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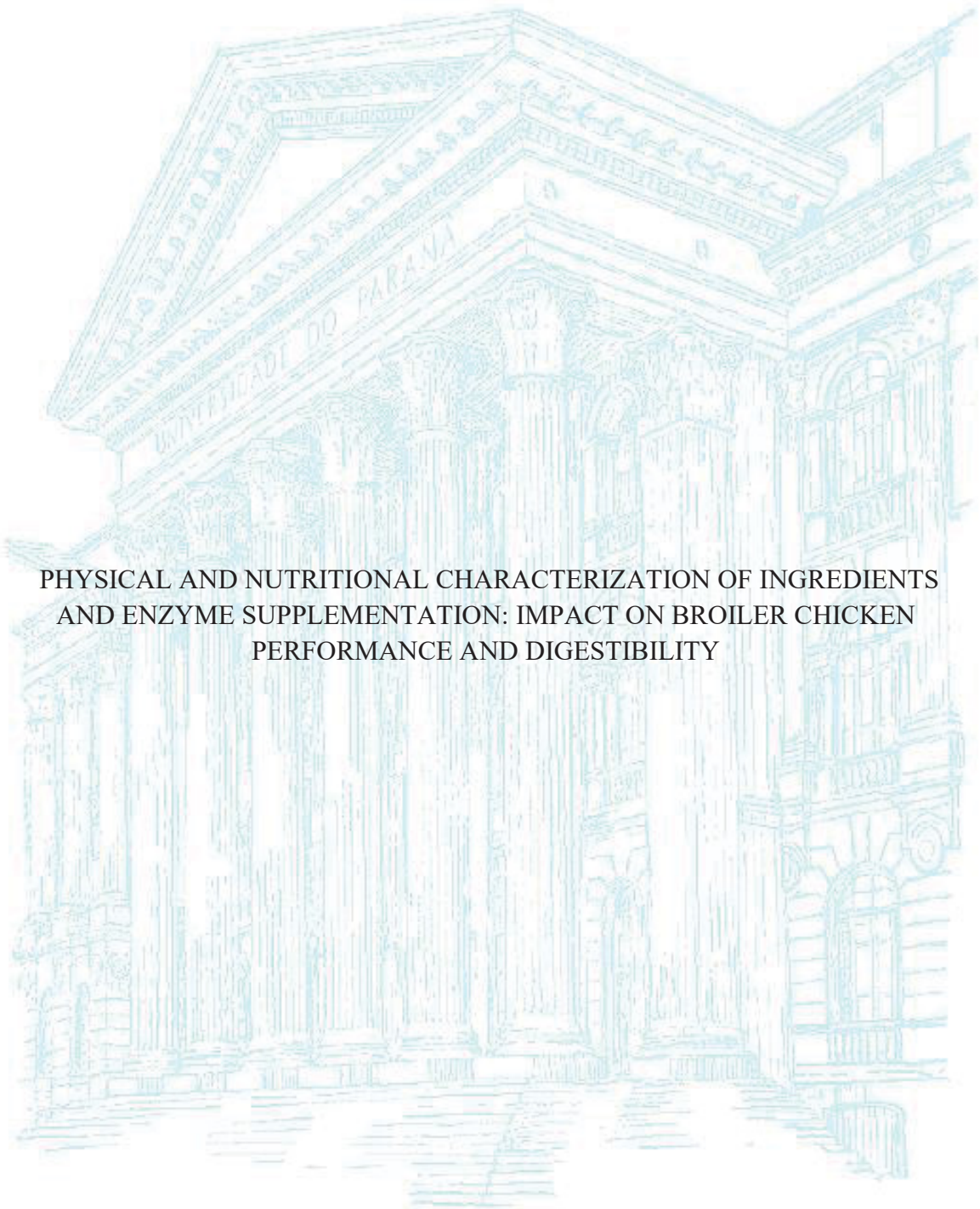

PHYSICAL AND NUTRITIONAL CHARACTERIZATION OF INGREDIENTS AND ENZYME SUPPLEMENTATION: IMPACT ON BROILER CHICKEN PERFORMANCE AND DIGESTIBILITY

CURITIBA
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PHYSICAL AND NUTRITIONAL CHARACTERIZATION OF INGREDIENTS AND ENZYME SUPPLEMENTATION: IMPACT ON BROILER CHICKEN PERFORMANCE AND DIGESTIBILITY

Tese apresentada ao curso de Pós-Graduação em Zootecnia (PPGZ/UFPR). Área de concentração em Nutrição e Produção Animal, Setor de Ciências Agrárias da Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Zootecnia.

Orientadora: Prof. Dra. Simone G. de Oliveira

Coorientador: Prof. Dr. Alex Maiorka

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação ZOOTECNIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **VIVIAN IZABEL VIEIRA**, intitulada: **Physical and nutritional characterization of ingredients and enzyme supplementation: impact on broiler chicken performance and digestibility**, sob orientação da Profa. Dra. SIMONE GISELE DE OLIVEIRA, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutora está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 19 de Março de 2025.

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“— Ma voi siete un geografo!
— Questo è esatto — disse il geografo — ma non sono mica un esploratore. Non ho esploratori al mio servizio.
Non è il geografo che va a fare la ricognizione delle città, dei fiumi, delle montagne, dei mari, degli oceani e dei deserti.”
(...)

Capitolo XV
Il Piccolo Principe

-

“Once you have a taste of the excitement of challenges and trying new things, your blindfold of the danger will fall from your eyes as you realize that your view of the "*scary*" life from inside your comfort zone is just an illusion, all part of the comfort zone's master plan to waste your life away without you experiencing a hell of a rollercoaster ride.” *Amber Lim*

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Agradeço a Deus por me dar sabedoria para compreender o que não esteve ao meu controle e por me guiar nas minhas escolhas. Sou profundamente grata à minha família, especialmente ao meu amado marido, que foi a pessoa mais me encorajou nessa jornada, desde o início esteve ao meu lado me impulsionando a seguir e ser persistente. Aos meus pais, meu irmão e sua família que todo o tempo sabiam da importância dessa etapa. Obrigada pela compreensão de que por muitos momentos que não pude estar presente fisicamente nesses últimos anos.

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Obrigada, *Vivian Vieira*.

RESUMO

Milho e farelo de soja (FS) são ingredientes amplamente utilizados nas dietas de frangos de corte e isso leva a questionamentos sobre qualidade e valor nutricional relacionados a vários fatores, como a origem da produção, características intrínsecas e metodologias de processamento. Apesar do uso de enzimas exógenas, como amilase e protease, ser uma estratégia estabelecida na nutrição de aves devido ao seu impacto positivo na digestibilidade e no desempenho, ainda há um espaço para novos estudos que busquem uma compreensão mais abrangente dos efeitos da inclusão dessas enzimas em dietas formuladas com ingredientes comumente utilizados, como milho e FS, porém provenientes de diferentes lotes com perfis nutricionais distintos. Sendo assim, dois experimentos foram conduzidos para investigar o efeito da amilase exógena em dietas de frangos de corte contendo milho de dois lotes diferentes (Capítulo II), e o impacto da protease exógena em dietas contendo farelo de soja de lotes distintos (Capítulo III). O primeiro estudo avaliou suplementação de amilase na dieta de frangos de corte formulada com milho de diferentes lotes e seu impacto no desempenho (consumo de ração, ganho de peso e conversão alimentar) e na digestibilidade dos nutrientes. Foi realizado um teste de dureza para avaliar e classificar cada uma das amostras de milho provenientes de lotes diferentes (A e B), e a microscopia eletrônica de varredura foi utilizada para investigar as variações físicas na estrutura dos grânulos de amido no endosperma farináceo e vítreo. Análises de composição química foram realizadas para avaliar ambos os tipos de milho. Os resultados mostraram diferenças estruturais entre os endospermas de milho A e B em termos de dispersão dos grânulos de amido. Em relação ao desempenho, não houve diferença para os parâmetros ($P>0,05$). Porém, a amilase melhorou a digestibilidade de nutrientes independentemente do tipo de milho ($P<0,05$). O segundo experimento investigou ação da protease no desempenho (consumo de ração, ganho de peso e conversão alimentar) e a digestibilidade ileal (CDI) de frangos de corte alimentados com dietas contendo FS de diferentes lotes (A e B). Análises físicas e químicas foram realizadas para avaliar cada lote de FS. A solubilidade da proteína em KOH e a avaliação da cor mostraram diferenças entre as amostras, assim como as imagens de microscopia. Nenhuma diferença foi encontrada ($P>0,05$) para os parâmetros de desempenho. As aves alimentadas com dieta contendo FS do lote A apresentaram melhores CDI de matéria seca, proteína, lisina, metionina e melhor aproveitamento de energia digestível ileal independentemente da suplementação de protease ($P<0,05$). A protease melhorou o CDI de proteína bruta e aminoácidos ($P<0,05$) independentemente do FS, e nenhuma interação foi observada ($P>0,05$), exceto para o CDI de tirosina ($P<0,05$). Em conclusão, no primeiro estudo, a suplementação de amilase aumentou a digestibilidade dos nutrientes para frangos de corte, independentemente da dieta com milho dos lotes A ou B. Além disso, no segundo estudo, o FS do lote A apresentou melhores indicadores de qualidade proteica, sugerindo um processo térmico adequado, e uma melhor utilização de nutrientes em frangos de corte.

Palavras-chaves: Aminoácidos; Digestibilidade; Nutriente; Super processamento; Solubilidade

ABSTRACT

Corn and soybean meal (SBM) are widely used ingredients in broiler diets, leading to questions about quality and nutritional value related to various factors, such as the origin of production, intrinsic characteristics, and processing methodologies. Despite using exogenous enzymes, such as amylase and protease, being an established strategy in poultry nutrition due to their positive impact on digestibility and performance, there is still room for new studies that seek a more comprehensive understanding of the effects of including these enzymes in diets formulated with commonly used ingredients, such as corn and SBM, but sourced from different batches with distinct nutritional profiles. Thus, two experiments were conducted to investigate the effect of exogenous amylase in broiler diets containing corn from two different batches (Chapter II), and the impact of exogenous protease in diets containing soybean meal from distinct batches (Chapter III). The first study evaluated the supplementation of amylase in the diet of broiler chickens formulated with corn from different batches and its impact on performance (feed intake, weight gain, and feed conversion) and nutrient digestibility. A hardness test was conducted to evaluate and classify each of the corn samples from different batches (A and B), and scanning electron microscopy was used to investigate the physical variations in the structure of the starch granules in the floury and vitreous endosperm. Chemical composition analyses were conducted to evaluate both types of corn. The results showed structural differences between the endosperms of corn A and B in terms of starch granule dispersion. Regarding performance, there was no difference between the parameters ($P>0.05$). However, amylase improved nutrient digestibility regardless of the type of corn ($P<0.05$). The second experiment investigated the protease effect on the performance (feed intake, weight gain, and feed conversion) and ileal digestibility (AID) of broilers fed diets containing SBM from different batches (A and B). Physical and chemical analyses were conducted to evaluate each batch of SBM. The solubility of the protein in KOH and the color evaluation showed differences between the samples, as well as the microscopy images. No difference was found ($P>0.05$) for the performance parameters. The birds fed with a diet containing SBM from batch A showed better AID of dry matter, protein, lysine, methionine, and better utilization of ileal digestible energy regardless of protease supplementation ($P<0.05$). The protease improved the AID of crude protein and amino acids ($P<0.05$) regardless of the SBM, and no interaction was observed ($P>0.05$), except for the AID of tyrosine ($P<0.05$). In conclusion, in the first study, the supplementation of amylase increased the nutrient digestibility for broilers, regardless of the corn diet in batches A or B. Furthermore, in the second study, the SBM of batch A showed better indicators of protein quality, suggesting an adequate thermal process and better nutrient utilization in broiler chickens.

Keywords: Amino acid; Digestibility; Nutrient; Overprocessed; Solubility

FIGURE LIST

CHAPTER I

CHAPTER II

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CHAPTER I

CHAPTER II

CHAPTER III

LIST OF ABBREVIATIONS, SYMBOLS AND UNITS

%	Percentage
Δa*	Changes in color red to green
Δb*	Changes in color yellow to blue
ΔE*	Total color difference
ΔL*	Changes in brightness
©	Copyright
µm	Micrometer
a*	Redness
ADP	Adenosine diphosphate
AIA	Ash insoluble ash
AID	Apparent ileal digestibility
AIDCP	Apparent ileal digestibility of crude protein
AIDDM	Apparent ileal digestibility of dry matter
α	Alpha
AME	Apparent metabolizable energy
ANOVA	Analysis of variance
ATTR	Apparent total tract retention
b*	Yellowness
BBI	Bowman-Birk factor
β	Beta
BWG	Body weight gain
Ca	Calcium
CEUA	Animal Use Ethics Committee
CF	Crude fiber
CIELab	Color space
cm	Centimeter
CP	Crude protein
d	Days
DAP	Days after pollination
DM	Dry matter
EE	Ether extract
ESBM	Enzymatically treated soybean meal
FCR	Feed conversion ratio
FI	Feed intake
Fig.	Figure
FYT	Phytase units
γ	Gamma
g kg^{-1}DM	Grams per kilogram of dry matter
g/kg	Grams per kilogram
GE	Gross energy
IDE	Ileal digestible energy
IF	Indigestible factor

Kcal/kg	Kilocalorie per kilogram
kDa	Kilodaltons
kg	Kilogram
KNU	Kilo-Novo α-amylase units
KNU/kg	Kilo-Novo α-amylase units per kilogram
KOH	Potassium hydroxide
KTI	Kunitz factor
kV	Kilovolt
L*	Brightness
LSCM	Laser scanning confocal microscopy
Lys	Lysine
m	Meter
MADS47	Transcription factor box 47
mbar	Milibar
Met	Methionine
Met+Cys	Methionine+Cysteine
mg	Miligram
mg/g	Miligram per gram
mm	Milimeter
N	Newton
NFP	New feed protease units
NFP/kg	New feed protease units per kilogram
ºC	Celsius
O2	Opaque-2
P	Phosphorus
PBF1	Prolamin box binding factor 1
P-value	Probability
RDS	Rapidly digestible starch
RS	Resistant starch
SBE	Starch branching enzyme
SBM	Soybean meal
SDS	Slow-digesting starch
SEM	Polled standard error mean
SGBSS1	Granule-bound starch synthase isoform 1
SS	Starch synthase
SSS	Soluble isoform of the enzyme starch synthase
Thr	Threonine
TIA	Trypsin inhibitor activity

SUMMARY

INTRODUCTION

The main ingredients in broiler chicken diets in Brazil are corn and soybean meal (SBM), which represent the largest energy and protein sources, respectively. As corn and soybean meal use is widespread, using exogenous enzymes has become a well-established nutritional strategy in broiler production over the years. This is due to the consistently good results in performance and nutrient utilization as reported by several studies (Cowieson and Adeola, 2005; Walk et al., 2019; Schramm et al., 2021; Salazar-Villanea et al., 2022; Stefanello et al., 2023; Zavelinski et al., 2024).

The batches of corn and SBM can present different nutritional characteristics due to location or the origin where they were produced, as well as the hybrids (Ibáñez et al., 2020; Aguirre et al., 2022; Stefanello et al., 2023), but also can be impacted by the thermal process strategies applied (Kaczmarek et al., 2013; Tousi-mojarrad et al., 2014; Ibáñez et al., 2020). This nutritional variation might impact on the effectiveness of enzymes, once according to Adeola and Cowieson, (2011), several factors, such as nutritional levels, enzyme characteristics, substrate concentrations, and the composition and quality of feed ingredients, might influence the use of exogenous enzymes.

However, few studies evaluate the effects of exogenous enzymes on performance and digestibility variables of broilers fed diets containing feed ingredients with different nutritional compositions, such as the case of corn batches (Stefanello et al., 2023; Vargas et al., 2023; Zavelinski et al., 2024), or dietary characteristics changes influenced by the origin of soybean meal production, as well as the thermal processing applied as reported by (García-Rebollar et al., 2016; Ibáñez et al., 2020; Liu et al., 2024). Thus, based on the factors mentioned above, there is a gap in current studies that presents an opportunity for new studies to provide a more comprehensive understanding of the effects on

performance and digestibility of broiler chickens fed diets containing widely used commercial enzymes and varying batches of ingredients such as corn and SBM.

In Chapter I, a literature review addresses the relevance of poultry production in Brazil to understand the importance of nutrition in this context. Additionally, a session will discuss topics about the characterization of corn grain, its physical structures, and issues related to nutritional value and its impact on nutrition. Points on the morphology and characterization of soybean grains were presented, as well as how the thermal processing strategies of soybean meal have a significant impact on the nutrition of broiler chickens, along with a brief discussion on anti-nutritional factors that affect the metabolism of monogastric animals and their relationship with the thermal processing of soybean meal. Regarding the nutritional quality of soybean meal, a session was dedicated to discussing protein quality indicators, such as analyses of protein solubility in KOH, urease activity, and trypsin inhibitor activity. To conclude the first chapter, a brief discussion on the importance of using exogenous enzymes in broiler diets, amylase, and protease, and the main advantages in terms of growth performance and nutrient utilization.

In Chapter II, a research trial investigated the impact of amylase supplementation on growth performance and nutrient digestibility of broiler chicken fed diets containing corn from two different batches. In this article, the objective was to evaluate endosperm characteristics through physicochemical analysis and the impact on nutrient utilization including crude protein and starch fractions digestibility. With this trial, we could confirm physical differences in starch granules distribution in the vitreous and floury endosperm of each corn, as well as significant differences in breakage force. The findings in this study indicated the effectiveness of amylase for nutrient utilization, regardless of corn type used in diets.

In Chapter III, an experiment was conducted to assess the effect of exogenous protease on growth performance and nutrient digestibility of broiler chickens fed two different batches of SBM with distinct protein quality indicators. We hypothesized that protease supplementation would increase the dietary value of soybean meals with different protein solubility, thereby increasing the digestibility of protein and amino acids. The scanning electron images, as well as the color measurements in samples of these soybean meals, demonstrated interesting findings regarding the quality and the impact of the thermal process that is applied in crushing plants and not only attributing this effect to the crop location.

In summary, Chapter IV provides a comprehensive and critical overview of my last three years of doctoral studies on corn, soybean meal, and enzymes used in broiler chicken nutrition, along with suggestions for future research topics.

CHAPTER I – LITERATURE REVIEW

1. The role of grains in broiler chicken production

Broiler chicken production is a representative endeavor of Brazilian agribusiness as one of the most relevant and distinguished activities on the global stage. In 2023, Brazil led the world exports, trading 5,139 million tons of chicken meat with more than 150 countries, mainly as broiler meat cuts, generating an income of around 10 billion dollars (ABPA, 2024). Brazil reached 14,833 million tons of broiler chicken meat produced in 2023, with the south region accounting for approximately 65% of this total (ABPA, 2024). The state of Paraná stands out as a leader in this activity, being responsible for 39.47% of the total chicken slaughter in Brazil and has a significant influence not only on production but also on export and the generation of direct and indirect jobs in the poultry industry.

The advancement of broiler chicken production in Brazil is driven by the growing consumer market and the availability of grains, especially corn and soybean meal, used in feed formulations. According to Sindirações (2023), about 84% of the estimated production of poultry feed, which was 42,6 million tons, was allocated to broiler chickens, totaling approximately 35,8 million tons. However, it is difficult to discuss the nutritional value of feed without referencing grain records, as they are closely interconnected. For example, in the year 2022, Brazil was responsible for about 11% of the global corn grain production, totaling approximately 126 million tons. The states of Mato Grosso and Paraná concentrated a large part of this production. These numbers place Brazil as the third largest producer of corn grains, behind only the United States and China, which together represent 54% of global production (Corn Explorer; USDA, 2022).

The main point for this research that created several questions was related to corn production in the year 2020, which was negatively impacted due to various factors, such

as delayed rains, diseases, and intense cold. According to the National Supply Company (CONAB, 2021), the 2020/2021 crop recorded a production of 85,7 million tons, representing a 16% reduction compared to the previous crop and being the lowest harvest since 2017/2018. In the state of Paraná, the crop production loss was even more pronounced, with a 58% drop in corn production, marking the largest historical reduction. The unfavorable scenario in grain stocks and their use in animal feed led Brazil to increase the import of corn from other countries, mainly from Argentina. In 2020, Brazilian companies imported a significant amount of Argentine corn to meet the demand for animal feed production.

Regarding the soybean meal (SBM) production indicators, Brazil is the world leader in soybean production, accounting for 39% of the global volume (152 million tons), with the states of Mato Grosso, Paraná, and Rio Grande do Sul as major producers (Soybean Explorer; USDA, 2022). According to ABIOVE (2022), 40 million tons of soybeans produced in Brazil were processed into meal, while more than 10 million tons were transformed into oil.

2. Corn grain

Corn (*Zeal mays L.)* is widely cultivated around the world as a source of food for humans and animals. It is one of the most used ingredients in broiler chicken diets, with inclusion recommendations that can reach up to 65% in the initial or growth phases (Rostagno et al., 2024) and this highlights the importance of knowing the nutritional value of this key ingredient in feed formulation. Corn is known as the main source of energy component (Aderibigbe et al., 2020a) due to its starch composition, but the protein content provided by corn is also relevant. In intensive broiler chicken production systems,

about 20% of the protein and 65% of the metabolizable energy in the initial diet formulations come from corn (Cowieson, 2005).

