet the assumption of normality of errors were subjected to logarithmic transformation. After ANOVA assumptions were reevaluated, the data were submitted to the Scott–Knott test.

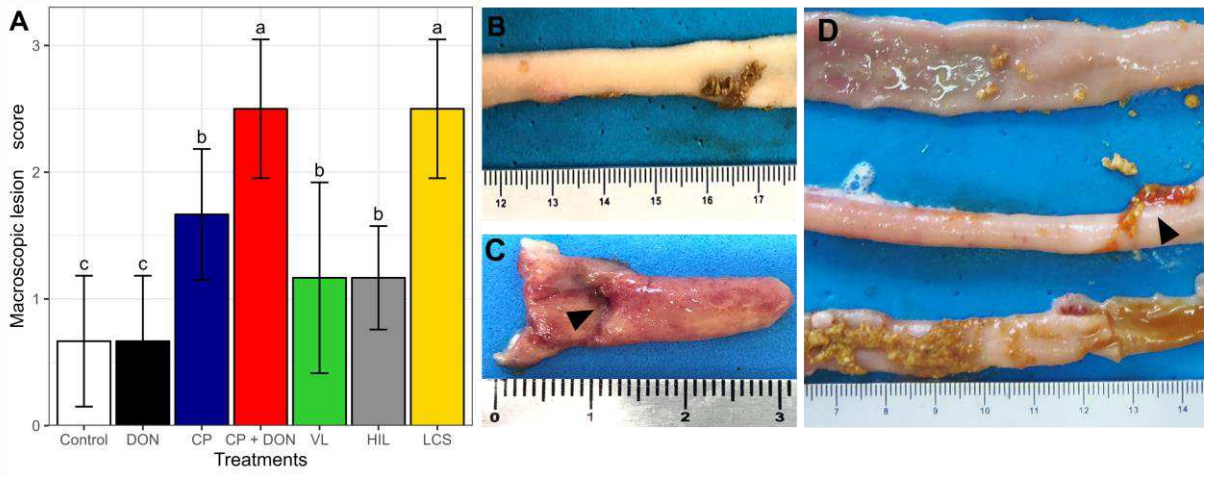
RESULTS

1. Effects of DON, C. perfringens, and Lactobacillus spp. Mixture on the Intestines

On the 23rd day, the intestines were macroscopically evaluated (Figure 1). The CP + DON group exhibited a worse intestinal gross appearance than the CP group. Among the *Lactobacillus* spp.-supplemented groups, VL and HIL induced the most effective protective effects. The observed changes included loss of intestinal tonus, hyperemia (Figure 1C), excessive mucus, the presence of yellow peeling content (Figure 1D), and, less frequently, a thick fibrinous mucus layer.

Figure 1. (A) Effect of *Lactobacillus* spp. mixture in the macroscopic aspect of the small intestine of broilers challenged with DON and *Clostridium perfringens*. Values are the means ± standard deviation of the mean. ANOVA followed by Scott–Knott multiple comparison test was used to determine statistical differences among groups. ^{a,b,c} Different letters indicate a statistical difference. (B) Control—normal gross aspect of intestinal mucosa. (C) CP + DON—altered gross aspect of intestinal mucosa, moderate hyperemia, and presence of an ulcer (arrowhead). (D) LV—gross aspect of the intestine from the viable *Lactobacillus* spp. mixture

group, discrete presence of yellow peeling content (arrowhead), and hyperemia and petechiae are observed. Control—uncontaminated diet. DON (deoxynivalenol)—diet with DON 19.3 mg kg^{-1}. CP (*Clostridium perfringens*)—uncontaminated diet + *C. perfringens* challenge. CP + DON—DON 19.3 mg kg^{-1} + *C. perfringens* challenge. VL—DON 19.3 mg kg^{-1} + *C. perfringens* challenge plus viable *Lactobacillus* spp. mixture. HIL—DON 19.3 mg kg^{-1} + *C. perfringens* challenge plus heat-inactivated *Lactobacillus* spp. mixture. LCS—DON 19.3 mg kg^{-1} + *C. perfringens* challenge plus *Lactobacillus* spp. mixture culture supernatant.



Intestinal morphometry is directly related to zootechnical performance (Schedle et al., 2008). In the jejunum, DON, CP, and CP + DON treatments reduced villus height and the villus: crypt ratio compared to the control and *Lactobacillus* spp.-supplemented groups. In the ileum, DON and CP treatments led to a reduction in villus height compared to the other groups, while no significant difference was observed in duodenal morphometry among the experimental groups (Table 1).

Table 1. Effect of *Lactobacillus* spp. mixture on villus height, crypt depth, villus: crypt ratio, microscopic lesion score, intraepithelial lymphocytes infiltration, and goblet cell count in the duodenum, jejunum, and ileum.

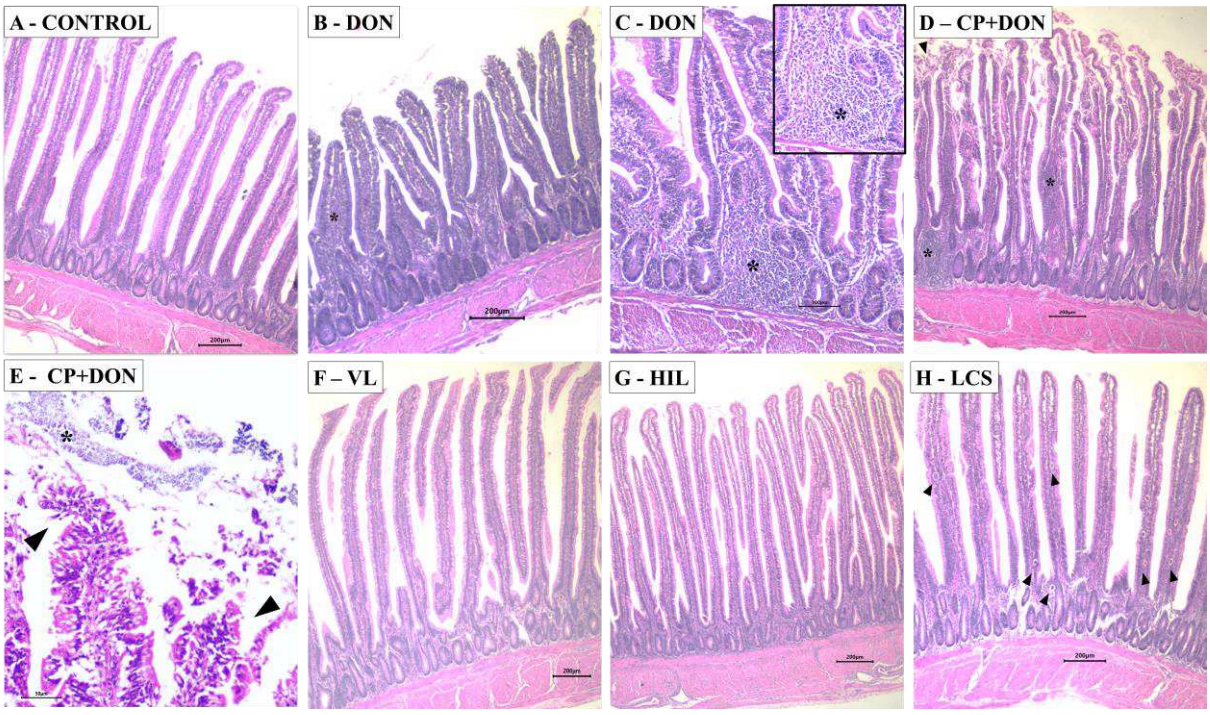
Treatment	Villus Height [µm]	Crypt Depth [µm]	Villi: Crypt Ratio	Microscopic Lesion Score	IEL	Goblet Cells
			Duodenum			
Control	1315.02 ± 189.85	145.91 ± 18.95	9.13 ± 1.78	7.50 ^{b} ± 1.22	NA	NA
DON	1216.70 ± 182.97	158.64 ± 19.96	7.84 ± 1.92	9.16 ^{b} ± 3.31	NA	NA
CP	1159.23 ± 202.50	133.11 ± 15.99	8.71 ± 1.19	11.83 ^{a} ± 5.81	NA	NA
CP + DON	1296.70 ± 258.16	148.60 ± 17.92	8.80 ± 1.93	15.40 ^{a} ± 3.71	NA	NA
VL	1299.06 ± 135.00	164.57 ± 43.96	8.22 ± 1.84	11.40 ^{b} ± 1.52	NA	NA
HIL	1352.86 ± 99.07	140.28 ± 13.89	9.76 ± 1.53	8.40 ^{b} ± 3.05	NA	NA
LCS	1180.26 ± 98.46	138.52 ± 14.33	8.55 ± 1.19	9.40 ^{b} ± 2.51	NA	NA
			Jejunum			
Control	970.52 ^{a} ± 159.33	133.87 ± 13.01	7.30 ^{a} ± 1.34	4.16 ^{b} ± 2.86	20.18 ^{b} ± 2.89	NA
DON	632.43 ^{b} ± 109.73	129.62 ± 12.69	4.89 ^{b} ± 0.83	8.16 ^{a} ± 1.47	25.68 ^{b} ± 2.66	NA
CP	840.82 ^{b} ± 232.02	126.74 ± 11.09	6.59 ^{b} ± 1.43	11.17 ^{a} ± 3.71	28.84 ^{a} ± 2.79	NA
CP + DON	789.68 ^{b} ± 77.20	127.23 ± 9.13	6.22 ^{b} ± 0.69	12.00 ^{a} ± 2.16	32.70 ^{a} ± 4.94	NA
VL	1075.19 ^{a} ± 154.24	122.39 ± 18.15	8.88 ^{a} ± 1.47	8.60 ^{a} ± 3.36	24.76 ^{b} ± 3.99	NA
HIL	961.53 ^{a} ± 225.59	131.70 ± 16.69	7.48 ^{a} ± 2.35	7.00 ^{b} ± 3.67	23.41 ^{b} ± 1.90	NA
LCS	901.30 ^{a} ± 94.33	113.33 ± 14.87	8.02 ^{a} ± 0.96	9.00 ^{a} ± 3.46	30.26 ^{a} ± 5.77	NA
			Ileum			
Control	750.29 ^{a} ± 49.07	123.43 ± 16.56	6.20 ± 1.21	4.50 ^{c} ± 1.87	18.98 ^{b} ± 1.07	66.97 ^{c} ± 5.87
DON	605.68 ^{b} ± 59.83	128.91 ± 25.98	4.86 ± 1.02	6.50 ^{b} ± 1.87	23.52 ^{a} ± 2.23	73.63 ^{c} ± 5.19
CP	671.15 ^{b} ± 107.38	118.87 ± 12.92	5.64 ± 0.62	8.67 ^{a} ± 3.93	26.11 ^{a} ± 5.50	87.34 ^{b} ± 11.32
CP + DON	713.88 ^{a} ± 86.42	115.98 ± 11.15	6.16 ± 0.58	9.00 ^{a} ± 1.87	26.75 ^{a} ± 2.47	88.54 ^{b} ± 9.36
VL	729.05 ^{a} ± 52.30	137.01 ± 13.66	5.34 ± 0.40	4.20 ^{c} ± 1.79	22.46 ^{a} ± 2.05	108.04 ^{a} ± 9.29
HIL	703.70 ^{a} ± 42.50	131.71 ± 10.05	5.35 ± 0.26	9.20 ^{a} ± 2.28	24.26 ^{a} ± 4.27	105.21 ^{a} ± 5.83
LCS	731.06 ^{a} ± 38.13	131.85 ± 16.41	5.59 ± 0.54	6.80 ^{b} ± 2.49	24.11 ^{a} ± 2.55	104.81 ^{a} ± 10.18

Values are the mean ± standard deviation. ANOVA followed by Scott–Knott multiple comparison test was used to determine significant differences among groups. ^{a,b,c} Different letters in the same column indicate a significant difference. Control—uncontaminated diet. DON (deoxynivalenol)—diet with DON 19.3 mg kg^{-1}. CP (*Clostridium perfringens*)—uncontaminated diet + *C. perfringens* challenge. CP + DON—DON 19.3 mg kg^{-1} + *C. perfringens* challenge. VL—DON 19.3 mg kg^{-1} + *C. perfringens* challenge, supplemented with viable *Lactobacillus* spp. mixture. HIL—DON 19.3 mg kg−1 + *C. perfringens* challenge, supplemented with heat-inactivated *Lactobacillus* spp. mixture. LCS—DON 19.3 mg kg−1 + *C. perfringens* challenge, supplemented with *Lactobacillus* spp. mixture culture supernatant. NA—not analyzed.

As sentinels of the intestinal barrier and local immune response, intraepithelial lymphocytes (IELs) were evaluated (Kaer; Olivares-Villagomez, 2018). In the jejunum, only DON ingestion did not induce an increase in IELs; however, broilers receiving CP or CP + DON showed increased IEL infiltrate (≈1.5-fold on average for both groups) compared to the control group, while the VL and HIL groups were similar to the control. In the ileum, all treatments induced a higher number of IELs compared to the control (Table 1). The number of goblet cells was evaluated in the ileum, and the *Lactobacillus* spp.-supplemented groups showed a higher abundance compared to the control, DON, CP, and CP + DON treatments.

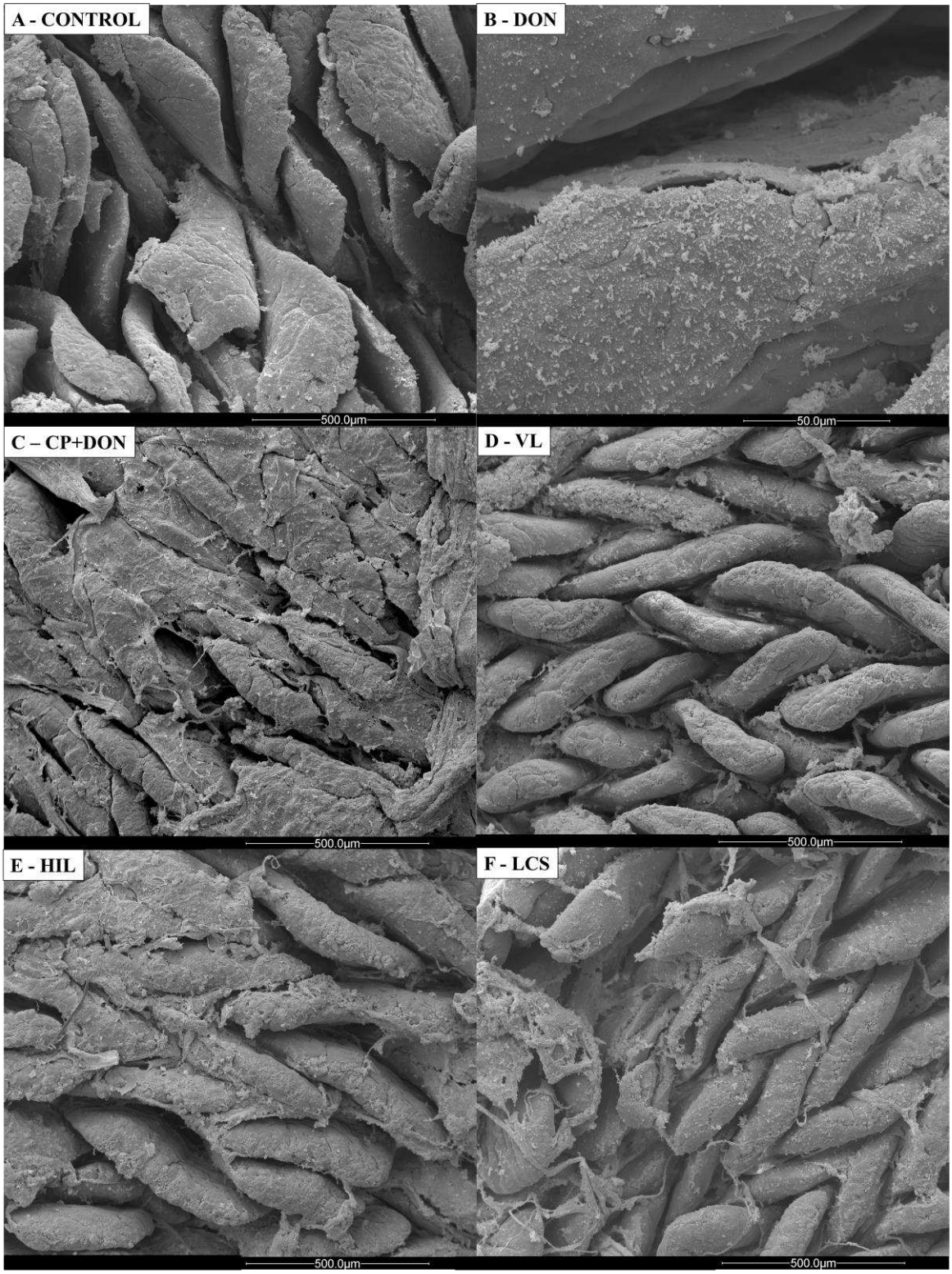
Morphological alterations induced by the different treatments were evaluated using a lesion score. In the duodenum, CP and CP + DON increased the lesion score by approximately 2.6-fold compared to the control, while the remaining treatments resulted in lesion scores similar to those of the control and DON groups. In the jejunum, all treatment groups showed higher scores than the control group, except for the HIL group. In the ileum, increased lesion scores were observed in all treatment groups except the VL group when compared to the control (Table 1). The most frequent changes observed in the histological evaluation included edema of the lamina propria, lymphocytic infiltrate, cell debris, apical necrosis, adhesion of bacteria on the villi surface, and cytoplasmic vacuolation of enterocytes (Figure 2).

