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UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
CAMPUS DE BOTUCATU

USO DE LIGNINA PURIFICADA NA DIETA DE POEDEIRAS COMERCIAIS CRIADAS EM DIFERENTES SISTEMAS E AMBIENTE TERMO ESTRESSANTE

ANA BEATRIZ SANTOS DE OLIVEIRA

Tese apresentada ao Programa de Pós-graduação em Zootecnia como parte das exigências para obtenção do título de Doutora em Zootecnia.

BOTUCATU – SP
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BIOGRAFIA DO AUTOR

ANA BEATRIZ SANTOS DE OLIVEIRA, filha de Maria de Lurdes Santos e Paulo Roberto de Oliveira, nasceu em Votuporanga, São Paulo, no dia 08 de janeiro de 1993. Em fevereiro de 2011, iniciou o curso de Zootecnia pela Universidade de São Paulo (USP). Bolsista da CNPq de fevereiro a julho de 2012 com o projeto de iniciação científica voltada para a área de piscicultura, de agosto a novembro do mesmo ano realizou estágio no setor de Avicultura da FZEA. Bolsista do RUSP de iniciação científica de agosto de 2012 a julho de 2013 na área de piscicultura, no mesmo período participou do grupo VZoo (Voluntários da Zootecnia - FZEA). Bolsista de iniciação científica da FAPESP de agosto de 2013 a julho de 2014 e de agosto a dezembro de 2014 bolsista de iniciação científica pela CNPq ambos na aréa de piscicultura. Realizou o estágio obrigatório em Mississippi State University (MSU) – USA no setor de Avicultura de março de 2015 a fevereiro de 2016. Mestre em Ciência pela ESALQ/USP na área de concentração de nutrição de aves. Em março de 2020 iniciou o curso de Doutorado em Zootecnia da Unesp – Faculdade de Medicina Veterinária e Zootecnia – Campus de Botucatu. No primeiro semestre de 2020 foi Pesquisadora Assistente na Mississippi State University (MSU) – USA no setor de Avicultura. Durante a Pós-graduação, atuou na área de nutrição e produção de aves. Hoje trabalha na Ingredion Corporation como Assessora Técnica Senior, atuando no time de Nutrição Animal com multiespécie.

DEDICATÓRIA

Á minha mãe, Maria de Lurdes, por ser tudo na minha vida.

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EPÍGRAFE

*"**Viver é partir, voltar e (re)partir***
Morte é quando a tragédia vira um costume
Partir, voltar e (re)partir
Pra diferença da qual ninguém 'tá imune
Viver é partir, voltar e (re)partir
Mas ouça de alguém que nasceu num tapume
Partir, voltar e (re)partir
É só na escuridão que se percebe os vagalumes".
(Emicida)

RESUMO GERAL

Três experimentos foram conduzidos para avaliar os efeitos dos níveis de lignina purificada (LP) em galinhas poedeiras. O primeiro experimento (**Exp. 1**) teve como foco o desempenho, saúde intestinal, qualidade e tempo de prateleira dos ovos de galinhas criadas em sistema convencional. No segundo experimento (**Exp. 2**), foram analisadas a produção, qualidade de ovos, morfologia e saúde intestinal de poedeiras em sistema *cage-free*. E no terceiro experimento (**Exp. 3**), o objetivo foi avaliar a produtividade, qualidade de ovos e morfologia intestinal de poedeiras em sistema *cage-free* sob elevadas temperaturas. Foram utilizadas 240 galinhas da linhagem Hy-Line W-36 com idade entre 31 e 43 semanas no **Exp. 1** e 300 galinhas, com idades entre 51 e 63 semanas no **Exp. 2** e 63 a 66 semanas no **Exp. 3**. Os animais foram submetidos a cinco tratamentos no **Exp. 1**, com seis repetições contendo oito aves por gaiola. No **Exp. 2 e 3**, foram utilizados dois tratamentos, com três repetições e 50 aves por baia. No **Exp. 1** os tratamentos consistiram em cinco dietas: uma dieta controle (DC) e quatro dietas testes suplementadas com LP nos níveis de 0,25%, 0,5%, 0,75% e 1%. Nos **Exp. 2 e 3** foram utilizados dois tratamentos sendo uma DC, e adotou-se o nível de destaque do **Exp. 1**, que foi o nível de 1% de suplementação de LP. **Exp.1:** Os tratamentos apresentaram $P<0,05$ em vários parâmetros ao longo dos ciclos experimentais. Destacam-se as diferenças na produção, peso, massa dos ovos e consumo de ração em diferentes ciclos. A concentração de malondialdeído (MDA) variou significativamente, indicando a influência positiva da adição de LP na redução da oxidação lipídica na gema do ovo. Além disso, a qualidade dos ovos, medida pela espessura da casca e gravidade específica, também foi afetada pelos tratamentos. **Exp. 2:** Nos resultados relacionados aos parâmetros produtivos e de desempenho observou-se que os tratamentos exibiram $P<0,05$ ao longo dos ciclos experimentais, impactando a produção, massa e peso dos ovos, bem como a proporção de ovos postos dentro e fora dos ninhos. Em relação aos parâmetros de qualidade dos ovos, os tratamentos também apresentaram $P<0,05$ ao longo dos ciclos experimentais, particularmente quanto à gravidade específica e espessura da casca. Notavelmente, a concentração de MDA demonstrou $P<0,05$ em todas as semanas experimentais. Nas análises relacionadas à morfologia e saúde intestinal, observaram-se $P<0,05$ no peso do baço e do jejuno, além do tamanho dos vilos presentes no jejuno. Entretanto, não se constatou efeito significativo nos níveis de ácidos graxos de cadeia curta (AGCC) em virtude da suplementação de LP. **Exp. 3:** Nos parâmetros produtivos e de desempenho, os tratamentos mostraram $P<0,05$. A suplementação de LP teve impactos negativos na conversão alimentar e na proporção de ovos postos dentro e fora dos ninhos. Quanto aos parâmetros de qualidade dos ovos, houve $P<0,05$, a suplementação de LP aumentou a porcentagem de casca, mas reduziu a

porcentagem de gema. A concentração de MDA apresentou P<0,05 durante um estresse agudo de calor. No entanto, não foi observado efeito (P>0,05) quando a exposição a altas temperaturas foi prolongada. Nas análises referentes à morfologia e saúde intestinal, notaram-se P<0,05 no comprimento dos vilos do duodeno. Entretanto, não se constatou efeito significativo nos níveis dos AGCC e nas análises dos órgãos internos devido a suplementação de LP. Conclui-se que, em um sistema criação convencional, a dosagem de 1% de LP foi segura e não afetou o desempenho das poedeiras. No entanto, no sistema *cage-free*, a inclusão de 1% de LP na dieta de galinhas resultou em desempenho inferior quando comparado ao DC. Contudo, sob condições de elevadas temperaturas ambientais, a suplementação de 1% de LP na dieta de galinhas em sistema *cage-free*, não afetou o desempenho das poedeiras. Porém, em ambos os sistemas de criação e condições ambientais foi observada melhora na qualidade dos ovos, devido ao seu efeito antioxidante, sem impacto na morfologia e histologia intestinal das galinhas.

Palavras-chave: antioxidante, sistemas de criação, qualidade de ovo, tempo de prateleira, ácidos graxos de cadeia curta

ABSTRACT

Three experiments were carried out to evaluate the effects of purified lignin (PL) levels in laying hens. The first experiment (**Exp. 1**) focused on the performance, intestinal morphology, quality, and shelf life of eggs from hens raised in a conventional system. In the second experiment (**Exp. 2**), production, egg quality, intestinal morphology, and histology of laying hens in a *cage-free* system were analyzed. The third experiment (**Exp. 3**), the objective was to evaluate productivity, egg quality and intestinal morphology and histology of layers in a *cage-free* system under high temperatures. 240 Hy-Line W-36 hens aged between 31 and 43 weeks were used in **Exp. 1** and 300 hens aged between 51 and 63 weeks in **Exp. 2** and 63 to 66 weeks in **Exp. 3**. The hens were subjected to five treatments in **Exp. 1**, with six replications containing eight birds per cage. In **Exp. 2 and 3**, two treatments were used, with three replications and 50 birds per pen. In **Exp. 1**, treatments consisted of five diets: a control diet (CD) and four test diets supplemented with PL at levels of 0.25%, 0.5%, 0.75% and 1%. In **Exp. 2 and 3**, two treatments were used, one DC, and the highlighted level of Exp. 1 was adopted, which was the 1% level of PL supplementation. **Exp.1:** The treatments showed $P<0.05$ in various parameters throughout the experimental cycles. Differences in production, weight, egg mass and feed intake in different cycles are highlighted. The concentration of malondialdehyde (MDA) varied significantly, indicating the positive influence of PL addition on reducing lipid oxidation in egg yolk. Furthermore, egg quality, measured by shell thickness and specific gravity, was also affected by treatments. **Exp. 2:** In the results related to production and performance parameters, it was observed that the treatments exhibited $P<0.05$ throughout the experimental cycles, impacting the production, mass, and weight of eggs, as well as the proportion of eggs laid inside and outside the nests. In relation to egg quality parameters, treatments also showed $P<0.05$ throughout the experimental cycles, particularly regarding specific gravity and shell thickness. Notably, MDA concentration demonstrated $P<0.05$ in all experimental weeks. In analyzes related to intestinal morphology and histology, $P<0.05$ were observed in the weight of the spleen and jejunum, in addition to the size of the villi present in the jejunum. However, there was no effect on the levels of short-chain fatty acids (SCFA) due to PL supplementation. **Exp. 3:** In productive and performance parameters, the treatments showed differences ($P<0.05$). PL supplementation had negative impacts on feed conversion ration and the proportion of eggs laid inside and outside the nests. Regarding egg quality parameters, there were variations ($P<0.05$). PL supplementation increased the percentage of shell but reduced the percentage of yolk. MDA concentration showed differences ($P<0.05$) during acute heat stress. Though no effect was observed ($P>0.05$) when exposure to high temperatures was prolonged. In the analyzes