The corn grain has a structure that can be divided into four parts: pericarp, endosperm, germ, and pedicel as illustrated in Figure 1 (Gwirtz and Garcia-Casal, 2014). These structures perform essential functions in the composition and nutritional properties of the grain. In summary, each part of the corn grain has a specific function, the pericarp is the outer layer that protects the endosperm, which is the main source of energy for plants and animals, this is where the starch granules are located. The corn germ, however, is the embryonic part rich in proteins and lipids. Finally, the pedicel is the supporting structure located at the base of the grain.

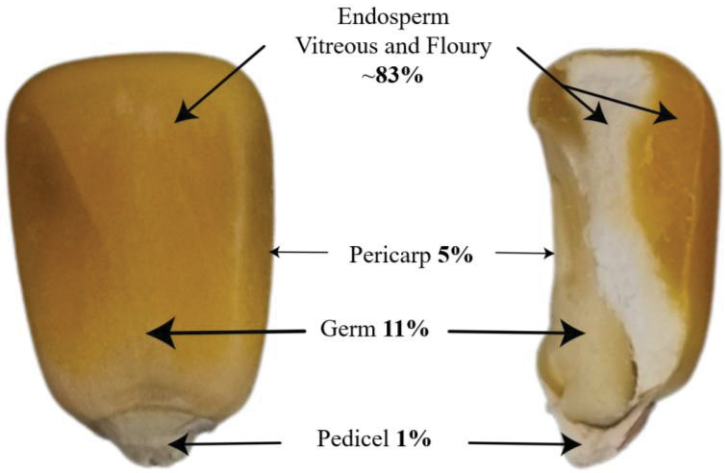

Figure 1. Corn grain parts

The corn grain development occurs over a period that varies between 50 and 60 days, from the moment of fertilization to the stage of complete maturation. In agronomic terms, this process is divided into six distinct stages, ranging from the emergence of the stigmas (functional female flowers) on the corncob to the physiological maturation phase of the grain. During each of these stages, important morphological and physiological

transformations occur that contribute to the final development of the grain and all its structures(Wu et al., 2022).

2.1. Pericarp

The pericarp is the outer layer that provides physical and chemical protection for the grain. It covers both the endosperm and the germ and is rich in fibers, vitamins, and minerals. In corn grains with vitreous endosperm, the pericarp represents about 6% of the total size, while in grains with floury endosperm, it represents approximately 6.5% (Gwirtz and Garcia-Casal, 2014; García-Lara et al., 2019).

The pericarp of the corn grain is composed of five distinct layers, which are the epidermis, mesocarp, cross cells, tubular cells, and husk. These layers extend from the inner portion, near the endosperm, to the outer part of the grain. The corn pericarp consists of approximately 50% heteroxylans, 20% cellulose, and 4% phenolic acids. The fraction of heteroxylans can be extracted from the pericarp using alkaline or acidic solutions, resulting in a product that contains up to 54% D-xylose, 35% L-arabinose, and 11% galactose. This composition evidences the presence of important components in the corn pericarp, which has different impacts on the structure and nutritional properties of the corn grain (Saulnier et al., 1995; Carvajal-Millan et al., 2007).

During the corn grain development, the pericarp tissue originates from the ovary wall after fertilization. As the grain matures, around 20 days after pollination (DAP), the cells of the pericarp tissue increase in width and divide, forming distinct inner and outer layers, and at this stage, these layers can already be identified. As the corn grain approaches maturity, the remaining pericarp cells degrade and accumulate a dark pigment, providing a characteristic coloration (García-Lara et al., 2019).

2.2. Endosperm

The endosperm, which is the central portion of the corn grain, has the main function of starch storage, providing energy both for the initial development of the plant and as an energy source in animal nutrition. Concerning a corn grain that has reached maturity, the endosperm is the predominant structure, occupying more than 70% of its total volume (Consonni et al., 2022). The starch granules are distributed throughout the endosperm, with their dispersion varying based on the surrounding protein matrix. Approximately 89% of the endosperm is composed of these starch granules, which have diameters ranging from 3 to 25µm (García-Lara et al., 2019).

The beginning of endosperm development occurs through double fertilization, in which the fusion of two haploid polar nuclei originating from the central cell of the embryo sac takes place, along with a haploid sperm nucleus from the pollen grain (Birchler, 1993). During the process of formation and development, the endosperm cells go through stages of cell formation and differentiation that are delineated according to the DAP (Olsen and Kalla, 1992).

The development of the corn grain endosperm is divided into four distinct phases: syncytium, cellularization, differentiation, and maturation. The syncytium, also known as the cenocytic phase, marks the beginning of this process with the rapid nuclear division until the formation of 32 free nuclei. Next, in the cellularization phase, the formation of cell walls around the nuclei occurs, approximately 4 DAP. The differentiation phase is characterized by the filling of the central vacuoles of the endosperm and the formation and development of the cell wall's outer layer, which transforms into the aleurone, occurring between 6 and 10 DAP. Finally, the maturation phase is marked by the deposition and maturation of the endosperm, with the increase in cell size, deposition of

starch, and storage protein. This stage is the longest, occurring 15 to 58 DAP, and represents the complete development of the endosperm (García-Lara et al., 2019).

The model described by Leroux et al. (2014) presents in detail the stages of endosperm development from day 0 to 12 DAP, including the cytological transitions during this period. In Figure 2, the diagram highlights the three initial stages of corn endosperm development, along with the main tissues, the transition time, and the location of differentiated cells.

During the period from 1 to 3 DAP, the corn grain exhibits a similar size and a small number of cells undergoing nuclear division. On the 4th DAP, there is an increase in the width of the endosperm. Upon reaching the 5th DAP, it is possible to observe that the endosperm is considerably larger, with cells filling its interior, and the embryo can already be identified. In the third stage, which occurs between 8 and 12 DAP, the endosperm grows rapidly, doubling its size every two days (Leroux et al., 2014).

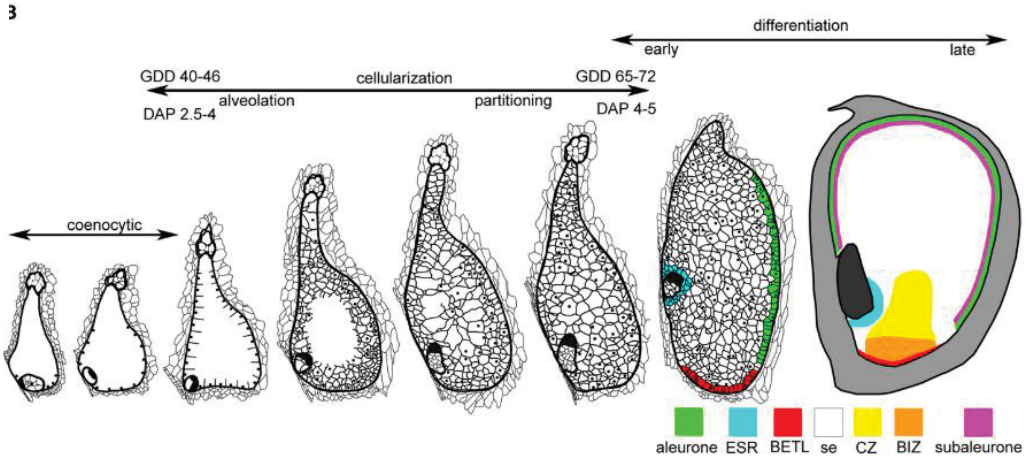

Figure 2. Cytological transitions, period, location, and cellular differentiation of corn endosperm

2.3. Germ

The germ or the embryonic part of the corn grain, is composed of a significant amount of lipids, proteins, and vitamins, making it an important nutritional component. Unlike the endosperm, the germ requires more time to develop, as at 22 DAP, it still has

a very small mass. The main function of the germ is the synthesis of proteins during the development and maturation of the grain. After 14 DAP, most of the germ proteins have already been synthesized (García-Lara et al., 2019).

Corn germ is considered a by-product obtained during the processing of corn and has a relevant nutritional composition, being rich in proteins and lipids. These are the two main components that are synthesized and stored during the development of the grain. According to Nasir (2009), the authors analyzed the proximate composition of six corn hybrids revealing variations not only in protein content (17% to 20%), but also in lipid content, which ranged from 33% to 38%.

In broiler chicken diets, corn germ is an option as a partial replacement ingredient for corn grain and oil, being an alternative source rich in amino acids, vitamins, and minerals. When available on the market at a competitive price compared to corn, the inclusion of up to 15% in the diet of growing broilers can be considered (Rostagno et al, 2024). According to the study conducted by Lopes et al. (2019), the inclusion of different levels of corn germ (40, 80, 120, 160, and 200g per kg of feed) indicated that lower levels of corn germ did not negatively affect the weight gain and feed conversion of the animals, demonstrating that its use for broilers is feasible. Despite the benefits, it is important to pay attention to the nutritional quality of the product. Due to its high oil content, the germ has a greater propensity for oxidation, which can affect its quality. Moreover, the fiber content of the germ, which can reach up to 5%, may reduce its digestibility by animals.

3. Starch granules

3.1. Synthesis

Approximately 90% of the corn endosperm, when it reaches the maturity stage, is composed of starch. According to García-Lara et al. (2019), starch deposition in the

endosperm begins around DAP 12. At this stage, there is intense metabolic activity, resulting in a significant acceleration in starch deposition between days 12 and 22. This period is characterized by a significant increase in the synthesis and deposition of starch in the endosperm. However, after the 22 DAP, the rate of starch deposition tends to gradually decrease.

Sucrose, a disaccharide composed of glucose and fructose, is a key molecule in the process of starch biosynthesis. Initially, sucrose is synthesized in the cytosol of plant cells. Subsequently, through the action of enzymes, sucrose is converted into glucose 1-phosphate within the amyloplasts. The enzyme ADP glucose pyrophosphorylase acts by converting glucose 1-phosphate into ADP glucose and additional glucose residues. (Nelson & Cox, 2017). These glucose residues, in turn, aggregate and form starch granules. In summary, the synthesis of this polysaccharide occurs by the addition of glucose units at the non-reducing ends. This process of converting sucrose into glucose 1-phosphate and the subsequent formation of starch granules is essential for the accumulation of starch in the corn endosperm.

As well described by Tester et al., (2004) the addition of glucose residues in the starch synthesis process is mediated by two distinct forms of an enzyme called starch synthase (SS). In the case of amylose chain synthesis, the active enzyme is the granule-bound starch synthase, isoform 1 (SGBSS1). In the formation of the amylopectin chain, this reaction occurs through the soluble isoform of the enzyme starch synthase (SSS). Moreover, at the branching points of starch, the addition of glucose is catalyzed by the starch branching enzyme (SBE).

3.2. Structure and organization

The characteristics of starch granules may vary according to the botanical species to which they belong. As demonstrated by Tester et al. (2004), the starch granules of

different cereals can exhibit a variety of shapes varying between round, lenticular, and polygonal as demonstrated in microscopy images of corn (Figure 3), potato (Figures 4 and wheat starch granules (Figure 5). In addition, these granules are distributed in distinct ways and have varying sizes, ranging from 1 to 150 μm.

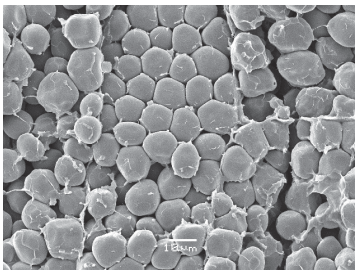

Figure 3. Corn starch granules

Source: The author (2024)

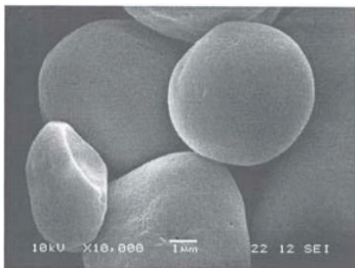

Figure 4. Potato starch granules

Source: Tomoaia-Cotisel et al., (2010)

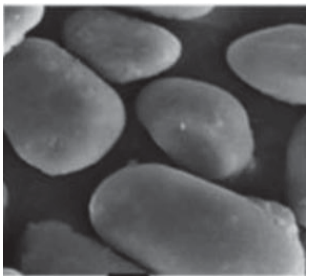

Figure 5. Wheat starch granules

Source: (Chakraborty et al., 2020)

Starch granules assume different shapes and are organized in various ways, depending on the type of endosperm. In the case of endosperm classified as vitreous, the corn starch granules have a polygonal shape and are organized in a more structured disposition, involved by a dense protein matrix. On the other hand, in the floury endosperm, the starch granules have a circular or oval shape and tend to be dispersed in the endosperm due to the sparse protein matrix that surrounds them (Vega-Rojas et al., 2016). As demonstrated by the scanning electron microscopy images, in the corn vitreous endosperm (Figure 6) is possible to notice a layer surrounding and compacting the starch granules, whereas, in the floury endosperm this layer is not so evident (Figure 7).

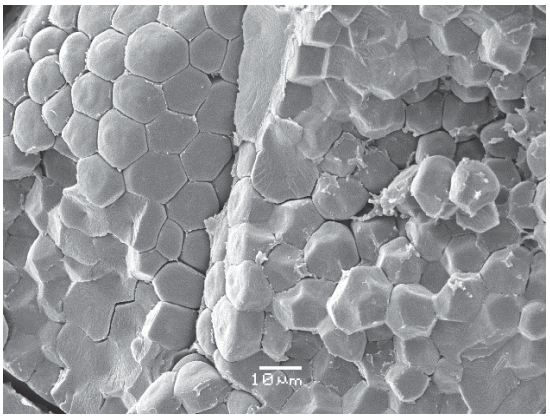

Figure 6. Corn starch granules in vitreous endosperm

Source: The author (2024)

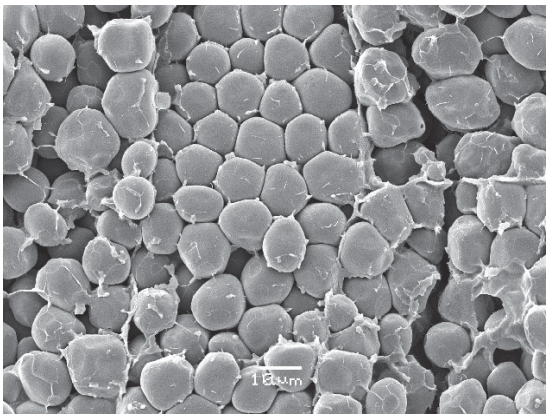

Figure 7. Corn starch granules in floury endosperm

Source: The author (2024)

Corn starch granules can be classified according to size as large or small, with diameter ranging from 2 to 30 µm (Pan and Jane, 2000; Tester et al., 2004). According to Svihus (2014a), the size of starch granules influence the degree of digestibility for birds. In summary, smaller granules are more easily digestible compared to larger granules due to the greater enzyme contact area. Furthermore, the author also mentioned the importance of the protein matrix that surrounds the granules, the denser this protein matrix, the lower the digestibility of the starch due to the difficulty it poses for the enzyme to directly access the starch granule.

Structurally, starch granules are composed of two main chains, amylose and amylopectin which represent approximately 98 to 99% of the dry weight (Tester et al. 2004). The relative proportion between amylose and amylopectin chains varies depending on the botanical species investigated. The structure of both chains has been extensively studied by researchers over an extended period (French, 1972; Gallant and Bouchet, 1986; Wang et al., 2014; Yang et al., 2019; Zhong et al., 2020).

The amylose chain has a linear and long structure, composed of 200 to 700 glucose units, and about 99% of amylose molecules are linked by α1-4 bonds. On the other hand, the amylopectin chain is a shorter structure, containing 15 to 25 glucose units, and it is

organized both linearly (95%), with α1-4 bonds, and branched (5%), with α1-6 bonds. (Tester et al., 2004; Nelson & Cox, 2017).

In terms of the proportion of amylose and amylopectin chains, there are three different classes for corn starch. The first, waxy starch has an amylose proportion of less than 15% and a higher amount of amylopectin. On the other hand, the high amylose corn starch has a minimum amylose content of 40% and a maximum amylopectin content of 60%. The most common, third type is normal corn starch, which has a range of 20% to 35% amylose and 65% to 80% amylopectin (Tester et al., 2004; Weber, 2009).

The chains of amylose and amylopectin distribute and form double helix structures that arrange into what are called amorphous and crystalline regions (Tester et al., 2004; Svihus, 2014a), where the amorphous region is formed by the branches of amylopectin that appear cleaner and more organized, and the crystalline region is formed by the linear part of amylose and amylopectin that intertwine their chains, as demonstrated in the model of diagrammatic representation of the lamellar structure described by Donald et al. (1997).

According to X-ray diffraction, patterns of crystallinity classification of the starch granule were identified, which vary according to the amount of water and the configuration of the double helix packing. The A-type A classification is described for cereal starches in general, where the crystalline material is more compact and condensed, and for every 12 glucose residues, there are 4 water molecules between the helices. The B-type represents the crystalline fraction for tubers and high amylose starches, where the double helix formations are arranged in parallel alignment less compactly with a more hydrated core, containing 36 water molecules for every 12 glucose residues (Tester et al., 2004; Denardin and Silva, 2009) (Figure 8).

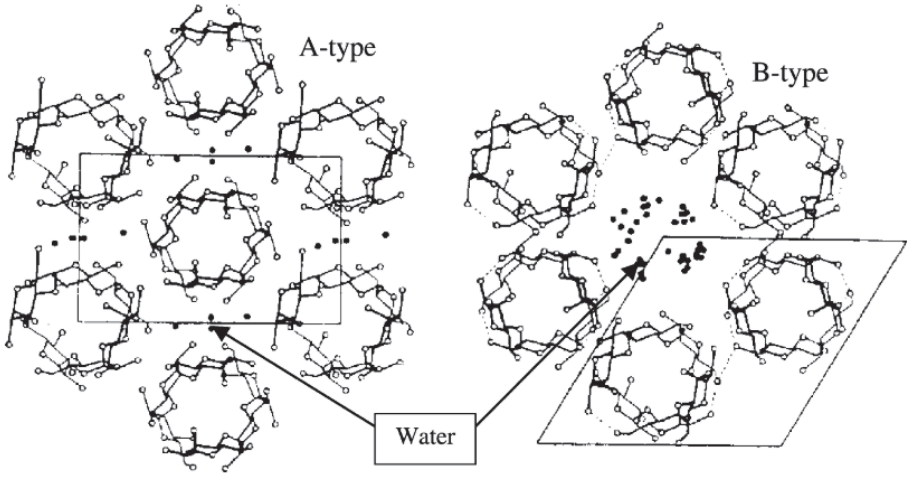

Figure 8.Polymorphs of crystallinity types A and B. Source: Tester et al., 2004

3.3. Other constituents

The digestibility of starch is related to its structural distribution in ingredients and the components associated with the granules (Zaefarian et al., 2015), as well as the proportion between the amylose and amylopectin chains as previously mentioned. In addition to amylose and amylopectin, starch granules are composed of lipids, proteins, and minerals (Baldwin, 2001; Tester et al., 2004). The interaction between these components associated with the granule can reduce the effectiveness of enzymes and, consequently, reduce the digestibility rate of starch in the gastrointestinal tract of broiler chickens.

The lipids associated with the starch granule appear in two forms: first, bound to the amylose chain as phospholipids and free fatty acids. And, the second form occurs when the lipids are located on the outer surface of the granules, being composed of phospholipids, free fatty acids, triglycerides, and glycolipids. These last lipids are derived from the amyloplast membrane and other non-starch sources (Tester et al., 2004).

Relatively low amounts of minerals, including calcium, magnesium, phosphorus, potassium, and sodium, are present. Among these, only phosphorus exhibits significant

functional activity in the starch granule, as it exists in the form of monoester phosphate, phospholipids, and inorganic phosphate (Tester et al., 2004).

A small amount of around 0.6% of protein is found in starch granules, and just like lipids, proteins are also present on the surface of the granule and in the matrix inside the granule, regardless of the botanical origin of the starch granule (Baldwin, 2001; Tester et al., 2004). The presence of pores on the surface of starch granules (Fannon et al., 1992) is closely related to the protein, these pores typically correspond to internal channels within the granule, varying in quantity, depth, and size (Fannon et al., 1993).

These channels that form within the granule are filled with protein molecules related to the adhesion of zeins, which are storage proteins found in the endosperm. When observed by laser scanning confocal microscopy (LSCM), these channels are easily identified (Gayral et al., 2016). This technique allows the visualization of structures that interact with the fluorescamine compound, which reacts with chains containing amino groups in their structure, resulting in a fluorescent coloration. The starch granules presented in vitreous endosperm exhibit a more intense color (Figure 9) than the granules in the floury endosperm, it occurs due to the higher presence of proteins both in the channels and in the layer that surrounds the starch granules (Figure 10).