Figure 2. Morphological changes induced by DON, CP, and *Lactobacillus* spp. mixture supplementation on broiler jejunal tissue. (A) Control: normal villi morphology. HE, bar 200 µm. (B) DON: villi atrophy and enhanced presence of inflammatory infiltrate (*). HE, bar 200 µm. (C) DON: enhanced presence of inflammatory infiltrate (*). HE, bar 100 µm. Insert: inflammatory infiltrate predominantly composed by mononuclear cells. HE, bar 50 µm. (D) CP + DON: focal area of necrosis (▲), enhanced presence of inflammatory infiltrate (*). HE, bar 200 µm. (E) CP + DON: focal area of apical necrosis with myriad of bacterial colonies attached to cell debris (*). HE, bar 50 µm. (F) Viable *Lactobacillus* spp. mixture: preserved villi morphology. HE, bar 200 µm. (G) Heat-inactivated *Lactobacillus* spp. mixture: preserved villi morphology. HE, bar 200 µm. (H) *Lactobacillus* spp. mixture culture supernatant: preserved villi morphology with different development stages of *Eimeria* spp. oocysts (▲). HE, bar 200 µm.



Scanning electron microscopy (Figure 3) was performed to illustrate the effects of *Lactobacillus* spp. mixture, DON, and CP challenge in the jejunum. The control group showed normal villus morphology, while in the DON and CP + DON groups, a thicker mucus layer was observed. The *Lactobacillus* spp. mixture groups showed preserved villi morphology, similar to the control.

Figure 3. Scanning electron microscopy images illustrating the effect of *Lactobacillus* mixture in the jejunum after DON and CP challenge. (A) Control—normal villi morphology. (B) DON—enhanced presence of mucus compared to the control group. (C) CP + DON—enhanced presence of mucus. (D) Viable *Lactobacillus*—preserved villi integrity. (E) Heat-inactivated *Lactobacillus*—preserved villi morphology. (F) *Lactobacillus* culture supernatant—preserved villi morphology.


A - CONTROL
500.0µm
B - DON
50.0µm
C – CP+DON
500.0µm
D - VL
500.0µm
E - HIL
500.0µm
F - LCS
500.0µm

2. Effects of DON, C. perfringens, and Lactobacillus spp. Mixture on Redox Status

Oxidative stress occurs when there is an imbalance between the production of radical species and the antioxidant response in the organism (Halliwell, 2007). Our study aimed to investigate the effects of DON and CP on the redox status. To achieve this, samples from the jejunum and ileum were used in lipid peroxidation (TBARS) and superoxide anion production (NBT) assays to assess the oxidative response. Additionally, GSH (reduced glutathione), ABTS (3-ethylbenzothiazoline-6-sulphonic acid) and FRAP (ferric reducing ability) assays were performed to evaluate antioxidant capacity.

In the jejunum, exposure to DON and CP + DON increased TBARS levels compared to the control, CP, and *Lactobacillus* spp.-supplemented groups. An increase in the NBT levels was detected in the CP, CP + DON, and *Lactobacillus* spp.-supplemented groups compared to the control and DON groups (Table 2). DON, CP, and CP + DON challenges reduced GSH levels compared to the control group, with the *Lactobacillus* spp.-supplemented treatments unable to restore GSH levels to those of the control. Regarding FRAP levels, the DON and control groups were similar, while the CP, CP + DON, and *Lactobacillus* spp.-supplemented groups showed higher levels in comparison. There were no significant differences in ABTS levels between the groups.

Table 2. Effect of *Lactobacillus* spp. mixture on the oxidative status in the small intestine. Values are the mean ± standard deviation of the mean. ANOVA followed by Scott–Knott multiple comparison test was used to determine statistical differences among groups. [a,b,c]Different letters in the same column indicate a statistical difference.

Treatment	TBARS	NBT	GSH	ABTS	FRAP
			Jejunum		
Control	0.04 [b] ± 0.03	6.67 [b] ± 3.19	2809.33 [a] ± 1215.38	0.70 ± 0.09	0.59 [b] ± 0.17
DON	0.08 [a] ± 0.05	7.56 [b] ± 2.30	1313.11 [b] ± 447.71	1.10 ± 0.18	0.75 [b] ± 0.25
CP	0.03 [b] ± 0.01	32.46 [a] ± 12.51	447.51 [c] ± 110.76	1.13 ± 0.44	1.65 [a] ± 0.56
CP + DON	0.05 [a] ± 0.01	24.51 [a] ± 13.54	458.97 [c] ± 20.38	1.11 ± 0.39	1.21 [a] ± 0.23
VL	0.03 [b] ± 0.01	32.85 [a] ± 27.97	593.57 [c] ± 75.59	1.00 ± 0.25	1.40 [a] ± 0.15
HIL	0.02 [b] ± 0.01	48.70 [a] ± 25.97	585.41 [c] ± 110.41	0.85 ± 0.25	1.55 [a] ± 0.33
LCS	0.03 [b] ± 0.01	25.55 [a] ± 16.27	400.20 [c] ± 107.88	1.37 ± 0.40	1.98 [a] ± 0.45
			Ileum		
Control	0.02 ± 0.007	6.81 [b] ± 0.64	1568.72 [a] ± 731.15	0.81 ± 0.17	1.28 ± 0.94
DON	0.04 ± 0.03	6.48 [b] ± 3.46	1702.98 [a] ± 412.50	0.64 ± 0.19	0.96 ± 0.49
CP	0.02 ± 0.008	30.80 [a] ± 8.62	588.39 [b] ± 120.34	0.89 ± 0.36	1.78 ± 0.57
CP + DON	0.03 ± 0.02	44.17 [a] ± 14.05	556.43 [b] ± 111.85	0.75 ± 0.35	0.97 ± 0.21
VL	0.02 ± 0.01	39.59 [a] ± 14.33	639.80 [b] ± 250.44	0.88 ± 0.22	1.18 ± 0.30
HIL	0.03 ± 0.005	36.29 [a] ± 13.26	606.61 [b] ± 87.30	0.57 ± 0.34	1.41 ± 0.52
LCS	0.03 ± 0.009	26.03 [a] ± 17.26	663.84 [b] ± 137.50	0.59 ± 0.34	0.93 ± 0.23

Control—uncontaminated diet. DON (deoxynivalenol)—diet with DON 19.3 mg kg^{-1}. CP (*Clostridium perfringens*)—uncontaminated diet + *C. perfringens* challenge. CP + DON—DON 19.3 mg kg^{-1} + *C. perfringens* challenge. VL—DON 19.3 mg kg^{-1} + *C. perfringens* challenge plus viable *Lactobacillus* spp. mixture. HIL—DON 19.3 mg kg^{-1} + *C. perfringens* challenge plus heat-inactivated *Lactobacillus* spp. mixture. LCS—DON 19.3 mg kg^{-1} + *C. perfringens* challenge plus *Lactobacillus* spp. mixture culture supernatant. Results are expressed as: TBARS (thiobarbituric acid reactive substances)—ΔOD A535-A532/mg of tissue; NBT (nitroblue tetrazolium)—OD/mg of protein; GSH (reduced glutathione)—nmol/mg of protein; ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)—nmol Trolox Eq/mg of tissue; FRAP (ferric reducing antioxidant power)—nmol Trolox Eq/mg of tissue.

In the ileum, both CP and CP + DON treatments significantly increased NBT levels by approximately 5.6-fold compared to the control and DON groups. However, the *Lactobacillus* spp.-supplemented treatments were unable to revert superoxide anion production to control group levels. Regarding GSH levels, CP and CP + DON treatments reduced it approximately 2.85-fold compared to the control and DON groups, with the *Lactobacillus* spp.-supplemented treatments showing no significant differences compared to the CP and CP + DON groups. No significant differences were observed among the TBARS, ABTS, and FRAP levels between the groups.

3. Effects of DON, C. perfringens, and Lactobacillus spp. on Intestinal Secretory IgA Levels

Intestinal secretory IgA levels were assessed at three time points: 7, 14, and 20 days. No significant difference was observed among the treatments ($p = 0.08$). However, with respect to time, a lower IgA concentration was observed at 7 days (1,165,620 ± 636,766 ng/mL), compared to that at 14 (1,488,459 ± 947,397 ng/mL) and 20 (1,610,248 ± 1,050,150 ng/mL) days ($p = 0.02$). No interaction between time and treatment was observed ($p = 0.20$).

DISCUSSION

Previous studies have shown that exposure to mycotoxins, such as DON alone or in combination with other mycotoxins like fumonisins, can exacerbate the occurrence of necrotic enteritis (NE) (Antonissen et al., 2014; Guo et al., 2021; Shanmugasundaram et al., 2022). In this study, we aimed to investigate the effects of different presentations [probiotic (viable cells), paraprobiotic (heat-inactivated cells), and postbiotic (heat-inactivated cells culture supernatant)] of a *Lactobacillus* spp. mixture on broiler chickens challenged with DON and *Clostridium perfringens* (CP), as previous research has suggested that *Lactobacillus* spp. strains may mitigate the negative effects of single challenge with mycotoxins or CP in the intestine (Kulkarni et al., 2022; Maidana; Souza; Bracarense, 2022).

Macroscopic evaluation of the intestines revealed that the CP + DON group exhibited a higher lesion score compared to the group challenged with CP alone, consistent with previous reports indicating that a DON-contaminated diet can increase the severity of NE cases (Antonissen et al., 2014; Shanmugasundaram et al., 2022). Among the *Lactobacillus* spp. groups, VL and HIL induced the lowest lesion scores. Microscopically, both DON and CP

exposure induced intestinal lesions, and the combination of both factors tended to increase the lesion score, although the difference was not significant. The *Lactobacillus* spp. treatments appeared to mitigate the morphological changes induced by both challenges.

Zootechnical performance is closely related to increased intestinal absorptive capacity. Villus height serves as an indicator of the absorption area, and the intestinal crypts are the sites of new enterocyte multiplication (Swatson et al., 2002; Van Loo, 2007). The jejunum and ileum were the intestinal segments most affected by both DON and CP challenges, showing a reduction in villus height and the villus: crypt ratio (only in the jejunum) compared to the control and *Lactobacillus* spp.-supplemented groups. Since the small intestine is the primary site of DON absorption, previous studies have reported impaired intestinal morphometry as a consequence of DON ingestion (Ghareeb et al., 2015; Souza et al., 2020). However, in this study, the combination of DON and CP did not worsen the intestinal morphometry compared to single challenge.

Intraepithelial lymphocytes (IELs) are key components of the intestinal barrier (Kaer; Olivares-Villagomez, 2018). In this study, exposure to CP increased the number of IELs in both the jejunum and ileum, regardless of DON administration. Similar findings were re-ported in naturally infected laying hens (Allaart et al., 2012). However, in the VL and HIL groups, the number of IELs was reduced to control levels in the jejunum but not in the ileum.

Contrary to previous reports (Wang; Hogan, 2019; Souza et al., 2020), DON exposure did not induce an increase in goblet cell density under light microscopy evaluation in this study. However, scanning electron microscopy revealed enhanced mucus presence in the DON-exposed group compared to the control group. *Lactobacillus* spp. supplementation increased the number of goblet cells compared to other groups. Excess mucus can predispose to NE; however, this finding was expected, as the microbiota can influence mucus layer development, and specifically, *Lactobacillus* spp. are known to contribute to strengthening the intestinal mucosal barrier function (Moore, 2016; Luis; Hansson, 2023; Ni et al., 2023; Nii et al., 2023).

Regarding oxidative stress in the jejunum, DON induced lipid peroxidation, and this effect was sustained after the CP challenge. However, the *Lactobacillus* spp. treatments de-creased the lipid peroxidation/MDA levels to control levels. Previous research has also reported DON-induced lipid peroxidation (Awad et al., 2014; Yang et al., 2017; Souza et al., 2020), which is associated with mitochondrial damage (Silva; Bracarense; Oswald, 2018). The NBT assay quantifies superoxide anions indirectly through their oxidative effects on NBT and is mainly produced by inflammatory cells (Baehner; Boxer; Davis, 1976). CP induced

inflammation, as confirmed by higher NBT levels, but the *Lactobacillus* spp. treatments did not reverse this effect. As a consequence of lipid peroxidation and inflammation, lower GSH levels were observed in all DON- and CP-challenged groups compared to control levels. These findings align with other studies that have reported the capacity of DON and CP to reduce intestinal antioxidant defense (Zhou et al., 2016; Cao et al., 2019; El-Houseiny; Khalil, 2020; Souza et al., 2020). The FRAP assay measures tissue ferric reducing ability, and CP-challenged groups showed higher FRAP levels, likely as a response to the inflammatory status and oxidative stress (Benzie; Strain, 1996).

The ileum is the final segment of the small intestine, and exposure to xenobiotics such as mycotoxins is lower than that in the proximal regions (Maresca, 2013). In this study, the ileum showed no change in the oxidative stress response after DON exposure. It is likely that the levels of mycotoxins were reduced due to the intestinal microbiota's detoxification activity (Yu et al., 2010). Organisms under long-term toxicity might induce adaptations to reduce the damage (Yunus et al., 2012; Dänicke; Brezina, 2013; Chen; Li; Lin, 2017). On the other hand, CP induced an inflammatory status resulting in higher levels of NBT and lower levels of GSH, and *Lactobacillus* spp. did not exert a protective effect.

In this study, concomitant exposure to DON and CP did not worsen most of the evaluated parameters compared to single challenge. However, the *Lactobacillus* spp. treatments, especially LV and HIL, were effective in mitigating tissue damage. Viable or heat-inactivated cells from one strain of *L. plantarum* used in this study have a recognized capacity to remove DON (Franco et al., 2011). The mechanism of action is still unclear, but based on research with similar microorganisms, it is hypothesized that viable cells can detoxify DON while heat-inactivated cells can bind to the mycotoxin, thereby reducing its toxic effects (El-Nezami et al., 2002; Qu et al., 2019; Zhai et al., 2019).

The *Lactobacillus* strains used in this experiment underwent in vitro evaluation, revealing their ability to antagonize the growth of *C. perfringens* (inhibition zone on spot on the lawn varying from 14 to 22 mm, data not shown). The protective effects of viable cells against CP-induced damage were likely a result of bacteriocin production targeting *C. perfringens*, whereas heat-inactivated cells might exert a prebiotic effect by modulating the gut microbiome and preventing the proliferation of pathogenic microorganisms (Qing et al., 2017; Cao et al., 2019; Adhikari et al., 2020; Emami et al., 2021; Shojadoost et al., 2022).