regarding morphology and histology intestinal, differences ($P<0.05$) were noted in the length of the Duodenum villi. However, no effect was found on SCFA levels and internal organ analyzes due to PL supplementation. In conclusion, in a conventional breeding system, the dosage of 1% PL was safe and did not affect the performance of the layers. Though, in the *cage-free* system, the inclusion of 1% PL in the hens diet resulted in lower performance when compared to DC. Nonetheless, under conditions of high environmental temperatures, supplementation of 1% PL in the diet of hens in a *cage-free* system did not affect the performance of the layers. Conversely, in both breeding systems and environmental conditions, an improvement in egg quality was observed, due to its antioxidant effect, without impact on the intestinal morphology and histology intestinal of the hens.

Keywords: antioxidant, breeding systems, egg quality, shelf life, short chain fatty acids

LISTA DE TABELAS

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

BW	Body Weight
BWG	Body Weight Gain
cm	Centimeters
EM	Egg mass
EP	Egg Production
EW	Egg weight
FCR	Feed Conversion Ratio
FI	Feed Intake
FMVZ	Faculdade de Medicina Veterinária e Zootecnia
g	Grams
IML	Interactive Matrix Language
kfg	Kilogram Force
kg	Kilos
m	Meters
MDA	Malondialdehyde
ml	Milliliters
mm	Millimeters
Na_2S	Sodium Sulfide
NaOH	Sodium Hydroxide
ng	Nanogram
PL	Purified Lignin
ROS	Reactive Oxygen Species
SCFA	Short Chain Fatty Acids
SP	São Paulo
t	Ton
UNESP	Universidade Estadual Paulista
µm	Microgram

SUMÁRIO

CAPÍTULO 1

INTRODUCTION

Poultry farming is an agricultural subsector that presents itself as one of the most developed activities in the world, with an active contribution to food and nutritional security, providing energy, protein, and essential micronutrients for the human population. Poultry meat and eggs are among the highest consumed animal protein sources globally (EL-HACK et al., 2020). Brazil has had significant climb in the *per capita* consumption of eggs in the last 20 years, as well as its production. A total of 52 billion eggs were produced in Brazil in 2022 (ABPA 2023).

Eggs are considered one of the richest foods nutrition wise for human consumption, as it is a source of essential proteins, vitamins, minerals, and fatty acids for people's daily diet (RÊGO et al., 2012). However, several factors influence the quality such nutritional treasure, making it impossible to take advantage of its full potential. Among these, we might list bird physiology, breeding system, number of hens per cage, frequency of collection, age of birds, nutrition, management conditions, health status, temperature and humidity, genetics, and birds' management (SACCOMANI, 2015).

The supplementation of poultry diets with natural products containing bioactive components has shown promising results in terms of improving the birds' physiology and consequently their productivity. Studies have shown that purified lignin has effects on gut microflora, animal performance and their ability to inhibit the growth of pathogenic enteric bacteria (RICKE et al., 1982; NELSON et al., 1994; GUARRERA, 1999; BAURHOO et al., 2007a; BAURHOO et al., 2008).

As a polyphenolic compound, purified lignin exhibits antioxidant properties by neutralizing reactive oxygen species and protecting cells against oxidative damage (FERNANDES et al., 2015; MARCHIORI et al., 2019; LIU et al., 2020). In addition, purified lignin also contains anti-inflammatory properties, reducing the inflammatory

response in a few conditions. These combined effects make purified lignin an optimistic option as a dietary additive, contributing to birds' health and welfare, as well as to optimizing productive performance (CATIGNANI; CARTER, 1982; BAURHOO et al., 2007a; OHISHI et al., 2016).

Heat stress causes severe physiological dysfunctions that can result in a decline in laying hen productivity (GOUS; MORRIS, 2005). High temperatures can be detrimental to laying hens, as it contributes to increased mortality, reduced number and quality of eggs and high formation of free radicals in the body and reproductive system (GOUS; MORRIS, 2005). The use of polyphenols may help laying hens by reducing the harmful effects produced during heat stress.

LITERATURE REVIEW

1. Egg Market

The egg market is a constantly growing sector driven by several factors, such as changing consumer preferences, demand for healthier food options and awareness of animal welfare practices (SANTOS, 2021a). Demand for eggs as a food has increased due to people's awareness of their health benefits, not to mention their culinary versatility and affordability compared to other protein sources (GODINHO JR et al., 2018; SANTOS et al., 2019; NASCIMENTO SILVA, 2020).

Additionally, the demand for eggs produced in sustainable farming systems and with animal welfare has increased significantly (CANDIDO, 2022). Consumers are increasingly concerned about the origin of their food and look for options that respect the environment and allow the animals to express natural behaviors (MAZZUCO, 2008; CARVALHO, 2019; REIS, 2022). As consumers become more aware of the origin of their food and seek more natural options, alternative farming systems, such as *cage-free*

and *free-range*, are gaining ground in the market (COUNCIL, 2009; DE OLIVEIRA et al., 2019).

Cage-free and *free-range* system are more ethical and sustainable options, which is aligned with consumer preferences that value responsible food production (PARKER AND SCRINIS, 2014; DE OLIVEIRA et al., 2019). These systems promote hens' freedom, allowing them to have contact with their natural environment, exercise and express their natural behaviors, which contributes to their welfare (REIS, 2022).

Eggs from these systems are marketed as superior quality products, which can result in higher demand and price in relation to eggs from conventional systems. Few consumers are willing to pay the extra dollar for eggs produced in such premium systems (BRIDI et al., 2020; DE OLIVEIRA et al., 2019). Therefore, the search for solutions that contribute to improving egg production and, at the same time, reduce costs are being widely explored.

It is important to emphasize that the transition to alternative breeding systems may require investments and adjustments in properties' infrastructure, in addition to demanding more complex management (ARNO, 2022). Producers might have to consider aspects such as biosecurity, proper management, balanced and preventive feeding, to guarantee the production of safe and high-quality eggs (DE SÁ et al., 2023; ARNO, 2022).

The egg market is on the rise, driven by the demand for nutritious, versatile, and accessible foods (DE SOUZA CUNHA et al., 2019). Awareness of nutritional benefits and sustainable rearing systems drive the adoption of alternative rearing systems, impacting the supply, quality, and commercialization of eggs in the market (BRIDI et al, 2020; MAHMOUD et al., 2022). Producers' response to these demands reflects the

constantly evolving dynamics of the egg production sector (ROCHA et al., 2008; DE SÁ et al., 2023).

2. Laying hens systems

2.1. Conventional system

The cage system came from the need to house each bird individually to allow individual recording of egg production and the disposal of unproductive hens. Afterwards, several birds were housed in a cage, this being the most common way of housing laying hens (REIS, 2022).

Keeping laying hens in cages offers significant benefits in terms of production control, management, and bird health. This results in economic advantages such as reduced labor required, less waste and feed costs. In addition, the use of cages allows the automation of egg feeding and collection, which facilitates handling (GUIMARÃES E DE ALMEIDA, 2021). The conventional system lowers the risks of dirty eggs, which in turn reduces the spreading of microbes and food that is more reliable (MAHMOUD et al., 2022; REIS, 2022).

For the conventional system, a minimum space of 310cm^2 per hen must be provided for three or more birds per cage when birds weigh less than 2.4kg (PAVAN et al., 2005). The feeder must be five cm wide per bird, while drinkers can be of the trough type (2.5cm/bird) or nipples (one for every eight birds). Cages must have floors with adequate inclination to move the eggs to the sideboard. It is essential to use caution when adjusting cage inclination, as an excessive angle can result in damage to the eggs due to breakage, while an insufficient inclination can lead to the accumulation of eggs at the bottom of the cages, increasing the amount of dirty eggs (PAVAN et al, 2005).

However, the conventional cage system has been questioned due to the restriction of movements and expression of birds' behaviors, due to the lack of space and compromised ambience, which would harm the welfare of the laying hens (GROOT and VIZÚ, 2021).