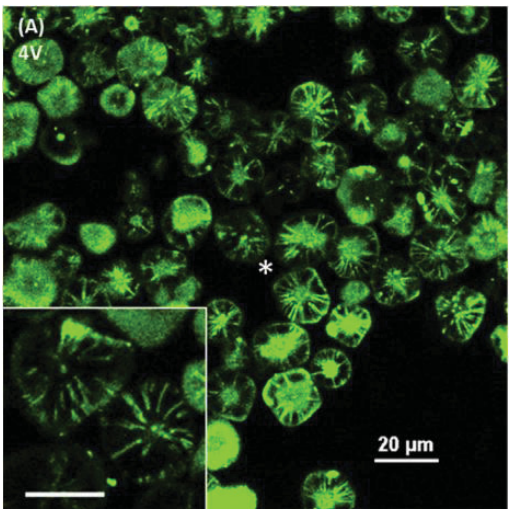

Figure 9. Presence of internal channels in corn starch granules in vitreous endosperm. Source: Gayral et al. (2016)

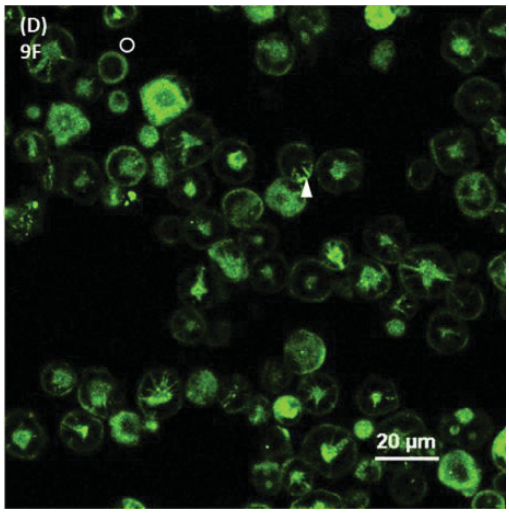

Figure 10. Presence of internal channels in corn starch granules in floury endosperm. Source: Gayral et al. (2016)

3.4.Hydrolysis and digestibility

The biochemical processes of starch hydrolysis begin with the action of pancreatic amylase in the duodenum and jejunum. At this stage, the glycosidic bonds in amylose and amylopectin are broken, forming products such as maltose, maltotriose, and dextrin. Amylose is broken down into maltose and maltotriose, while amylopectin is converted into maltose, maltotriose, and dextrin (Moran, 1985). All these compounds are subsequently hydrolyzed into glucose. Glucose is then absorbed by the intestinal wall, where part of it is oxidized to provide immediate energy to the organism, while another part is transported by the portal vein to be used as an energy source by other tissues or stored in the form of glycogen as an energy reserve can be converted into fatty acids through the process of lipogenesis and subsequently stored in the form of triglycerides in adipose tissue (Nelson and Cox, 2008; Zaefarian et al., 2015).

The starch digestibility involves a series of endogenous enzymes such as alpha-amylase, maltase, and isomaltose (Carré, 2004), and most of the starch is hydrolyzed in the duodenum by the action of alpha-amylase secreted by the pancreas. The complete digestion of starch occurs through the process of total hydrolysis when starch is broken

down into glucose monomers that are subsequently absorbed by the epithelial cells located in the intestinal villi. (Cowieson, 2005). Quantitatively, starch is the most important component of broiler chicken diets and represents more than half of the metabolizable energy that the animal consumes (Svihus, 2011, 2014b).

Even though the endogenous digestive enzymes of broiler chickens can hydrolyze the glycosidic bonds of starch (Englyst et al., 1996). The ratio between amylose and amylopectin is one of the factors that can affect digestibility in the gastrointestinal tract. This is justified due to the structural differences between the two chains, as in amylose, the surface available for enzyme action is smaller and the number of hydrogen bonds linking the glucose molecules in the chain is greater, making it more cohesive and less susceptible to amylase action. On the other hand, amylopectin, due to its more branched structure, enhances the enzyme's action and consequently has greater digestibility (Zaefarian et al., 2015).

The genetic selection of broiler chicken strains with the main objective of body weight gain and feed efficiency is directly related to feed consumption, and this has been mentioned previously by Siegel and Wisman (1966) and confirmed recently by Zuidhof et al., (2014). Furthermore, the genetic selection carried out in recent decades has resulted in an improvement in the utilization of dietary energy (Tallentire et al., 2016), which is explained by the improvement in digestive and absorptive capacity. In the study conducted by Zuidhof et al., (2014) comparing three strains of broiler chickens, the authors reported that growth rates increased by 400% and feed conversion ratio reduced by 50% comparing strains from 1957 and 2005. Moreover, this study also demonstrated that the amount of feed to produce chicken meat was reduced by 50% in almost 50 years of genetic selection. The rate and extension of starch digestibility is variable according to several factors, including the type of feedstuffs (Weurding et al., 2001; Selle et al., 2021)

368 the proportion of rapid and slow starch (Weurding et al., 2003) and the days of age related
369 to the immaturity of the gastrointestinal tract (Uni et al., 1995; Bassi et al., 2023).

370 **3.5. Classification**

371 As reported by Englyst and Hudson (1996), starch can be classified into three
372 groups based on tests that simulate the gastric and intestinal environment. The first group
373 is rapidly digestible starch (RDS), where the release of glucose occurs within 20 minutes
374 of ingestion. The second is classified as slow-digesting starch (SDS), in which the release
375 of glucose occurs between 20 and 120 minutes. The third group is resistant starch (RS),
376 which corresponds to the total starch minus the amount of glucose released within 120
377 minutes.

378 The RS fraction does not undergo enzymatic digestion and can be subdivided into
379 four categories. The first of these is RS type 1, which is inaccessible to enzymatic
380 digestion due to the physical form of the food, which hinders the action of pancreatic
381 enzymes as it is encapsulated within a protein matrix. The second category is RS type 2,
382 which refers to native granules that exhibit a certain degree of resistance to digestibility
383 due to the intrinsic characteristics of the granule itself. The third is RS type 3, which
384 represents retrograded starch, formed by the action of processing where the starch
385 undergoes cooling after the gelatinization process. Finally, the fourth category is RS type
386 4, which refers to starch that has undergone structural chemical changes (Brown, 1996;
387 Raigond et al., 2015; Zaefarian et al., 2015).

388 The digestibility rate of these fractions varies depending on the specific site within
389 the gastrointestinal tract. For instance, the RDS is primarily digested up to the jejunum,
390 whereas the SDS continues to be metabolized down in the ileum. This differentiation in
391 digestion sites highlights the importance of understanding the distinct roles of various
392 starch fractions within the digestive system. In the study performed by Weurding et al.,

(2003), the authors investigated broiler chicken diets containing high and low amounts of SDS. The group fed 5.2% of SDS presented better performance than the group fed 0.7% of SDS. This study indicated the importance of feeding a minimum of dietary SDS to maintain a lower glucose response, which results in reduced energy utilization for metabolism reactions, particularly into lactate conversion, and a gradual insulin response that is closely related to protein deposition metabolism.

4. Protein

Although the primary energy source in broiler chicken diets comes from the starch concentration in corn, the protein derived from this feed ingredient also plays an important role in nutrition. Most of the protein in corn grains is concentrated in the endosperm, accounting for about 70% of the total protein found in the grain (Larkins, 2018). The protein fraction is also present in other portions, such as the germ (20%) and other components (10%).

The corn endosperm contains various proteins, with glutelin making up around 30% of the total protein content, albumin and globulin together account for about 6%. However, the most abundant and significant group of proteins in the corn endosperm are the prolamins or the zein group. Zeins are storage proteins that provide amino acids to the developing embryo during germination, and this group of proteins represents 60% (Coleman and Larkins, 1999; Larkins, 2018).

4.1. Alpha zein: characterization and structure

The protein in the endosperm is represented by the matrix that surrounds the starch granules. When considering the portion of vitreous endosperm, the starch granules are surrounded by a rigid and dense protein matrix, consequently, the starch granules become

more compacted and condensed. On the other hand, in the floury endosperm fraction, the granules are more dispersed because the protein matrix is not so prominent.

The chemical classification of zeins is based on their degree of solubility, which is defined as the maximum amount of a substance that completely dissolves in each amount of solvent (Gong et al., 2007). This solubility varies among several types of zeins, influencing their properties and potential applications, for example, in general, zein is an alcohol-soluble protein used as an industrial polymer, or as coatings for food and pharmaceuticals, biodegradable plastics, and fibers. (Shukla and Cheryan, 2001). About 60% of the endosperm protein is composed by zeins, which can be divided into four categories: alpha, beta, gamma, and delta zeins. Alpha zein represents between 60% and 70% of the total amount of zeins. (Larkins, 2018).

From a structural perspective, alpha zein has a cylindrical and asymmetric conformation, organizing into bi or three-dimensional layers supported by disulfide bridges (Tatham et al., 1993), with a molecular weight ranging between 19 and 22Kda (Momany et al., 2006) and has 240 to 245 amino acid residues. The chain of alpha zein has a molecular weight of 22Kda formed by 240 amino acid residues (Figure 11) (Jumper et al., 2021; Varadi et al., 2022).

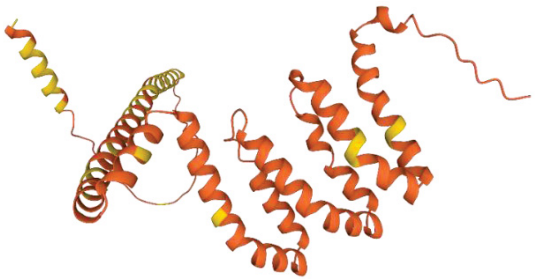

Figure 11.Representation of the three-dimensional structure of the alpha zein protein. (Source: AlphaFold Code Q00919_MAIZE).

Although the biosynthesis mechanisms of zeins have already been addressed and described (Larkins and Hurkman, 1978; Salamini, 1984; Coleman and Larkins, 1999),

recently, the processes of alpha zein synthesis in the endosperm have been detailed by Li and Song, (2020). In summary, the synthesis of alpha zein is mediated by three transcription factors: the prolamin box binding factor 1 (PBF1), which is responsible for encoding a factor that regulates and controls zein expression; the opaque 2 (O2) factor, which is related to the regulation of specific zein production in the endosperm; and the transcription factor box 47 (MADS47), which is responsible for modulating the expression of multiple genes involved in cell signaling and gene transcription. The alpha zein synthesis occurs by the ribosomes in the rough endoplasmic reticulum and is stored in organelles called protein bodies. The entire biosynthesis process involves a series of molecular reactions ranging from transcription in the cell nucleus, translation, modification of the chain structure, storage, accumulation, and maturation within the protein bodies.

Gayral et al., (2016) evaluated 13 inbred corn lines to investigate the relationship between vitreousness and protein composition. The vitreousness varied within a range of 73.14 to 85.01% and the average zein content in vitreous endosperm found was 7.3% and in the floury endosperm was 4.2%. The authors reported no correlation between beta and gamma zeins with vitreousness (Figure 12A), on the other hand, a negative correlation was observed for zein concentration and vitreousness for both endosperm regions (Figure 12A).

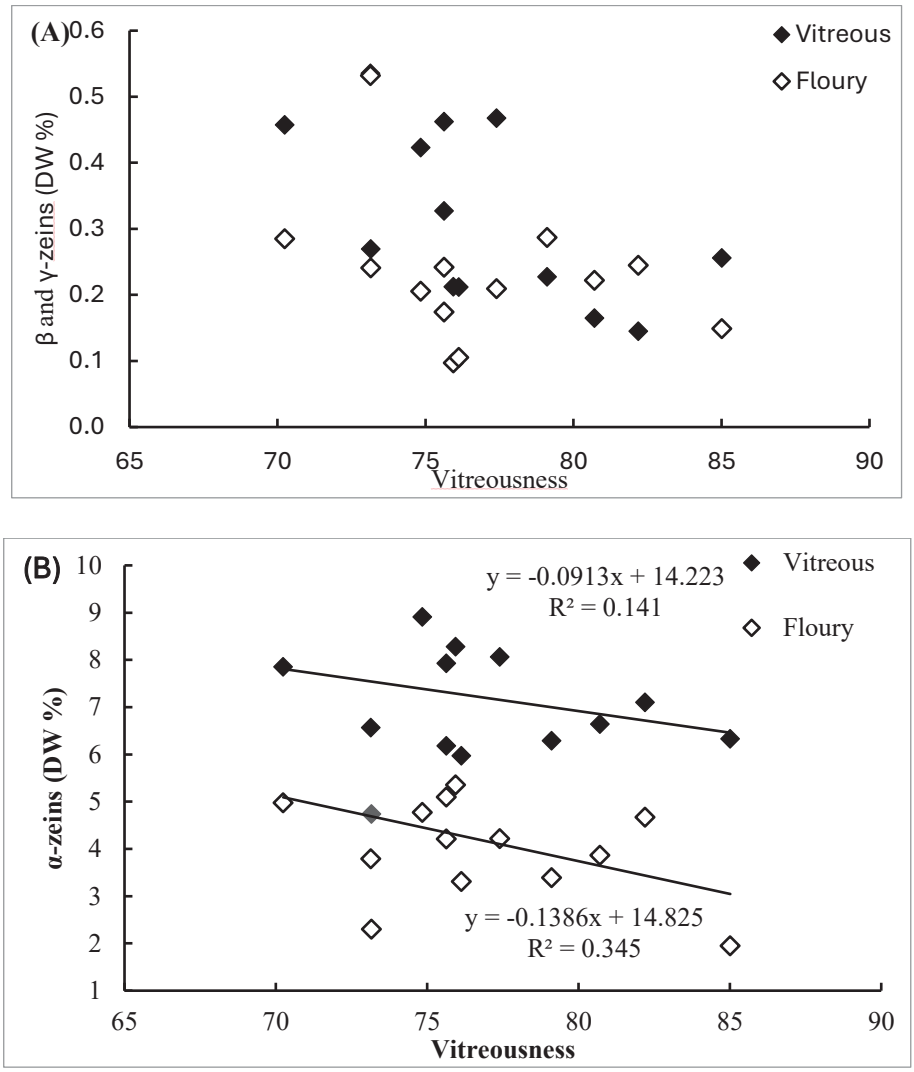

Figure 12.(A): Vitreous and floury endosperm β and γ-zeins contents from the 13 inbred lines vs. grain vitreousness. (B): Vitreous and floury endosperm α-zeins contents from the 13 inbred lines vs. grain vitreousness.

5. Soybean seed

Soybean (*Glycine max* L.) is an Asian-origin legume produced on a large scale worldwide due to its nutritional composition (Medic et al., 2014). Among the cereal and other legume species produced globally, soybean has the highest protein and second-highest oil content (Liu, 1997).

The fruit of soybean, also known as the pod, contains one to five seeds (Matsuo et al., 2017). These seeds when processed into soybean meal, represent the main source of protein for monogastric animals once this meal may contain levels of crude protein

between 45% and 48%. According to Rostagno et al. (2024), the recommended inclusion of SBM in broiler chicken diets is up to 35%, making it the primary dietary protein source.

5.1. Morphology

The soybean seed is composed of two distinct parts, the outer coating or hull and the embryo. The embryo, representing 90% of the total seed weight, is composed of two cotyledons and the embryonic axis, which, in turn, is made up of the radicle, hypocotyl, and epicotyl (Medic et al., 2014) (Figure 13).

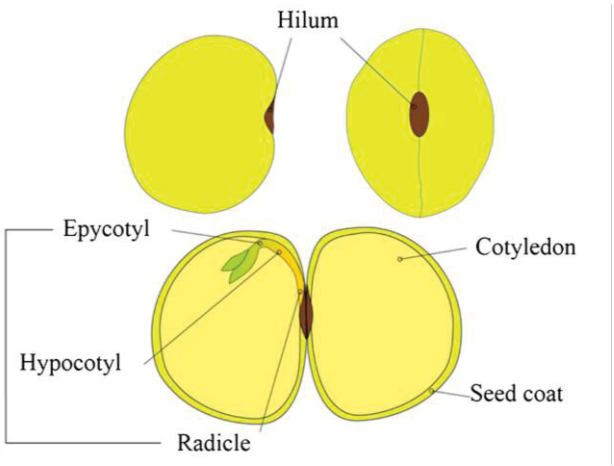

Figure 13. Soybean seed parts. (Adapted from Medic et al., (2014))

Commercially, the cotyledon portion is the most important, as it contains the protein bodies and lipid bodies (or spherossomes; spherical organelles surrounded by a thin membrane), which respectively are the structures that store protein and lipids in the grain (Medic et al., 2014) as demonstrated in Figure 14. The protein and lipid bodies are structures located in the cytoplasm, and the diameter of each one is in a range of 2 – 10 µm for protein bodies and around 0.2 – 0.5 µm for oil bodies.

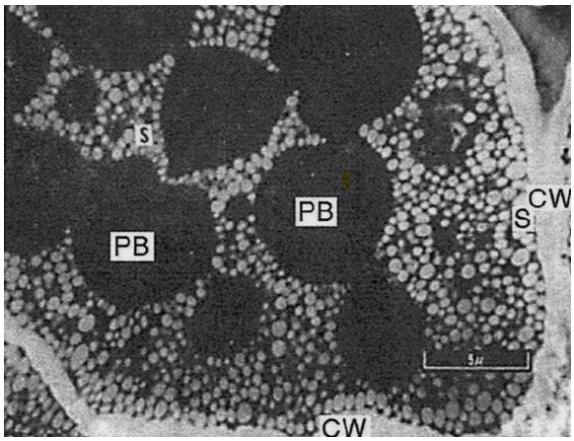

Figure 14. Protein bodies (PB) and lipid bodies (S) of soybean seed under electron microscope. (Adapted from Medic et al. (2014))

The maturation of soybean seeds is divided into five stages (Wilson, 1987), starting with cell division, which occurs in the first 15 days, until the end of day 60 post-flowering when the seed begins to decrease its starch content and moisture level. Regarding the portion that is relevant to nutritional values, the protein and lipid bodies begin to form in the third stage of seed maturation, which occurs from 26 to 36 days post-flowering; during this same period, 50% of the lipid content is deposited. When the seed reaches the fourth stage of maturation, which lasts from 36 to 52 days post-flowering, it is the moment when these structures increase in size and occupy almost the entire volume of the cotyledon. The protein bodies occupy about 60% of the total volume of the cotyledons, and the lipid bodies range between 18 and 22% (Tombs, 1967; Krishnan, 2008; Zaaboul et al., 2022).

5.2. Composition: carbohydrates and proteins

As reported by Assefa et al., (2019), the composition of soybean grains, both quantitatively and qualitatively, is influenced by several factors, including genetics, cultivation environment, and management practices during cultivation. The nutritional value, particularly the amino acid composition, is affected by genetic and environmental conditions such as water stress, temperature, and plant nutrition. Additionally, the thermal processes applied during soybean crushing also play a significant role in determining the

499 final nutritional quality of the soybean grains (Rotundo and Westgate, 2009; Rotundo et
500 al., 2016).

501 In terms of whole grain composition, soybeans contain approximately 40% crude
502 protein, 21% lipids, 5% minerals, and 34% carbohydrates. Among the carbohydrates
503 present in soybean grains, oligosaccharides are of significant nutritional importance,
504 especially for monogastric animals. Oligosaccharides make up about 6% of the total
505 carbohydrate fraction and are further divided into several types, including sucrose,
506 stachyose, raffinose, and verbascose (Medic et al., 2014). These specific oligosaccharides
507 can influence the digestive processes, performance, and overall health of monogastric
508 animals as demonstrated by Teague et al., (2023). The author used concentrations of
509 oligosaccharides that are commonly expected in feed formulation and showed that
510 increasing levels of raffinose and stachyose in broiler chicken diets can impact
511 performance, intestinal function, and microbiota.

512 Proteins, which are the main component of soybean seeds, have a significant
513 impact on animal nutrition. These proteins can be categorized into storage proteins and
514 anti-nutritional factors. The storage proteins, primarily beta-conglycinin (Figure 15) and
515 glycinin (Figure 16), make up about 70% of the total protein content. On the other hand,
516 anti-nutritional factors, such as Bowman-Birk and Kunitz inhibitors, constitute more than
517 6% of the total protein content (Medic et al., 2014). This topic will discuss the importance
518 of storage proteins. Following that, the impact and implications of the Bowman-Birk and
519 Kunitz inhibitors on animal nutrition will be explored.

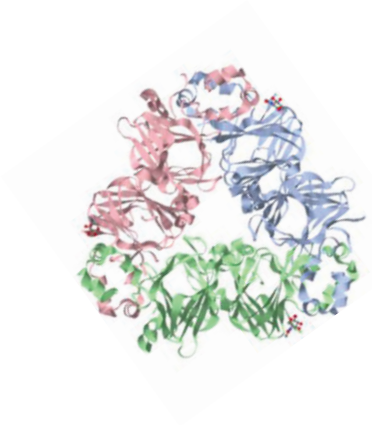

Figure 15. Structure of beta conglycinin.
Source: Protein Data Bank. Code:1IPJ

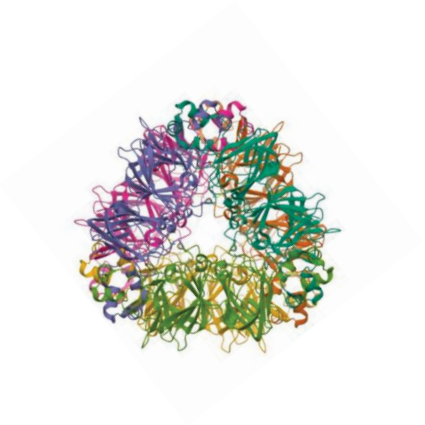

Figure 16.Structure of glycinin.
Source: Protein Data Bank. Code: 1OD5

Beta-conglycinin and glycinin are important because provide intermediate metabolites during germination, and the presence of these storage proteins ensures that the seed has a readily available source of amino acids. Beta-conglycinin is a glycoprotein composed of three subunits: alpha, alpha', and beta with a molecular weight of around 180 kDa. Otherwise, glycinin is formed by six subunits with a molecular weight of 375 kDa, the hexamer chain is composed of two trimers connected by hydrophobic, electrostatic, hydrogen, and ionic bonds. Nutritionally, glycinin contains more methionine and cysteine per subunit compared to beta-conglycinin. Glycinin subunits have a total of 36 cysteine residues and 24 methionine residues, while beta-conglycinin subunits have only 2 cysteine residues and 5 methionine residues (Medic et al., 2014; Sui et al., 2021).