CONCLUSIONS

Additional studies are required to clarify the mechanisms of action of the evaluated *Lactobacillus* spp. strains and establish an industrial process for producing and transforming these strains into a commercial product for animal consumption. This is especially crucial as the model employed in this trial (oral gavage) is not applicable in commercial poultry farming. Nonetheless, it can be inferred that these strains exhibited a protective effect, mitigating a significant portion of the intestinal damage induced by DON and *C. perfringens*.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1. Experimental design. Experimental groups: Control—uncontaminated diet. DON (deoxynivalenol)—diet with DON 19.3 mg kg^{-1}. CP (*Clostridium perfringens*)—uncontaminated diet + *C. perfringens* challenge. CP + DON—DON 19.3 mg kg^{-1} + *C. perfringens* challenge. LV—DON 19.3 mg kg^{-1} + *C. perfringens* challenge plus viable *Lactobacillus* spp. mixture. HIL—DON 19.3 mg kg^{-1} + *C. perfringens* challenge plus heat-inactivated *Lactobacillus* spp. mixture. LCS—DON 19.3 mg kg^{-1} + *C. perfringens* challenge plus *Lactobacillus* spp. mixture culture supernatant.

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Informed Consent Statement: Not applicable.

Data Availability Statement: All data are present in the article.

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6 MANUSCRIPT B – GUAR GUM AS GALACTOMANNAN SOURCE INDUCES DYSBIOSIS AND REDUCES PERFORMANCE IN BROILER CHICKENS AND DIETARY B-MANNANASE RESTORES THE GUT HOMEOSTASIS[2]

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ABSTRACT

Galactomannans are abundant non-starch polysaccharides in broiler feed ingredients. In broilers, diets with high levels of galactomannans have been associated with innate immune response stimulation, poor zootechnical performance, nutrient and lipid absorption, and excessive digesta viscosity. However, data about its effects on the gut microbiome are scarce. *β*-mannanases are enzymes that can hydrolyze *β*-mannans, resulting in better nutrient utilization. In the current study, we have evaluated the effect of guar gum, a source of galactomannans, supplemented to broiler diets, either with or without *β*-mannanase supplementation, on the microbiota composition, in an attempt to describe the potential role of the intestinal microbiota in *β*-mannanase-induced gut health and performance improvements. One-day-old broiler chickens (n = 756) were randomly divided into three treatments: control diet, guar gum supplemented diet (1.7 %), or guar gum supplemented diet + *β*-mannanase

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(Hemicell® 330g/ton). The zootechnical performance, gut morphometry, ileal and caecal microbiome, and short-chain fatty acid concentrations were evaluated at different time points. The guar gum supplementation decreased the zootechnical performance, and the *β*-mannanase supplementation restored performance to control levels. The mannan-rich diet induced dysbiosis, with marked effects on the caecal microbiota composition. The guar gum supplemented diet increased the caecal abundance of the genera *Lactobacillus*, *Roseburia, Clostridium sensu stricto 1* and *Escherichia-Shigella,* and decreased *Intestinimonas, Alistipes*, *Butyricicoccus* and *Faecalibacterium.* In general, dietary *β*-mannanase supplementation restored the main microbial shifts induced by guar gum to levels of the control group. In addition, the *β*-mannanase supplementation reduced caecal isobutyric, isovaleric, valeric acid and branched chain fatty acid concentrations as compared to the guar gum supplemented diet group, suggesting improved protein digestion and reduced caecal protein fermentation. In conclusion, a galactomannan-rich diet impairs zootechnical performance in broilers and results in a diet-induced dysbiosis. *β*-mannanase supplementation restored the gut microbiota composition and zootechnical performance to control levels.

KEY WORDS: beta-mannanase, guar gum, microbiota, mannan, gut health.

INTRODUCTION

It is estimated that by 2030, poultry meat consumption will represent 41% of all meat protein sources worldwide, driven by the efficient production and the lack of cultural and religious hurdles (Oecd; Fao, 2021). One of the major potential problems for the future is to keep the production cost at low levels. Currently, feed represents >60% of the final production cost (Noblet; Wu; Choct, 2022), so dietary additives that can improve feed utilization are of great value for the poultry industry. One of the most widely used dietary additives to enhance digestibility are carbohydrate-degrading enzymes, such as xylanases (Zhang et al., 2014). These enzymes are targeting plant cell wall components, more specifically soluble non-digestible non-starch polysaccharides (NSP), considered to be anti-nutritional factors (Jezierny; Mosenthin; Bauer, 2010; Amerah, 2015). NSP have *β*-glycosidic bonds that cannot be digested by monogastric animals, and data clearly show negative correlations between digestibility and dietary NSP concentrations (Jaworski et al., 2015). High concentrations of soluble NSP increase

small intestinal viscosity, decrease passage rate, favor the expansion of harmful microbiota, such as *Escherichia coli* and *Clostridium perfringens,* reduce animal performance, and affect the intestinal microbiota composition (Hussain; Rehman; Khalid, 2012; Shojadoost; Vince; Prescott, 2012; Latorre et al., 2015; Jha et al., 2019; Bushra et al., 2020).

While arabinoxylans are the most well-known NSP, there are a variety of other NSPs that can negatively affect animal performance (Căpriţă; Căpriţă; Julean, 2010; Kermanshahi; Shakouri; Daneshmand, 2018). As an example, *β*-mannans (galactomannans and glucomannans) are present in different concentrations in many feed ingredients. Dehulled soy bean meal (48% crude protein), one of the most important ingredients in poultry diets, has about 2.8-10 g/kg of *β*-mannans (Hove et al., 2018). Guar gum meal typically contains between 20-80g/kg of galactomannans (mannose backbone with galactose side-chains), and is often used as *β*-mannan-rich source to evaluate the effect of *β*-mannans and *β*-mannan degrading enzymes in experimental trials (Hussain; Rehman; Khalid, 2012; Saeed et al., 2019).

In humans, galactomannan ingestion has been related to glycemic index reduction in diabetics, alleviation of the irritable bowel syndrome symptoms, prebiotic effects, and improvement of short-chain fatty acid (SCFA) production (Singh; Singh; Arya, 2018; Rao; Quartarone, 2019; Miao et al., 2021). However, in broilers, diets with high concentrations of plant-derived galactomannans are associated with poor performance, reduced feed intake, nutrient and lipid absorption, innate immune response stimulation and excessive digesta viscosity (Lee; Bailey; Cartwright, 2003; Shirouchi et al., 2011; Shastak et al., 2015).

β-mannanases are enzymes synthesized by strains of *Aspergillus niger*, *Paenibacillus lentus*, *Bacillus subtilis,* or *Trichoderma longibrachiatum* (Li, Y.-F. et al., 2014; Saeed et al., 2019). These enzymes can hydrolyze *β*-1,4-glycosidic linkages in *β*-mannans resulting in a better nutrient utilization, improvement of innate immune responses, reduced intestinal viscosity and lower pathogen proliferation (Hussain; Rehman; Khalid, 2012). The effects of *β*-mannans and *β*-mannanase feed supplementation on performance and gut histology in chickens have been described previously (Maisonnier et al., 2003; Zou; Qiao; Xu, 2006; Caldas et al., 2018; Latham et al., 2018). As NSP-degrading enzymes typically cause release of smaller oligosaccharides that can be used by the intestinal microbiota, it might be that shifts in microbial composition contribute to the observed health effects when *β*-mannanases are added to poultry diets (Hussain; Rehman; Khalid, 2012; Saeed et al., 2019). However, data on the effect of *β*-mannans and *β*-mannanase on the intestinal microbiome are lacking. In the current study, we evaluate the effect of guar gum (GG) supplementation to the diet of broilers, either or without

β-mannanase, on the microbiota composition, in an attempt to describe the potential role of the intestinal microbiota in *β*-mannanase-induced gut health and performance improvements.

MATERIAL AND METHODS

Animal Trial

A total of 756 1-day-old Ross-308 broiler chicks were randomly divided in three treatments (12 pens per treatment with 21 animals each): control diet; guar gum diet (GG); guar gum diet+*β*-mannanase (GG+E) (Hemicell® 330 g/ ton of feed) and housed on solid floors covered with wood shavings following European Union Directive 2007/ 43/EC (EU, 2007). Water, feed (Table 1) and heating were provided according to broiler guidelines (Aviagen, 2018). At 1, 14, 21, 28, and 35 days of age the animals and feed leftovers were weighed per pen to calculate the feed conversion ratio (FCR), body weight (BW), daily feed intake (DFI) and average daily gain (ADG). At 14 and 28 days of age, one animal per pen (n = 12 birds/treatment) was euthanized by an intravenous overdose of 20% sodium pentobarbital (Kela, Hoogstraten, Belgium), according to Annex I to the Council Regulation (EC) No 1099/2009 (EC, 2009), and content from ileum and caecum was collected and stored at -20°C, while part of the duodenum and ileum were fixed in 4% buffered formalin for 24 hours.

Table 1- Composition of the experimental diets

Ingredients (g/kg)	Control diet		GG diet	
	Starter	Grower	Starter	Grower
Maize	610.40	625.85	588.60	606.40
Guar gum	-	-	17.90	16.50
Soya bean meal	310	300	314	303
Full fat soya bean	2	-	2	-
Animal fat	25	25	25	25
Soybean oil	10	12.50	10	12.50
Premix	5	5	5	5
Lime fine (38% Ca)	15	13	15	13
Dicalcium phosphate	10.10	6.90	10.10	6.90
Sodium bicarbonate	2.70	2.20	2.70	2.20
L-lysine HCL	3.20	2.90	3.10	2.85
DL-methionine	3.25	2.90	3.25	2.90
L-threonine	1.25	1.10	1.25	1.10
Calculate composition (g/kg)				
ME (MJ/kg)	12.86	13.03	12.59	12.79
Crude protein	203.9	199.4	204	199.2
Crude fat	67.8	70.4	66.9	69.6
Starch	402.4	412.4	388.2	399.7
Sugars	43.7	42.9	43.7	42.8
Ca	9.0	7.8	9	7.8
Available P	4.8	4.2	4.8	4.2

The starter diet was provided from day 1 until day 14, followed by grower diet until day 35. ME- metabolizable energy. Hemicell® 330 g per ton of feed was added only to the group that received the GG diet supplement with *β*-mannanase. Premix composition per kg of product: vitamin A 10,000 IU; vitamin D_3 2,500 IU; vitamin E 50 mg; vitamin K_3 1.5 mg; vitamin B_1 2.0 mg; vitamin B_2 7.5 mg; vitamin B_6 3.5 mg; vitamin B_{12} 20 µg; niacin 35 mg; D-pantothenic acid 12 mg; choline chloride 460 mg; folic acid 1.0 mg; biotin 0.2 mg; iron 80 mg; copper 12 mg; manganese 85 mg; zinc 60 mg; iodate 0.8 mg; selenium 0.15 mg. GG- guar gum.

Histological Analysis

The formalin-fixed tissue segments (n= 12/treatment/intestinal segment) were embedded in paraffin, 5 µm sections obtained, deparaffinized and stained with Hematoxylin-Eosin (HE). Duodenal and ileal villus length and crypt depth were assessed by random measurement of 15 villi and crypts using a PC-based image analysis system (Leica Application Suite V4.1, Leica, Diegem, Belgium). Afterwards the villus to crypt ratio was calculated.

Molecular Analysis

DNA extraction. DNA was extracted from 100 mg caecal and ileal content of one bird per pen (12 birds/treatment), using the hexadecyltrimethylammonium bromide (CTAB) method according to Kowalchuk et al. (1998) with small modifications. Briefly the intestinal content was suspended in 0.5 mL CTAB (Sigma Aldrich, St. Louis, MO, USA) buffer 5% (w/v), 0.35 M NaCl, 120 mM K_2HPO_4) and 0.5 mL phenol–chloroform-isoamyl alcohol (25:24:1). The mixture was homogenized by grinding with 0.5 g unwashed glass beads (Sigma-Aldrich) in a bead beater (2x3 min 30 Hz for ileal content and 2x2 min, 22.5 Hz for caecal content; TissueLyser II; Qiagen, Hilden, Germany) with a 30 s interval between shakings. Samples were centrifuged for 10 min at 8000 rpm and 300 µL of the supernatant was transferred to a new tube. A re-extraction from the remaining content was performed by adding 0.25 mL CTAB buffer and homogenizing and centrifuging the sample as described above. An equal volume (0.6 mL) of chloroform-isoamyl alcohol (24:1) was added to the supernatant collected in order to remove the phenol from the samples. The mixture was further centrifuged at 16000 *g* for 10 s. Nucleic acids were precipitated with 1.2 mL of polyethyleenglycol-6000 solution (30% w/v; 1.6 M NaCl) for 2 h at room temperature. Samples were centrifuged (13000 *g*, 20 min, 4°C) and the pellet was washed twice with 1 mL of ice-cold ethanol (70% v/v). The obtained pellet was dried and resuspended in 100 µL de-ionized water (LiChrosolv Water, Merck, Darmstadt, Germany). The quality and the concentration of the DNA was examined spectrophotometrically using NanoDrop (Termo Scientifc, Waltham, MA, USA). Only samples with a 260/280 purity value above 1.7 were selected.

16S rRNA sequencing and bioinformatics. The extracted DNA was diluted to 20 ng/µl and the V3-V4 hypervariable region of the 16S rRNA gene was amplified using the gene-specific primers (Table 2), as described by Aguirre et al. (2019). The final barcoded libraries were pooled at an equimolar concentration of 5 nM and sequenced with 30 % PhiX spike-in using the Illumina MiSeq v3 technology (2 × 300 bp, paired-end) by the Ghent University next generation sequencing facility NXTGNT. After demultiplexing of the amplicon dataset and deletion of the barcodes, optimal trimming parameters were determined using the python-based application FIGARO (Weinstein et al., 2019). All further processing was performed in R (v4.1.2) (Bunn; Korpela, 2013). Raw sequence reads were trimmed, quality-filtered and dereplicated using the *DADA2* algorithm (v1.14.0) (Callahan et al., 2016). An initial amplicon sequence variant (ASV) table was constructed before chimeras were identified using the *removeBimeraDenovo* function. Finally, taxonomy was assigned using *DADA2*'s native naïve

Bayesian classifier against the Silva database (v138) (Quast et al., 2013). To construct a phylogenetic tree, multiple sequence alignment was performed using the *DECIPHER* (v2.14.0) algorithm (Wright, 2015), after which a neighbor-joining tree was constructed using *PHANGORN* (v2.7.0) (Schliep, 2011). This neighbor-joining tree was used as the starting point to fit the final GTR+G+I (Generalized time-reversible with Gamma rate variation) maximum likelihood tree. The resulting phylogenetic tree and ASV table were loaded into *Phyloseq* (v1.28.0) (Mcmurdie; Holmes, 2013), after which potential contaminant chloroplastic and mitochondrial ASVs were removed from the data set. Potential contaminant DNA reads originating from the DNA extraction or library preparation buffers were identified based on both the DNA concentration and prevalence of the ASVs in the negative control samples (DNA extraction controls) using *decontam* (v1.14.0) (Davis et al., 2018) and removed from the final dataset.

Quantitative PCR. To confirm the main microbial shifts that were identified using 16S rRNA gene analysis, a *q*PCR for families *Enterobacteriaceae* and *Ruminococcaceae* and genus *Lactobacillus* was performed. Additionally, a *q*PCR quantifying the number of genes encoding the butyryl-CoA:acetate CoA-transferase was performed using the CFX384 BioRad detection system (BioRad, Nazareth-Eke, Belgium). Each reaction was done in triplicate in a 12 µl total reaction mixture using 1x SensiMix SYBR No-ROX mix (Bioline, Kampenhout, Belgium), 0.5 µM final primer concentration (2.5 µM for butyryl-CoA: acetate-CoA transferase enzyme), 2 µl of (20 ng/µl) DNA and de-ionized water to complete the reaction volume. A standard curve was included in triplicate for each primerset. The amplification program consisted of 1 cycle at 95°C for 10 min, 40 cycles of 30 s at 95°C, followed by the annealing temperature, described in table 2 for each primerset, for quantifying the number of gene copies encoding butyryl-CoA: acetate CoA-transferase a 3-steps protocol was used 1 cycle at 95°C for 10 min, 40 cycles of 30 s at 95°C, 30 s at 53ºC and 30 s at 72ºC. The fluorescent products were detected at the last step of each cycle. A melting curve analysis was done after amplification and was obtained by slow heating from 60°C to 95°C at a rate of 0.5°C/5 s to confirm the specificity of the reaction. The primer sequences are described in table 2.