2.2. *Cage-free* system

Global egg consumers demonstrate growing concern about animal welfare, reflected in the preference for eggs from production systems that adopt *cage-free* farming practices for laying hens. They believe that birds need to be loose, scratch, take sand baths, flap their wings, lay eggs in nests, and walk freely to express their natural behavior (PORTELA et al., 2019).

The *cage-free* system is monitored and must comply with animal welfare guidelines and the requirements of certifiers, as well as other systems for raising *free-range* chickens. *Cage-free* creations need, to contain perches, nests, lines for drinkers and feeders inside (DE MOURA et al., 2022).

Compared to the conventional system, the *cage-free* system requires a change in birds' management and, therefore, more labor. In Brazil, there is no specific welfare legislation for the breeding of laying hens in a *cage-free* system (PORTELA et al., 2019).

Brazil has protocols for agricultural practices that meet international standards; among them are the Protocol of Egg Production Practices, prepared by the Brazilian Association of Animal Protein (UBA, 2008) and Technical Circular nº 49 – Production Practices in the Commercial Position (EMPRESA BRASILEIRA DE PESQUISA AGROPECUÁRIA - EMBRAPA, 2006). Certified Humane Animal Care (2018) established the guidelines to produce eggs in a *cage-free* system, to obtain certification for the property.

The *cage-free* system is the most trending alternative, as it is easier to implement, due to the possible adaptation of the broiler breeding, adding nests for egg production (PORTELA et al, 2019). However, there are some research reports on negative impacts on production characteristics, including laying percentage, reduced consumption, cannibalism, mortality, and egg quality, causing financial losses. losses (Elson and Croxall, 2006; Englmaierova et al., 2014; Jones et al., 2015; Karcher et al., 2015; Dong et al., 2017).

In order to mitigate possible challenges in poultry production, it is advisable to adapt the composition of the diet through the inclusion of zootechnical additives, such as probiotics, prebiotics, acidifiers, polyphenols, organic acids, among others. This approach aims to promote preventive health, resulting in the preservation of production efficiency and egg quality.

3. Obtaining Purified Lignin

Lignin is a complex and abundant component found in the cell wall of woody plants (NEUTELINGS, 2011; BES et al., 2019; DORTE, 2019). In recent years, obtaining and purifying lignin has attracted significant interest due to its potential as a valuable renewable resource to produce a wide range of chemical, material, and food products (HUANG et al., 2019; FERNANDES, 2021).

Lignin purification is an essential process to obtain high quality lignin from lignocellulosic feedstocks. There are several methods for purifying lignin, the most common being the Kraft method, the Soda method and the Organosolve method (TORRES et al., 2020; KNAPP, 2020). Each one of these involves specific steps to separate lignin from other biomass components.

The lignin purification process by the Kraft method is widely used in the pulp and paper industry (MARTINS, 2023). In this method, lignin is extracted during the process of cooking wood in an alkaline solution of sodium sulfide and sodium hydroxide. During cooking, lignin dissolves and is separated from cellulose and hemicellulose fibers. After that, the lignin is recovered from the alkaline solution through acid precipitation. This process results in obtaining Kraft lignin, which has high purity, but may also contain some unwanted components, such as residues of chemical products used in the process (MAHMOOD et al., 2018; TORRES et al., 2020; KNAPP, 2020; MARTINS, 2023).

The Soda method, also known as the mild alkaline purification method, is another process used to purify lignin (SANTOS, 2021). In this method, lignin is extracted from biomass using an alkaline sodium hydroxide solution. The dissolved lignin is then recovered by acidification, without the need for harsh chemicals. This process results in a lignin with a lower degree of chemical modification compared to Kraft lignin, maintaining some natural properties of the original lignin (MAHMOOD et al., 2018; TORRES et al., 2020; SANTOS, 2021b).

The Organosolve method is a purification process that uses organic solvents in combination with an acid separating agent (SALVE, 2020). In this method, the biomass is treated with an organic solvent, such as ethanol or acetone, in a pressurized atmosphere and elevated temperature. The solvent dissolves lignin and other soluble components, while cellulose and hemicellulose remain insoluble. After that, the lignin is separated from the solvent through distillation or precipitation, resulting in a purified lignin (MAHMOOD et al., 2018; SALVE, 2020).

Each lignin purification method has specific advantages and disadvantages, depending on the desired application and the desired properties of the lignin. The choice

of the most appropriate purification method depends on characteristics of the raw material, the lignin purity requirements, and the intended applications (MAHMOOD et al., 2018; HUANG et al., 2019; SALVE, 2020). There are continuous advances in research for new lignin purification methods, to develop better efficiency and quality of purified lignin, making it a promising alternative for a wide range of industrial applications (NEUTELINGS, 2011; MARTINS, 2023).

Purified lignin has been studied in the animal feed industry as an additive with the potential to improve the quality of animal diets. Its inclusion in feed can bring significant benefits as a feed additive, especially due to its antioxidant properties and the capacity to improve the intestinal health and animal welfare (BAURHOO et al., 2007a; MAHMOOD et al., 2018). Research in this area aims to explore and better understand the potential of purified lignin as a promising option for improving animal health and performance through its use in feed formulation (BEZERRA, 2019; RÖHE et al., 2020; SUN et al., 2022).

4. Polyphenols in the diet of laying hens

Polyphenols are bioactive compounds found in a variety of plant foods such as fruits, vegetables, grains, herbs, and spices (SCICUTELLA et al., 2021; LIMA, 2021). These compounds have been the subject of increasing interest due to their potential health benefits and their antioxidant and anti-inflammatory properties. In the diet of laying hens, the inclusion of polyphenols can have positive effects in several areas, from the health of the birds to the quality of the eggs produced (LIPORI, 2019; ZHOU et al., 2021; ABD EL-HACK et al., 2023).

Studies have shown that the supplementation of the diet of laying hens with polyphenols can improve hens' performance. This includes an increase in laying rate,

higher egg weight, increase percentage of production and improved egg quality (WANG et al., 2017; OMER et al., 2019; FAN et al., 2021; LIMA, 2021). Polyphenols have antioxidant properties, which means they can help reduce oxidative stress in bird cells, protecting them against damage caused by free radicals (MARCHIORI et al., 2019; LIU et al., 2020; WANG et al., 2020). Furthermore, these compounds may also have anti-inflammatory effects, aiding the overall health of birds (OHISHI et al., 2016; LIPORI, 2019).

The inclusion of polyphenols in the diet of laying hens can also influence the quality of eggs produced. Studies have shown that polyphenol supplementation can improve yolk color, making it more vibrant and attractive to consumers (LIPORI, 2019; ZHU et al., 2020; FAN et al., 2021). In addition, polyphenols can help strengthen eggshells, reducing the incidence of broken shells and improving structural strength. This is important to ensure eggs' integrity and commercialization (EL-MOTAAL et al., 2008; GOLIOMYTIS et al., 2018; FERNANDES, 2022).

Another benefit of including polyphenols in the diet of laying hens is the transfer of these compounds to the eggs (LIPORI, 2019). The polyphenols present in the food consumed by birds are absorbed and metabolized by their organism, being deposited in the tissues and, consequently, the eggs (WILLIAMSON and CLIFFORD, 2017). Which means that those who consume these eggs can also benefit from the bioactive compounds, gaining the potential positive health effects associated with polyphenols (FERNANDES, 2022; VLAICU and PANAITE, 2022).

4.1. Purified lignin polyphenols

Purified lignin is composed of a variety of polyphenols, which are derived from the polymerization of phenolic units. The main polyphenols found in purified lignin include

(FENGEL and WEGENER, 1983; CONSTANT et al., 2016; BEZERRA, 2019; HUANG et al., 2019):

- Syringyl: It is one of the most common polyphenols in lignin. It has a structure based on syringyl units, which are methoxylated phenolic units.
- Guaiacyl: It is also an important component in purified lignin. Its structure is based on guaiacyl units, which are hydroxylated phenolic units.
- P-Hydroxyphenyl: It is a polyphenol derived from p-hydroxyphenyl units, which are hydroxylated phenolic units.

These polyphenols are present in the complex three-dimensional structure of lignin, which is formed by linking phenolic units through carbon-carbon and carbon-ether bonds (BEZERRA, 2019). The proportion and exact composition of polyphenols may vary depending on the source of lignin and the purification method used (LIPORI, 2019).

It is important to highlight that purified lignin can be obtained from different sources of biomass, such as wood, agricultural residues, and other lignocellulosic materials. Each biomass source may have a slightly different polyphenol composition, which will influence the properties and applications of the resulting purified lignin.

5. Egg quality

Egg quality is a crucial issue for both poultry producers and consumers. Egg quality is influenced by several factors, including bird genetics, age, nutrition, handling conditions, bird health and production environment (DE OLIVEIRA et al., 2019). Egg quality assessment covers different characteristics, such as size, weight, shell color, white and yolk quality, nutrient content, and freshness (RODRIGUES et al., 2019).