From the nutritional point of view, beta-conglycinin and glycinin have a negative impact acting like potential allergens for monogastric, especially for piglets that have an immune system more sensitive and capable of recognizing these compounds (Zheng et al., 2014; Wang et al., 2023a) in comparison to broiler chickens. The studies evaluating the effects in broiler chickens are not so explored as in piglets, but according to Recoules et al., (2017) beta-conglycinin and glycinin were identified in the jejunum digesta of

broiler chickens fed soybean meal as the primary source of dietary protein, which means both storage proteins were partially digested.

Technics such as thermal processing, fermentation, high hydrostatic pressure, and irradiation can be used to reduce the negative effects of beta-conglycinin and glycinin on soybean meal for monogastric diets (Pi et al., 2021). The study conducted by Wang et al., (2011) evaluated soybean meal processed (ESBM: enzymatically treated soybean meal) by fermentation and protease hydrolysis and the effect of partial replacement for traditional soybean meal on the growth performance, nutrient digestibility, and immune response of broiler chickens. In terms of composition, the ESBM presented 0 and 4 g/kg (dry matter basis) of beta-conglycinin and glycinin, respectively. Whereas the traditional soybean meal investigated presented 13 and 48 g/kg (dry matter basis) of beta-conglycinin and glycinin, respectively. For performance, they observed a linear increase in feed consumption, weight gain, and feed conversion ratio, and the digestibility of dry matter, crude protein, and energy was improved with increased levels of ESBM.

5.3. Thermal process of soybean meal

The co-product resulting from the crushing of soybean grains is known as soybean meal, and it is widely used in the animal nutrition industry. In Figure 17, the processes to which soybeans are subjected before being transformed into the meal used in animal feed are briefly described. These processes encompass stages of grain processing, along with specific treatment steps for soybean meal, including toasting, cooling, and grinding.

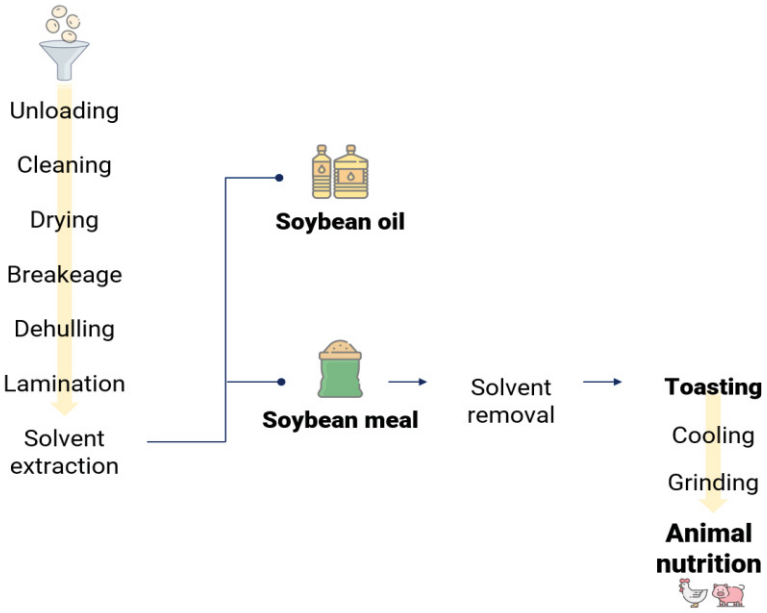

Figure 17.Stages of processing soybeans to obtain soybean meal used in animal nutrition.

Soybean meal is widely used in broiler chicken diets, not only because it is cultivated on a large scale but also due to its high protein and energy content (Stein et al., 2008). In comparison to other oilseed meals, soybean meal presents a more advantageous nutritional composition in terms of metabolizable energy, crude protein content, and amino acid digestibility as described by Rostagno et al. (2024) in Table 1.

Table 1. The values of apparent metabolizable energy, crude protein content, and apparent ileal digestibility of amino acids in oilseeds meals for broiler chickens

Item	Soybean meal	Sunflower meal	Rapeseed meal
AME[1], Kcal/kg	2,296	1,795	1,743
CP[2], %	46.5	33.0	36.2
Amino acid digestibility[3], %			
Lysine	91.4	82.8	80.7
Methionine	92.4	91.2	89.7
Threonine	87.1	82.6	80.3
Tryptophane	90.0	86.0	84.8
Arginine	93.2	91.8	90.2

[1]Apparent metabolizable energy; [2]Crude protein; [3]Standardized ileal amino acid digestibility.

Raw soybean meal contains antinutritional factors, some of which are heat labile. Therefore, it is necessary to submit soybean meal to a thermal process treatment before using it in feed formulation. This process helps eliminate the components that impact negatively on nutrient absorption. Although heat treatment does not address all

antinutritional factors, it is effective in reducing those that are heat-sensitive, thereby improving the nutritional quality of soybean meal (Liener, 1994; Bellaver and Snizek, 1999).

In summary, these anti-nutritional factors can be divided into two groups: those that are heat-stable (oligosaccharides, non-starch polysaccharides, and phytic acid) and those that are inactivated by thermal processing (trypsin inhibitors, lectins, glycinin, and beta-conglycinin) (Medic et al., 2014). The main anti-nutritional factors inactivated during thermal processing, the protease inhibitors known as Bowman-Birk and Kunitz factors, will be better discussed next.

5.4. Anti-nutritional factors: Bowman-Birk and Kunitz factors

The two main anti-nutritional compounds in soybean meal that are inactivated during thermal processing are protease inhibitors, known as the Bowman-Birk factor and Kunitz factor. The Bowman-Birk was first described by Bowman (1946) and later characterized by Birk and Gertler (1963). The Kunitz factor was described by Kunitz, (1946), who discovered that a new crystalline soybean protein became readily digestible by pepsin, chymotrypsin, or trypsin after denaturation. Combined, these two compounds represent approximately 6% of the total soluble protein content in soybean seeds (Mittal et al., 2021).

Both molecules are formed by amino acid residues, but their structure differ in some aspects, according to Liu (1997), the Bowman-Birk factor (BBI) has a molecular weight of 8kDa, with 2 active sites, 7 disulfide bonds, and a total of 71 amino acid residues. The Kunitz factor (KTI) has a molecular weight of 20 kDa, 1 active site, 2 disulfide bonds, and its structure is formed by 181 amino acid residues.

In terms of thermal resistance, DiPietro and Liener, (1989) reported that in purified BBI and KTI, the resistance behavior is different in raw soybean meal. In the

conditions of their study, the KTI was inactivated after 180 minutes, and BBI, after 360 minutes of heat exposure, was only 25% inactivated. The study conducted by Xu et al. (2012) reported that there are distinct modes and conditions for the deactivation of BBI and KTI. Heat more easily induces the incorporation of KTI into protein aggregates, including small proteins like globulins and albumin, as well as larger proteins like glycinin and beta-conglycinin. This is due to the presence of disulfide bonds and/or non-covalent interactions, causing KTI to lose its protease inhibitor property. In contrast, BBI tends to maintain its natural conformation due to the greater stability provided by a higher number of disulfide bonds.

Another factor that explains the greater stability of BBI is the study conducted by He et al., (2017), which discusses the amino acid chains in the structure. The authors report that there is a disparity in the stability of two subdomains of BBI, showing that these subdomains are not equally heat resistant. Furthermore, it was observed that some amino acids are more sensitive to heat (cysteine, serine, and lysine) and are more easily degraded. Thus, the conformation of the BBI chain changes gradually during heat exposure, requiring more time.

6. Quality protein indicators: Thermally processed soybean meal

As mentioned earlier, it is recommended that soybean meal undergo to thermal processing before being used for monogastric feeds due to the presence of anti-nutritional factors such as BBI and KTI. There are variations in the thermal process applied in soybean meal, so there is no standardization of the processes and total quality assurance of all products. Mendes et al., (2004) reported that both under-processing and over-processing modify the nutritional value and cause distinct effects on animal performance due to the quality of the nutritional composition.

Therefore, it is possible to use laboratory analysis methodologies to determine if the thermal processing of soybean meal was carried out satisfactorily, preserving the nutrients and inactivating anti-nutritional factors. Next, some of the analyses will be discussed, and the impact of the measured variables on the nutrition of broiler chickens.

6.1. Visual color measurement

A preliminary visual test can be used to identify the intensity of color changes due to the thermal process of soybean meal. These changes can occur due to the intensity of the temperature applied. More aggressive thermal processing can trigger the formation of compounds that cause brown/caramel coloration in varying intensities, this occurs due to a series of reactions known as maillard reactions or caramelization.

In the sequence of three stages of reactions, the complexation of the carbonyl group of reducing sugars and the free amine group of amino acids, peptides, or proteins occurs, leading to the formation of compounds, resulting in the loss of nutritional quality of the protein, especially when it comes to the lysine content (Starowicz and Zieliński, 2019). In general, melanoidin reduces the efficacy of proteases due to this complexation of compounds and blocking the intestinal absorption of protein and amino acids (Fu et al., 2020).

The visual perception of the color change of processed over or under soybean meal can be evaluated by a non-invasive method of determining color intensity following the CIElab system. This method can be employed in the investigation of the coloration of foods that present variable characteristics regarding regular or irregular surfaces, porosities, transparency, and even colors distributed in layers (Macdougall, 2010).

To identify practical changes in the coloration of the same material, the variables of luminosity (L) are estimated in the range from 0 (white) to 100 (black), and the chromatic coordinates of variation between the shades of red (+a*) and green (-a*), and

642 the variations between yellow (+b*) and blue (+b*), as illustrated in the three-dimensional
643 model below (Figura 18).

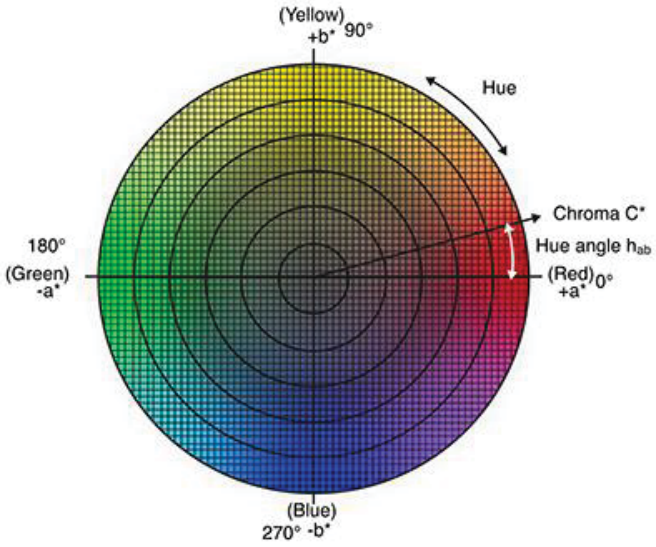

Figure 18. Three-dimensional model to exemplify the CIE L*a*b* color space. (Source: Konika Minolta, Undestanding the space color).

644 The interpretation of color variations of a material is an important point, and
645 several tests can be used in this evaluation. However, a disadvantage of this type of test
646 is that it is not possible to correlate the evaluator's interpretation with those obtained by
647 the colorimeter (Gazibarić et al., 2024), thus the authors suggested a method based on the
648 variation of the deltaE (ΔE*) value that relates to the variation in the distance between
649 two colors.

650 This value of ΔE* is determined by the variations observed in the L*, a*, and b*
651 axes, as described by Zielinska and Markowski (2012), with this measured value it is
652 possible to determine the degree of visual perception of the total color difference between
653 two samples and determine the degree of visual sensitivity following a scale that ranges
654 from $\Delta E<0.2$ (the degree of difference between the samples is not visible) to $\Delta E>6$ (the
655 degree of difference between the samples is significant and easily recognizable)
656 (Zielinska and Markowski, 2012; Gazibarić et al., 2024).

This methodology is widely used in the assessment of color perception in products such as chicken, goose, beef, and goat meat (Wang et al., 2016; Henriott et al., 2020; Song et al., 2023; Wereńska, 2024). However, studies on this perception analysis as a visual tool for evaluating the thermal processing of soybean meal are scarce, few studies evaluated the L*, a*, and b* axis values in samples of dry distillers' grains (Batal and Dale, 2006; Liu, 2008) and even a preliminary study on the identification of the geographical origin of soybean meal via NIR and visual coloration (Núñez-Romero et al., 2009).

6.2. Protein solubility in KOH

The protein solubility test in KOH is an analysis that was developed to evaluate both under-processing (Araba and Dale, 1990a) and over-thermal processing (Araba and Dale, 1990b). This technique is adopted by the industry precisely because it is easy to execute, low-cost, and provides quick results. Even though there is variation in the established standard in the processing industry, high-quality soybean meal should present protein solubility in KOH between 78 and 85% (Van Eys, 2012).

In practical terms, low-solubility soybean meal or that which undergoes more aggressive thermal processing will present a more subdued caramel color. Unlike high-solubility soybean meal that was processed less aggressively, the meal will present a lighter shade. The values obtained when are above 85% indicate possible under-processing, while values below 70% suggest over-processing of the sample (Araba e Dale, 1990b). Parsons et al., (1991) reported that in vitro methodology is a reliable indicator for estimating the quality of soybean meal in vivo, providing information about the thermal process. Despite the negative correlation between *in vitro* methods and *in vivo* outcomes, such as performance and digestibility, there is a significant gap in research

681 providing reliable predictions and strong correlations between KOH protein solubility
682 and *in vivo* parameters.

683 The occurrence of excessive thermal processing of soybean meal indicates
684 excessive protein denaturation, as well as the complexation of amino acids and reducing
685 sugars. As a result, the product loses nutritional quality due to the lower availability of
686 amino acids for the animal. In summary, the protein loses biological value, resulting in
687 lower efficiency in the absorption of amino acids by the gastrointestinal tract.

688 The review conducted by Ibáñez et al., (2020) shows that there is a great disparity
689 in the protein solubility of KOH among soybean meals from various origins.
690 Approximately 40% of the samples obtained in South American countries, such as Brazil
691 and Argentina, revealed solubility values equal to or lower than those recommended for
692 satisfactory thermal processing. These results indicate noticeable variations in the quality
693 of thermal processing between different geographical regions. In addition, Thakur and
694 Hurburgh, 2007), emphasizes the importance of considering the quality of thermal
695 processing when evaluating the protein solubility of soybean meal.

696 This variability in the nutritional value of soybean meal protein has been observed
697 by other authors even in Brazil, for example, Jardim (2019) conducted a survey and
698 analyzed 283 samples of soybean meal produced in Brazil and found that just over 52%
699 of them showed protein solubility values in KOH between 80.1 and 85%. The author also
700 reports that 1.06% of the analyzed samples showed solubility above 90.1%, indicating
701 under thermal processing, and about 3% of the analyzed material showed solubility below
702 75%, indicating that the samples were over-processed.

703 **6.3. Urea activity test**

704 A second chemical quality assessment of soybean meal is the urease activity
705 analysis, which, through the change in pH of the sample, will indicate whether there was

706 under thermal processing, and the methodology adopted is described in Caprita et al.,
707 (2010). In general, raw soybean grains should show a pH variation between 2.0 and 2.5
708 according to Butolo (2002). After the toasting process, it is expected that the pH variation
709 for soybean meal will be between 0.05 and 0.25 to ensure that adequate thermal
710 processing has occurred and that anti-nutritional factors have been deactivated.

711 According to Van Eys (2012), the urease test should indicate a pH variation of
712 less than or equal to 0.10 in samples that comply with proper processing standards. In
713 Brazil, the MAPA (1993) established that this variation can be from 0.05 to 0.25, and the
714 higher this value, the greater the presence of urease in the sample, indicating that the meal
715 was not exposed to the appropriate temperature and for the right amount of time. Several
716 studies show that there is considerable variability in the urease activity levels observed in
717 soybean meals from various countries, including Brazil. This diversity indicated in the
718 studies suggests the existence of a wide variation in thermal processing conditions (Lee
719 et al., 2009; Ibáñez et al., 2020)

720 The color of soybean meal can serve as an indicator of thermal processing quality
721 when combined with urease activity results, which correlate with broiler chicken
722 performance, according to the study by Mc Naughton et al., (1981). The authors reported
723 that broiler chickens fed with soybean bean meal with less intense color (+a* = 3.21; red
724 intensity) and urease activity of 0.19 showed a higher feed conversion rate at 21 days of
725 age compared to others. The authors also showed that this same soybean meal sample
726 exhibited the highest protease inhibitor activity, suggesting under-thermal processing.

727 **6.4. Trypsin inhibitor activity**

728 The analysis of trypsin inhibitor activity (TIA) in soybean meal is an important
729 procedure to evaluate the concentration of BBI and KTI, and when these components are

730 found at undesirable levels, they can cause harmful impacts on the physiology and
731 performance of the animals.

732 In Table 2, some studies are presented relating different concentrations of TIA
733 and effects on performance, intestinal health, and organ weight, among other variables.
734 The presence of BBI and KTI measured by TIA analysis is a well-documented
735 methodology (JL Collins, 1976; Smith et al., 1980; Vagadia et al., 2017; Liu and Ruiz,
736 2021) and this allows the understanding of how the compounds act, the mechanism of
737 action in the organism, and the correlation of the results of this analysis with performance
738 variables. In general, the presence of trypsin inhibitors affects nutrient digestibility and,
739 consequently, performance, as will be demonstrated in the following studies.

740 Raw soybean meal can present TIA values around 27mg/g (Palliyeguru et al.,
741 2011), however, the acceptable limit after thermal processing has already been defined as
742 4mg/g (Clarke and Wiseman, 2005) and values below the established limit are shown to
743 be more promising and less deleterious, as reported in the studies by Kuenz et al., (2022)
744 The authors showed that TIA values equal to or less than 3mg/g are less harmful in terms
745 of intestinal integrity, protein digestibility, and specifically the performance of broiler
746 chickens.

747 In the study by Hoffmann et al., (2019) when evaluating 34 soybean meals with
748 TIA values between 0.25 and 23.6mg/g in the diet of broiler chickens in two growth
749 phases, the authors found that the negative effects of TIA were present even in samples
750 classified below the recommended limit of 4.0mg/g. A concentration of 2.6 mg/g of TIA,
751 considered common in commercially available soybean meal, can linearly increase the
752 feed conversion rate. This occurs due to a high correlation between TIA and feed
753 conversion (R^2=0.77).

754

755 **Table 2.** Summary of studies that investigated the relationship between trypsin inhibitor
756 activity and key nutritional variables of importance in broiler chicken.

References	TIA content (mg/g)	Effects
Perez-Maldonado et al., (2003)	0.15 – 3.2	↓[1]Feed intake; ↓Body weight gain High feed:gain; ↑[2]Liver and pancreas weight
Palliyeguru et al., (2011)	1.90 – 3.72	↓Feed intake; ↓Body weight gain; ↓CP digestibility High feed:gain ↑ Intestinal lesions and proliferation of *C. perfringens*
Heger et al., 2016)	2.60 – 2.73	↓Feed intake; ↓Body weight gain; ↓Carcass and breast yeald High feed:gain ; ↑Pancreas weight
Hemetsberger et al., (2021)	0.50 – 5.10	↓Feed intake; ↓Body weight gain High feed:gain ; ↑Organs weigh
Kuenz et al., (2022)	0.25 – 23.6	↓Feed intake; ↓Body weight gain; High feed:gain
Adeleye and Oyeniyi (2023)	2.27 – 9.20	The correlations between in vitro protein digestibility, residual trypsin inhibitor activity, and protein solubility in KOH. The residual activity of the trypsin inhibitor was positively correlated with protein solubility in KOH and in vitro protein digestibility.

[1]Decreased; [2]Increased.

757 Recently, Kuenz et al. (2022), investigated the impact of TIA on nutrient
758 digestibility and the performance of broiler chickens and found that as TIA values
759 increased, all evaluated performance variables showed adverse effects. For each increase
760 of 1mg/g of TIA, there was a decrease of 15g in live weight, 16.5g in average weight at
761 22 days of age, and 5.7g in total feed intake, while feed conversion increased by 0.015,
762 this reinforces that, at present, there is no safe threshold for trypsin inhibitors.

763 This same behavior was observed by Palliyeguru et al. (2011) when evaluating
764 the inclusion of raw soybeans, characterized by high TIA concentration, in the diet of
765 broiler chickens. The results showed negative impacts on performance and crude protein

digestibility. Furthermore, the authors also reported that higher levels of TIA caused intestinal lesions in the segments of the duodenum, jejunum, and ileum.

6.5. Reactive lysine

As SBM undergoes thermal processing, a variety of chemical reactions may occur that can negatively affect its nutritional value. During this process, high temperatures and prolonged exposure can lead to the degradation of nutrients such as amino acids. These reactions can reduce the availability and digestibility of nutrients, potentially compromising the effectiveness of SBM as a protein source. One strategy applied in industry is the determination of reactive lysine, considering that this amino acid is the sensitive marker of high-temperature processes.

In summary, lysine is the most susceptible amino acid due to its free side chain that is not connected to other amino acids. This part of the chain connects with reducing sugars and forms a Schiff base in the presence of heat. With continuous heating, the Schiff base turns into an Amadori compound, and with ongoing heating, this Amadori compound turns into melanoidins. These melanoidins are cyclic compounds and have the lysine destroyed, which means that the reduced sugar attached to the nitrogen in the amino group of the lysine side chain makes the lysine unreactive and unavailable for protein synthesis.

Reactive lysine is the amount of lysine available for digestion and absorption, which has a free side chain amino group and can be either free or protein-bound, and it is detected by methods as fluorodinitrobenzene, dye binding, guanidination, and others (Rutherfurd, 2015). This reduction in the availability of lysine can be harmful to monogastric animals since lysine is the second limiting amino acid for broiler chickens and the first for pigs, and is also involved in several metabolic paths such as protein synthesis, hormone production, and immunity (Liao et al., 2015).