Table 2- Primer sequences and annealing temperatures used for quantification of the respective taxa and the butyryl-CoA CoA-transferase gene, in *q*PCR reactions

Target	Primer	Sequence	Annealing temperature and time	Reference
V3-V4 region of the 16s rRNA gene	*S-D-Bact-0341-b-S-17*	Fw- 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACA GCCT ACGGGNGGCWGCAG 3'	55ºC, 30"	Klindworth et al. (2013)
	S-D-Bact-0785-a-A-21	Rv – 3' GTCTCGTGGGCTCGGAGATGTGTATAAGAG ACAGGACTACHVGGGTATCTAATCC 5'		
Enterobacteriaceae	*Eco1457F*	Fw – 5' CATTGACGTTACCCGCAGAAGAAGC 3'	55º C, 1'	Bartosch et al. (2004)
	Eco1652R	Rv – 3' CTCTACGAGACTCAAGCTTGC 5'		
Lactobacillus spp.	*Lacto-16S-F*	Fw – 5' GGA ATC TTC CAC AAT GGA CG 3'	60º C, 1'	Abdulamir et al. (2010)
	Lacto-16S-R	Rv – 3' CGC TTT ACG CCC AAT AAA TCC GG 5'		
Ruminococcaceae	*sg-Clept-F*	Fw – 5' GCACAAGCAGTGGAGT 3'	60º C, 1'	Matsuki et al. (2004)
	sg-Clept-R3	Rv – 3' CTTCCTCCGTTTTGTCAA 5'		
Butyryl-CoA:acetate CoA-transferase	*BCoATscrF*	Fw – 5' GCIGAICATTTCACITGGAAYWS ITGGCAYATG 3'	53º C, 30"	Louis;Flint (2007)
	BCoATscrR	Rv – 3' CCTGCCTTTGCAATR TCIACRAANGC 5'		

Fw- forward, Rv- reverse.

Short-Chain Fatty Acid (SCFA) and Branched-Chain Fatty Acid (BCFA) Analysis

SCFA and BCFA were extracted from 200 mg of caecal content with diethyl ether and measured using a GC-2014 gas chromatograph (Shimadzu, 's-Hertogenbosch, the Netherlands) (Eaton et al., 1998; De Weirdt et al., 2010; Boesmans et al., 2018). The results are expressed as μmol of SCFA or BCFA per gram of caecal content.

Statistical Analysis

Statistical analysis of zootechnical performance parameters, intestinal morphology, *q*PCR and SCFA data was performed using GraphPad Prism (version 7.04, San Diego, USA). Assumption of homoscedasticity (Bartlett's test and Brown-Forsythe test) was evaluated and when this assumption was met ($p > 0.05$), ANOVA, at a 5% of significance level, followed by Tukey's test, was performed. Due to the lack of homoscedasticity, the SCFA, BCFA and *q*PCR values were log transformed and further subjected to ANOVA.

Statistical analysis of the gut microbiota results was performed using R (version 4.2.1). The microbial alpha diversity (number of observed ASVs and the Shannon diversity index) was calculated using *phyloseq* (v1.18.0). The effect of the dietary treatment on the microbial alpha diversity was assessed using a Kruskall-Wallis test, followed by a Dunn's post hoc test. Prior to beta diversity analysis, the 16S sequencing data was transformed to portions. The Bray-Curtis distance was used as a measure for the microbial beta diversity. The dispersion (variance) in the beta diversity was calculated using the *betadisper* function in the *vegan* package (Dixon, 2003). ANOVA showed no difference in variances between the groups. Significant differences in the community composition between the groups were determined through a permutational multivariate analysis of variance using distance matrices (PERMANOVA), using the *adonis2* function in *vegan*. In case a significant effect of the diet was observed, pairwise comparison between the diets was performed using the function *pairwise.perm.manova* from the *RVAideMemoire* package and Bonferroni corrected p-values were reported (Hervé, 2022). Differentially abundant taxa (phyla, families or genera) in the ileal or caecal microbiome at the different sampling days were identified by applying DESeq2 on the non-rarefied community composition data (Love; Huber; Anders, 2014). Significant differences were obtained using a Wald test followed by a Benjamini-Hochberg multiple hypothesis correction.

RESULTS

Guar gum reduces animal performance and dietary β-mannanase supplementation restores performance to control group level

Through the experimental period GG supplementation impaired broiler performance (Table 3). The body weight of the animals fed a GG-supplemented diet was significantly lower at all ages as compared to the animals fed the control diet, and *β*-mannanase supplementation restored the body weight to control levels. Overall from 0-35 days, GG supplementation significantly reduced the ADG with 7.31 g/day and increased FCR with 0.14, relative to the control group ($p<0.0001$), while the animals that received dietary *β*-mannanase as an additive to the GG diet, had an ADG and FCR that was not different from the animals fed the control diet (Table 3).

Table 3- The body weight in grams (BW) at day 1, 14, 28, 35 and 42, and feed conversion ratio (FCR), daily feed intake (DFI), and daily weight gain (DWG) measured at 4 time intervals, for animals fed a control diet or a diet supplemented with guar gum, either with or without *β*-mannanase supplementation at 330g/ton feed. Values are the means for 12 pens of 21 chickens ± standard deviation of the mean. ANOVA, followed by Tukey multiple comparison test, was used to determine statistical differences among groups

	Control	**GG**	**GG+E**	**Control vs GG**	**Control vs GG+E**	**GG vs GG+E**
	Mean ± Standard Deviation			**Adjusted p-value**		
BW 1 d	42.04 ± 1.10	42.31 ± 0.85	42.07 ± 0.35	0.8103	0.9978	0.8441
BW 14 d	490.80 ± 13.23	457.60 ± 15.09	497.90 ± 12.34	<0.0001	0.4186	<0.0001
BW 21 d	961.50 ± 27.68	901.20 ± 22.38	960.60 ± 29.12	<0.0001	0.9967	<0.0001
BW 28 d	1,551 ± 54.27	1,428 ± 51.13	1,544 ± 12.20	<0.0001	0.9364	<0.0001
BW 35 d	2,320 ± 138.30	2,038 ± 75.62	2,302 ± 112.30	<0.0001	0.9203	<0.0001
Period 1-14 days						
DFI (g/bird)	35.74 ± 0.96	34.66 ± 1.20	36.06 ± 1.51	0.0997	0.8081	0.0252
ADG (g/day/bird)	31.86 ± 0.89	29.35 ± 1.22	32.43 ± 0.88	<0.0001	0.3721	<0.0001
FCR	1.12 ± 0.02	1.18 ± 0.03	1.11 ± 0.03	0.0001	0.7025	<0.0001
Period 15-21 days						
DFI (g/bird)	86.97 ± 3.08	85.34 ± 3.07	86.09 ± 3.38	0.4319	0.7808	0.8323
ADG (g/day/bird)	67.16 ± 2.80	63.01 ± 2.17	65.46 ± 4.76	0.0150	0.4519	0.2028
FCR	1.29 ± 0.02	1.35 ± 0.03	1.32 ± 0.05	0.0020	0.3052	0.0782
Period 22-27 days						
DFI (g/bird)	123.80 ± 3.45	123.20 ± 7.33	124.80 ± 3.68	0.9616	0.8805	0.7368
ADG (g/day/bird)	84.13 ± 5.17	74.90 ± 4.48	81.86 ± 3.29	<0.0001	0.4237	0.0013
FCR	1.47 ± 0.06	1.64 ± 0.08	1.52 ± 0.05	<0.0001	0.1504	0.0003
Period 28-35 days						
DFI (g/bird)	165.90 ± 8.06	157.30 ± 5.32	168.90 ± 8.50	0.0202	0.5812	0.0015
ADG (g/day/bird)	108.10 ± 12.81	85.29 ± 9.53	106.10 ± 11.95	<0.0001	0.9115	0.0003
FCR	1.55 ± 0.15	1.86 ± 0.160	1.60 ± 0.14	<0.0001	0.6454	0.0006
Overall 0-35 days						
DFI (g/bird)	83.63 ± 2.46	81.24 ± 2.54	84.10 ± 2.69	0.0720	0.8960	0.0264
ADG (g/day/bird)	61.15 ± 3.35	53.84 ± 2.51	60.22 ± 3.01	<0.0001	0.7248	<0.0001
FCR	1.37 ± 0.04	1.51 ± 0.04	1.39 ± 0.03	<0.0001	0.2643	<0.0001

GG- guar gum; GG+E- guar gum + *β*-mannanase.

Effects of guar gum and β-mannanase supplementation on gut morphometry

The intestinal morphometry was evaluated at 14 and 28 days in both the duodenum and ileum segments (Table 4). At the duodenum level, no significant changes in villus height or crypt depth were observed between groups at both ages. In the ileum, GG ingestion significantly increased the villus height and the villus: crypt ratio as compared to the control group at 14 and 28 days, while both parameters did not differ between the β-mannanase group and the control.

Table 4- Duodenal and ileal villus height, crypt depth, villus:crypt ratio and $CD3^+$ T-cell infiltration, at 2 time points, for animals fed either a control diet or a diet supplemented with guar gum, either with or without *β*-mannanase supplementation at 330g/ton feed. Values are the means of 12 animals (one/pen/treatment) ± standard deviation. ANOVA, followed by Tukey multiple comparison test, was used to determine statistical differences among groups

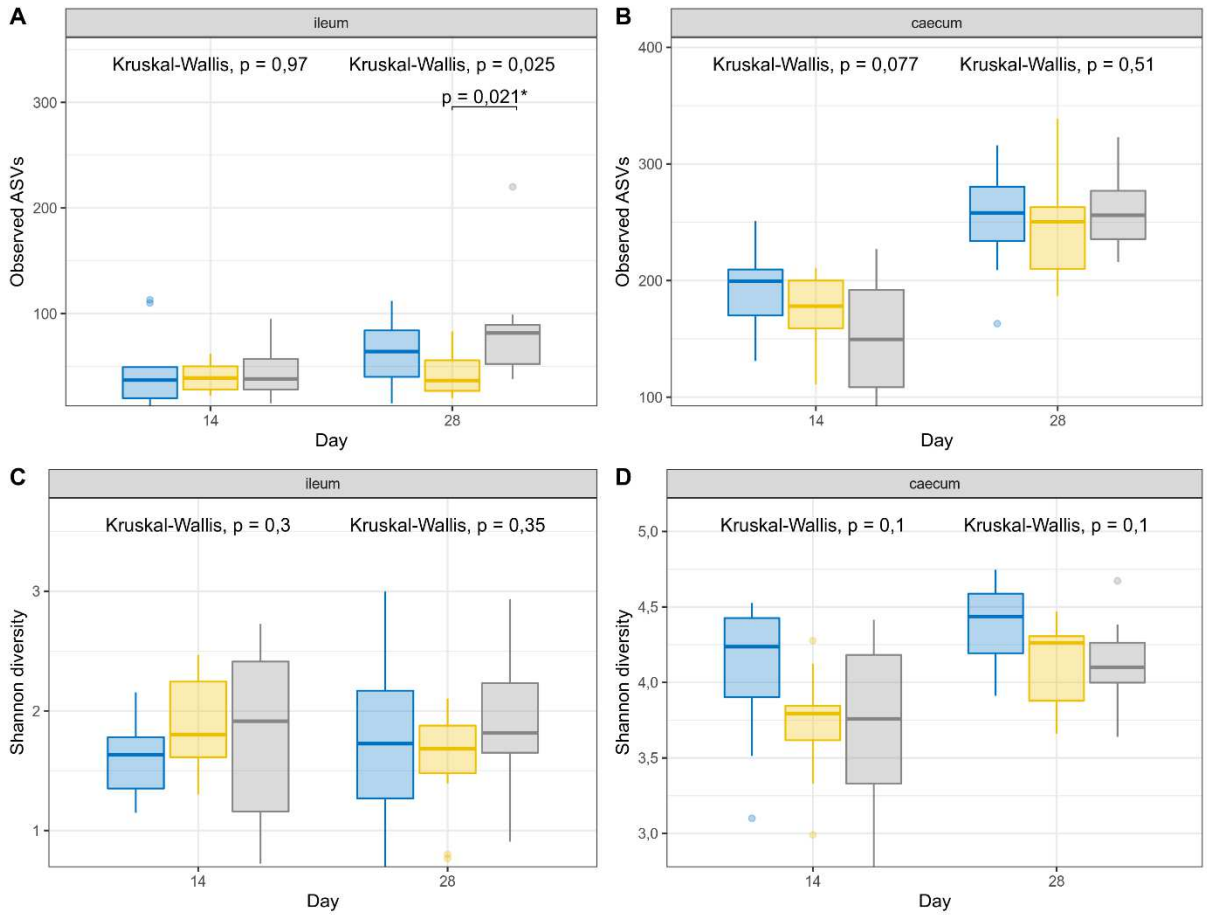
	Control	GG	GG+E	Control vs GG	Control vs GG+E	GG vs GG+E
	Mean ± Standard Deviation			Adjusted p-value		
Duodenum 14 days						
Villus height µm	1,470 ± 267.60	1,419 ± 143.20	1,484 ± 125.20	0.7848	0.9836	0.6817
Crypt depth µm	184.80 ± 33.15	195.00 ± 24.59	179.90 ± 32.23	0.6871	0.9174	0.4464
Villi: crypt ratio	8.20 ± 2.01	7.34 ± 0.87	8.46 ± 1.47	0.3593	0.9089	0.1841
Duodenum 28 days						
Villus height µm	1,874 ± 215.20	1,860 ± 219.60	1,720 ± 307.80	0.9898	0.3050	0.3723
Crypt depth µm	180.60 ± 34.57	180.80 ± 22.57	176.10 ± 42.90	>0.9999	0.9455	0.9410
Villi: crypt ratio	10.71 ± 2.16	10.38 ± 1.39	10.12 ± 2.26	0.9138	0.7521	0.9461
Ileum 14 days						
Villus height µm	389.20 ± 72.86	509.90 ± 117.40	416.70 ± 77.53	0.0076	0.7439	0.0455
Crypt depth µm	119.70 ± 38.66	110.90 ± 29.47	122.50 ± 27.87	0.7848	0.9768	0.6613
Villi: crypt ratio	3.22 ± 0.70	4.69 ± 0.827	3.51 ± 0.860	0.0003	0.6553	0.0031
Ileum 28 days						
Villus height µm	489 ± 124.40	640 ± 167	615.80 ± 130.50	0.0354	0.0880	0.9084
Crypt depth µm	112.20 ± 36.18	114.60 ± 26.68	116.70 ± 28.57	0.9793	0.9323	0.9856
Villi: crypt ratio	4.51 ± 0.85	5.60 ± 0.76	5.42 ± 0.95	0.0103	0.0364	0.8611

GG- guar gum; GG+E- guar gum + *β*-mannanase; $CD3^+$ T-cell- expressed as percentage of mucosal area positive for T-cells $CD3^+$ lymphocytes infiltration.

Effects of guar gum and β-mannanase supplementation on the ileal and caecal microbiota composition

The composition of the ileal and caecal microbiome was evaluated at 14 and 28 days of age. The microbial richness was evaluated through the number of observed ASVs and the estimated community diversity (Shannon index) in each sample (Figure 1). In the ileum, no significant changes were observed at 14 days. At 28 days GG ingestion reduced the microbial richness (p= 0.021) as compared to GG+E. No differences in α-diversity metrices were seen between groups in the caeca (Figure 1 B, D).