Nutrition plays a key role in egg quality, as a balanced and adequate diet is essential to provide the necessary nutrients for the proper development of eggs (GARCIA and GOMES, 2019). The most important nutrients in the diet of laying hens include proteins, amino acids, lipids, vitamins, and minerals (RODRIGUES et al, 2019). Deficiencies or excesses of nutrients can negatively affect egg quality (GARCIA and GOMES, 2019).

The quality of the eggshell is a fudamental aspect. Composed mainly of calcium carbonate and proteins, and it plays a crucial role as a natural protection for the internal contents of the egg (SAMPAIO et al., 2022). In addition, the shell has a physical protection function, contributing to the quality and safety of food.

Furthermore, egg freshness is an essential factor for food quality and safety. Freshness depends on several factors, such as the rearing system, age and nutrition of the hens and storage of the eggs (OLIVEIRA et al., 2020; MORAES, 2021). Adequate refrigeration is necessary to prolong eggs' shelf life, thus reducing the proliferation of microorganisms and quality degradation (DE OLIVEIRA et al., 2019; SAMPAIO et al., 2022).

Egg shelf life is a critical variable in the poultry industry, with a direct impact on egg quality and food safety (DIAS, 2020). Knowledge about what affects the shelf life of eggs is essential to ensure the sale of safe and high-quality products to consumers (SILVA, 2022). Several factors can affect eggs' shelf life, consequently influencing their quality and freshness over time.

Eggs initial quality is an essential factor. High quality eggs, with intact shells and without defects, tend to have a longer shelf life than low quality ones, which may have cracks in the shell, facilitating the entry of microorganisms and accelerating deterioration (DE MEDEIROS et al. al., 2017).

The age of the laying hen also plays an important role. Eggs from older hens tend to be of lower quality, with more fragile shells and more liquid whites, which can reduce shelf life compared to eggs from younger hens (CAMARGO, 2019). The proper handling of eggs, from collection to storage is utmost importance. Eggs must be handled with care, avoiding falls and impact that could damage the shell. In addition, hygiene during the collection process is essential to prevent contamination by dirt or waste (DE OLIVEIRA et al., 2019).

Microbiological control is essential to ensure egg quality and freshness. The presence of microorganisms can lead to deterioration of eggs, and storage at a higher ambient temperature accelerates chemical reactions and the multiplication of microorganisms, hence reducing eggs' shelf life (SILVA, 2022). Control measures must be implemented through the type of system, handling, nutrition, and storage, to prevent external and internal bacterial contamination (BRITO, 2022). Controlling these factors throughout the production process is critical to maximizing shelf life and ensuring the delivery of fresh, high-quality products to consumers.

6. Heat stress in laying hens production

Heat stress is a significant challenge in poultry production, negatively affecting its welfare, health, and performance. During prolonged exposure to high temperatures, chickens face difficulties in regulating their body temperature, which leads to a series of physiological and behavioral changes (CARVALHO, 2019; MARINHAGO, 2020).

During heat stress period, birds can experience a series of negative physiological responses, such as increased body temperature, changes in energy metabolism, immune dysfunction and increased oxidative stress (MAHMOUD et al., 1996; MASHALY et al., 2004). Oxidative stress occurs when there is an imbalance between the production of

reactive oxygen species (ROS) and the body's antioxidant capacity, leading to cell damage (LARA and ROSAGNO, 2013; VANDANA et al., 2021).

The effects of heat stress on production and reproduction in chickens are significant. The reduction in feed intake result in less weight gain, reduced production, and compromised egg quality, consequential in economic losses (CARVALHO, 2019; PEREIRA, 2022). In addition, there is interference in the development and functioning of the reproductive organs, affecting the function of the ovaries and egg production (IRÚN and TECH, 2021; PEREIRA, 2022).

Hens exposed to heat stress exhibit adaptive behavioral responses such as seeking out cooler areas, reduced physical activity, reduced social interaction, and changes in feeding patterns and water consumption (APPLEBY et al., 2004; BHADAURIA et al., 2014).

Another important aspect to be considered is the effect of heat stress on the intestinal microbiota of chickens. Elevated temperature can lead to changes in the composition and balance of the microbiota, which compromises intestinal health and nutrient absorption (LARA and ROSAGNO, 2013; CARVALHO, 2019; VANDANA et al., 2021). This can lead to an increased susceptibility to infection and an overall reduction in hens health.

Nutrition plays a crucial role in mitigating heat stress. The formulation of balanced diets, with adequate levels of nutrients and supplementation of antioxidants, can help reduce the damage caused by heat stress (LOPES, 2019) Antioxidants such as vitamin C, vitamin E and polyphenols have protective properties that help birds to deal with oxidative stress resulting from heat stress (MELLO, 2020; SOUZA, 2020).

The use of dietary polyphenol supplementation may help reduce heat-induced oxidative stress. They act as antioxidants, neutralizing ROS and protecting cells against oxidative damage. In addition, polyphenols have anti-inflammatory properties, which can

help lower the inflammatory response caused by heat stress (HALLIWELL, 2008; PROCHÁZKOVÁ et al., 2011; SURAI, 2014; LIPIŃSKI et al., 2017; HU et al., 2019).

Several studies have demonstrated the beneficial effects of dietary polyphenols during heat stress in poultry. For example, the supplementation of plant extracts rich in polyphenols in diets for broiler chickens exposed to heat stress has shown improvements in productive performance, reduction of oxidative stress, modulation of immune response and improvement of intestinal integrity (LIU, HE et al., 2014; OKE et al., 2017; LUO et al., 2018).

In egg production, the inclusion of polyphenols in the diet has also shown positive effects during heat stress. Studies have reported improvements in egg production, eggshell quality, egg lipid profile and yolk oxidative stability (DOSOKY et al., 2021). In addition, polyphenols can also improve the birds' immune response, strengthening their ability to deal with infectious challenges during heat stress (NAWAB et al., 2019).

While the results are encouraging, it is important to note that the effectiveness and effects of polyphenols can vary depending on source, concentration, combination with other dietary ingredients, and duration of supplementation (HU et al., 2019). Therefore, more research is needed to determine the best strategies for using polyphenols in poultry diets during heat stress, considering the specific needs of each species and production phase. This highlights the importance of adding polyphenols to the diet of laying hens.

Thus, the present study aimed to evaluate the effects of including purified lignin in the diet of commercial laying hens in different systems and heat-stressful environments by evaluating productive performance (production, weight and mass of egg, feed intake and feed conversion ratio); internal and external quality of the eggs (lipid peroxidation of the yolk, egg specific gravity, shell thickness, shell strength and percentage of albumen,

yolk and shell); morphology of the digestive tract (weight and length); histology of the small intestine and concentration of short-chain fatty acids in the cecal content.

In Chapter 2 contains the study that aimed to evaluate the effects of purified lignin as a feed additive in the diet of laying hens from 31 to 43 weeks, to measure the productive performance, gut morphology, egg quality, and shelf life, entitled: "Effect of supplementation of laying hens from 31 to 43 weeks of age with a diet containing purified lignin as an additive on laying performance, egg quality and shelf life", which was written by in accordance with the standards of the **Journal of Applied Poultry Research (JAPR)**.

In Chapter 3 contains the study that objective was to verify the production, quality, and composition of eggs from hens supplemented or not with purified lignin in the *cage-free* system, entitled: "Effect of purified lignin on performance, egg quality, intestinal morphology, and histology of laying hens on *cage-free* system", which was written by in accordance with the standards of the **Journal of Applied Poultry Research (JAPR)**.

In Chapter 4 contains the study that the premise was evaluate the effects of purified lignin and its antioxidant potential in the diet of laying hens in a *cage-free* system, under thermal stress, through productive, physiological and egg quality parameters, entitled: "Effect of purified lignin on performance, egg quality, gut morphology, and histology of laying hens at *cage-free* system under heat stress", which was written by in accordance with the standards of the **Journal of Applied Poultry Research (JAPR)**.

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CAPÍTULO 2

"Effect of supplementation of laying hens from 31 to 43 weeks of age with a diet containing purified lignin as an additive on laying performance, egg quality and shelf life."