It is recommended that the ratio of reactive lysine: total lysine be higher than 90% after the thermal process, which suggests less heat damage and greater nutritional value. In the study of Kim et al., (2012), the authors investigated the impact of heat treatment on the lysine content in autoclaved soybean meal and confirmed that by increasing the time of autoclaving at 135℃, the losses of reactive and total lysine were more evident (Figure 19).

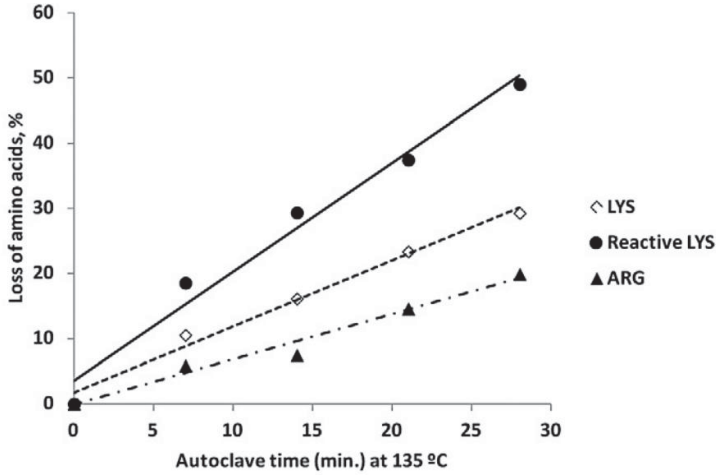

Figure 19. Loss of amino acid including total lysine and reactive lysine of soybean meal autoclaved at 135 degrees celsius from 0 to 30 minutes (Kim et al., 2012).

The use of the reactive lysine method as a practical determination in industry and it is feasible according to Neoh et al. (2023), the authors evaluated 70 samples of soybean meal from different countries and demonstrated that there are reasonable correlations between reactive lysine and practical quality analysis applied such as protein solubility of KOH (R^2=0.70), protein dispersibility index (R^2=0.60), and color L (R^2=0.55).

In the studies conducted by Oliveira et al. (2020), the authors investigated two different batches of SBM, the first was autoclaved at 110° C for 15 and 30 minutes, and the second batch of autoclaved SBM at 150°C for 3, 6, 9, 12, 15, and 18 minutes, since the digestibility parameters were reduced only in diets containing SBM autoclaved at 150° C, the authors emphasized that soybeans crushing plants might avoid apply thermal process with more than 110° C, intending to reduce the risk of over-processing.

6.6. Arrangement of cluster aggregation caused by thermal processing

The thermal processing conditions for soybean meal may induce cluster aggregation involving specific temperature and moisture content parameters. The more aggressively the heat treatment is applied, it may impact significantly the structural properties and aggregation behavior of soybean proteins, particularly β-conglycinin, and glycinin, resulting in solid structures characterized by denser aggregation which difficult the enzyme access during the digestion process (He et al., 2016; Li et al., 2020; Zhang et al., 2022; Ju et al., 2023).

According to Zhang et al. (2022) the heat treatment at 160 °C for 10 to 30 minutes is effective for inducing aggregation in soybean proteins, particularly when the moisture content is around 10.68% and 46.29%. These clusters, or protein-aggregated, form solid structures with negligible pores as demonstrated by Li et al. (2020), in this study, the authors investigated the pre-denaturation degree of soybean protein isolated submitted in a range of temperatures from 60 to 100˚C for 50 seconds and reported through microscope images a stabilized network formation and large aggregates from samples of soybean protein isolated partially at 90˚C (Figure 20) or completely pre-denatured at 100˚C (Figure 21).

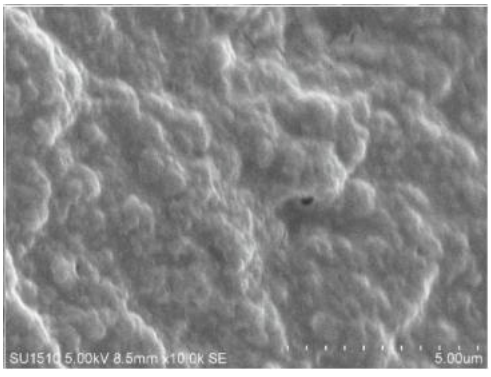

Figure 20. Aggregated structure of soybean protein isolated pre-denatured at 90˚C (Li et al., 2020)

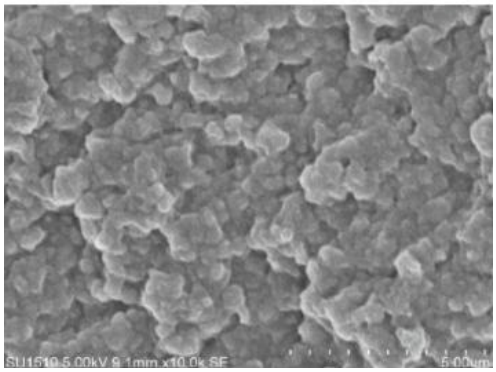

Figure 21. Aggregated structure of soybean protein isolated pre-denatured at 100˚C (Li et al., 2020)

7. The use of exogenous enzymes in broiler chicken diets

Exogenous enzymes are classified as zootechnical additives, which have the function of improving animal performance by nutrient utilization (MAPA, 2004). Biochemically, enzymes are defined as biomolecules that act as catalysts, controlling the speed and regulating reactions that convert a specific substrate into a product.

The effectiveness of enzymes is influenced by four factors, such as the enzyme activity characteristic of each type and origin, the exposure time of the substrate to the enzyme presence, the pH of the portion of the gastrointestinal tract where each enzyme will act, and finally, having knowledge of the specific type of substrate for each enzyme (Scopes, 2002; Ao et al., 2008; Angel and Sorbara, 2014). Throughout this topic, two classes of digestive enzymes used in broiler chicken nutrition will be discussed as a strategy to improve the digestibility and utilization of carbohydrates and protein.

7.1. Amylase

Amylase is an enzyme of the hydrolase class that is responsible for the digestion of starch, breaking down starch molecules into units of simple sugars. Commercially, there are two types of amylases on the market: alpha-amylase and beta-amylase. In the case of broiler chickens, alpha-amylase is naturally an endogenous enzyme and also is a commonly used exogenous enzyme in diets due to the digestion and metabolism reactions of starch, mainly derived from corn, which will serve as an energy source (Gracia et al., 2003; Cowieson et al., 2019). The alpha-amylase in conditions of neutral pH breaks the alpha 1-4 chains and releases a mixture of maltose, dextrin, and oligosaccharides. Beta-amylase, on the other hand, is frequently used in industrial processes, especially in the production of beers and distilled beverages during the stage of producing fermentable sugars from the starch contained in malt (Bathgate, 2016; Gomaa, 2018), further under

852 slightly acidic pH conditions (between 5 and 6.5), the chain is primarily broken down into
853 maltose.

854 Amylase is divided into 13 enzymes of the glycoside hydrolase group of microbial
855 origin (Reddy, 2003), where each one specifically hydrolyzes one or more substrates. In
856 broiler chicken diets, the most commonly used is alpha-amylase (E.C: 3.2.1.1), an enzyme
857 of class 3 that represents the group of hydrolases (catalyze hydrolysis reactions by
858 breaking bonds through the addition of water), subclass 2 that acts on glycosidic
859 compounds, belonging to subclass 1 of enzymes because it breaks bonds between two
860 glucose residues, and finally, it has the identifier digit 1 referring to alpha-amylase which
861 uses starch as a substrate (IUBMB, 1961).

862 There are numerous benefits to using amylase in broiler chicken diets, the
863 inclusion of alpha-amylase brings good results in animal performance, nutrient
864 digestibility of the diet, and the impacts on intestinal integrity and health parameters. In
865 terms of performance, the use of alpha-amylase is already a well-established strategy.
866 Several studies conducted in the 1980s and 1990s showed that the use of amylase was
867 promising in improving the performance of broiler chickens and in the utilization of
868 dietary nutrients (Richter et al.; Oh and Mak, 1980; Neskar, 1987; Rodriguez-Castanon,
869 1988; Zanella et al., 1999).

870 However, in recent years, research on enzymes in animal nutrition has provided
871 evidence that the results in broiler chicken production can be significantly improved, not
872 only because of advances in genetic selection but also by the advances in nutrition
873 research and the development of new enzyme generations. The use of alpha-amylase for
874 broiler chickens, especially when the birds are fed corn-based diets, is the main energy
875 source. Results show the effects of alpha-amylase supplementation on average weight
876 gain in the initial, growth, and final phases, as well as improving the feed conversion rate

and feed efficiency of these animals (Aderibigbe et al., 2020b; Liu et al., 2020; Ma et al., 2020; Córdova-Noboa et al., 2021; Schramm et al., 2021).

The dietary nutrient digestibility also is improved with the inclusion of alpha-amylase. Studies show that these effects are observed due to the increase in total starch digestibility (Córdova-Noboa et al., 2021), ileal energy digestibility (Liu et al., 2020; Ma et al., 2020; Schramm et al., 2021), as well as ileal starch digestibility. (Ma et al., 2020; Schramm et al., 2021; Bassi et al., 2023).

In addition to improving performance and digestibility, the use of amylase can increase the diversity of the intestinal microbiota, mainly of bacteria from the Bacteroidetes and Firmicutes phyla (Yin et al., 2018) that are responsible for the fermentation of non-starch polysaccharides. Parameters related to the intestinal integrity and functionality of broiler chickens also showed promising results. It was observed that the use of exogenous amylase is capable of increasing amylase activity in portions of the small intestine, such as in the jejunum (Ma et al., 2020) and just over 3 times in the ileum. (Bassi et al., 2023).

The use of alpha-amylase can also affect the morphology of the intestine and other organs. Aderibigbe et al., (2020b) showed that the height of the villi in the jejunal portion is significantly greater in chickens that received exogenous amylase in their diet. Furthermore, the authors reported a decrease in the viscosity of the digesta, even under the condition that these animals received a diet with a low concentration of non-starch polysaccharides, which may have led to better nutrient utilization.

7.2. Protease

Proteases are divided into classes according to specific characteristics and mechanisms of action. Based on this concept, proteases are classified into six groups: aspartic, glutamic, and metalloproteases, cysteine, serine, and threonine proteases. The

first three have a water molecule in their active site and the others, in turn, have cysteine, serine, and threonine residues as their active site (López-Otín and Bond, 2008).

More than one-third of proteases are grouped in the serine protease class, which act as endopeptidases dependent on aspartate, histidine, and serine residues (Di Cera, 2009). The class of serine endopeptidase, known as subtilisin (EC 3.4.21.62), produced by a genetically modified strain of *Bacillus Licheniformis*, will be addressed (De La Huebra, 2022).

According to the IUBMB (1992) description, subtilisin, registered as (EC 3.4.21.62), is an enzyme of the hydrolase class (3), subclass 4, which refers to a serine peptidase, that is, it uses a serine residue at its site to catalyze reactions; its sub-subclass is 21 because it is an endopeptidase that acts on breaking bonds within a polypeptide chain, and it is identified with the number 62 within the sub-subclass of endopeptidases where each enzyme receives its own identification number.

In the last 20 years, research investigating the use of protease in broiler diets and its effects has grown significantly. Thus, another digestive enzyme has shown promise in improving performance parameters and nutrient digestibility in broiler diets. Research on the inclusion of protease in broiler diets was more focused on the evaluation of enzyme complexes, such as the use of cocktails with xylanase, amylase, and protease, for example. These studies indicated that the use of exogenous enzymes was an effective nutritional strategy in terms of costs in poultry nutrition (Cowieson and Adeola, 2005) in addition, it was capable of improving the nutritional value of diets from the initial phase (Cowieson and Ravindran, 2008).

Other studies were developed and found that the use of protease proved effective in improving performance parameters, such as feed intake, body weight gain, feed conversion (Yu et al., 2007; Peek et al., 2009; Zavelinski et al., 2024), as well as feed

927 efficiency (Yadav and Sah, 2005), due to the improvement in protein digestibility (Yadav
928 and Sah, 2005; Yu et al., 2007; Cowieson and Ravindran, 2008), not only in diets based
929 on corn and soybean meal but also in diets with the inclusion of sorghum and (Selle et
930 al., 2010) or using dry distillers' grains (Yan et al., 2012), and finally the protease can
931 also be used for economic, and environmental reasons (Doskovič et al., 2013; Lee et al.,
932 2023).

933 Another benefit observed with the supplementation of protease in the diet of
934 broiler chickens refers to intestinal health and integrity (Kamel et al., 2015) found that in
935 addition to improving the digestibility of crude protein, the inclusion of protease affected
936 the reduction of clostridia species in the ileum of broilers, the height of villi, and the
937 villus/crypt ratio in the duodenum. This same effect on intestinal morphology was
938 observed by Cowieson et al., (2017), along with the fact that supplementation with
939 protease also affected the dynamics of intestinal secretion and absorption through changes
940 in mucin secretion and the improvement in the integrity of cell junctions in the intestine.

941 In addition to research that reinforces the effect on nutrient digestibility (Amerah
942 et al., 2017; Mahmood et al., 2017; Mohammadigheisar and Kim, 2018), Aderibigbe et
943 al., (2020c) recently conducted a study investigating the effect of protease in diets
944 containing levels of purified trypsin inhibitors, finding that protease has a significant
945 effect independent of the concentration of inhibitors added. This was observed in
946 performance variables, but mainly in issues of digestibility and absolute pancreas weight
947 at 21 days of age.

948 Regarding the gut microbiota, (Yi et al., 2024) demonstrated that protease was
949 able to regulate metabolism and the community of microorganisms in the intestines of
950 broiler chickens, mainly by increasing the production of short-chain fatty acids from the
951 metabolic activity of the bacteria *Eubacterium*, *Alistipes*, and *Rikenella*. The authors also

952 report that the inclusion of protease influences the production of metabolites that are
953 beneficial for the animal's health, such as the production of pentadecanoic acid and
954 caproic acid, aimed at promoting cell integrity, providing energy, and helping to control
955 the population of microorganisms in the gastrointestinal tract.

8. CONSIDERATIONS

In terms of nutritional value, there is a significant difference in the composition of corn and SBM used in broiler diets. Therefore, it is increasingly important to have knowledge and understanding of these variables when we talk about nutrition and nutrient utilization by production animals. Knowing the composition of corn used in feed production and understanding how the structural differences in the endosperm region can impact the utilization of starch, protein, and consequently energy, should be considered a strategic cost that will directly reflect on profits. The same principle can be applied to soybean meal, knowing the nutritional value of the ingredient can increase nutrient utilization, consequently improving gains in animal production.

Evaluating the use of enzymes considering the influence of factors related to dietary nutrients is a challenge and can lead to more assertive strategies being adopted at a point where the mechanisms of enzyme action on a specific substrate are better understood. Therefore, understanding how all these factors of nutritional quality of ingredients and the use of enzymes is necessary and becomes increasingly important when discussing nutrition, nutrient utilization, and zootechnical gains. Still, emphasizing the importance of adopting techniques for evaluating ingredients used in diets for broiler chickens.

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CHAPTER II - The impact of amylase supplementation on growth performance and nutrient digestibility for broiler chicken fed diets containing corn from different batches

1. Abstract

Corn is the major source of energy in broiler chicken diets. However, the physicochemical characteristics inherent to the grain may impact nutrient utilization as well as performance. In this context, the use of exogenous amylase may help mitigate the negative impact of different corn types on the nutritional value and digestibility in broiler chickens. This study aimed to evaluate the effects of amylase supplementation on broiler chickens fed diets containing corn from different batches, focusing on its impact on growth performance, nutrient digestibility, and energy utilization. A total of 360 male Cobb broilers from 14 to 26 d of age were randomly distributed in 2 x 2 factorial arrangement with 2 corns (A and B) and 2 amylase doses: 0 and 80 KNU per kg of feed, totalling 4 treatments and 9 replicates of 10 broiler chickens each. A hardness test was performed to evaluate the breakage force, and scanning electron microscopy was used to investigate structure variations of starch granules in the floury and vitreous endosperm. Proximate analyses were performed to evaluate both corn types. Growth performance variables were evaluated from 14 to 26 days of age. From 19 to 21 d, excreta samples were collected to determine apparent total tract retention (ATTR) of nutrients, and ileal digesta to determine apparent ileal nutrient digestibility (AID) at 26 d. Both corn presented different breakage force ($P<0.05$), hard corn B showed higher breakage force (217 N) compared to corn A (166 N). Visual evidence through scanning electron microscopy images revealed structural differences between floury and vitreous endosperms from corn samples, corn A showed starch granules more dispersed in the floury endosperm and a less compact granule distribution in the vitreous endosperm. No differences ($P > 0.05$) were found in body weight gain, feed intake, or feed conversion ratio from 14 to 26 d of age among the treatments. Amylase increased AID of DM, CP, and ileal digestible energy, and ATTR of DM and CP ($P<0.05$) regardless of corn

endosperm hardness. No interactions were observed between corn types and amylase supplementation (P>0.05) on growth performance or nutrient digestibility. In conclusion, amylase supplementation increased nutrient digestibility for broiler chickens regardless the corn type.

Keywords: Digestibility; Endosperm; Proline; Starch; Vitreous

2. Implications

Corn is the major ingredient in poultry diets and a crop highly diffused around the world. The investigation of the nutritional characteristics of this widespread ingredient is a potential tool in broiler chicken nutrition. Although prior research has identified the effectiveness of amylase supplementation in corn-soybean meal diets for broiler chickens, the impact of nutritionally different corn investigated in this paper elucidated the importance of proximate analysis evaluations and digestibility assays in feed ingredient evaluations.

3. Introduction

As the ingredient that represents the majority of monogastric feed formulations, corn is also known as the main energy supply in broiler chicken diets (Aderibigbe et al., 2020a), approximately 65% of metabolizable energy in the starter diet formulation comes from corn (Cowieson, 2005) due to starch stored in the endosperm.

Among the several factors influencing nutrient utilization responses, the nutritional value of the diet emerges as the most pronounced impact for broiler chickens. Using exogenous enzymes is a method to improve performance and nutrient digestibility and an effective nutritional strategy (Cowieson, 2005). Amylase which belongs to a class of carbohydrases is well-stated in poultry nutrition due to enhancement of the

digestibility of nutrients (Aderibigbe et al., 2020b; Córdova-Noboa et al., 2021; Schramm et al., 2021) and can also improve the efficiency of energy utilization (Stefanello et al., 2019; Cowieson et al., 2019).

Feeding broiler chickens corn with a high proportion of soft or floury endosperm is closely related to greater nutrient digestibility and growth performance (Vargas et al., 2023a; b) compared to corn with harder or vitreous endosperm where the amount of prolamins that surrounds starch granules is greater and limit the enzyme access which influences the nutritional value of the grain for broiler chickens (Kaczmarek et al., 2013).

Although corn is widely used by the poultry industry, as well as dietary amylase supplementation, research on corn nutritional value and type/origin is still a potential field of study in order to explore effects on performance variables and nutrient digestibility (Yegani and Korver, 2013; Stefanello et al., 2023; Vargas et al., 2023b). Moreover, understanding the nutritional and morphological characteristics inherent to corn endosperm is required for poultry nutritionists to provide more accurate diets. Therefore, the objective of our study was to investigate corn endosperm characteristics through physicochemical evaluations of the endosperm and determine the effect of the dietary inclusion of corn types combined with amylase supplementation on growth performance and nutrient digestibility from 14 to 26 days of age.

4. Materials and methods

4.1. Animal facilities, experimental design, and diets

Experimental procedures were previously approved by the Animal Use Ethics Committee of the Federal University of Paraná. The experiment was conducted in Curitiba, PR, Brazil (25º25’40’’S and 49º16’23’’W). A total of 360 male broiler chickens from a commercial hatchery (Cobb 500; BRF Global S.A, Castro, PR, Brazil) were

allocated in metabolic battery cages from 14 to 26 d of age. Each cage measured 0.98 m in length x 0.90 m in width x 0.35 m in height and was equipped with nipple drinkers and gutter feeders. Metal trays covered with plastic canvas were placed under each cage for excreta collection. Broiler chickens received water and feed ad libitum during the experimental period. Room temperature was set at and reduced by 0.5º C each day up to 19.5ºC on day 26.

The broiler chickens were allocated into a completely randomized block design in a 2 x 2 factorial arrangement, which consisted of 2 corn batches (A and B) and 2 amylase doses (inclusion of 0 and 80 KNU per kg of feed) totalling 4 treatments and 9 replicates of 10 broiler chickens each. Each cage was considered an experimental unit and treatments varied according to the corn endosperm hardness and enzyme supplementation.

The experimental diets based on corn-soybean meal were formulated and fed in mash form. The ingredients and nutritional composition of the experimental diet are presented in Table 1. To measure the total tract retention from day 19 to 21 and apparent nutrient digestibility of nutrients at day 26, 1% of acid-insoluble ash (Celite 400; Celite Corp. Lompoc. CA) was added to all diets as an indigestible marker.