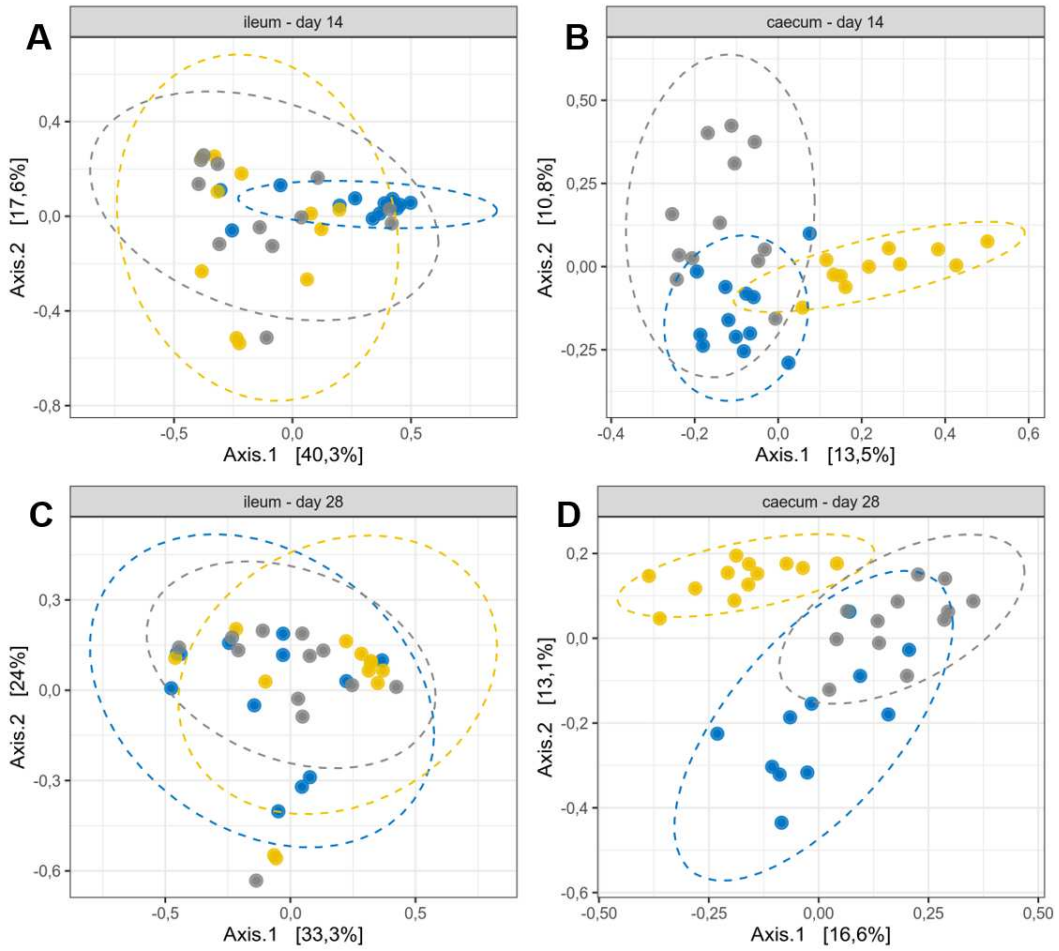
Figure 1- Ileal and caecal bacterial α-diversity measurements (A, B: observed ASVs; C, D: Shannon diversity) at 2 time points (14 or 28 days of age) among groups of animals fed a control diet or a diet supplemented with guar gum, either or not supplemented with β-mannanase at 330g/ton feed. Values are the means of 12 animals (one/pen/treatment)



● Control; ● Guar gum; ● Guar gum+β-mannanase.

Bray–Curtis dissimilarity metric was used to investigate β-diversity in the ileum and caecum between the treatment groups. In the ileum at 14 days a significant shift in the microbial communities was observed, in which 18% of the variation was due to the treatment. The β-diversity of the GG ($p= 0.0033$) and GG+E ($p= 0.0291$) groups were significantly different from the control group (Figure 2 A). β-mannanase supplementation of the GG diet did not affect the ileal microbial composition, as no difference between the GG and GG+E group could be observed ($p= 0.394$). No differences were observed in the ileum at 28 days. In the caeca, a significant difference in the microbial communities was observed at 14 days, in which 17% of the variation could be justified by the treatment, and all groups differed from each other (Figure 2 B). At 28 days 22% of the difference in caecal microbial community composition could be attributed to the dietary treatment, and all groups differed from each other (Figure 2 D).

Figure 2- Principal coordinate analysis plot of bacterial *β*-diversity based on Bray-Curtis dissimilarities. Ileal and caecal bacterial *β*-diversity measurements at 2 time points among groups of animals fed a control diet or a diet supplemented with guar gum, either or not supplemented with *β*-mannanase at 330g/ton feed. Values are the means of 12 animals (one/pen/treatment)



● Control; ● Guar gum; ● Guar gum+*β*-mannanase. Each dot represents an individual chicken microbiome. *β*-Diversity. Bray–Curtis dissimilarity metric. **A-** Ileum 14 days: significant separation in the microbial communities (p= 0.002), 18% of the difference is attributed to the treatments, GG (p= 0.0033) and GG+E (p= 0.0291) differed from control. **B-** Caecum 14 days: a significant difference of the microbial communities was observed (p= 0.001), 17% of the difference can be attributed to the treatments, all groups differ from each other (control vs GG p= 0.0003; control vs GG+E p= 0.0006; GG+E vs GG p= 0.0003). **C-** Ileum 28 days: there was no statically difference among the microbial communities (p= 0.219). **D-** Caecum 28 days: a significant difference (p= 0.001) among the groups was observed. The treatments explain 22% of the variation between the samples, all groups differ from each other (control vs GG p= 0.0003; control vs GG+E p= 0.0006; GG+E vs GG p= 0.0003).

The phylum *Firmicutes* was the most abundant phylum in both intestinal segments at both ages (> 90%). In the ileum on 14 days, GG group had a significantly higher relative abundance of the phylum *Proteobacteria* as compared to the control group (6.2% vs 0.08%, p= <0.0001). Adding *β*-mannanase to the GG group (GG+E) resulted in a significantly lower relative *Proteobacteria* abundance as compared to the GG group (1.7% vs 6.2%, p= 0.0264) (Table 5). No significant differences were observed in the ileum at 28 days, at phylum level.

In the caecum at both 14 and 28 days, GG significantly increased the relative abundance of *Actinobacteriota* and *Proteobacteria* and reduced *Bacteroidota* as compared to the control group. *β*-mannanase supplementation (GG+E) resulted in a significant decrease in *Actinobacteriota* and *Proteobacteria* and an increase in *Bacteroidota* relative abundance as compared to the GG group. No significant differences were observed between the control and GG+*β*-mannanase groups at phylum level.

At genus level in the ileum at 14 days, GG supplementation increased the relative abundance of *Bifidobacterium*, *Streptococcus*, *UCG-008*, *Tyzzerella*, *Escherichia-Shigella*, and reduced *Enterococcus*, *Family_Peptostreptococcaceae* and *Romboutsia*, as compared to the control group (Table 6). The *β*-mannanase supplementation was able to restore all these shifts to control levels. In the ileum at 28 days GG supplementation increased *Tyzzerella* abundance and decreased *Erysipelatoclostridium*, *Butyricicoccus*, *Faecalibacterium*, *Family_Ruminococcaceae* and *Romboutsia,* and again *β*-mannanase supplementation restored these changes to control levels (Table 6).

In the caecum, at 14 days of age, GG supplementation caused significant changes in 24 bacterial genera as compared to the control group, and dietary *β*-mannanase was effective to restore 20 (83.33%) of these to control levels (Table 7). The main changes induced by GG were an increase in genera belonging to the families *Lactobacillaceae* (*HT002*, *Lactobacillus* and *Limasilobacillus*), *Streptococcacea* (*Streptococcus*), *Lachnospiraceae* (*Marvinbryantia* and *Roseburia*) and *Enterobacteriaceae* (*Escherichia-Shigella*) and a decrease in some genera from the families *Oscillospiraceae* (*Intestinimonas* and *Family_oscillapiraceae*), *Ruminococcaceae* (*Anaerotruncus*, *Caproiciproducens*, *DTU089*, *Family_Ruminococcaceae*, *Incertae Sedis* and *Negativibacillus*) and *Peptostreptococcaceae* (Family_*Peptostreptococcaceae*).

At 28 days, in the caecum, GG supplementation caused significant changes in 38 bacterial genera as compared to the control group, and dietary *β*-mannanase restored 36 to control levels (Table 7). Relative to the control group, GG significantly increased the relative abundance of genera belonging to the families *Clostridiaceae* (*Clostridium sensu stricto 1*),

Lactobacillaceae (*HT002* and *Lactobacillus*), *Lachnospiraceae* (*[Eubacterium] hallii group*, *Blautia*, *Family_Lachnospiraceae*, *Frisingicoccus*, *Lachnospira*, *Roseburia* and *UC5-1-2E3*) and *Enterobacteriaceae* (*Escherichia-Shigella*), and reduced genera from the families *Bacillaceae* (*Bacillus* and *Family_Bacillaceae*), *Butyricoccaceae* (*Butyricicoccus*, *UCG-008* and *UCG-009*), *Oscillospiraceae* (*Family_Oscillospiraceae*, *Flavonifractor*, *Intestinimonas*, *Oscillospira* and *UCG-005*) and *Ruminococcaceae* (*Anaerofilum*, *Anaerotruncus*, *Family_Ruminococcaceae*, *Fournierella* and *Paludicola*).

In general, *β*-mannanase dietary supplementation was able to restore the main microbial shifts induced by GG and restore the gut microbiome to levels of the control group.

To confirm specific microbial changes induced by the diets, *q*PCR analysis was done to quantify the abundance of specific families and genera in caecal content. Regarding the family *Enterobacteriaceae* no significance was observed at both ages, however, at 14 days the GG group had a trend towards higher levels (p= 0.0709) (Figure 3). The family *Ruminococcaceae* was significantly reduced in the GG group at 14 days relative to the control. The abundance of the *Lactobacillus* genus was significantly higher in the GG group as compared to the control group at 14 and 28 days, which is in accordance with the 16SrRNA gene data.

The gene encoding butyryl-CoA: acetate CoA-transferase, which estimates the butyrate-producing ability of the microbiota (Louis; Flint, 2007), was also quantified. At 28 days a significant decrease was found in the GG group relative to the control group.

Table 5- Mean abundance of ileal and caecal phyla in the microbiota at 2 time points among groups of animals fed a control diet or a diet supplemented with guar gum, either or not supplemented with *β*-mannanase at 330g/ton feed. Values are the means of 12 animals (one/pen/treatment). DESeq2 analysis at 5% of significance level, was used to determine statistical differences among groups

Phylum	**CTR**	**GG**	**GG + E**	**CTR vs GG**	**CTR vs GG + E**	**GG vs GG + E**
	Mean abundance (%)			**adjusted *p*-value**		
			Ileum 14 days			
Actinobacteriota	2.69	3.78	6.16	>0.05	>0.05	>0.05
Bacteroidota	0.01	0	0.02	>0.05	>0.05	>0.05
Firmicutes	97.21	90.01	92.07	>0.05	>0.05	>0.05
Proteobacteria	0.09	6.21	1.75	< 0.0001	0.0316	0.0264
			Ileum 28 days			
Actinobacteriota	4.33	5.94	5.03	>0.05	>0.05	>0.05
Bacteroidota	0.04	0	0.16	>0.05	>0.05	>0.05
Firmicutes	95.10	92.30	92.87	>0.05	>0.05	>0.05
Proteobacteria	0.52	1.76	1.92	>0.05	>0.05	>0.05
			Caecum 14 days			
Actinobacteriota	0.25	0.67	0.33	<0.0001	>0.05	<0.0001
Bacteroidota	6.04	1.00	4.65	<0.0001	>0.05	<0.0001
Firmicutes	91.73	92.29	92.06	>0.05	>0.05	>0.05
Proteobacteria	1.96	6.02	2.94	0.0163	>0.05	>0.05
			Caecum 28 days			
Actinobacteriota	0.17	1.14	0.26	<0.0001	>0.05	0.0001
Bacteroidota	8.86	3.95	8.28	<0.0001	>0.05	0.0001
Firmicutes	90.25	92.30	90.51	<0.0001	>0.05	0.0001
Proteobacteria	0.71	2.59	0.94	0.0047	>0.05	0.0071

Table 6- Differentially abundant genera in the ileal microbiota at 2 time points among groups of animals fed a control diet or a diet supplemented with guar gum, either or not supplemented with *β*-mannanase at 330g/ton feed. Values are the means of 12 animals (one/pen/treatment). DESeq2 analysis at 5% of significance level, was used to determine statistical differences among groups

Phylum	Class	Family	Genus	CTR	GG	GG + E	CTR vs GG	CTR vs GG + E	GG vs GG + E
				Mean abundance (%)			adjusted *p*-value		
ILEUM 14 days									
Actinobacteriota	*Actinobacteria*	*Bifidobacteriaceae*	*Bifidobacterium*	0.0004	0.0427	0.0086	0.0384	0.3273	1
Firmicutes	*Bacilli*	*Enterococcaceae*	*Enterococcus*	13.2080	0.9023	6.5246	0.0034	1	0.0073
Firmicutes	*Bacilli*	*Erysipelatoclostridiaceae*	*Erysipelatoclostridium*	0.0719	0.0104	0.0319	0.0571	1	0.6122
Firmicutes	*Bacilli*	*Lactobacillaceae*	*Lactobacillus*	66.8353	47.8278	49.2388	0.0571	1	0.4713
Firmicutes	*Bacilli*	*Lactobacillaceae*	*Weissella*	0	0.0082	0.0574	0.2450	0.0104	0.3590
Firmicutes	*Bacilli*	*Staphylococcaceae*	*Staphylococcus*	0.1599	0.0837	0.3014	0.4290	0.0104	0.1463
Firmicutes	*Bacilli*	*Streptococcaceae*	*Streptococcus*	2.8872	19.8697	7.4034	0.0226	0.3273	1
Firmicutes	*Clostridia*	*Butyricicoccaceae*	UCG-008	0	0.1665	0	<0.0001	1	<0.0001
Firmicutes	*Clostridia*	*Clostridiaceae*	*Candidatus Arthromitus*	2.5497	1.1646	9.3110	0.1829	1	0.0192
Firmicutes	*Clostridia*	*Lachnospiraceae*	*Tyzzerella*	0.0028	1.3470	0.0176	0.0046	1	0.4713
Firmicutes	*Clostridia*	*Peptostreptococcaceae*	*Family_Peptostreptococcaceae*	0.5989	0.0160	0.1894	0.0125	1	0.0046
Firmicutes	*Clostridia*	*Peptostreptococcaceae*	*Romboutsia*	0.2374	0	0.0415	<0.0001	1	<0.0001
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriaceae*	*Escherichia-Shigella*	0.0879	6.2105	1.7457	0.0034	0.1327	0.9838

ILEUM 28 days									
Firmicutes	*Bacilli*	*Erysipelatoclostridiaceae*	*Erysipelatoclostridium*	0.0124	0.0003	0.1467	0.0210	1	0.0104
Firmicutes	*Clostridia*	*Butyricicoccaceae*	*Butyricicoccus*	0.0389	0	0.1264	0.0011	1	0.0104
Firmicutes	*Clostridia*	*Clostridiaceae*	*Clostridium sensu stricto 1*	4.0782	5.0610	0.0358	0.9294	0.2173	0.0104
Firmicutes	*Clostridia*	*Lachnospiraceae*	*[Ruminococcus] torques group*	0.2051	0.0134	0.6417	0.0987	1	0.0347
Firmicutes	*Clostridia*	*Lachnospiraceae*	*Tyzzerella*	0	0.3303	0	<0.0001	1	<0.0001
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Faecalibacterium*	0.2367	0.0050	0.6200	0.0347	1	0.0347
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Family_Ruminococcaceae*	0.0563	0.0025	0.2794	0.0443	1	0.0735
Firmicutes	*Clostridia*	*Peptostreptococcaceae*	*Romboutsia*	4.2001	0.1062	1.0038	0.0009	1	0.0735

Only bacterial families with minimum relative abundance > 0.05% in at least one group are reported. The taxonomic classification and relative abundance of each family are shown.