Effect of supplementation of laying hens from 31 to 43 weeks of age with a diet containing purified lignin as an additive on laying performance, egg quality and shelf life

A.B.S. Oliveira[1] *

[1]School of Veterinary Medicine and Animal Science, São Paulo State University, Botucatu, SP, 18610-034, Brazil

Key words: phenolic, antioxidant, egg quality, shelf life, egg production

Primary audience: nutritionists, researchers, and feed formulators

* Corresponding author: ana.bs.oliveira@unesp.br

SUMMARY

The objetive was evaluate the effects of purified lignin (PL) on the performance, intestinal morphology, quality, and shelf life of eggs from hens raised in a conventional system. 240 Hy-Line W-36 hens aged between 31 and 43 weeks were used to compose five treatments, with six replications containing eight birds per cage. The five diets consisted of a control diet and four test diets supplemented with PL at levels of 0.25%, 0.5%, 0.75% and 1%. The egg production of laying hens was measured per day, feed intake, egg weight, egg mass and feed conversion ratio were recorded at the end of 21 days. One egg produced on the last three days of each cycle, total of tree eggs per replicate, were analyzed to evaluate interior and exterior egg quality parameters. The malondialdehyde (MDA) content was measured in the egg yolk. At the end of the experimental period the two hens per replicate were sacrificed to determine the weight of the internal organ and short-chain fatty acids (SCFA) through the collect of cecal contents. On the last day of this study, five eggs per replicate were stored at the interval of 0, 10, 20 and 30 days to determine shelf life. The treatments showed differences ($P<0.05$) in various parameters throughout the experimental cycles. Differences in production, weight, egg mass and feed intake in different cycles are highlighted. The MDA varied significantly, indicating the positive influence of PL addition on reducing lipid oxidation in egg yolk. Furthermore, egg quality, measured by shell thickness and specific gravity, was also affected by treatments. In conclusion, the dose of 10.0g of PL/kg was considered safe and equivalent to the control treatment, not affecting the performance, and had better feed conversion ratio of laying hens in the period from 31 to 43 weeks. This inclusion also proved to be beneficial in preventing the deterioration of lipids present in the eggs, resulting in greater oxidative stability and, consequently, contributing to better egg quality and shelf life.

DESCRIPTION OF PROBLEM

Egg production is a vital industry around the world, with the demand for eggs continuing to grow. However, the composition of the feed provided to laying hens has a major impact on productivity and egg quality. In current poultry production, finding new alternatives for feed supplementation that improve animal health and the quantitative and qualitative parameters of production has become one of the highest priorities (Gerzilov et al., 2015).

Consequently, the global demand for poultry products has led to the need to discover alternative additives that act in the biological modulation of the microflora. In this context, there is a growing interest in the use of alternatives with an antimicrobial and antioxidant profile.

In the literature, the potential of purified lignin is demonstrated due to its antioxidant properties, as it is a complex phenolic polymer (Dizhbite et al., 2004). Lignin is essential for the structure of plant cell walls, making it the second most abundant natural compound on the planet, after cellulose (Boudet and Grima-Pettenati, 1996). However, in the cellulose pulping and paper production industry, lignin can be recovered as a by-product during the sulfitation of wood, thus being presented in its pure form (Baurhoo et al., 2007).

One of the lignin obtaining processes is by the Kraft method, which uses sodium hydroxide and sodium sulfide to extract lignin from the cellulose in the wood fibers. Due to the process, lignin in its purified form has chemical structures that differ from native lignin. The lignin obtained from the Kraft process is rich in guaiacyl and syringyl subunits, these compounds along with p-hydroxyphenyl are the three main subunits of

the phenolic fraction, known for their sensory, antioxidant properties (Constant et al., 2016; Fengel and Wegener, 1983).

Polyphenolic fragments have a wide pharmacological activity, as they are characterized by their low toxicity. Due to the presence of hydroxyl groups, they can neutralize free radicals in the body and stabilize materials, effectively preventing chain reactions of these radicals and slowing or inhibiting metabolic processes (Xin-yue et al., 2021; Bernatoniene and Kopustinskiene, 2018). The synergy between polyphenols and other active substances has aroused increasing interest in research into their antioxidant activity.

Antioxidants are involved in protecting animal health, supporting the immune system, and increasing animal productivity (Alagawany et al., 2015). The hydroxyl and methoxy functional groups present in the lignin structure have antioxidant activity with the ability to eliminate or stabilize reactions induced by free radicals in cells under oxidative stress (Ayyachamy et al., 2013; Espinoza-Acosta et al., 2016; Liao et al., 2020; Solihat et al., 2021). In addition, the use of compounds with antioxidant characteristics improves egg quality through oxidative stability and consequently longer shelf life (Kara et al., 2016).

However, there is few knowledge about the use of purified lignin (PL) on the performance and quality of laying hens eggs. Given the above, the premise of this study was to evaluate the effects of purified lignin as a feed additive in the diet of laying hens from 31 to 43 weeks, to measure the productive performance, gut morphology, egg quality, and shelf life.

MATERIAL AND METHODS

The experimental procedures were approved by the Institutional Animal Care and Use Committee, of the School of Veterinary Medicine and Animal Sciences of the São Paulo State University (FMVZ/UNESP), Botucatu, SP, Brazil (protocol number: 0155/2020).

1. Animals, Diets, and Housing

The study was conducted at the facilities of the FMVZ/UNESP (geographical coordination: latitude 22° 49' 07" S and longitude 48° 24' 40" W). In total, 240 commercial Hy-Line W-36 laying hens at 31 weeks old with uniform body weight were used in the experiment. The experimental period was subdivided into 4 cycles of 21 days, totaling 84 experimental days. The hens were reared in a Californian aviary (conventional system), with metallic cages measuring 1.00m long x 0.45m deep x 0.40m high, divided into two compartments and four hens in each totaling eight hens per cage. The cages are individually equipped with metal feeders and nipple drinkers. Feed and water were provided *ad libitum* during the experiment.

The laying hens were submitted to five experimental diets, with six replications of eight hens each. The control group was fed a corn-soybean meal-basal diet (Table 1) in mash form without purified lignin. The remaining four groups were given the basal diet supplemented with purified lignin at 2.5 g/kg, 5 g/kg, 7.5 g/kg, and 10 g/kg. The nutrition program consisted of one isonutritive diet formulated to meet the nutrients requirements for layer hens adapted according to the recommendations of the breeder (Hy-Line W-36, 2011) and Rostagno et al. (2017).

The experiment was carried out between 31 to 43 weeks of age (12 weeks experimental period). The lighting regimen was 16 hours of continuous light per day from 06:00 to

22:00. The laying hens were kept in optimal and standard bio-climatic and welfare conditions.

Table 1 – Composition of basal diet.

Ingredient	Amount (g/kg)
Corn, grain	635.36
Soybean meal (45% CP)	243.81
Limestone	95.3
Di - Calcium phosphate	12.73
DL - Methionine	4.55
Sodium chloride	4.25
Vitamins-minerals premix[1]	4.00
Total	1000
Calculated chemical composition	
Dry Matter (%)	89.6
Crude Protein (%)	16.4
Methionine (%)	0.70
Lysine (%)	0.82
Ether Extract (%)	2.4
Calcium (%)	4.0
Phosphorus Total (%)	0.52
Metabolizable energy (MJ/kg)	11.4

[1] Provided per kilogram of diet: vitamin A, 8800 IU; vitamin D3, 2500 IU; vitamin E, 11 IU; vitamin K3, 2.2 IU; thiamin, 1.5 mg; riboflavin, 4 mg; nicotinic acid, 7.85 mg; pantothenic acid, 34.65 mg; pyridoxine, 2.46 mg; folic acid, 0.48 mg; vitamin B12, 4 mg; manganese 74.4 mg; zinc, 64.67 mg; iron, 75 mg; copper, 6 mg; iodine, 0.86 mg; selenium, 0.2 mg; choline chloride, 200 mg.

The purified lignin used in this study was extracted from *Eucalyptus urograndis* wood, being a by-product of the Kraft process of cellulose production (reaction of wood with sodium hydroxide (NaOH) + sodium sulfide (Na_2S)), in the form of a brown powder. The product was composed of lignin (> 95%), total phenolic compounds (4.6%): syringyl-OH (2.3%), guaiacyl-OH (condensed) (1.11%), guaiacyl-OH (non-condensed) (1%) and hydroxyphenyl-OH (0.14%); pH 3 - 5, molar mass 1300 to 1400, moisture (1.92%), ash (2.42%), 61.1% total carbon, 31.3% oxygen, 5.5% hydrogen, 1.7% sulfur and 0.4% nitrogen and 0.99% carbohydrates. Purified lignin was added on top and to ensure that the diets remained isonutritive, sand was supplemented, as an ingredient with no nutritional value, to ensure uniform dilution of all diets used in the study.

2. Performance and Egg Quality

All hens were weighed by replicate group (eight hens) at 31st and 43rd week of age. The egg production (%) was obtained by recording the number of eggs produced daily throughout the experimental period. The feed intake and feed conversion ratio were recorded in the end of each cycle (21 days). The feed conversion ratio (FCR) was expressed in grams of feed consumed per grams of eggs produced. All eggs collected in the last day of each period were weighed to the nearest 0.01g. The average egg weight was multiplied by the total number of eggs produced during the experimental period, thus obtaining the total egg mass. This value was then divided by the total number of hens per replicate for each 21-day period.

One egg produced on the last three days of each cycle, total of tree eggs per replicate, were analyzed to evaluate interior and exterior egg quality parameters. Specific gravity was measured by immersing eggs produced in the last three day of each period in saline solutions of 1.050 to 1.100g/ml (Moreng and Avens, 1990), at 0.005 intervals. The eggs were classified according to their specific gravity when they floated (Albino et al., 2014). Eggshells were washed and dried at 60°C for 48 hours, after each one was weighed. Shell thickness was measured to the nearest 0.01mm in tree points at the middle-transversal area of the shell, from which an average measure expressed in millimeters (mm) using a digital caliper. Shell strength, evaluated with the support of a texturometer TA. XT plus (Texture Analyzer TA-XT Plus, Surrey, England), equipped with a 75mm probe (p/2) and test speed of $1mm/s^{1}$, which was expressed in kfg/cm^{2}. Percentage of yolk, albumen, and shell were calculated by the ratio between the mass of the respective constituents and the mass of the egg.