1681 Table 1. Ingredients and nutritional composition of the experimental diet for broiler
1682 chickens (g/kg as fed).

Ingredients	Experimental diet
Corn	641.9
Soybean meal	302.8
Soybean oil	18.4
Dicalcium phosphate	6.7
Limestone	9.1
Salt	2.5
Sodium bicarbonate	1.8
DL – Methionine	2.5
L – Lysine HCl	1.7
L – Threonine	0.2
L – Valine	0.2
Phytase[1]	0.0
Mycotoxin adsorbent	0.7
Mineral supplement[2]	0.5
Vitamin supplement[3]	1.0
Celite[4]	10.0
Calculated values	
Crude protein	195.0
Digestible Lysine	10.7
Digestible Methionine	5.1
Digestible Methionine + Cysteine	8.0
Digestible Threonine	7.1
Calcium	8.0
Total phosphorus	4.6
Available phosphorus	4.0
Sodium	1.9
Metabolizable energy, kcal/kg	3,140

1683 [1] RONOZYME HiPhorius inclusion of 2000 FYT per kg of feed.
1684 [2] Composition per kg of feed: Manganese (65.0 mg); Iron (50.0 mg); Zinc (65.0 mg); Copper (10.0 mg);
1685 Iodine (1 mg); Selenium (0.39 mg).
1686 [3] Supplementation per kg of feed: Vitamin A (14.300 UI); Vitamin D3 (5.200 UI); Vitamin E (71.5 UI);
1687 Vitamin B1 (2.99 mg); Vitamin B2 (9.10 mg); Vitamin B6 (5.20 mg); Pantothenic Acid (15.6 mg); Biotin
1688 (0.325 mg); Vitamin K3 (3.9 mg); Folic Acid (2.60 mg); Nicotinic Acid (78.0 mg); Vitamin B12 (32.5
1689 mcg).
1690 [4] Indigestible marker (Celite 400; Celite Corp. Lompoc. CA).

The amylase used was a commercial granulated thermostable product (IUB N° 3.2.1.1, Ronozyme HiStarch, Novozymes A/S Bagsverd, Denmark), which contains a minimum activity of 600 kilo-Novo α-amylase units (KNU) per g of product, produced by submerged fermentation of *Bacillus licheniformis*. One KNU is the amount of enzyme that releases 5.26 g of starch in one hour. Amylase activity in feed samples was measured at Biopract GmbH, Berlin, Germany.

4.2. Corn characteristics

A hardness test was performed on each corn using a universal machine (DL 30,000; Software Test Version 3.04; EMIC, São José dos Pinhais, Brazil) with a load cell Trd 22 with penetration force at a speed of 0.6 mm/min. Intact corn kernels samples were previously separated, and each kernel with no cracks or previous signs of breaks was selected. Then, each selected kernel was placed on a metal plate individually, and a perpendicular force was applied in the centre of the opposing face of the germ zone. The breakage force values obtained for each sample were expressed in Newton (N).

For the investigation of structural differences in the starch granules in the floury and vitreous endosperm, corn kernels were cross-sectionally sliced with a carbon steel blade (0.34 mm thickness x 13.92 mm width x 19.90 mm length), attached on metal stubs and coated with a thin gold-palladium cover (approximately 15 nm thickness). Samples were then observed using the JEOL JSM-6360 LV scanning electron microscope (JEOL Ltd., Tokyo, Japan) at an acceleration potential of 10 kV. Magnifications of 1000x were used for each corn endosperm sample.

Representative samples were collected to perform proximate analysis to evaluate the nutritional value of both corn. DM content was determined using a drying oven at 105°C for 72 hours. The CP (Method 954.01), ether extract (EE; Method 920.39), crude

fiber (CF; Method 962.09), ash content (Method 942.0), calcium (Ca; Method 927.02) and phosphorus (P; Method 965.17) were determined according to the Association of Official Analytical Chemists (AOAC, 2005). The gross energy (GE) for both samples was determined using an adiabatic calorimetric bomb (IKA Model C2000; Parr Instrument Co., Moline, IL). The concentrations of amino acids were determined by high-performance liquid chromatography (Hewlett-Packard 1100, Waldbronn, Germany) following the procedures described by White et al., (1986) and Hagen et al., (1989).

4.3. Growth performance

Growth performance was evaluated from 14 to 26 days of age. Broiler chickens were weighed by cages on days 14 and 26 to determine body weight and calculate body weight gain (BWG) by the difference measured on day 26 and the initial on day 14. The feed allowance and the amount remaining in the feeders (feed refusal) were weighed on days 14 and 26 to determine feed intake (FI). Feed conversion ratio (FCR) was calculated as the ratio between FI and BWG and corrected to the weight of dead broiler chickens.

4.4. Total tract retention and apparent nutrient digestibility assays

Following the 5d period of adaptation to the experimental diets, excreta samples were collected twice daily (08:30 and 17:30) from each cage using the partial method for 3 days (19 to 21 d of age). With the aid of plastic spatulas and tweezers, a sample of about 30% of the excreta was randomly collected avoiding contamination with feed and feathers. At the end of the excreta collection, the samples were packed in plastic containers and stored in the freezer until analysis.

At day 26, 6 broiler chickens per experimental unit (total of 216 broiler chickens, which represents 60% of all broiler chickens housed) were randomly selected, then

euthanized by cervical dislocation, and the abdominal cavity opened immediately to expose the digestive tract. For the ileal content removal, the reference gut segment was defined as 4 cm below Meckel's diverticulum and 4 cm above the ileum-cecum-colon junction. The digesta was collected by slightly pushing, then placed into plastic containers previously identified, frozen at -18ºC, and freeze-dried (Christ Alpha 1-4 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) up to a 7×10^{-2} mbar vacuum pressure until analysis.

Excreta samples were thawed at ambient temperature, homogenized and sub-samples were collected. Excreta aliquots were then placed in a drying oven at 55°C until constant weight. Feed, excreta, and ileal samples were grounded to 1mm to measure DM content by drying oven at 105°C, GE value was estimated using an adiabatic calorimetric bomb (IKA Model C2000; Parr Instrument Co., Moline, IL) and CP (Method 954.01) as described by AOAC (2005). Acid-insoluble ash (AIA) was used as an indigestible marker in digestibility calculations, and AIA content was determined according to the methodology described by Scott and Boldaji (1997). To estimate the indigestible factor (IF) in the calculations was used the ratio between AIA dietary concentration and AIA excreta or ileal content concentration.

The apparent total tract retention (ATTR) of nutrients was calculated according to the following equation: ATTR (%) = [Nutrient in the diet – (Nutrient in the excreta*IF)]/Nutrient in the diet. The apparent ileal digestibility (AID) of nutrients was calculated following the equations: AID (%) = [Nutrient in the diet - (Nutrient in the ileal digesta*IF)]/Nutrient in the diet. Apparent metabolizable energy (AME) and ileal digestible energy (IDE) were calculated using the following formula: AME or IDE (kcal/kg DM) = GE in the diet – (GE in excreta or ileal digesta x IF).

4.5. Statistical analysis

Corn hardness test data were analysed by Shapiro-Wilk test and then compared by Student t-test ($P<0.05$). Data obtained from digestibility assays were analysed by the normality of the residues using the Shapiro-Wilk test. When detected normal distribution data were subjected to a two-way ANOVA at a 5% significance level, including the two main factors (corn endosperm hardness and amylase supplementation) and their interactions. When observed significant effect of the factors or interaction between them, the averages were compared by the Tukey test at 5% probability. All the statistical procedures were carried out using the linear model of the ExpDes.pt package (Experimental Designs Package, Belo Horizonte, Minas Gerais, Brazil) on R software (R Foundation for Statistical Computing, Vienna, Austria).

5. Results

5.1. Dietary amylase recovery and corn characteristics

The amylase recovery in the experimental diets supplemented was 98 KNU/kg and 57 KNU/kg of feed for the soft corn and hard corn diets, respectively. In contrast, no amylase was detected in non-supplemented diets. Corn types were classified according to endosperm hardness as soft and hard due to the minor and greater breakage force ($P < 0.05$), respectively 166 and 217 N, suggesting the presence of an endosperm significantly more resistant or not to fracture (Table 2).

Table 2. Hardness test of soft and hard corn used in the experimental diet for broiler chickens.

Item	Corn		*P*-Value
	A[1]	B[2]	
Hardness, N[3]	166	217	<0.001
SEM[4]	7.4	7.6	

[1] Data represents the mean of 56 kernels.
[2] Data represents the mean of 55 kernels.
[3] Newton.
[4] Polled standard error mean.

The visual analysis through scanning electron microscopy of the floury and vitreous endosperm of corn A and B has shown structural changes in starch granules between them. In floury endosperm from corn A (Fig. 1A), round-shaped starch granules were more dispersed compared to the corn B floury endosperm (Fig. 1C). Moreover, the vitreous endosperm from corn A (Fig. 1B) highlighted a less cohesive distribution of polyhedral-shaped starch granules when compared with the corn B vitreous endosperm (Fig. 1D) where starch granules are denser.

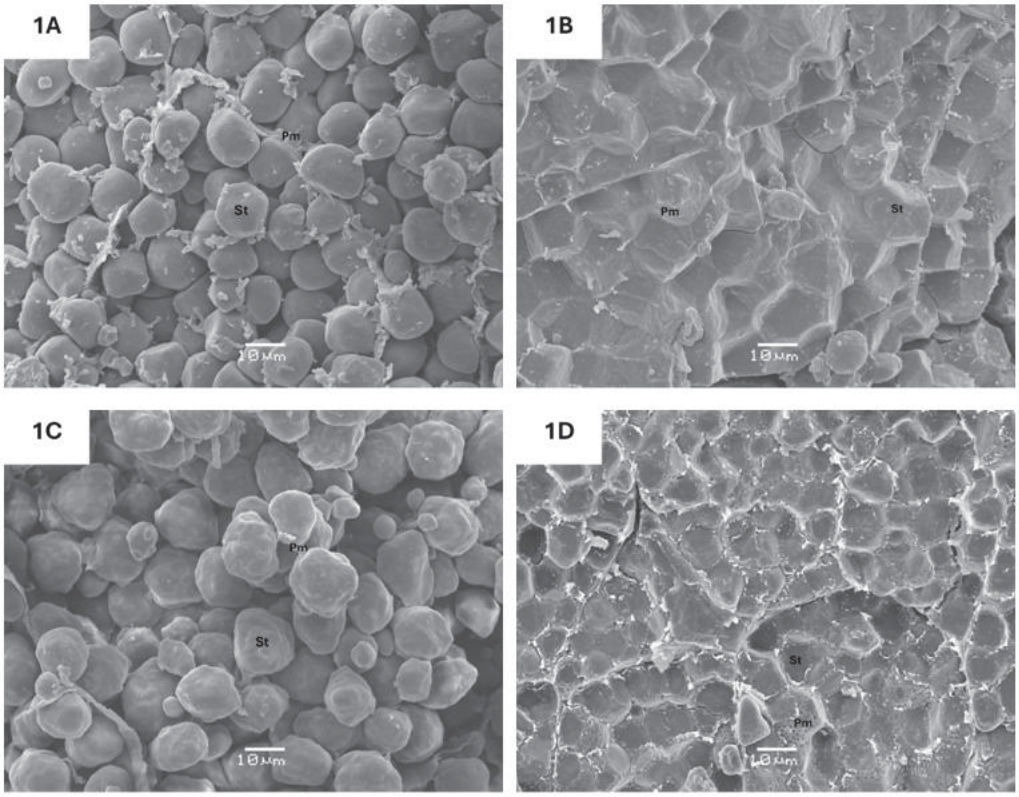

Figure 1. Scanning microscopy images of starch granules (St) and protein matrix (Pm) from floury (Fig. 1A) vitreous (Fig. 1B) endosperms of corn A, and floury (Fig. 1C) vitreous (Fig. 1D) endosperms of corn B in magnification of 1000x . Starch granules in floury endosperm are spherical while in vitreous are polyhedric. Protein matrix in the floury endosperm is thinner than in the vitreous endosperm.

1801

1802 The proximate analysis of both corn samples, including amino acids, is outlined
1803 in Table 3. The corn A showed a more noticeable GE content compared to the hard sample
1804 (3,732 vs. 3,569 Kcal/kg). The corn B showed greater content of CP compared to soft
1805 (78.8 vs. 72.9 g kg^{-1} DM), as well as the concentration of essential amino acids such as
1806 arginine, histidine, methionine, and valine. For amino acids, only the amount of glutamine
1807 was lower in corn B compared with the A.

1808 Table 3. Analyzed nutrient composition of corn from different batches

Item	Corn	
	A	B
Component, g/kg (DM basis, unless otherwise indicated)		
Total ash	9.7	13.0
Ether extract	45.1	42.1
Crude fiber	12.9	15.0
Calcium	0.5	0.4
Phosphorus	1.9	2.6
Crude protein	72.9	78.8
Gross energy, kcal/kg	3,732	3,569
Essential AA[1]		
Arginine	3.8	4.3
Histidine	2.3	2.6
Isoleucine	2.8	2.6
Leucine	9.3	8.4
Lysine	2.9	2.5
Methionine	1.2	1.7
Threonine	3.2	2.8
Valine	3.8	4.0
Nonessential AA		
Alanine	5.9	6.1
Aspartic Acid	5.3	5.8
Cysteine	1.2	1.6
Glutamic Acid	14.0	13.4
Glycine	3.1	3.6
Proline	6.8	7.0
Serine	3.7	3.7

1809

1810 **5.2. Growth performance**

1811 Growth performance results are summarized in Table 4. BWG, FI, and FCR of
1812 broiler chicken were not affected by corn type ($P > 0.05$). Dietary supplementation of
1813 amylase did not alter any growth performance variable. No interaction was detected
1814 between corn type and amylase supplementation for growth performance ($P > 0.05$).

1815 Table 4. Effect of amylase supplementation and different corn batch (A or B) on growth performance of broiler chickens from 14 to 26 d of age.

	Treatments											
	Corn		Amylase, KNU/kg[1]		Corn A		Corn B			*P*-value		
Item	A	B	0	80	0	80	0	80	SEM[2]	Corn	Amylase	Corn x Amylase
BWG[3], g	785	784	774	795	782	788	766	802	9.0	0.941	0.278	0.422
FI[4], g	1133	1147	1126	1154	1131	1136	1122	1173	10.6	0.511	0.197	0.287
FCR[5], g/g	1.444	1.465	1.457	1.453	1.447	1.442	1.467	1.464	0.0063	0.112	0.734	0.951

1816 [1] KNU = kilo-Novo α-amylase units per kg of feed.
1817 [2] Pooled standard error of the mean.
1818 [3] Body weight gain.
1819 [4] Feed intake.
1820 [5] Feed conversion ratio.

5.3. Apparent ileal digestibility nutrient and total tract retention

Supplementation of dietary amylase increased the AID of DM ($P<0.05$) and CP ($P<0.05$) (Table 5). The different corn types only affected the AID of DM ($P<0.05$), which was greater for broiler chickens fed corn A diet. Using amylase improved IDE regardless of corn diet A or B ($P<0.05$). No interactions were observed between corn type and amylase supplementation for AID of nutrients ($P>0.05$) and IDE ($P>0.05$).

Excreta analysis has shown amylase supplementation increased the ATTR of DM ($P<0.05$) and CP ($P<0.05$) compared to non-supplemented diets, regardless of corn type (Table 6). The corn A or B has no effect on ATTR of DM and CP ($P>0.05$) or AME ($P>0.05$), as well as no interaction between corn and amylase inclusion was observed for ATTR and AME results.

Table 5. Effect amylase supplementation and different corn batch (A or B) on apparent nutrient ileal digestibility and ileal digestible energy of broiler chickens.

Item	Treatments								SEM[2]	*P*-value		
	Corn		Amylase KNU/kg[1]		Corn A		Corn B					
	A	B	0	80	0	80	0	80		Corn	Amylase	Corn x Amylase
AIDDM[3], %	70.44	66.6	66.3	70.74	67.66	72.21	64.94	68.25	0.99	0.041	0.019	0.538
AIDCP[4], %	84.11	82.57	82.06	84.61	82.83	83.37	81.29	83.74	0.53	0.118	0.012	0.992
IDE[5], Kcal/kg DM	3,323	3,290	3,211	3,402	3,211	3,436	3,212	3,368	40.2	0.665	0.018	0.655

[1] KNU = kilo-Novo α-amylase units per kg of feed.
[2] Pooled standard error of the mean.
[3] Apparent ileal digestibility of dry matter.
[4] Apparent ileal digestibility of crude protein.
[5] Ileal digestible energy.
Data represents the means of 54 broiler chickens per treatment.

Table 6. Effect amylase supplementation and different corn batch (A or B) on apparent total tract retention and apparent metabolizable energy of broiler chickens.

Item	Treatments								SEM[2]	*P*-Value		
	Corn		Amylase KNU/kg[1]		A		B					
	A	B	0	80	0	80	0	80		Corn	Amylase	Corn x Amylase
ATTRDM[3], %	73.86	73.51	72.14	75.23	71.49	75.53	72.79	74.93	0.64	0.772	0.015	0.436
ATTRCP[4], %	71.36	71.11	69.03	73.44	69.17	73.55	68.89	73.34	0.94	0.894	0.020	0.984
AME[5], Kcal/kg DM	3,584	3,506	3,502	3,589	3,545	3,567	3,559	3,601	27.1	0.145	0.108	0.507

[1] KNU = kilo-Novo α-amylase units per kg of feed.
[2] Pooled standard error of the mean.
[3] Apparent total tract retention of dry matter.
[4] Apparent total tract retention of crude protein.
[5] Apparent metabolizable energy.
Data represents the means of 54 broiler chickens per treatment.

6. Discussion

Starch granules in floury endosperm showed a more disorderly orientation compared to vitreous endosperm, which has a more cohesive starch granule distribution. This could be explained by the protein matrix disposal in each type of endosperm and closely associated with breakage force. As reported by Borrás et al. (2022) protein matrix in vitreous endosperm from hard corn hybrid is denser and more abundant when compared to a dent hybrid.

As already reported by Landry et al. (2004), in floury endosperm the distribution of protein matrix is lighter than in vitreous endosperm, specifically the zein content which represents 48.2% in floury and 78.8% in vitreous. Zein content greatly influences vitreousness, a correlation was observed between the two corn variables investigated by the authors indicating a positive association. As the zein concentration is considered high, the values of vitreousness tend to increase as well (Kljak et al., 2018), and corn kernel tends to present a harder texture and break force when compared to soft variety. As stated by Blandino et al. (2010), the combined effect of chemical and physical characteristics holds a potential impact on corn hardness.

Corn B had noticeably greater CP value and some amino acid concentrations within the group associated with the zein matrix. Zein is the major storage protein in corn kernels and its composition is well recognized and described by several authors (Gianazza et al., 1977; Argos et al., 1982; Momany, et al., 2006; Anderson and Lamsal, 2011; Leroux et al., 2014; Larkins, 2019). Zein protein chains are rich in hydrophobic amino acids such as leucine, proline, glutamine, and alanine (Matsushima et al., 1997; Shukla and Cheryan, 2001) and in the present study, corn B showed alanine and proline concentrations greater than corn A, but lower concentration of lysine content. This characteristic composition in hard corn type has also been reported by Shukla and

1873 Cheryan (2001) and is highly related to endosperm texture and nutrient digestibility
1874 (Gerde et al., 2017; Kljak et al., 2018; Zurak et al., 2020).

1875 The effects on BWG, FI, and FCR in this study were not significant. As reported
1876 by Yegani and Korver (2013) when comparing three different corn sources and enzyme
1877 supplementation, the authors also did not find significant differences in growth
1878 performance variables. Recently, the study conducted by Córdova-Noboa et al., (2021)
1879 also found no significant response for growth performance when broiler chickens were
1880 fed corn with different kernel hardness and enzyme supplementation. This lack of
1881 response on performance may be related to the extent of dietary energy, the experimental
1882 diets in the present study had the same energy density (metabolizable energy; 3,140
1883 kcal/kg). Positive effects of improvement in growth performance due to enzyme
1884 supplementation in diets with different nutrient densities were previously reported
1885 (Cowieson and Ravindran, 2008; Pasquali et al., 2017; Stefanello et al., 2017; Attia et al.,
1886 2022).

1887 ATTR of DM was affected by corn type, broiler chicken fed a corn A diet
1888 presented greater nutrient absorption efficiency, which is possibly related to the
1889 endosperm characteristics and easier enzyme access to starch granules. However, this
1890 variable may be affected by several other elements, the presence of exogenous enzymes,
1891 nutritional factors, sex, genetics, and feed processing techniques (Ziaei et al., 2007;
1892 Teixeira Netto et al., 2019; Yang et al., 2020; Šimić et al., 2023; Stefanello et al., 2023).
1893 Broiler chickens fed A or B corn diet had no enhancement on IDE, in contrast, exogenous
1894 amylase showed greater value of IDE compared to a non-supplemented diet. That
1895 indicates that even if corn presented a similar composition, it could lead to varying levels
1896 of energy utilization (Gehring et al., 2013).

The values of IDE are closely related to the salt-soluble protein content, and this variable suggests the susceptibility of the protein matrix and starch granules to enzyme action (Gehring et al., 2012) and also is positively correlated with the zein content in corn kernels as reported by Malumba et al., (2009). Liu et al., (2020) evaluated nutrient utilization by broiler chickens fed diets formulated using corn from different batches and their study showed an impact of corn batches on IDE, in addition, the authors also suggested that exogenous amylase can reduce the variability in IDE.

The increase in DM and CP utilization at both ileal and total tract retention levels obtained through the supplementation of amylase induces a greater utilization of nutrients. Similar results were obtained by other studies evaluating the effects of dietary amylase (Amerah et al., 2017; Stefanello et al., 2019; Ma et al., 2020; Giacobbo et al., 2021; Schramm et al., 2021). Liu et al. (2020) recently reported that amylase supplementation could be a strategy to modify intestinal physiology, inducing greater nutrient absorption as well as the effect of inhibition of amylase pancreatic synthesis.