Table 07- Differentially abundant genera in the caecal microbiota at 2 time points among groups of animals fed a control diet or a diet supplemented with guar gum, either or not supplemented with *β*-mannanase at 330g/ton feed. Values are the means of 12 animals (one/pen/treatment). DESeq2 analysis at 5% of significance level, was used to determine statistical differences among groups

Phylum	Class	Family	Genus	CTR	GG	GG + E	CTR vs GG	CTR vs GG + E	GG vs GG + E
				Mean abundance (%)			adjusted *p*-value		
CAECUM 14 days									
Actinobacteriota	*Actinobacteria*	*Bifidobacteriaceae*	*Bifidobacterium*	0	0.2836	0.0008	<0.0001	<0.0001	0.0220
Actinobacteriota	*Actinobacteria*	*Corynebacteriaceae*	*Corynebacterium*	0.0075	0.0189	0	0.5852	NA	0.0220
Actinobacteriota	*Coriobacteriia*	*Eggerthellaceae*	*Eggerthella*	0.0520	0.1896	0.0235	0.0069	0.5446	0.0002
Firmicutes	*Bacteroidia*	*Bacteroidaceae*	*Bacteroides*	1.7703	0.3534	1.0694	0.4688	0.3784	0.0353
Bacteroidota	*Bacteroidia*	*Rikenellaceae*	*Alistipes*	4.2728	0.6545	3.5875	<0.0001	0.5750	<0.0001
Firmicutes	*Bacilli*	*Bacillaceae*	*Bacillus*	0.7770	0.0806	0.2986	0.0170	0.2633	0.4078
Firmicutes	*Bacilli*	*Lactobacillaceae*	*HT002*	1.0569	2.7934	0.7001	0.0069	0.5446	0.0002
Firmicutes	*Bacilli*	*Lactobacillaceae*	*Lactobacillus*	3.9770	11.3182	3.8157	<0.0001	0.7878	<0.0001
Firmicutes	*Bacilli*	*Lactobacillaceae*	*Limosilactobacillus*	0.0547	0.4844	0.0984	0.0006	0.7842	0.0220
Firmicutes	*Bacilli*	*Streptococcaceae*	*Streptococcus*	0.0830	7.7199	0.4476	<0.0001	0.3490	<0.0001
Firmicutes	*Clostridia*	*Defluviitaleaceae*	*Defluviitaleaceae UCG-011*	0.1487	0.4659	0.0962	0.1933	0.7045	0.0415
		[Eubacterium]	*Family_[Eubacterium]*						
Firmicutes	*Clostridia*	*coprostanoligenes group*	*coprostanoligenes group*	1.8682	5.6657	3.4489	0.0170	0.4980	0.1555
Firmicutes	*Clostridia*	*Lachnospiraceae*	*Family_Lachnospiraceae*	2.6678	2.5480	2.2075	0.4118	0.4546	0.0460
Firmicutes	*Clostridia*	*Lachnospiraceae*	*Frisingicoccus*	0.0296	0.2682	0	0.1802	0.0093	<0.0001
Firmicutes	*Clostridia*	*Lachnospiraceae*	*Marvinbryantia*	0.4971	2.3199	0.4942	0.0245	0.6922	0.0566
Firmicutes	*Clostridia*	*Lachnospiraceae*	*Roseburia*	0.0059	0.6579	0.0477	0.0418	0.4546	0.3246

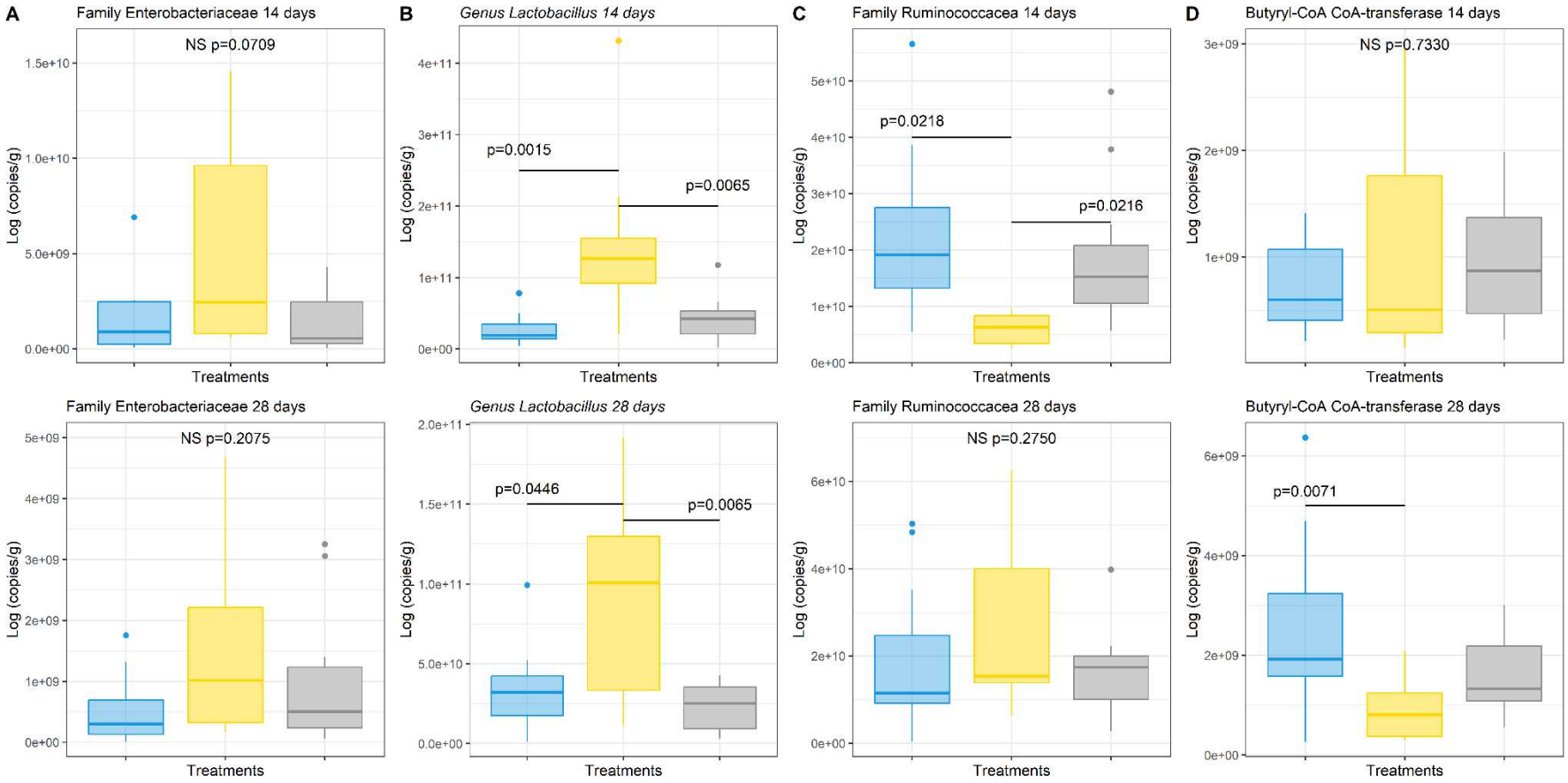
Firmicutes	*Clostridia*	*Lachnospiraceae*	*Tyzzerella*	3.1932	2.4835	1.0642	0.9897	0.0093	0.0087
Firmicutes	*Clostridia*	*Monoglobaceae*	*Monoglobus*	1.3950	3.5698	1.5500	0.0457	0.9020	0.0307
Firmicutes	*Clostridia*	*Oscillospiraceae*	*Family_Oscillospiraceae*	2.5308	0.7232	0.9058	0.0457	0.0086	0.5377
Firmicutes	*Clostridia*	*Oscillospiraceae*	*Flavonifractor*	0.4751	0.2950	1.4531	0.8243	0.0043	0.0003
Firmicutes	*Clostridia*	*Oscillospiraceae*	*Intestinimonas*	5.9153	0.4555	1.7173	<0.0001	0.0026	0.0566
Firmicutes	*Clostridia*	*Oscillospiraceae*	*Oscillibacter*	0.6241	0.3149	3.3616	0.3680	0.0577	0.0003
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Anaerotruncus*	2.8432	0.0803	0.5591	0.0009	0.3936	0.0410
Firmicutes	*Clostridia*	*Ruminococcaceae*	*CAG-352*	0.0016	0.3842	0.0591	0.0032	0.2258	0.2259
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Caproiciproducens*	0.0241	0	0.0941	0.0221	0.2939	0.0007
Firmicutes	*Clostridia*	*Ruminococcaceae*	*DTU089*	0.6647	0.1800	1.8231	0.0992	0.2633	0.0003
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Family_Ruminococcaceae*	6.0167	2.0304	5.1728	0.0244	0.3936	0.2973
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Fournierella*	0.0950	0.0411	0.0013	0.7692	0.0093	0.0410
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Incertae Sedis*	4.7382	1.7320	12.2317	0.0244	0.0382	<0.0001
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Negativibacillus*	0.9024	0.2068	0.8357	0.0049	0.5767	0.0401
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Subdoligranulum*	1.2818	5.5883	0.6227	0.0020	0.3784	<0.0001
Firmicutes	*Clostridia*	*Peptostreptococcaceae*	*Family_Peptostreptococcaceae*	0.0419	0	0.0905	<0.0001	0.8898	0.0003
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriaceae*	*Escherichia-Shigella*	1.9624	6.0210	2.8657	0.0049	0.8246	0.0120
			CAECUM 28 days						
Actinobacteriota	*Actinobacteria*	*Bifidobacteriaceae*	*Bifidobacterium*	0	0.6695	0	<0.0001	1	<0.0001
Actinobacteriota	*Coriobacteriia*	*Eggerthellaceae*	*CHKCI002*	0.1540	0.3567	0.2271	0.0385	0.6846	0.2387
Bacteroidota	*Bacteroidia*	*Rikenellaceae*	*Alistipes*	4.9440	2.0440	3.4186	0.0039	0.6444	0.0665
Firmicutes	*Bacilli*	*Bacillaceae*	*Bacillus*	1.1595	0.2377	0.8679	0.0417	0.9439	0.1153
Firmicutes	*Bacilli*	*Bacillaceae*	*Family_Bacillaceae*	0.1304	0	0.0796	<0.0001	0.9687	<0.0001
Firmicutes	*Bacilli*	*Lactobacillaceae*	*HT002*	0.3678	1.9823	0.1494	0.0105	0.6651	0.0001
Firmicutes	*Bacilli*	*Lactobacillaceae*	*Lactobacillus*	0.9118	4.8281	0.8689	0.0003	1	0.0004

Firmicutes	*Bacilli*	*Streptococcaceae*	*Streptococcus*	7.9427	16.0682	6.0690	0.0982	0.9326	0.0186
Firmicutes	*Clostridia*	Butyricicoccaceae	*Butyricicoccus*	6.5845	2.0123	4.3069	0.0003	0.5928	0.0186
Firmicutes	*Clostridia*	Butyricicoccaceae	UCG-008	0.0397	0	0.0046	0.0288	0.6444	0.2325
Firmicutes	*Clostridia*	Butyricicoccaceae	UCG-009	0.1720	0.0328	0.1117	0.0026	0.7532	0.0253
Firmicutes	*Clostridia*	*Christensenellaceae*	*Christensenellaceae R-7 group*	4.2309	4.1057	2.3629	0.9887	0.1443	0.0349
Firmicutes	*Clostridia*	*Christensenellaceae*	*Family_Christensenellaceae*	0.0459	0.0094	0.0363	0.0480	1	0.0665
Firmicutes	*Clostridia*	*Clostridiaceae*	*Clostridium sensu stricto 1*	0.0062	0.2236	0.0026	0.0367	0.7863	0.0023
Firmicutes	*Clostridia*	*Defluviitaleaceae*	*Defluviitaleaceae UCG-011*	0.3430	0.4338	0.1992	0.3254	0.5929	0.0186
Firmicutes	*Clostridia*	*[Eubacterium] coprostanoligenes group*	*Family_[Eubacterium] coprostanoligenes group*	2.1012	4.6901	3.1611	0.0039	1	0.0031
Firmicutes	*Clostridia*	*Hydrogenoanaerobacterium*	*Family_Hydrogenoanaerobacterium*	0.0119	0.0020	0.0427	0.2526	0.6142	0.0167
Firmicutes	*Clostridia*	*Lachnospiraceae*	*[Eubacterium] hallii group*	0.1646	0.4483	0.2148	0.0155	0.9326	0.0599
Firmicutes	*Clostridia*	*Lachnospiraceae*	*[Eubacterium] ventriosum group*	0.0997	0.0135	0.0038	0.0340	0.0006	0.1633
Firmicutes	*Clostridia*	*Lachnospiraceae*	*Blautia*	1.7790	4.3392	0.8162	0.0059	0.3236	<0.0001
Firmicutes	*Clostridia*	*Lachnospiraceae*	*Dorea*	0.3769	0.2576	0.0072	0.6096	<0.0001	<0.0001
Firmicutes	*Clostridia*	*Lachnospiraceae*	*Family_Lachnospiraceae*	1.9547	2.6942	2.3210	0.0043	0.3236	0.2824
Firmicutes	*Clostridia*	*Lachnospiraceae*	*Frisingicoccus*	0.1599	0.6978	0.0476	0.0518	0.5180	0.0004
Firmicutes	*Clostridia*	*Lachnospiraceae*	*Lachnoclostridium*	1.4047	0.9023	1.5288	0.2635	0.7863	0.0498
Firmicutes	*Clostridia*	*Lachnospiraceae*	*Lachnospira*	0.0110	0.3710	0.0267	<0.0001	0.4807	<0.0001
Firmicutes	*Clostridia*	*Lachnospiraceae*	*Lachnospiraceae NK4A136 group*	0.6699	0.8944	0.1580	0.7390	0.1044	0.0047
Firmicutes	*Clostridia*	*Lachnospiraceae*	*Marvinbryantia*	0.8277	1.5084	0.4420	0.1196	0.5928	0.0031
Firmicutes	*Clostridia*	*Lachnospiraceae*	*Roseburia*	0.0045	0.2859	0.0218	0.0043	0.6142	0.0835
Firmicutes	*Clostridia*	*Lachnospiraceae*	*Sellimonas*	1.0880	1.1354	0.4360	0.6137	0.0006	<0.0001
Firmicutes	*Clostridia*	*Lachnospiraceae*	*Tyzzerella*	2.9827	1.1992	1.5099	0.0315	0.3886	0.5791
Firmicutes	*Clostridia*	*Lachnospiraceae*	*UC5-1-2E3*	0.1178	0.4007	0.1730	0.0253	0.6611	0.1791

Firmicutes	*Clostridia*	*Monoglobaceae*	*Monoglobus*	0.7497	4.2514	0.5809	<0.0001	0.9439	<0.0001
Firmicutes	*Clostridia*	*Oscillospiraceae*	*Family_Oscillospiraceae*	2.5731	1.0761	2.9011	0.0182	0.5787	0.0023
Firmicutes	*Clostridia*	*Oscillospiraceae*	*Flavonifractor*	0.6582	0.2162	0.6112	0.0203	1	0.0090
Firmicutes	*Clostridia*	*Oscillospiraceae*	*Intestinimonas*	2.8395	0.5787	2.7148	<0.0001	1	<0.0001
Firmicutes	*Clostridia*	*Oscillospiraceae*	*Oscillibacter*	0.5280	0.3254	0.7632	0.4564	0.3973	0.0186
Firmicutes	*Clostridia*	*Oscillospiraceae*	*Oscillospira*	0.2566	0.0622	0.1776	0.0315	0.9439	0.0319
Firmicutes	*Clostridia*	*Oscillospiraceae*	*Pseudoflavonifractor*	0.1228	0.0291	0.1126	0.0658	1	0.0399
Firmicutes	*Clostridia*	*Oscillospiraceae*	UCG-005	5.2923	2.4787	5.0788	0.0431	0.9687	0.0845
Firmicutes	*Clostridia*	Peptococcaceae	Family_*Peptococcaceae*	0.4498	0.7504	0.5115	0.0431	0.9326	0.1516
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Anaerofilum*	0.2865	0.0282	0.0984	0.0272	0.6444	0.2236
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Anaerotruncus*	0.8918	0.0214	0.4581	<0.0001	0.6444	<0.0001
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Angelakisella*	0.0094	0.0013	0.0526	0.2987	0.6533	0.0348
Firmicutes	*Clostridia*	*Ruminococcaceae*	*CAG-352*	0	0.1907	0	<0.0001	1	<0.0001
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Caproiciproducens*	0.0172	0.0025	0.0372	0.0550	0.7296	0.0032
Firmicutes	*Clostridia*	*Ruminococcaceae*	*DTU089*	0.4591	0.3197	0.6368	0.5802	0.3973	0.0265
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Faecalibacterium*	12.9897	7.6660	21.6605	0.2828	0.2132	0.0007
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Family_Ruminococcaceae*	5.0240	2.6720	5.4050	0.0059	0.9439	0.0006
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Fournierella*	0.9912	0.1160	0.1417	<0.0001	0.0006	0.7409
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Incertae Sedis*	1.7218	1.7541	4.2044	0.6137	0.0006	0.0017
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Paludicola*	0.4965	0.1426	0.3843	0.0368	0.9687	0.0665
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Subdoligranulum*	3.0761	6.0777	1.3110	0.1316	0.3236	0.0004
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriaceae*	*Escherichia-Shigella*	0.7102	2.5917	0.9393	0.0182	0.7865	0.0938

Only bacterial families with minimum relative abundance > 0.05% in at least one group are reported. The taxonomic classification and relative abundance of each family are shown.