The malondialdehyde (MDA) content was measured in the egg yolk, this analysis occurred through the colorimetric method using a spectrophotometer (722N, Shanghai Precision and Scientific Instrument Co. Ltd. Shanghai, P.R. China). For this parameter, one egg per replicate was collected at the end of each cycle and analyzed using the assay kits purchased from Nanjing Jiancheng Institute of Bioengineering (Nanjing, Jiangsu, P.R. China) and the procedures accordingly. All samples were measured in triplicate, at appropriate dilutions, to give activities of the enzymes in the linear range of standard curves constructed with pure enzymes. The values are expressed in ng per g.

On the last day of this study, five eggs per replicate (total of 120 eggs) were stored to determine shelf life. Six eggs per treatment were collected at storage interval of 0, 10, 20 and 30 days, for evaluation of egg specific gravity, strength and thickness of the shell and percentage of yolk, albumen, and shell.

3. Internal Organs

At the end of the experimental period (43 weeks of age), 12 hens from each treatment were randomly selected (two hens per replicate), the birds were weighed, anesthetized, and euthanized by decapitation. The weight of the proventriculus, gizzard, pancreas, liver, and spleen were recorded, and the values were expressed in relation to the body weight of each hen (percentage of live body weight). The duodenum, jejunum and ileum were removed to determine the relative weight and length, the latter expressed in relation to the total size of the intestine.

4. Short Chain Fatty Acids (SCFA) from the Cecum

The cecum was also removed from the hens to collect of cecal contents from all the euthanized hens (total of 12 hens per treatment). The cecal contents were collected into plastic centrifuge tubes separately per birds and stored at -20°C until the analysis of short

chain fatty acids. Approximately 0.1g of the cecal pool per treatment was subsequently squeezed out into an falcon 10ml, pipetted 1.6ml of the cecal contents sample; 0.4ml of a 3:1 solution of 25% metaphosphoric acid (Vetec Química Fina Ltda., Rio de Janeiro-RJ) with 98-100% formic acid (Merck KGaA, Darmstadt, Germany) plus 0.2ml of a solution of 100mm 2-ethyl-butyric acid (internal standard; MW = 116.16; CAS 88-09-5; Sigma Chemie Gmbh, Steinheim, Germany) and mixed thoroughly to obtain a uniform pooled sample (Maia et al., 2012). The concentrations of short chain fatty acids (acetic, propionic, butyric, isobutryic, valeric and isovaleric acids) were determined by gas-liquid chromatography as described by Ferreira et al. (2015). Analysis was performed with an HP-FFAP capillary column chromatograph (19091F-112; 25m, 0.320mm, 0.50μm, J&W Agilent Technologies Inc., Palo Alto, CA, USA). Each sample peak profile was integrated and quantified against an internal placed in the same sample.

5. Statistical Analysis

Effects of treatment on productive performance, intestinal morphology and egg quality were analyzed using a completely randomized design submitted to ANOVA by PROC GLIMMIX (SAS, 2019). Orthogonal polynomial contrasts were used to evaluate linear and quadratic effects of increasing inclusion level of PL in diets on each measurement. The IML procedure (SAS, 2019) was used to generate orthogonal coefficients for unequally spaced contrasts. Using the Levene and Shapiro-Wilk test, the data were evaluated for homogeneity of variance ($P > 0.05$) and normal distribution ($P < 0.05$). Statistical significance was considered at the level of 5%.

RESULTS

1. Performance

The initial and final body weight (BW), feed intake (FI), feed conversion ratio (FCR) and egg production (EP), weight (EW), and mass (EM) data are presented in Table 2. From the 31st to the 34th week, first cycle, EW and EM showed a quadratic (P=0.006 and P=0.006) response to the treatments. The hens fed diets supplemented with PL produced egg with lower mass (P=0.047) and had lower feed intake (P=0.024) compared to the hens submitted to the control diet.

Between 34 and 37 weeks of age, second cycle, EW and EM showed a quadratic (P=0.002 and P=0.002) response to the treatments. Laying hens supplemented with PL had lower feed intake (P=0.026), egg weight (P=0.002) and mass (P=0.008) than control. Egg weight of laying hens consuming control and 10g of PL/kg diets were similar but had higher (P=0.002) value compared to 5.0g of PL/kg and laying hens consuming diet 2.5g of PL/kg had lower (P=0.026) egg mass compared to the laying hens fed the control diet.

In the 3rd cycle, 37 to 40 weeks, EW, and EM showed a quadratic (P=0.007 and P=0.039) response to the diets, whereas EP and FI were linear (P=0.041 and P=0.015). The supplementation of purified lignin decreased egg production (P=0.066) and feed intake (P=0.009) of laying hens compared to birds submitted to the control diet. Egg weight was higher (P=0.039) to 10g of PL/kg when compared to 5.0g of PL/kg. Egg mass was higher (P=0.015) to control diet and 10g of PL/kg birds than 7.5g of PL/kg hens.

The last cycle, 40 to 43 weeks, there were no effects of dietary purified lignin on EP, EM, FI, final BW and BWG. There was a quadratic (P=0.001) effect of PL on egg weight. In addition, laying hens consuming diet 5.0g of PL/kg had lower (P=0.008) egg weight

compared to the laying hens fed the control diet. In all the weeks FCR was not significant between treatments.

Regarding the whole experimental period, it was observed that the variable EP showed differences (P=0.028) between the PL inclusion levels, in which the dosages of 2.5 and 10.0g of PL/kg had better result when compared with the level of 7.5g of PL/kg. In relation to the egg weight variable, there was a difference (P=0.001), the levels of 10.0g of PL/kg and control presented higher values compared to levels of 2.5 and 5.0g of PL/kg. This variable demonstrated a quadratic effect (P=0.001) of levels.

In addition, the parameters of egg mass and feed intake also showed a quadratic (P=0.001 and P=0.044) effect, indicating a superiority of the treatment control compared to the level of 2.5g of PL/kg for the EM variable and 5,0g of PL/kg to FI. As for feed conversion, a linear (P=0.014) effect was observed between treatments (P=0.007), with the treatment containing an inclusion of 10.0g of PL/kg presenting a better conversion compared to the control treatment.

Table 2 – Effects of purified lignin supplementation on the performance parameters of laying hens[1]

Period	Item	Experimental diets					SEM	Probabilities			
		Purified Lignin (g/kg of diet)						Control x PL[2]	Model	Linear	Quadratic
		0	2.5	5.0	7.5	10.0					
Weeks 31 to 34	Egg production, %	94.8	95.5	94.7	94.6	95.2	0.171	0.653	0.491	0.925	0.772
	Egg weight (g)	61.8	60.8	60.1	61.2	61.9	0.217	0.098	0.063	0.729	**0.006**
	Egg mass (g/bird per day)	58.8	57.4	57.3	57.5	58.4	0.207	**0.047**	0.079	0.681	**0.006**
	Feed intake (g/bird per day)	114	108	109	108	109	0.890	**0.024**	0.240	0.150	0.108
	Feed conversion ratio	1.97	1.90	1.92	1.90	1.88	0.013	0.225	0.669	0.226	0.927
	Initial body weight (g)	1565	1548	1580	1563	1586	9.937	0.874	0.803	0.450	0.722
Weeks 34 to 37	Egg production, %	94.1	94.2	94.4	94.3	94.7	0.202	0.580	0.936	0.437	0.907
	Egg weight (g)	64.2a	62.5bc	62.1c	62.4bc	63.7ab	0.221	**0.002**	**0.002**	0.347	**0.002**
	Egg mass (g/bird per day)	60.7a	59.0ab	58.4b	59.0ab	60.0ab	0.247	**0.008**	**0.026**	0.377	**0.002**
	Feed intake (g/bird per day)	114	110	112	112	111	0.739	**0.026**	0.192	0.196	0.224
	Feed conversion ratio	1.89	1.89	1.89	1.95	1.86	0.012	0.885	0.223	0.879	0.244
Weeks 37 to 40	Egg production, %	95.5a	95.4ab	94.7ab	94.0b	95.1ab	0.188	0.066	**0.048**	**0.041**	0.065
	Egg weight (g)	64.8ab	64.4ab	63.4b	63.7ab	65.3a	0.257	0.331	**0.039**	0.816	**0.007**
	Egg mass (g/bird per day)	61.5a	60.4ab	61.5ab	59.5b	61.8a	0.254	0.162	**0.015**	0.780	**0.039**
	Feed intake (g/bird per day)	120	114	114	112	112	1.022	**0.009**	0.078	**0.015**	0.193
	Feed conversion ratio	1.92	1.89	1.88	1.85	1.84	0.015	0.145	0.395	0.054	0.962
Weeks 40 to 43	Egg production, %	94.1	95.2	93.9	93.0	95.1	0.297	0.819	0.106	0.861	0.277
	Egg weight (g)	65.5a	63.8ab	63.4b	63.5ab	65.0ab	0.254	**0.008**	**0.015**	0.428	**0.001**
	Egg mass (g/bird per day)	61.5	61.4	60.4	59.3	60.4	0.312	0.142	0.198	0.054	0.352
	Feed intake (g/bird per day)	123	120	119	119	119	0.965	0.149	0.691	0.284	0.353
	Feed conversion ratio	2.01	1.94	1.98	1.99	1.98	0.014	0.382	0.788	0.939	0.534
	Final body weight (g)	1712	1690	1718	1697	1741	9.947	0.992	0.553	0.369	0.339
	Body weight gain (g)	146	157	138	134	155	6.026	0.976	0.720	0.878	0.503
Weeks 31 to 43	Egg production, %	94.6ab	95.0a	94.5ab	94.1b	95.0a	0.106	0.860	**0.028**	0.746	0.233
	Egg weight (g)	63.9a	62.7b	62.7b	63.1ab	63.8a	0.165	**0.001**	**0.001**	0.961	**0.001**