7. Conclusions

In conclusion, structural differences were found between corn A and B endosperms, either floury or vitreous. Despite amylase doses, broiler chickens fed diet containing corn A showed greater apparent ileal digestibility of dry matter. However, amylase supplementation improved ileal digestibility, as well as total tract retention measurements, the use of amylase has a positive effect, regardless of the corn type. Using 80 KNU of amylase per kg of feed, promoted greater apparent ileal digestibility and total tract retention of dry matter, crude protein, and energy.

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CHAPTER III - The effect of exogenous protease and soybean meal batches on growth performance and nutrient digestibility for broiler chickens

1. Abstract

The thermal processing of soybean meal (**SBM**) can reduce its protein quality by decreasing solubility. Exogenous protease may help mitigate these negative effects on broiler chicken's protein digestibility. This study investigated how protease affects performance and digestibility in broiler chickens fed SBM from different batches. A total of 360 chickens from 14 to 26 days of age were randomly assigned in a 2 x 2 factorial arrangement with 2 SBM (batch A and B) and 2 protease doses (0 and 30,000 NFP/kg of diet), totaling 4 treatments with 9 replicates and 10 birds each. The SBM samples were analyzed for proximate composition, protein quality indicators, color assessment, and microscopy images. From 19 to 21 days of age, excreta were collected to determine apparent total retention (**ATTR**) of DM, CP, and metabolizable energy (**AME**). On day 26, ileal content was collected to estimate the apparent ileal digestibility (**AID**) of DM, CP, amino acids, and digestible energy (**IDE**). Feed intake, body weight gain, and feed conversion ratio were evaluated. Protein solubility in KOH was different for SBM from batches A and B. Total color difference demonstrated a noticeable change in SBM samples. Microscopy images showed disorganized structures and asymmetric pores in particles from the SBM of batch A. However, insignificant pores and large cluster aggregates were observed in particles from batch B. No differences were found ($P>0.05$) for feed intake, and body weight, whereas feed conversion ratio was better for birds fed SBM from batch A ($P<0.05$). SBM batches influenced ($P<0.05$) AID of DM, CP, lysine, methionine, and IDE regardless of protease supplementation. Protease impacted the AID of CP and amino acids ($P<0.05$) irrespective of the SBM, and no interaction was observed ($P>0.05$), except for the AID of tyrosine ($P<0.05$), which was greater for birds fed SBM of batch A plus protease. In conclusion, SBM from batch A had better protein quality

indicators, suggesting an appropriate thermal process, and had improved nutrient utilization in broiler chickens.

Keywords: Amino acid; Cluster; Protein; Overprocessed; Solubility

2. Implications

Protein is one of the most expensive macronutrient in broiler nutrition. Traditionally, the protein source in feed formulation is soybean meal (SBM), and regarding nutritional quality, the processes applied in the crushing soybean industry may impact the protein quality indicators. This study emphasized that protease meliorated protein and amino acid utilization by birds. Furthermore, this investigation demonstrated differences in SBM particles, and this might impact nutrient digestion and performance. The knowledge of the nutritional value of ingredients commonly used in broiler nutrition is a distinctive strategy in precision nutrition.

3. Introduction

Soybean meal (**SBM**) is considered the major source of protein in broiler chicken diets. The nutritional composition and the protein quality of SBM are variable and affected by a combination of factors, such as the origin of the SBM (García-Rebollar et al., 2016; Grieshop and Fahey, 2001; Ibáñez et al., 2020; Lee et al., 2009) and the thermal process applied in the crushing soybean industry. As described by Van Eys (2012), the appropriate thermal processing of SBM should result in a range of 78 to 85% of KOH protein solubility, urease activity pH unit rise should be within 0.000 to 0.100, trypsin inhibitor concentration should be from 1.75 to 2.50 mg/g, and visually the recommendation is that the SBM should present a color variation between light tan and light brown.

2097 The overheating process causes Maillard reactions, which is correlated with the
2098 decrease of nutrient digestibility due to the formation of protein cross-linking reactions
2099 that occur in advanced stages and the formation of Maillard reaction products, such as the
2100 glycosylated lysine derivates, which reduces lysine bioavalability (Dozier et al., 2011). A
2101 non-optimal thermal process applied in SBM, specifically an over-heated, may negatively
2102 impact protein quality indicators, such as KOH protein, reducing protein and amino acids
2103 digestibility, resulting in negative effects on growth performance and health parameters
2104 of broiler chickens (Hemetsberger et al., 2021; Ravindran et al., 2014; Rocha et al., 2014;
2105 Tousi-mojarrad et al., 2014).
2106 A visual assessment of overheated SBM can be conducted by identifying the
2107 brown pigment produced by the Maillard reaction. This brown hue confirms the presence
2108 of compounds known as melanoidins formed in the third stage of Maillard reactions by
2109 the complexation of reducing sugars and protein or amino acids (Wang et al., 2011). Thus,
2110 the melanoidin in overheated SBM reduces the efficacy of proteolytic enzyme since the
2111 compound shows high resistance against digestive enzymes and blocks the intestinal
2112 absorption sites reducing the digestibility of protein and amino acids (Fu et al., 2020;
2113 Mauron, 1990).
2114 The use of exogenous protease is widely acknowledged by monogastric
2115 nutritionists as an effective strategy for enhancing the digestibility of crude protein and
2116 amino acids, resulting in improved energy efficiency and utilization (Cowieson et al.,
2117 2020, 2019; McCafferty et al., 2022; Min et al., 2019; Munezero and Kim, 2022).
2118 According to Ravindran (2013), feed enzymes can present variability in responses and
2119 are not always predictable due to several factors such as the content and quality of
2120 nutrients which vary among ingredient batches. Hence, the results of recent studies
2121 evaluating a sfericase protease demonstrated positive effects on performance and

digestibility for broiler chickens (Lee et al., 2023; Liu et al., 2024; Stefanello et al., 2024; Vieira et al., 2022) given that acts like a complementary to endogenous digestive protease. Classified as an endopeptidase from the largest family of serine protease, in the subtilisin subfamily A (Rawlings, et al., 2018), this protease has an active site similar to trypsin and chymotrypsin even with the disparity in their molecular arrangements (Mala et al., 1998).

While several studies investigated the effectiveness of protease supplementation, only a few such as Liu et al. (2024), evaluated different SBM batches in broiler chicken diets and explored potential interactions with protease. Thus, our objective was to assess the effect of exogenous protease on growth performance and nutrient digestibility of broiler chickens fed SBM from two different batches focused on protein quality indicators. We hypothesized that protease supplementation would increase the dietary value of SBM with different protein solubility, thereby increasing the digestibility of protein and amino acids.

4. Materials and methods

4.1. Broiler chickens, experimental design, and experimental diets

All experimental procedures were previously approved by the Animal Use Ethics Committee of the Universidade Federal of Paraná (Protocol number 061/2021). A total of 360 male broiler chickens (Cobb500; BRF S.A, Castro, PR, Brazil) from 14 to 26 days of age, vaccinated for Marek's disease at the hatchery, were assigned in 4 treatments with 9 replications of 10 broiler chickens each in a completely randomized block design and allocated in metabolic battery cages.

Broiler chickens were allocated following a 2 x 2 factorial arrangement, containing 2 different SBM batches (A and B) and 2 protease doses (0 and 30,000 NFP/kg of diet). Treatments varied according to SBM batches and protease supplementation. The

2147 experimental diet was based on maize-soybean meal and fed in mash form. Ingredients
2148 used and nutritional composition calculated and analyzed are summarized in Table 1.

2149 Table 1. Ingredients, calculated and analyzed nutritional composition of broiler chicken
2150 experimental diets.

Item, %	SBM Batch A		SBM Batch B	
	0 NFP[1]/kg	30,000 NFP/kg	0 NFP/kg	30,000 NFP/kg
Ingredients				
Maize	64.19	64.19	64.19	64.19
Soybean meal	30.28	30.28	30.28	30.28
Soybean oil	1.84	1.84	1.84	1.84
Dicalcium phosphate	0.67	0.67	0.67	0.67
Limestone	0.91	0.91	0.91	0.91
Sodium chloride	0.25	0.25	0.25	0.25
Sodium bicarbonate	0.18	0.18	0.18	0.18
DL-Methionine 99%	0.25	0.25	0.25	0.25
L-Lysine 99%	0.17	0.17	0.17	0.17
Threonine 98.5%	0.02	0.02	0.02	0.02
Valine 96.5%	0.02	0.02	0.02	0.02
Mycotoxin adsorbent	0.07	0.07	0.07	0.07
Mineral premix[2]	0.05	0.05	0.05	0.05
Vitamin premix[3]	0.10	0.10	0.10	0.10
Phytase[4]	0.005	0.005	0.005	0.005
Protease[5]	0.00	0.005	0.00	0.005
Celite[6]	1.00	1.00	1.00	1.00
Calculated nutrient composition				
Metabolizable energy, kcal/kg	3,140	3,140	3,140	3,140
DM	88.20	88.30	88.10	88.00
CP	19.50	19.50	19.50	19.50
Digestible Lys	1.07	1.07	1.07	1.07
Digestible Met	0.51	0.51	0.51	0.51
Digestible Met+Cys	0.80	0.80	0.80	0.80
Digestible Thr	0.71	0.71	0.71	0.71
Calcium	0.80	0.80	0.80	0.80
Total Phosphorus	0.46	0.46	0.46	0.46
Available Phosphorus	0.40	0.40	0.40	0.40
Sodium	0.19	0.19	0.19	0.19
Analyzed nutrient composition				
Crude protein	18.50	19.20	19.60	19.30
Calcium	0.77	0.74	0.79	0.76
Total phosphorus	0.42	0.49	0.48	0.45
Sodium	0.21	0.23	0.22	0.25

[1] NFP = New feed protease unit.
[2] Composition per kg of feed: Manganese, 65.0 mg; Iron, 50.0 mg; Zinc, 65.0 mg; Copper, 10.0 mg; Iodine, 1 mg; Selenium, 0.39 mg.
[3] Supplementation per kg of feed: Vitamin A, 14.300 UI; Vitamin D3, 5.200 UI; Vitamin E 71.5 UI; Vitamin B1, 2.99 mg; Vitamin B2, 9.10 mg; Vitamin B6, 5.20 mg; Pantothenic Acid, 15.6 mg; Biotin, 0.325 mg; Vitamin K3, 3.9 mg; Folic Acid; 2.60 mg; Nicotinic Acid, 78.0 mg; Vitamin B12, 32.5 mcg.
[4] Ronozyme HiPhorius with 40,000 phytase units (FYT) per g of product (IUB N°3.1.3.26, HiPhoriusTM 40, DSM, Nutritional Products, Switzerland).
[5] Ronozyme ProAct 360 with 600,000 protease units (NFP) per g of product (IUB N°3.4.21.62, Ronozyme ProAct 360; DSM Nutritional Products, Kaiseraugst, Switzerland).
[6] Indigestible marker (Celite®, Celite Corp. Lompoc, CA, US).

In all experimental diets a commercial granulated thermostable phytase was used, the product is encoded by the 6-phytase gene from Aspergillus oryzae containing a minimum activity of 40,000 phytase units per g of product (IUB N°3.1.3.26, HiPhoriusTM 40, DSM, Nutritional Products, Switzerland). One phytase unit is defined as the amount of enzyme that releases 1 μmol of inorganic phosphate from phytate per minute at pH 5.5 and 37°C (Bampidis et al., 2023a). Inclusion of 50 g per t of diet provides 2,000 phytase units per kg of diet.

The protease tested was a novel commercial granulated thermostable enzyme (IUB N°3.4.21.62, Ronozyme ProAct 360; DSM Nutritional Products, Kaiseraugst, Switzerland) produced by a genetically modified strain of Bacillus licheniformis, which contains 600,000 new feed protease (NFP) units per g. One NFP unit is defined as the amount of enzyme that releases approximately1μmol of p-nitroaniline from 1 mM substrate (N-Succinyl-Ala-Pro-Phe p-nitroanilide) per minute at pH 9.0 and 37°C (Bampidis et al., 2023b). Protease inclusion was established as 50 g per t of diet, providing 30,000 NFP units per kg of diet. Protease activity in experimental diet samples was measured at Biopract GmbH, Berlin, Germany.

4.2. Housing and husbandry

Broiler chickens were allocated in metabolic battery cages (0.98 m in length x 0.90 m in width x 0.35 m in height; measure of each cage) equipped with gutter feeders and nipple drinkers. Under each cage, a metal tray was recovered with a plastic sheet for excreta collection. During the experimental period, water and feed were supplied ad libitum. Each cage was considered an experimental unit. A program of incandescent light was established as 14 hours of light and 10 hours of dark. Room temperature was set at 26°C and reduced by 0.5°C per day up to 19.5°C on day 26.

4.3. SBM proximate analysis and protein quality indicators

Samples of each batch of SBM were collected to conduct proximate analysis to evaluate the nutritional components. The content of DM was determined using a drying oven for 72 hours at 105ºC, as well as CP (Method 954.01), total ash content (Method 942.0), ether extract (Method 920.39), crude fiber (Method 962.09), calcium (Method 972.02) and phosphorus content (Method 965.17) determined according to Association of Official Analytical Chemists (AOAC, 2005). Gross energy was measured using an adiabatic calorimetric bomb (IKA Model C2000; Parr Instrument Co., Moline, IL).

To assess the protein quality indicators of both SBM batches, samples were collected to analyze urease activity (Method BA 9-58) according to AOCS (2017a) and trypsin inhibitor activity (Method BA 12-75) as described by AOCS (2017b), KOH protein solubility (Araba and Dale, 1990), and amino acid concentrations were determined using high-performance liquid chromatography (Hewlett-Packard 1100, Waldbronn, Germany) as described by Hagen et al. (1989) and White et al. (1986).

4.4. Color measurement

A representative sample of SBM batches A and B was submitted to a rotary mill (Pulverisette 14, Fritsch GmbH, Germany) equipped with a 0.5 mm sieve, at an operation speed of 20,000 rpm. Milled samples were subsequently homogenized, collected, identified, and stored in plastic bags for further color measurement assessment. A portable Minolta colorimeter (Chroma CR-10, Konica Minolta Sensing, Tokyo, Japan) was used to measure the surface color of SBM batch samples. Color space, also known as the CIELab system was expressed in values of brightness (L^*), and chromatic coordinates (a^* and b^*). Where $+a^*$ represents the red direction, $-a^*$ is the green, $+b^*$ is the yellow, and $-b^*$ is the blue direction.

Total color difference (ΔE^*) was evaluated to estimate the level of human sight perception between two samples, using the following equations: $\Delta E^* = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}}$. The individual changes in color parameters (L^*, a^*, b^*) between SBM batches A and B were calculated as $\Delta L^* = L_1^* - L_2^*$; $\Delta a^* = a_1^* - a_2^*$, and $\Delta b^* = b_1^* - b_2^*$. Where L_1^*, a_1^*, b_1^* represent color values of SBM from batch A (used as a reference for ΔE^* calculation) and L_2^*, a_2^*, b_2^* are the values for SBM batch B samples.

4.5. Scanning electron microscopy images

Scanning electron microscopy images were assessed for batches A and B to determine structural variations in SBM particles, such as aggregates and pores. Samples were attached to standard aluminum stubs and covered with a thin layer of gold-palladium coating (about 15 nm thickness) and then observed using the JEOL JSM-6360 LV scanning electron microscope (JEOL Ltd., Tokyo, Japan) at a potential acceleration of 10 kV, and magnifications of 1000x were used for each SBM batch sample analyzed.

4.6. Apparent total tract retention and apparent ileal digestibility assays

At days 19, 20, and 21 of age, an amount of 30% of the excreta sample was randomly collected twice daily (0830 and 1730) from each cage following the partial method and avoiding feed and feather contamination. All samples were identified, stored in plastic bags, and immediately frozen at -18°C. Excreta samples were thawed at room temperature and placed in a drying oven for 72 hours at 55°C for further analysis.

At day 26, 6 broiler chickens from each replication (a total of 216 broiler chickens) were randomly selected, euthanized by cervical dislocation, and then eviscerated for ileal digesta removal. The ileal fraction was separated and defined as 4 cm below Meckel's diverticulum and 4 cm above the ileum-cecum-colon junction. All ileal digesta content was collected by gently pushing, polled, and placed into plastic containers identified and immediately frozen at -18°C and freeze-dried (Christ Alpha 1-4 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) up to a 7×10^{-2} mbar vacuum pressure.

Diets, excreta, and ileal content were grounded 1 mm to determine gross energy content using a calorimetric bomb (IKA Model C2000; Parr Instrument Co., Moline, IL), DM content measured in a drying oven at 105°C per 12 hours, and CP (Method 954.01) determined according to the Association of Official Analytical Chemists (AOAC, 2005). Calcium (Method 927.02), Sodium (Method 969.23), and total Phosphorus (Method 965.17) were analyzed in feed according to AOAC (2005). Acid-insoluble ash was added at a proportion of 1% in the experimental diet and used as an indigestible marker component in the calculation. The acid-insoluble ash content was determined as described by (Scott and Boldaji, 1997). The experimental diets and ileal content analysis of amino acids composition were performed by high-performance liquid chromatographic (Hewlett-Packard 1100, Waldbronn, Germany) according to the methodologies described by Hagen et al. (1989) and White et al. (1986).

Based on the results obtained, the apparent total tract retention (ATTR) and apparent ileal digestibility (AID) of nutrients were calculated according to the following equation: digestibility (%) = 100 – (100 x (nutrient concentration in excreta or ileal digesta/nutrient concentration in feed) x (indigestible marker concentration in feed/ indigestible marker concentration in excreta or ileal digesta)). Furthermore, apparent metabolizable energy (AME) and ileal digestible energy (IDE) were calculated using the following formula: AME or IDE (kcal/kg DM) = Gross energy in the diet – (Gross energy in excreta or ileal digesta x (indigestible marker concentration in feed/ indigestible marker concentration in excreta or ileal digesta)).

4.7. Growth performance

Growth performance variables were recorded from 14 to 26 days of age. Broiler chickens were weighed by cage to estimate body weight gain, measured by the difference between the end (26 d) and the initial (14 d) of the experimental period. All the feed allowance and refusal were weighed during the whole period to determine feed intake. The feed conversion ratio was calculated as the ratio between feed intake and body weight gain and adjusted by the weight of dead broiler chickens.

4.8. Statistical analysis

The comparison between instrumental color data from SBM batches was made using a Student t-test ($P<0.05$). Data obtained from growth performance and digestibility assays were tested for normality using the Shapiro-Wilk test. When the requirements were attended, a two-way ANOVA at a 5% significance level was conducted, including the two main factors (SBM batches and protease supplementation) and their interactions. When significant, a Tukey test was performed to compare the

means at 5% probability. All the statistical procedures were carried out using the linear model of the ExpDes.pt package (Ferreira et al., 2022) on R software (R Foundation for Statistical Computing, Vienna, Austria).

5. Results

5.1. Dietary enzyme recovery and SBM proximate analysis

The protease recovery was 40,331 and 39,613 new feed protease units per kg for diets containing the SBM from batches A and B, respectively. In non-supplemented diets, no protease was detected. The gross energy values, KOH protein solubility, and urease activity were greater in SBM from batch A when compared to batch B, while the opposite was observed for trypsin inhibitor activity and CP content. Indispensable amino acids showed higher concentrations in SBM from batch B, specifically the amounts of arginine, leucine, phenylalanine, histidine, isoleucine, valine, lysine, and methionine. However, SBM from batch A presented a greater concentration of all dispensable amino acids (Table 2).

2295 Table 2. Proximate analysis and quality protein indicators of soybean meal (SBM) from
2296 different batches tested in experimental diets for broiler chicken

Item (%)	SBM Batch A	SBM Batch B
Crude protein	46.4	46.9
Total ash	5.79	5.85
Ether extract	1.86	1.45
Crude fiber	2.22	2.64
Calcium	0.29	0.20
Phosphorus	0.63	0.64
Gross energy, Kcal/kg	4,059	4,031
Protein quality indicators		
Urease activity, ΔpH	0.09	0.01
KOH protein solubility	85.7	75.5
Trypsin inhibitor activity, mg/g	3.47	3.65
Indispensable amino acids		
Arginine	3.67	3.73
Histidine	1.24	1.26
Isoleucine	2.34	2.45
Leucine	3.81	3.98
Lysine	3.64	3.89
Methionine	0.57	0.58
Threonine	2.33	2.28
Valine	2.37	2.44
Phenylalanine	2.49	2.60
Dispensable amino acids		
Alanine	2.26	2.24
Aspartic Acid	5.78	5.57
Cystine	0.73	0.72
Glutamic Acid	9.10	8.82
Glycine	2.13	2.07
Proline	2.53	2.50
Serine	2.56	2.49

2297

2298 **5.2. Color measurement and scanning electron images**

2299 The CIELab space coordinates and the total difference between both batches of
2300 SBM showed significant differences for L*, a*, and b* values from SBM batches ($P < 0.001$; Table 3). A contrast between SBM from batches A and B demonstrated in L*
2301 values showed a medium-light yellow-brown hue for SBM from batch A (Fig. 1a) and a
2302 more pronounced medium golden-brown hue in SBM batch B (Fig. 1b).
2303

Table 3. Instrumental color of CIE*Lab* space coordinates and total color difference of soybean meal (SBM) batches tested broiler chicken experimental diets.