Figure 3- *q*PCR analysis from caecal content at day 14 (first line) and day 28 (second line) for animals fed a control diet or a diet supplemented with guar gum, either or not supplemented with *β*-mannanase at 330g/ton feed. Values are the means of 12 animals (one/pen/treatment) ± standard deviation of the mean. ANOVA, followed by Tukey multiple comparison test, was used to determine statistical differences among groups



Y axis – results are expressed as log of copies per gram of caecal content. Treatments: Control; Guar gum; Guar gum+*β*-mannanase. NS – non-significant.

Guar gum and β-mannanase supplementation affect SCFA and BCFA concentrations in the caeca

SCFA and BCFA concentrations in caecal content were analyzed at 14 and 28 days of age (Table 8). At 14 days of age *β*-mannanase supplementation reduced propionate, valeric, isovaleric, isobutyric and BCFA levels, as compared to the GG group. At 28 days GG increased acetic and isocaproic acid levels relative to the control group, and again a reduction in propionate, caproic, valeric, isovaleric, isobutyric, isocaproic and BCFA levels were induced by the enzyme, relative to the GG group levels.

Table 8- SCFA concentrations in caecal content at 2 time points among groups of animals fed a control diet or a diet supplemented with guar gum, either or not supplemented with *β*-mannanase at 330g/ton feed. Values are the means of 12 animals (one/pen/treatment) ± standard deviation. ANOVA, followed by Tukey multiple comparison test, was used to determine statistical differences among groups

SCFA	CTR	GG	GG + E	CTR vs GG	CTR vs GG + E	GG vs GG + E
	Mean ± SD (µmol/g)			adjusted *p*-value		
CAECUM 14 days						
Acetic Acid	64.78 ± 22.60	66.12 ± 15.05	69.15 ± 21.53	0.9860	0.8560	0.9310
Butyric Acid	10.37 ± 4.99	10.04 ± 4.67	10.65 ± 5.97	0.9871	0.9909	0.9579
Propionic Acid	8.59 ± 5.22	7.68 ± 2.97	2.90 ± 0.29	0.9943	0.0001	0.0001
Caproic Acid	0.31 ± 0.02	0.32 ± 0.02	0.31 ± 0.01	0.8284	0.9943	0.8669
Isobutyric Acid	0.79 ± 0.19	0.92 ± 0.22	0.67 ± 0.14	0.2028	0.2943	0.0077
Isovaleric Acid	0.81 ± 0.24	1.00 ± 0.22	0.64 ± 0.12	0.0726	0.1486	0.0006
Isocaproic Acid	0.25 ± 0.01	0.25 ± 0.01	0.25 ± 0.01	0.9655	0.9977	0.9774
Valeric Acid	1.14 ± 0.31	1.22 ± 0.30	0.76 ± 0.25	0.8024	0.0073	0.0016
Total SCFA	90.33 ±31.53	87.66 ± 20.3	85.99 ± 27.36	0.9695	0.9180	0.9879
BCFA	1.91 ± 0.52	2.19 ± 0.42	1.57 ± 0.26	0.2713	0.1206	0.0033
CAECUM 28 days						
Acetic Acid	58.25 ± 9.62	65.01 ± 17.51	41.41 ± 17.32	0.5426	0.0504	0.0040
Butyric Acid	8.49 ± 2.81	14.62 ± 9.53	14.58 ± 12.36	0.2677	0.2723	>0.9999
Propionic Acid	9.93 ± 2.73	11.94 ± 4.92	7.50 ± 4.17	0.4963	0.3656	0.0334
Caproic Acid	0.30 ± 0.01	0.32 ± 0.01	0.30 ± 0.01	0.0579	0.9652	0.0292
Isobutyric Acid	0.88 ± 0.17	0.93 ± 0.32	0.61 ± 0.11	0.9865	0.0064	0.0035
Isovaleric Acid	0.91 ± 0.26	1.07 ± 0.49	0.67 ± 0.25	0.7524	0.1175	0.0211
Isocaproic Acid	0.24 ± 0.01	0.26 ± 0.02	0.25 ± 0.01	0.0242	0.9679	0.0421
Valeric Acid	1.06 ± 0.18	1.19 ± 0.16	0.87 ± 0.29	0.3485	0.1311	0.0052
Total SCFA	80.12 ± 10.74	95.55 ± 28.97	67.75 ± 37.17	0.3847	0.5659	0.0635
BCFA	2.04 ± 0.43	2.29 ± 0.78	1.48 ± 0.25	0.7004	0.0175	0.0019

GG- guar gum; GG+E- guar gum + *β*-mannanase; SCFA- short-chain fatty acid; BCFA- branched-chain fatty acid. Quantification limit: acetic acid- 18.43 µmol/g, butyric acid- 1.45 µmol/g; propionic acid- 1.47 µmol/g; caproic acid- 0.29 µmol/g; isobutyric acid- 0.43 µmol/g; isovaleric acid- 0.42 µmol/g; isocaproic acid- 0.24 µmol/g; valeric acid- 0.43 µmol/g; total SCFA- 23.73 µmol/g; BCFA- 0.88 µmol/g.

DISCUSSION

Guar gum is produced from guar, a drought resistant legume. The by-product guar meal may contain up to 45% protein and is used as a feed ingredient, but it contains a high concentration of *β*-mannans, considered to be an anti-nutritional factor, that leads to animal performance losses (Saeed et al., 2017). This was confirmed in our trial, and *β*-mannanase supplementation was able to restore the BW and FCR to control levels. The negative effects of GG on zootechnical performance was already observed in previous studies (Lee; Bailey; Cartwright, 2003; Mishra et al., 2013; Rama Rao et al., 2014). The poor performance is attributed to the high intestinal viscosity induced by GG, that reduces the passage rate, increases the satiety, reduces the feed intake (also observed in this study), and also impairs the nutrient absorption (Maisonnier et al., 2003; Owusu-Asiedu et al., 2006). According to previous studies the positive effects of dietary *β*-mannanase supplementation on broiler performance is a consequence of a higher ileal digestibility of carbohydrates and amino acids (Caldas et al., 2018; Latham et al., 2018; Gomez-Osorio; Oh; Lee, 2021; White et al., 2021).

Villus morphology was not negatively affected by GG supplementation to the diets. GG increased the ileal villus height as compared to the control group. Diets containing high levels of NSP significantly increase the length and weight of the gastrointestinal tract (Jorgensen et al., 1996). Maisonnier et al. (2003) also reported an increase in the villus height as a consequence of dietary GG supplementation and suggested that this finding is an adaptative process counteracting the negative effects of the diet, attempting to improve nutrient uptake.

Dysbiosis of the gut microbiome is a disruption in the balance, diversity, and function of intestinal microbial communities (Perez; Dorsen; Squires, 2019). In the current study guar gum ingestion caused changes in microbial diversity and composition in the caecal and ileal microbiome. Guar gum ingestion increased the relative abundance of the *Proteobacteria* phylum (ileum 14 d, caecum 14 and 28 d). A high abundance of phylum *Proteobacteria* is a hallmark of dysbiosis and poor chicken performance (Shin; Whon; Bae, 2015; Kollarcikova et al., 2019). The poor performance observed in the GG group in this study, might thus be associated with a diet-induced dysbiosis. The GG supplementation increased the relative abundance of bacterial families and genera known to contain opportunistic pathogens. *Escherichia-Shigella* (ileum 14 d, caecum 14 and 28d) and *Clostridium sensu stricto* 1 (encompassing *C. perfringens*) (caecum 28 d) are the most well-known examples of bacterial members that were increased by dietary GG supplementation. The *β*-mannanase

supplementation restored this shift to control levels. High levels of *Enterobacteriaceae* may increase intestinal lipopolysaccharide concentrations, often associated with increases in gut permeability, triggering inflammation (Bibbò et al., 2016). Guar gum supplementation also increased enterotoxigenic *Escherichia coli* proliferation in post-weaning piglets (Mcdonald et al., 1999). In chickens, an increase of *Escherichia-Shigella* has been associated with a poor zootechnical performance (Rubio et al., 2015; Han et al., 2016). Similarly, in the present study GG supplementation impaired the zootechnical performance. Another important change was observed in the genus *Lactobacillus* that was increased in GG group as compared to the others, a finding that was confirmed by qPCR. β-mannanase supplementation restored the levels to control values. *Lactobacillus* is a galactomannan fermenter and lactate producer (Ali et al., 2022). When high quantities of non-digestible carbohydrates reach the large intestine, they will be fermented and unusual high amounts of organic acids (lactic acid and SCFA) can be produced, resulting in a lower luminal pH, affecting the microbial composition (Petersen, 2005). While lactic acid bacteria are known for their prebiotic properties, intestinal overgrowth has been related to poor intestinal health in multiple studies (Park et al., 2021; Dey; Ray Chaudhuri, 2022; Slanzon et al., 2022).

Our data show that in the caecum, the mannan-rich diet increased the relative abundance of the genus *Bifidobacterium* and *Roseburia*, known by their capacity to ferment complex polysaccharides that have escaped proximal digestion (Jandhyala et al., 2015). This result supports our hypothesis of dysbiosis as consequence of poor ileal digestibility. The relative abundance of specific members of the *Ruminococcaceae* and *Lachnospiraceae* were reduced when GG was added to the broiler diets. These families contain butyrate producing bacteria, of which a subset use lactate, and are shown to be of crucial importance to maintain gut health (Parada Venegas et al., 2019). Examples of beneficial bacterial genera from the *Ruminococcaceae* family that are negatively affected by GG supplementation (and restored to normal levels after supplementation with the *β*-mannanase) are *Butyricicoccus* and *Faecalibacterium*. These are known as butyrate-producing microorganisms that are highly anti-inflammatory, improve intestinal integrity because of effects on tight junction protein expression, and associated with optimal animal performance (Onrust et al., 2015; Bedford et al., 2017; Sikandar et al., 2017). As an example, *Butyricicoccus* supplementation to broiler diets has been shown to improve animal performance in a diet-induced dysbiosis models and was shown to reduce necrotic enteritis caused by *C. perfringens* (Eeckhaut *et al.,* 2016). Also, it reduced the caecal abundance of *Escherichia*, an association seen in our study as well. While

butyrate production has been hypothesized as the main driver for beneficial effects related to these bacterial genera, we did not observe effects on SCFA production, although a lower caecal (28 d) abundance of the gene encoding butyryl-CoA acetate CoA-transferase was observed in the GG supplemented group.

Isobutyric, isovaleric, valeric acids and BCFA are the result of protein fermentation (Fan et al., 2015). They were increased in the group that received the GG diet as compared to the group receiving the GG+*β*-mannanase. Probably the higher abundance of protein-derived BCFAs in the caecal content of the GG group is a consequence of poor protein absorption in the upper intestine.

In conclusion, guar gum diet supplementation impairs the broilers' zootechnical performance and for the first time a detailed effect of a mannan-rich diet on the broiler's gut microbiome has been described. GG causes a shift towards increases in opportunistic pathogens and reductions in beneficial microbiota and *β*-mannanase supplementation was able to restore the effects on the microbiota composition to control levels. Our results indicate that *β*-mannanase is a viable feed additive that can be used to counteract negative effects of mannan-rich feed ingredients on the microbiota composition.

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7 GENERAL DISCUSSION

This study encompasses three challenges widely distributed in poultry farming worldwide: mycotoxins, non-starch polysaccharides, and necrotic enteritis. All are critical issues in animal production systems. Here, they were studied separately; however, in field conditions, they might occur simultaneously, with an increasing incidence due to global warming.

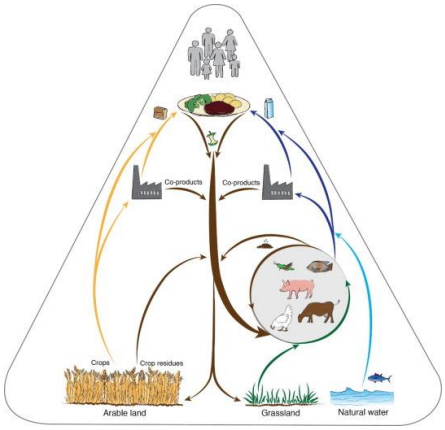
Global warming results from intense human activity with an excess of carbon dioxide and other pollutants in the atmosphere that trap heat, resulting in higher global surface temperatures (Al-Ghussain, 2019). A prediction study reveals that in Europe, if the global temperature increases by 3ºC, the estimated economic impact in the agriculture, public water supply, power generation, commercial shipping and on buildings and infrastructure will be about 17.3 billion euros, with the Mediterranean and Atlantic regions being the most affected. This means that by 2100, drought-related losses will be five times higher (Cammalleri et al., 2020).

Besides production-related losses, in wheat, for example, drought periods are directly related to arabinoxylan content (NSP), and a negative relationship between arabinoxylan and starch content is also observed (Coles et al., 1997). NSPs are macromolecules resistant to degradation by endogenous enzymes in monogastric animals, directly impacting animal zootechnical performance (Căpriţă; Căpriţă; Julean, 2010; Saeed et al., 2019). Another consequence of global warming is the increasing crop contamination with mycotoxins, in Europe, for example, an increase is expected on DON contamination of wheat, and AFLA contamination of maize (Medina; González-Jartín; Sainz, 2017).

Global warming will directly increase mycotoxin-crop contamination and reduce agricultural yields (Medina; González-Jartín; Sainz, 2017; Wang, J. et al., 2018). Drought periods are related with NSP deposition on cereals (Coles et al., 1997; Hussen, 2020). Both factors impact animal gut integrity (Jorgensen et al., 1996; Andretta et al., 2011; Awad; Ghareeb; Böhm, 2012). Approximately 40% of global arable land is used to produce feed crops for farm animals, and 21-24% of poultry feed is composed of human-edible protein sources (PAS et al., 2021). A circular food production system is an alternative to reduce food production-related environmental impact and to meet the nutritional demands of the growing world's population (Figure 7). In this system, cropland is primarily used for human food production, while farm animals receive diets based on alternative feed ingredients. However, such diets do not have the same digestibility as conventional ones, and solutions such as feed

enzymes are needed (Pas et al., 2021; Bikker; Jansman, 2023).

Figure 7. The biophysical concept of circularity: arable land is primarily used for food production; biomass unsuitable for direct human consumption is recycled as farm animal feed; and crop residues and manure are utilized to maintain soil fertility.



Source: Van Zanten; Van Ittersum;De Boer (2019).

In the first manuscript of this doctoral dissertation, the effects of a DON-contaminated diet on NE challenge were discussed. Diets rich in animal protein and NSP with high water retention capacity are predisposing factors to NE (Branton et al., 1997; Moore, 2016). Kim et al. (2022) described that mannanase supplementation on wheat-based diets ameliorates production losses induced by NE. Based on that, further studies could be conducted to evaluate the potential effect of the β-mannanase used in the second manuscript of this thesis facing NE challenge.