Egg mass (g/bird per day)	60.6a	59.4b	59.5ab	59.6ab	60.3ab	0.169	**0.008**	**0.010**	0.726	**0.001**
Feed intake (g/bird per day)	118a	115ab	113b	114ab	114ab	0.616	**0.002**	**0.022**	**0.009**	**0.044**
Feed conversion ratio	1.95a	1.91ab	1.90ab	1.91ab	1.89b	0.008	**0.007**	0.079	**0.014**	0.365

[ab]Means within a row with different superscripts differ significantly (P<0.05).

[1] Data are means for six replicates of eight laying hens/replicate.

[2] Contrast of the control treatment versus all purified lignin (PL) treatments.

2. Egg Quality

The parameters used to evaluate the quality of the eggs during the experimental period were MDA, specific gravity of the egg, shell thickness and strength, and percentage of yolk, albumen, and shell (Table 3 and 4). These parameters are fundamental measures to evaluate the quality of eggs in terms of their composition and structural integrity.

During all experimental cycles, not statistically differences were observed between the variables shell strength and percentage of egg yolk and shell ($P>0.05$). This indicates that these parameters were not significantly affected by the different treatments during stages of development of laying hens throughout the cycles.

In the first cycle, which comprised weeks 31 to 34, a statistically difference ($P=0.001$) was observed for the variable albumen. Treatment with 10.0g of PL/kg resulted in a higher percentage of albumen compared to the other treatments. In this case, the relationship between the inclusion of PL and the percentage of albumen showed a linear ($P=0.001$) pattern, with the percentages raising as the amount of PL increased.

In the second cycle, which spanned weeks 34 to 37, the albumen value exhibited a result of a quadratic ($P=0.019$) pattern. This means that the relationship between the inclusion of PL and the percentage of albumen did not follow a linear pattern, but rather a pattern of increase followed by decrease and lastly another increase.

In the third cycle, which covered weeks 37 to 40, the specific gravity of the eggs showed a statistically difference ($P=0.024$) when compared to the control treatment versus treatments with PL, demonstrating a superior value compared to treatments with PL. Furthermore, the relationship between PL inclusion and egg specific gravity exhibited a quadratic ($P=0.012$) pattern.

In the last cycle, which comprised weeks 40 to 43, a statistically difference (P=0.017) was also observed for the specific gravity value of the eggs. In this case, the relationship between the inclusion of PL and the specific gravity of the eggs showed a quadratic (P=0.009) value. The treatment with the inclusion of 5.0g of PL/kg obtained the highest specific gravity value and statistically differed from the treatment with the inclusion of 10.0g of PL/kg.

Egg shell thickness also showed differences (P=0.018) during the last experimental cycle (40 to 43 weeks). The relationship between PL inclusion and shell thickness exhibited both a linear and a quadratic pattern (P=0.009 and P=0.034). In this context, the treatments with inclusion of 5.0 and 7.5g of PL/kg resulted in smaller and statistically significant values of shell thickness compared to the control treatment.

While the experimental weeks, statistical analysis showed differences between treatments (P < 0.001) to MDA. The treatment without inclusion of PL consistently showed the highest MDA value in all cycles, showing statistically differences (P<0.001) in relation to all other treatments. In contrast, the treatment with inclusion of 10.0g of PL/kg presented the lowest MDA value throughout the experimental period. However, in the first week (31 to 34 weeks of age of the hens), it was statistically equivalent to the treatments with the inclusion of 2.5 and 7.5g of PL/kg. In the other weeks, all treatments with PL inclusion showed equal MDA values.

The results obtained suggest a linear (P<0.001) relationship between the treatments, indicating that the increase in the inclusion of purified lignin in the diets of laying hens may be associated with an increase in the levels of MDA in the analyzed egg yolks.

Table 3 – Effects of purified lignin supplementation on egg quality of laying hens[1]

Period	Egg quality	Experimental diets					SEM	Probabilities			
		Purified Lignin (g/kg of diet)						Control x PL[2]	Model	Linear	Quadratic
		0	2.5	5.0	7.5	10.0					
Weeks 31 to 34	Egg specific gravity (g/ml)	1.095	1.093	1.091	1.092	1.092	0.645	0.153	0.616	0.202	0.428
	Shell thicknees (mm)	0.390	0.393	0.390	0.392	0.397	0.003	0.677	0.934	0.557	0.722
	Shell strength (kgf)	4.74	4.98	4.76	4.78	4.61	0.067	0.832	0.574	0.343	0.331
	Shell, %	9.96	10.17	9.88	9.96	9.94	0.048	0.830	0.448	0.471	0.800
	Yolk, %	26.2	26.4	26.5	26.1	25.8	0.132	0.965	0.483	0.220	0.179
	Albumen, %	63.1c	63.4bc	63.4bc	63.8b	64.5a	0.115	**0.001**	**<.0001**	**<.0001**	0.079
Weeks 34 to 37	Egg specific gravity (g/ml)	1.094	1.095	1.095	1.095	1.094	0.257	0.477	0.303	0.338	0.132
	Shell thicknees (mm)	0.400	0.396	0.392	0.400	0.395	0.002	0.293	0.488	0.624	0.490
	Shell strength (kgf)	4.60	4.66	4.65	4.76	4.68	0.050	0.503	0.910	0.480	0.730
	Shell, %	9.99	9.97	9.90	9.95	9.84	0.045	0.499	0.873	0.356	0.888
	Yolk, %	25.7	25.7	25.9	25.8	25.2	0.192	0.909	0.822	0.519	0.374
	Albumen, %	64.5	64.2	64.0	64.2	65.7	0.203	0.961	0.077	0.083	**0.019**
Weeks 37 to 40	Egg specific gravity (g/ml)	1.093	1.091	1.091	1.091	1.092	0.345	**0.024**	0.109	0.573	**0.012**
	Shell thicknees (mm)	0.392	0.390	0.383	0.385	0.388	0.002	0.254	0.549	0.344	0.248
	Shell strength (kgf)	4.60	4.77	4.57	4.56	4.76	0.070	0.739	0.795	0.858	0.681
	Shell, %	9.82	9.90	9.68	9.58	9.77	0.070	0.633	0.675	0.423	0.576
	Yolk, %	26.8	26.0	26.1	26.1	26.7	0.198	0.277	0.625	0.951	0.142
	Albumen, %	63.5	64.0	64.0	64.2	63.5	0.224	0.477	0.203	0.965	0.234
Weeks 40 to 43	Egg specific gravity (g/ml)	1.091ab	1.090ab	1.094a	1.090ab	1.088b	0.521	0.865	**0.017**	0.083	**0.009**
	Shell thicknees (mm)	0.406a	0.392ab	0.390b	0.387b	0.390ab	0.002	**0.001**	**0.018**	**0.009**	**0.034**
	Shell strength (kgf)	4.50	4.27	4.24	4.23	4.39	0.069	0.222	0.694	0.618	0.179
	Shell, %	9.89	9.72	9.86	9.71	9.70	0.065	0.424	0.851	0.441	0.965
	Yolk, %	27.0	26.9	26.5	27.3	26.9	0.190	0.845	0.853	0.913	0.729
	Albumen, %	62.9	63.4	63.8	63.4	63.3	0.223	0.395	0.857	0.698	0.334

[ab]Means within a row with different superscripts differ significantly (P<0.05).

[1] Data are means for six replicates of 15 eggs/replicate.

[2] Contrast of the control treatment versus all purified lignin (PL) treatments.