Item	SBM Batch A	SBM Batch B	SEM[2]	*P*-value
Lightness (*L**)	76.4	68.2	0.667	<0.001
Redness (*a**)	3.69	7.79	0.336	<0.001
Yellowness (*b**)	30.7	32.6	0.138	<0.001
Total color difference (Δ*E**)[3]	9.40 ± 1.05			

[1] CIELab color space was used to determine coordinates *L** [black (0) to white (100)], *a** [green (-) to red direction (+)], *b** [blue (-) to yellow direction (+)].
[2] Pooled standard error of the mean.
[3] Mean and standard deviation of total color difference (Δ*E**) of SBM from batch A compared to SBM from batch B.
Data represents the means of 20 samples of each SBM.

The total color difference between the samples of SBM batches was recognizable and visible by human sight and demonstrated a visually apparent change caused by differences in the thermal process applied in each SBM batch. Images obtained from scanning electron microscopy showed variations in structural organization in SBM particles from batches A and B. The image of the sample from batch A (Fig. 1c) emphasized clear differences, such as disorganized cluster structures and uneven pores, whereas the image from batch B (Fig. 1d) revealed denser structures characterized by large cluster aggregates and insignificant pores.

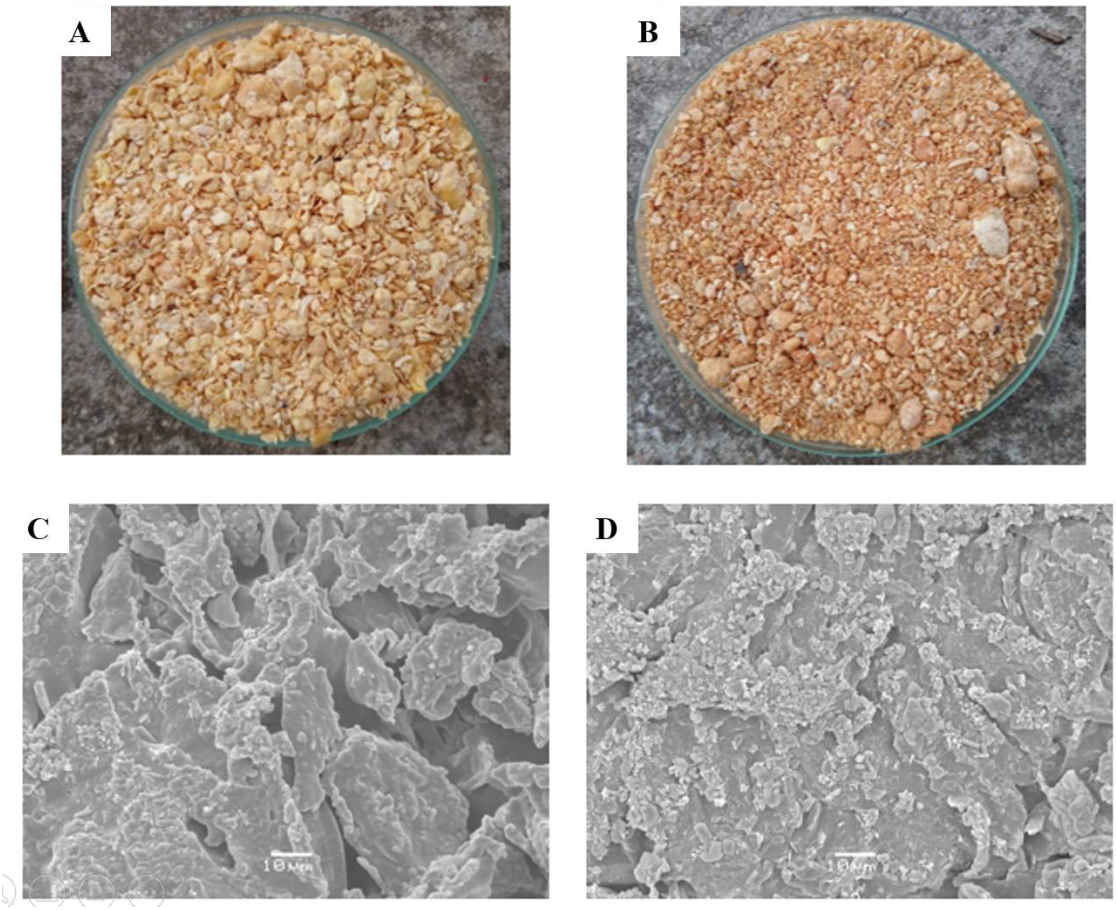

Figure 2. Particles of soybean meal (SBM) from different batches indicating visual color differences and microscopic structural organization. SBM from batch A (A) showed a slightly lighter hue in comparison with batch B (B). Scanning electron microscopy images of SBM samples from batches A and B at 1000 x magnification, highlighted visual variations with small aggregates, disorganized structures, and irregular pores (C), in contrast to the formation of a denser structure, with large aggregates and negligible pores (D). [© Vivian I. Vieira, 2024. All rights reserved.]

2321

2322 **5.3. Total tract retention and ileal nutrient digestibility**

2323 Excreta sample analysis demonstrated that SBM batches and protease
2324 supplementation did not affect the coefficients of ATTR and AME (P>0.05; Table
2325 4). No interaction between SBM batches and protease supplementation was observed
2326 for ATTR and AME (P>0.05; Table 4). Nutrient ileal digestibility coefficients and
2327 IDE are presented in Table 4. SBM batch significantly affects AID of DM, CP, and
2328 energy (P<0.05; Table 4). Protease supplementation did not affect (P>0.05; Table 4)

2329 the AID of DM and energy, although, increased the ileal digestibility of CP (P<0.05)
2330 regardless of SBM batch. No interactions were observed between SBM batches and
2331 protease supplementation on AID of DM, CP, and IDE (P>0.05; Table 4).
2332 The AID of lysine and methionine were higher in SBM batch A (P<0.05;
2333 Table 5), whereas the AID of other amino acids was significantly improved by
2334 protease supplementation (P<0.05; Table 5). An interaction between SBM batches
2335 and protease was observed for the AID of tyrosine (P<0.05; Table 5). Broiler
2336 chickens fed with SBM from batches A and B with protease supplementation
2337 exhibited greater tyrosine utilization compared to non-supplemented animals.

Table 4. Effect of exogenous protease on apparent total tract retention, metabolizable energy, ileal nutrient digestibility, and ileal digestible energy in broiler chickens fed diets with different soybean meal sources.

Item	SBM Batch A		SBM Batch B			*P*-Value		
	0 NFP[1]/kg	30,000 NFP/kg	0 NFP/kg	30,000 NFP/kg	SEM[2]	SBM	Protease	SBM x Protease
Apparent total tract retention								
Dry matter, %	74.2	75.1	72.8	73.4	0.006	0.209	0.547	0.884
Crude protein, %	72.5	72.2	68.9	72.0	0.009	0.308	0.444	0.352
Metabolizable energy, Kcal/Kg	3,590	3,621	3,559	3,526	22.91	0.185	0.986	0.491
Ileal nutrient digestibility								
Dry matter, %	70.6	72.6	69.2	64.9	0.010	0.024	0.110	0.552
Crude protein, %	84.1	86.2	81.3	84.2	0.006	0.031	0.023	0.715
Ileal digestible energy, Kcal/Kg	3,426	3,520	3,258	3,349	38.79	0.028	0.219	0.984

[1] NFP = New feed protease units.

[2] Pooled standard error of the mean.

Data represents the means of 54 broiler chickens per treatment.

Table 5. Effect of exogenous protease on apparent ileal digestibility of amino acids measured in 26-day-old in broiler chickens fed diets fed diets with different soybean meal sources.

Item	SBM Batch A		SBM Batch B			*P*-Value		
	0 NFP[1]/kg	30,000 NFP/kg	0 NFP/kg	30,000 NFP/kg	SEM[2]	SBM	Protease	SBM*Protease
Alanine	84.3	88.0	84.4	87.6	0.005	0.822	<0.001	0.724
Aspartic acid	87.4	90.5	87.3	89.0	0.004	0.267	0.002	0.325
Cysteine	86.1	89.3	86.9	88.6	0.004	0.974	0.005	0.374
Glutamic acid	88.9	91.7	88.6	90.6	0.003	0.223	<0.001	0.556
Glycine	78.2	83.2	78.9	82.5	0.006	0.976	<0.001	0.505
Proline	81.8	86.3	81.4	85.2	0.006	0.492	<0.001	0.762
Serine	81.5	86.4	81.6	85.5	0.006	0.676	<0.001	0.639
Tyrosine	84.7[b]	89.5[a]	85.8[b]	87.0[ab]	0.005	0.397	0.001	0.034
Arginine	89.5	92.0	89.2	91.4	0.003	0.424	<0.001	0.815
Histidine	83.4	87.2	84.0	85.0	0.005	0.363	0.008	0.105
Isoleucine	83.3	87.5	84.2	86.6	0.009	0.973	<0.001	0.294
Leucine	83.5	87.8	83.5	86.8	0.005	0.565	<0.001	0.601
Lysine	90.6	92.8	90.1	91.2	0.003	0.025	0.001	0.216
Methionine	95.7	96.9	94.7	96.0	0.002	0.005	<0.001	0.809
Phenilalanine	85.0	89.2	85.6	88.3	0.005	0.861	<0.001	0.383
Treonine	76.9	82.3	77.0	80.9	0.007	0.589	0.001	0.582
Valine	81.7	86.6	82.9	85.5	0.005	0.952	<0.001	0.221

[1] NFP = New feed protease units.
[2] Pooled standard error of the mean.
[a, b] Means within a row with superscript, differ significantly (P <0.05).
Data represents the means of 54 broiler chickens per treatment.

5.4. Growth performance

The growth performance variables from 14 to 26 days of age presented that broiler chickens fed with SBM from batch A had a lower feed conversion ratio ($P<0.05$; Table 6) regardless of protease supplementation. Exogenous protease did not affect body weight gain, feed intake, or feed conversion ratio ($P>0.05$; Table 6). No interaction between SBM batches and protease was observed in broiler chicken growth performance in this study ($P>0.05$; Table 6).

Table 6. Effect of exogenous protease on growth performance in broiler chickens from 14 to 26 days of age fed diets fed diets with different soybean meal sources.

Item	SBM Batch A		SBM Batch B			*P*-Value		
	0 NFP[1]/kg	30,000 NFP/kg	0 NFP/kg	30,000 NFP/kg	SEM[2]	SBM	Protease	SBM x Protease
Body weight gain, g	809	828	766	804	10.0	0.125	0.191	0.637
Feed intake, g	1155	1175	1122	1167	11.6	0.371	0.177	0.598
Feed conversion ratio, g/g	1.429	1.421	1.467	1.451	0.008	0.034	0.451	0.783

[1] NFP = New feed protease units.
[2] Pooled standard error of the mean.
Data represents the means of 90 broiler chickens per treatment.

6. Discussion

The data showed that SBM from batch A had greater KOH solubility in comparison to SBM from batch B (85.71% vs. 75.53%), as well as greater urease activity (0.09 vs. 0.01, respectively). Although the difference in trypsin inhibitor activity was not so perceptible (3.47 vs. 3.45 mg/g, respectively), the other two indicators demonstrated a discrepancy in thermal processing between SBM samples. In addition, the values of KOH solubility and trypsin inhibitor activity are in agreement with Erdaw et al. (2016), the authors reported that KOH solubility and trypsin inhibitor should be a maximum of 87% and 3.45 mg/g, respectively.

Several factors influence the protein quality indicators of SBM, but the heat applied during processing in crushing soybean industry has a noticeable impact. When adequately processed, trypsin inhibitors are inactivated and other parameters involving protein quality, texture, color, and nutrient digestibility are improved in SBM (Krička et al., 2003; Vagadia et al., 2017). As reported by Liu and Ruiz (2021) two of the protein quality indicators, urease activity and trypsin inhibitor activity decreased rapidly between 20 and 35 minutes at 163°C, indicating a strong positive correlation between them for the first 40 minutes of thermal processing of soybean.

Indeed, the SBM from batches A and B used in experimental diets presented different nutritional characteristics, such as the amino acid composition that barely varied between SBM batches, otherwise, KOH solubility values were more pronounced. The same behavior was demonstrated by Liu et al. (2024) and others similar results focused on SBM produced and processed in different origins (Aguirre et al., 2022; García-Rebollar et al., 2016; Ibáñez et al., 2020). According to the authors, the country of origin influences the chemical composition, while the impact of thermal processing, particularly on protein quality parameters, should not be overlooked as a key factor. Although the

main target for crushing soybean plants is protein and fiber content (Demarco et al., 2020), protein quality indicators assessment is important to ensure adequate protein digestibility to broiler chickens, especially considering that SBM is the main or the unique protein source for poultry.

The changes observed in color parameters evidenced that SBM batches were submitted to different thermal processes. The values evidenced by the CIELab color space corroborate the finding of Hemetsberger et al., (2021), the authors demonstrated that SBM submitted to higher temperatures during processing showed lower values of L* and higher values of a* indicating a darker hue. According to Dozier et al. (2011), the apparent differences in color are caused by overprocessing, resulting in a browning hue, which is formed in the third stage of the Maillard reaction due to the presence of low-molecular-weight color products (Yu et al., 2023).

In the case of SBM overprocessing, the presence of these compounds is confirmed through the color space assessment because the rising temperature causes a drastic decrease in L*, a noticeable reduction in the b*, and a change in the a* values, as mentioned by Yu et al. (2023). According to Mukherjee et al. (2019), this browning hue is a result of the decreasing L* value, which is a response to the recrystallization of sugars and the existence of darker compounds.

It has been shown by Zhang et al. (2022) that the heat treatment applied in SBM can induce protein denaturation, which may be beneficial due to the dissociation of protein structure (Li et al., 2020; Scott et al., 1997). Moreover, once the heat treatment applied in SBM is not appropriate, this protein denaturation can be related to the reduced availability of amino acids, due to the complexation with free sugars, which reduces the protein solubility. As mentioned by Cowieson and Ravindran, (2008), solubility plays a

key role in digestibility, it means that, once more nutrients are available, better will be digestibility and utilization by the animal.

In the current study, the particles of SBM presented different structural aggregations, and this may be associated with the intensity of the thermal processing and the behavior of protein chains. Sulphide bonds, hydrophobic interactions, and electrostatic interactions are related to thermal aggregate formation. When a thermal process is applied, protein fractions of soybean (β-conglycinin and glycinin) can form solid structures characterized by denser aggregation and negligible pores, making it difficult the access digestive enzymes (He et al., 2016; Ju et al., 2023; Li et al., 2020; Zhang et al., 2022).

Although growth performance variables were not affected by SBM batches and protease supplementation in this study, digestibility results differed between treatments. The AID of DM, CP, IDE, and AID of the lysine and methionine were influenced by SBM batches. Broiler chickens fed with SBM from batch A, which presented greater protein quality indicators, showed better nutrient utilization indexes regardless of protease supplementation. These results are consistent with other findings which reported noticeable improvements in nutrient digestibility when broiler chickens were fed diets containing SBM of better protein quality indicators (Aguirre et al., 2022; Saleh et al., 2020; Silva et al., 2022; Sung et al., 2023; Yi et al., 2024).

Several studies investigating the use of exogenous protease reported improvements in CP and amino acid digestibility for broiler chickens as a complement of endogenous protease (Angel et al., 2011; Fru-nji et al., 2011; Lee et al., 2023; Silva et al., 2022). Even though no improvement in IDE was observed by the protease in this study, the enhancement in CP and amino acid ileal digestibility might be due to protein solubility levels, which were within or close to the range 78 to 85% (Van Eys, 2012). In addition,

the amino acid concentrations in both SBM were similar, as noticed by Silva et al. (2022) when investigating SBM from different origins.

7. Conclusions

In conclusion, broiler chickens fed diets formulated with SBM from batch A presented better feed conversion ratio and ileal digestibility of DM, CP, and energy. The proximate analysis performed in SBM samples showed that batch A presented superior protein solubility in KOH and better nutritional composition parameters, such as gross energy, compared to SBM from batch B. SBM sample characterization indicated that particles from batch A were visually and physically different from batch B. Besides color parameters indicating changes in thermal processing applied, microscopy images of SBM particles from batch A showed a structure less compacted and with numerous pores, which may be easier to protease access during digestive processes. The use of exogenous protease in a dosage of 30,000 NFP per kg of diet improved CP and amino acid digestibility, regardless of the SBM batch used.

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CHAPTER IV - Implications

While Brazil produces large amounts of grains, the methodologies that the industry employs in the processing of these ingredients might impact nutrient utilization by broiler chickens, because the nutritional parameters follow a pattern but are not consistent due to several technical factors during processing. Considering that, there is potential for further investigations of how the use of exogenous enzymes could interact with these ingredients and improve digestibility.

The use of amylase and protease is well documented in the literature as a strategy to improve digestibility and performance when broiler chickens are fed corn and soybean meal-based diets (Gracia et al., 2003; Schramm et al., 2021; Lee et al., 2023). In contrast, studies investigating these exogenous enzymes in diets containing feedstuffs ingredients with different nutritional values and the impact on digestibility and performance are not so vast (Stefanello et al., 2023; Zavelinski et al., 2024).

In Chapter II, we investigated the nutritional characteristics of corn from two different batches used in a diet formulated for broiler chickens and supplemented with amylase. Although the proximal composition was very similar between them, the hardness test, and the microscopy images detected differences that did not impact growth performance. However, the corn type influenced the digestibility of dry matter which might be related to the starch content present in each corn batch. In this case, it is expected that the soft corn used presents a high amount of rapidly digestible starch and a low amount of resistant starch, resulting in a greater starch apparent ileal digestibility.

The animal nutrition industry could consider that all batches of corn could have the same or at least very similar proximate composition. However, specific parameters such as starch fraction composition and zein content can impact nutrient utilization. It is erroneous to assume that all the corn batches are identical. Several factors, including those

related to starch and protein composition, as well as the drying and storage processing techniques applied could affect nutrient digestibility. This highlights the importance of further investigations into the nutritional value of ingredients.

This same perspective was explored in Chapter III, the study emphasized that protease improved protein and amino acid digestibility. However, it demonstrated differences in protein quality indicators of SBM from different batches, as well as structural variations in SBM particles through scanning electron microscopy images. As expected, the SBM with high protein solubility in KOH presented greater apparent ileal digestibility of dry matter, protein lysine, and methionine, as well as ileal digestible energy. It means, the SBM submitted to a less aggressive thermal process, which might preserve better the nutrients in comparison to the SBM submitted to a more intense temperature and processing time.

There are a variety of papers that investigated different batches of SBM (Thakur and Hurburgh, 2007; Lopez et al., 2020; Liu et al., 2024). Most of them are focused on nutritional variations according to the origin of production, but not specifically regarding protein quality indicators. This allows different interpretations of nutritional quality. These studies are approached in a broad and generalized manner, making it seem that every SBM produced in a determined country has the same process and nutritional composition.

This major area of study about feedstuff ingredient quality has a noticeable impact not only on broiler chickens but also on animal nutrition in general. If we know each ingredient used for feed formulation, we have more chances to successfully reach good production results. In the case of Brazilian broiler chicken production, this advantage of being one of the biggest corn and soybean producers worldwide, allows the country to produce high-quality animal protein using excellent feedstuff ingredients.

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ANNEX I – Protocol approved by the Animal Ethics Committee of the Federal University of Paraná (CEUA-SCA/UFPR) number 061/2021.

UNIVERSIDADE FEDERAL DO PARANÁ
SETOR DE CIÊNCIAS AGRÁRIAS
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o protocolo número 061/2021, referente ao projeto de pesquisa "**Uso de amilase e protease sobre a digestibilidade de amido, energia e aminoácidos em dietas para frangos de corte formuladas a base de milhos e farelos de soja com diferentes características nutricionais**", sob a responsabilidade de **Alex Maiorka** – 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 (CEUA) DO SETOR DE CIÊNCIAS AGRÁRIAS DA UNIVERSIDADE FEDERAL DO PARANÁ - BRASIL, com grau 2 de invasividade, em 07/12/2021.

Finalidade	Pesquisa
Vigência da autorização	Dezembro/2021 até Dezembro/2022
Espécie/Linhagem	*Gallus gallus domesticus* (ave)
Número de animais	1224
Peso/Idade	1 dia/45g
Sexo	Macho
Origem	Incubatório comercial em Carambeí, Paraná, Brasil

*A autorização para início da pesquisa se torna válida a partir da data de emissão deste certificado.

CERTIFICATE

We certify that the protocol number 061/2021, regarding the research project "**Use of amylase and protease on the digestibility of starch, energy and amino acids in broiler diets based on corn and soybean meal with different nutritional characteristics**" under **Alex Maiorka** – which includes the production, maintenance and/or utilization of animals from Chordata phylum, Vertebrata subphylum (except Humans), for scientific or teaching purposes – is in accordance with the precepts of Law nº 11.794, of 8 October 2008, of Decree nº 6.899, of 15 July 2009, and with the edited rules from Conselho Nacional de Controle da Experimentação Animal (CONCEA), and it was approved by the ANIMAL USE ETHICS COMMITTEE OF THE AGRICULTURAL SCIENCES CAMPUS OF THE UNIVERSIDADE FEDERAL DO PARANÁ (Federal University of Paraná, Brazil), with degree 2 of invasiveness, on 2021, December 7th.

Purpose	Research
Validity	December/2021 to December/2022
Specie/Line	*Gallus gallus domesticus*
Number of animals	1224
Weight/Age	1day old/ 45g
Sex	Male
Origin	Commercial hatchery in Carambeí, Paraná, Brazil

*The authorization to start the research becomes valid from the date of issue of this certificate.

Curitiba, 07 de dezembro de 2021

Maity Zopollatto

Vice-Coordenadora
Comissão de Ética no Uso de Animais
AG - UFPR

Comissão de Ética no Uso de Animais do Setor de Ciências Agrárias - UFPR