In this thesis, a guar gum diet was used as a rich source of galactomannans. However, in Europe and Brazil, this feed ingredient is not common. In some African and Asian countries, as well as parts of South America, guar gum or palm kernel meals (both rich in mannans) are options to be used as alternative ingredients to soybean meal (Gresta et al., 2017). Therefore, in this context, β-mannanase supplementation might be an essential tool to improve animal performance in diets containing alternative feed ingredients (Sundu; Kumar; Dingle, 2006; Wilkinson; Young, 2020; Azizi et al., 2021).

In the era of molecular microbiology, it is accepted that microorganisms can be either beneficial or harmful depending on the situation. A practical example of this is exposed in this doctoral dissertation.

Lactobacillus spp. are widely used as probiotic strains in poultry farming. Many beneficial effects are attributed to its supplementation, such as improvements in growth

performance, intestinal morphometry, immune status, competitive exclusion of pathogenic microorganisms, and mycotoxin detoxification, among others (Franco et al., 2011; Wang et al., 2018; Souza et al., 2020; Wu et al., 2021). Ileum is one of the main sites of *Lactobacillus* colonization in the gut (Un-Nisa et al., 2022). Manuscript A described the beneficial effects of *Lactobacillus* spp. supplementation, probably due to small intestine colonization, sites of action DON and *C. perfringens* (Abd El-Hack, 2022; Maresca, 2013).

However, in the second article, an excess of the same bacterial genus showed negative effects on the intestinal microbiome, especially in the caeca. *Lactobacillus* are known to ferment carbohydrates into lactic acid as a major end product, which may lower the pH of the intestinal environment, affecting microbial composition (Petersen, 2005).

Although this research investigated high levels of NSP, the mycotoxin deoxynivalenol, and necrotic enteritis separately, in field conditions, these problems might occur simultaneously, with ongoing increases due to global warming. Thus, the solutions proposed for both challenges demonstrated efficiency.

With these results, it is expected that further research could be developed with a mixture of *Lactobacillus* spp. strains aiming at the development of a commercial product to mitigate DON and CP losses in poultry farming. Also, experimental work should be conducted to identify the metabolites and action mechanism of cell-free supernatants and to investigate the probable absorption capacity of heat-inactivated cells.

Another perspective is that the use of a β-mannan-rich diet supplemented with β-mannanase becomes more frequent in regions that use more alternative ingredients for feeding poultry, reducing production costs and competition with human nutrition for commodities like corn, for example. Furthermore, future research should be performed to investigate the effects of β-mannanase supplementation on NE challenges.

8 CONCLUSIONS

Our research findings indicate that *Lactobacillus* spp. strains isolated from the Avian Medicine Laboratory at UEL exhibit varying abilities to inhibit the growth of *C. perfringens in vitro*. Among these strains, we selected the top three based on their inhibition halo for further investigation in this project.

In our study, we found that both viable and heat-inactivated forms of a *Lactobacillus* spp. mixture, as well as its culture supernatant, preserved the macroscopic intestinal appearance of broilers exposed to DON and CP. Microscopically, the *Lactobacillus* spp. mixture also ameliorated the damage caused by the challenge. Notably, exposure to DON did not compromise the intestinal or humoral immune responses of the broilers. Furthermore, the *Lactobacillus* spp. mixture did not exhibit any discernible immunomodulatory effects.

In our work, we observed that DON primarily induced oxidative stress in the jejunum, while the *Lactobacillus* spp. mixture effectively maintained the redox balance in this region. However, CP also triggered oxidative stress, and in this instance, the *Lactobacillus* spp. mixture showed limited efficacy in mitigating small intestine oxidative damage.

Simultaneous exposure to DON and CP did not exacerbate most of the assessed parameters compared to individual challenges. Nevertheless, treatments involving *Lactobacillus* spp., particularly LV and HIL, demonstrated effectiveness in mitigating tissue damage.

The effects of non-starch polysaccharides were also investigated. Inclusion of β-mannan (1.7 %) to broilers diet reduced BWG, DFI and increased FCR negatively impacting zootechnical performance. As a compensatory mechanism to improve nutrient absorption, the ileal villus height was increased in the group supplemented with a guar gum diet. Probably, through better nutrient digestibility, dietary β-mannanase supplementation restored gut morphometry and zootechnical performance to control levels.

β-mannan-rich diet-induced intestinal dysbiosis in both the fore and hindgut, this was evidenced by the increase in some hallmark's microorganisms such as the phylum *Proteobacteria*, the genders *Escherichia-Shigella* and *Lactobacillus* in the caeca, for example. These results evidence that an excess of undigested carbohydrates in the intestine modulates the microbial population. Despite the negative impacts of the experimental diet on the intestinal microbiome β-mannanase supplementation showed to be efficient in restoring the intestinal microbiome to control levels.

The levels of short-chain fatty acids (SCFs) were also influenced by a β-mannan-rich

diet. An increase in SCFs related to protein fermentation was observed in the treatment that received the guar gum diet without the enzyme supplementation. Guar gum is a rich protein source, this result indicates poor protein absorption in the upper intestine, with a higher availability to cecal microbiota fermentation. These findings illustrate that the utilization of β-mannan-rich feed ingredients without β-mannanase supplementation incurs production losses.

Based on our results, we conclude that supplementation with *Lactobacillus* spp. and β-mannanase offers efficient alternatives for mitigating economic losses caused by challenges related to non-starch polysaccharides (NSP), necrotic enteritis (NE), and deoxynivalenol (DON).

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10 CURRICULUM VITAE

Marielen de Souza was born on October 19th, 1993, in Jacarezinho city, located within the state of Paraná, Brazil, into a family with a longstanding agrarian heritage. From an early age, her fascination with veterinary sciences was palpable.

Upon entering high school, she pursued education at a technical institution, culminating in the acquisition of an agricultural technician diploma in 2010. It was during this period, catalyzed by internships and the influence of her grandfather, a poultry farmer himself, that her passion for poultry sciences took root.

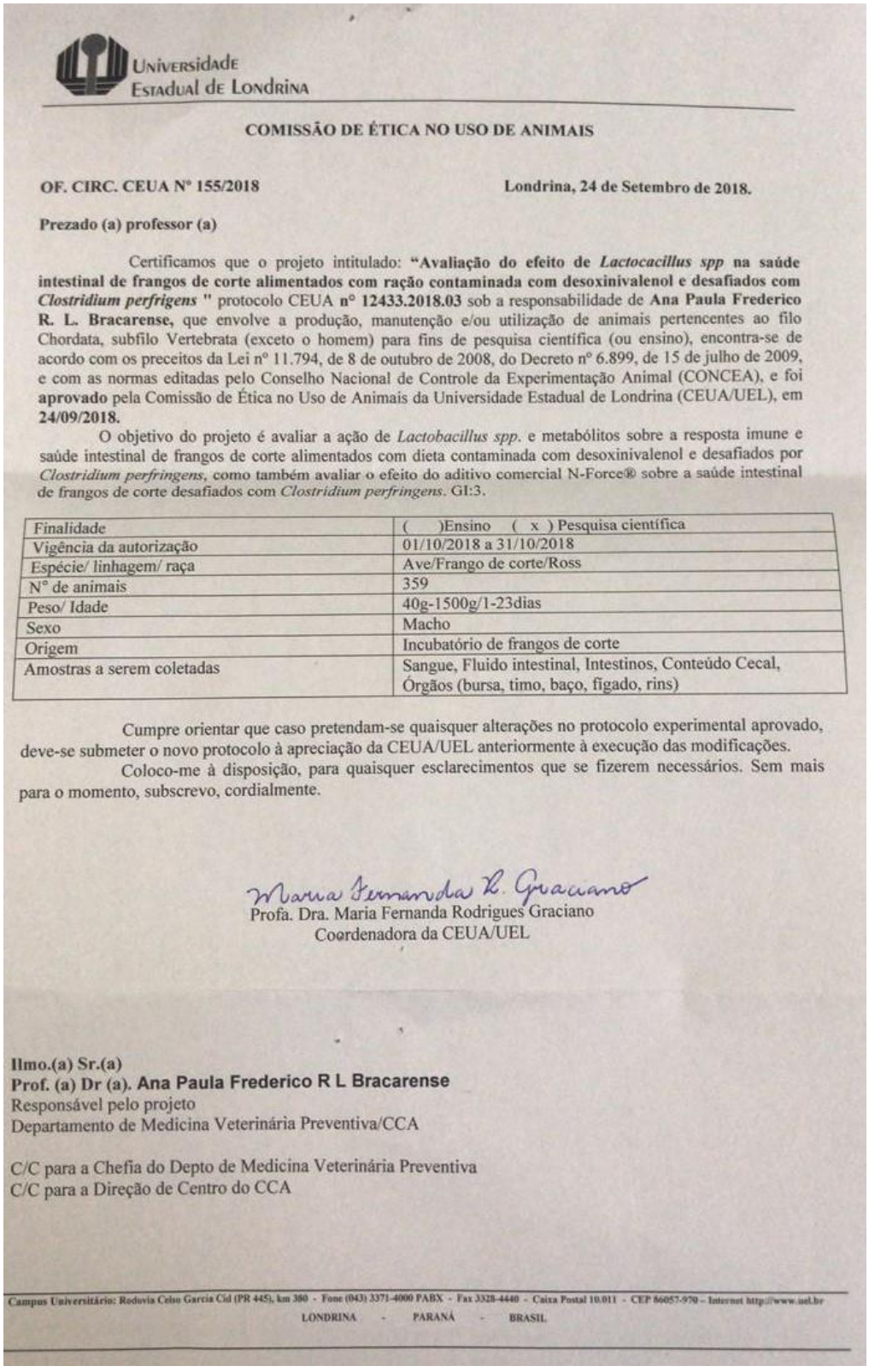
In 2015, she graduated from Universidade Estadual de Londrina (UEL) with a bachelor's degree in veterinary medicine, she undertook a mandatory internship at a commercial poultry hatchery and breeders' farm, further deepening practical knowledge in the field. Following this, embarked on a Residency in Avian Medicine at UEL, under the mentorship of Prof. Dr. Ana Angelita S. Baptista, graduating in 2017. The year 2020 heralded the defense of her master's thesis, titled "Effects of Lactobacillus spp. in broilers challenged with deoxynivalenol," under the guidance of Prof. Dr. Ana Paula F. R. L. Bracarense.

In March 2020, she commenced her doctoral journey in Animal Science at UEL, once again under the esteemed supervision of Prof. Dr. Ana Paula F. R. L. Bracarense. The following year, she embarked on an internship with the Livestock Gut Health Team (LiGHT) at UGent, under the supervision of Prof. Dr. Filip Van Immersseel. This collaboration culminated in the successful application and approval of a joint Ph.D. program between UEL and UGent in 2022.

Her doctoral research endeavors primarily focused on evaluating strategies to enhance the intestinal health of broilers in the face of antinutritional, toxic, and sanitary challenges commonly encountered in poultry farming, including non-starch polysaccharides, the mycotoxin Deoxynivalenol, and Necrotic Enteritis. Throughout her academic odyssey, she collaborated with esteemed research groups to publish 14 manuscripts in various scientific peer-reviewed journals and disseminated her findings at numerous poultry congresses both domestically and internationally, thereby contributing to the scholarly discourse in avian sciences.

ANNEX I

Institutional approval from the Ethics Committee in Animals Use (CEUA - UEL)



Universidade Estadual de Londrina

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 155/2018 **Londrina, 24 de Setembro de 2018.**

Prezado (a) professor (a)

Certificamos que o projeto intitulado: **"Avaliação do efeito de *Lactocacillus spp* na saúde intestinal de frangos de corte alimentados com ração contaminada com desoxinivalenol e desafiados com *Clostridium perfrigens* "** protocolo CEUA **nº 12433.2018.03** sob a responsabilidade de **Ana Paula Frederico R. L. Bracarense,** que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovado** pela Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA/UEL), em **24/09/2018.**

O objetivo do projeto é avaliar a ação de *Lactobacillus spp*. e metabólitos sobre a resposta imune e saúde intestinal de frangos de corte alimentados com dieta contaminada com desoxinivalenol e desafiados por *Clostridium perfringens*, como também avaliar o efeito do aditivo comercial N-Force® sobre a saúde intestinal de frangos de corte desafiados com *Clostridium perfringens*. GI:3.

Finalidade	( )Ensino (x) Pesquisa científica
Vigência da autorização	01/10/2018 a 31/10/2018
Espécie/ linhagem/ raça	Ave/Frango de corte/Ross
Nº de animais	359
Peso/ Idade	40g-1500g/1-23dias
Sexo	Macho
Origem	Incubatório de frangos de corte
Amostras a serem coletadas	Sangue, Fluido intestinal, Intestinos, Conteúdo Cecal, Órgãos (bursa, timo, baço, figado, rins)

Cumpre orientar que caso pretendam-se quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação da CEUA/UEL anteriormente à execução das modificações.

Coloco-me à disposição, para quaisquer esclarecimentos que se fizerem necessários. Sem mais para o momento, subscrevo, cordialmente.

Maria Fernanda R. Graciano

Profa. Dra. Maria Fernanda Rodrigues Graciano
Coordenadora da CEUA/UEL

Ilmo.(a) Sr.(a)
Prof. (a) Dr (a). Ana Paula Frederico R L Bracarense
Responsável pelo projeto
Departamento de Medicina Veterinária Preventiva/CCA

C/C para a Chefia do Depto de Medicina Veterinária Preventiva
C/C para a Direção de Centro do CCA

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ANNEX II

Figure S1. Experimental design. Experimental groups: Control—uncontaminated diet. DON (deoxynivalenol)—diet with DON 19.3 mg kg^{-1}. CP (*Clostridium perfringens*)—uncontaminated diet + *C. perfringens* challenge. CP + DON—DON 19.3 mg kg^{-1} + *C. perfringens* challenge. LV—DON 19.3 mg kg^{-1} + *C. perfringens* challenge plus viable *Lactobacillus* spp. mixture. HIL—DON 19.3 mg kg^{-1} + *C. perfringens* challenge plus heat-inactivated *Lactobacillus* spp. mixture. LCS—DON 19.3 mg kg^{-1} + *C. perfringens* challenge plus *Lactobacillus* spp. mixture culture supernatant.

Control
0-23 days uncontaminated diet
Sterile PBS via oral gavage 14, 16,17, 18,19, 20, 21 and 22 days
Sterile MRS broth via oral gavage 2, 3, 5, 7, 9, 11, 13, 15, 17 and 19 days

DON 19.3 mg kg^{-1}
0-6 days uncontaminated diet / 7-23 days DON-contaminated diet (19.3 mg kg^{-1})
Sterile PBS via oral gavage 14, 16,17, 18,19, 20, 21 and 22 days
Sterile MRS broth via oral gavage 2, 3, 5, 7, 9, 11, 13, 15, 17 and 19 days

CP
0-23 days uncontaminated diet
14 days 10 fold *Eimeira* spp. and Gumboro oral vaccin
Fresh CP culture via oral gavage 16,17, 18,19, 20, 21 and 22 days
Sterile MRS broth via oral gavage 2, 3, 5, 7, 9, 11, 13, 15, 17 and 19 days

CP + DON
0-6 days uncontaminated diet / 7-23 days DON-contaminated diet (19.3 mg kg^{-1})
14 days 10 fold *Eimeira* spp. and Gumboro oral vaccin
Fresh CP culture via oral gavage 16,17, 18,19, 20, 21 and 22 days
Sterile MRS broth via oral gavage 2, 3, 5, 7, 9, 11, 13, 15, 17 and 19 days

VL and HIL
0-6 days uncontaminated diet / 7-23 days DON-contaminated diet (19.3 mg kg^{-1})
14 days 10 fold *Eimeira* spp. and Gumboro oral vaccin
Fresh CP culture via oral gavage 16,17, 18,19, 20, 21 and 22 days
Viable or heat-inactivated *Lactobacillus* spp. mixture via oral gavage 2, 3, 5, 7, 9, 11, 13, 15, 17 and 19 days

LCS
0-6 days uncontaminated diet / 7-23 days DON-contaminated diet (19.3 mg kg^{-1})
14 days 10 fold *Eimeira* spp. and Gumboro oral vaccin
Fresh CP culture via oral gavage 16,17, 18,19, 20, 21 and 22 days
Lactobacillus spp. mixture culture supernatant via oral gavage 7, 9, 11, 13, 15, 17 and 19 days