Table 4 – Effects of purified lignin supplementation malondialdehyde (MDA) content in egg yolk of laying hens[1]

Egg quality	Period	Experimental diets					SEM	Probabilities			
		Purified Lignin (g/kg of diet)						Control x PL[2]	Model	Linear	Quadratic
		0	2.5	5.0	7.5	10.0					
MDA[3] (ng/g)	Weeks 31 to 34	32.0a	25.3bc	28.1b	26.4bc	23.9c	0.080	**<.0001**	**0.000**	**0.000**	0.237
	Weeks 34 to 37	25.0b	22.5c	28.1a	21.3c	20.7c	0.077	**0.004**	**<.0001**	**<.0001**	**0.001**
	Weeks 37 to 40	25.4a	21.4ab	23.3ab	20.5ab	19.1b	0.069	**0.009**	**0.020**	**0.004**	0.898
	Weeks 40 to 43	22.2a	5.9d	20.8a	12.9b	8.3c	0.175	**<.0001**	**<.0001**	**<.0001**	0.567

[ab]Means within a row with different superscripts differ significantly (P<0.05).

[1]Data are means for six replicates of five eggs/replicate.

[2] Contrast of the control treatment versus all purified lignin (PL) treatments.

[3]MDA - Malondialdehyde

3. Internal Organs

The effect of inclusion of purified lignin on the relative weight are in the Table 5, where proventriculus, pancreas, liver, spleen, jejunum, and ileum, as well as the relative size of the small intestine (duodenum, jejunum, and ileum), were not significant (P>0.05).

However, regarding to the relative weight of the gizzard, a linear (P=0.023) effect was observed. The treatment with inclusion of 7.5g of PL/kg showed a higher relative gizzard weight compared to the control treatment.

In addition, the relative Duodenum weight also had a linear (P=0.015) effect. The treatment with the inclusion of 10.0g of PL/kg resulted in a relative weight of the duodenum superior to the treatments with the inclusion of 2.5g of PL/kg and the treatment without inclusion.

4. Short Chain Fatty Acids (SCFA) from the Cecum

The concentrations of short chain fatty acids (acetic, propionic, butyric, isobutryic, valeric and isovaleric acids) are expressed on Table 6. The results showed that the inclusion of purified lignin had significant effects on the total short chain fatty acids and propionic acid analyzed. For both the treatment with 10g of PL/kg resulted in higher concentrations when compared to the ones with 5 and 7.5g of PL/kg.

However, for butyric and isobutyric acid, the treatment with 10g of PL/kg has also resulted in P=0.042 showing a higher concentration, when compared with treatment with 5.0g of PL/kg (P=0.042) and 7.5g of PL/kg (P=0.023), respectively.

On the other hand, acetic acid and valeric acid did not present statistically differences (P=0.061 and 0.140) between treatments. Overall, a quadratic (P<0.05) effect was

observed for all short-chain fatty acids analyzed, indicating a non-linear variation in their concentrations in relation to the inclusion of purified lignin.

Table 5 – Effects of purified lignin supplementation on the internal organs parameters of laying hens[1].

Internal organs	Experimental diets					SEM	Probabilities			
	Purified Lignin (g/kg of diet)						Control x PL[2]	Model	Linear	Quadratic
	0	2.5	5.0	7.5	10.0					
Length										
Duodenum, %	20.5	20.7	19.8	22.1	21.9	0.357	0.433	0.213	0.097	0.464
Jejunum, %	41.7	44.6	45.1	42.2	43.6	0.668	0.191	0.427	0.747	0.251
Ileum, %	37.7	36.1	36.1	35.5	35.8	0.544	0.185	0.726	0.283	0.491
Weigth										
Proventriculus, %	0.32	0.33	0.33	0.33	0.33	0.005	0.757	0.991	0.863	0.670
Gizzard, %	1.8b	1.88ab	1.88ab	2.13a	1.94ab	0.035	0.053	**0.039**	**0.023**	0.266
Pancreas, %	0.18	0.19	0.19	0.20	0.19	0.004	0.140	0.337	0.128	0.534
Liver, %	2.20	2.43	2.23	2.37	2.29	0.048	0.322	0.542	0.757	0.512
Spleen, %	0.09	0.10	0.10	0.10	0.09	0.003	0.701	0.822	0.888	0.338
Duodenum, %	0.65b	0.74ab	0.65b	0.70ab	0.80a	0.018	0.060	**0.014**	**0.015**	0.206
Jejunum, %	1.45	1.55	1.38	1.45	1.45	0.037	0.928	0.742	0.717	0.912
Ileum, %	1.12	0.99	1.19	1.03	1.02	0.042	0.521	0.594	0.567	0.778

[ab]Means within a row with different superscripts differ significantly (P<0.05).

[1] Data are means for six replicates of two laying hens/replicate.

[2] Contrast of the control treatment versus all purified lignin (PL) treatments

Table 6 – Effects of purified lignin supplementation on the concentrations of short chain fatty acids (SCFAs) from the cecum of laying hens.

Concentrations of SCFAs	Experimental diets					SEM	Probabilities			
	Purified Lignin (g/kg of diet)						Control x PL[2]	Model	Linear	Quadratic
	0	2.5	5.0	7.5	10.0					
SCFA (mmol/L of wet cecal content)										
Total SCFA	30.9ab	26.1ab	22.3b	19.8b	38.4a	2.051	0.332	**0.024**	0.488	**0.003**
Acetic acid	23.6	19.2	16.4	17.3	27.5	1.440	0.287	0.061	0.520	**0.005**
Propionic acid	4.95ab	4.02ab	3.51b	3.66b	6.91a	0.377	0.564	**0.014**	0.109	**0.002**
Butyric acid	1.20ab	1.29ab	0.83b	0.93ab	2.08a	0.154	0.803	**0.042**	0.159	**0.027**
Isobutyric acid	0.18ab	0.12ab	0.11ab	0.06b	0.23a	0.018	0.224	**0.023**	0.628	**0.003**
Valeric acid	0.51	0.49	0.41	0.50	0.68	0.034	0.844	0.140	0.121	**0.048**
Isovaleric acid	0.50a	0.29ab	0.22b	0.26b	0.38ab	0.031	**0.003**	**0.019**	0.144	**0.002**

[ab]Means within a row with different superscripts differ significantly (P<0.05).

[1] Data are means for six replicates of two laying hens/replicate.

[2] Contrast of the control treatment versus all purified lignin (PL) treatments

5. Egg Shelf Life

During the shelf-life test of 0, 10, 20 and 30 days (Table 7), the parameters of shell strength and percentage of yolk and albumen did not show differences between treatments. However, the specific gravity of the eggs showed a statistical difference (P=0.006) between the treatments when analyzed at time 0 of the evaluation. The treatment with inclusion of 7.5g of PL/kg showed a lower specific gravity compared to the treatment with 5g of PL /kg.

The percentage of shell varied between treatments at time 0 and 10 days, but only showing a quadratic (P=0.050) effect at the 10-day analysis. The difference among the treatments stabilized at the 20 and 30-day analyses. For the shell thickness variable, there was a statistical difference (P=0.010 and P=0.044) between the treatments at the 0 and 20-day time analyses, demonstrating a linear effect for this period, as well. After 20 days, the treatment without PL showed a lower value of shell thickness compared to the treatments with 2.5, 7.5, and 10g of PL/kg. The difference between treatments stabilized at the 30-day analysis.

MDA values showed a linear and quadratic (P=0.001 and P=0.001) effect for the 0, 10, 20 and 30-day analyses. There was also a difference (P<0.001) between treatments, where the treatment without PL showed a high value of MDA when compared to the treatments with the addition of PL. These results indicate that the inclusion of purified lignin may influence specific gravity, shell percentage, shell thickness and MDA values of the analyzed eggs.

Table 7 – Effects of purified lignin supplementation on egg shelf life of laying hens[1].

Shelf Life - Egg quality	Days	Experimental diets					SEM	Probabilities			
		Purified Lignin (g/kg of diet)									
		0	2.5	5.0	7.5	10.0		Control x PL[2]	Model	Linear	Quadratic
Egg specific gravity (g/ml)	0	1.092ab	1.091ab	1.099a	1.087b	1.093ab	1.064	0.849	**0.006**	0.687	0.428
	10	1.084	1.081	1.081	1.080	1.081	0.628	0.078	0.472	0.197	0.193
	20	1.073	1.075	1.072	1.075	1.072	0.706	0.728	0.475	0.623	0.490
	30	1.055	1.056	1.053	1.055	1.053	0.653	0.801	0.443	0.436	0.810
Shell strength (kgf)	0	4.60	4.58	4.97	4.80	4.65	0.108	0.729	0.967	0.681	0.699
	10	4.72	4.90	4.47	4.66	4.64	0.086	0.878	0.790	0.904	0.840
	20	4.76	5.06	4.74	4.69	4.61	0.083	0.755	0.781	0.768	0.985
	30	4.90	4.77	4.45	4.59	4.55	0.115	0.302	0.756	0.314	0.539
Yolk, %	0	26.0	26.8	26.5	25.7	26.5	0.201	0.434	0.440	0.995	0.736
	10	28.1	27.6	27.9	27.3	27.7	0.293	0.554	0.944	0.634	0.724
	20	29.4	28.8	28.2	28.3	29.6	0.339	0.471	0.683	0.998	0.157
	30	27.5	29.5	28.2	29.4	28.3	0.315	0.088	0.224	0.494	0.158
Albumen, %	0	64.1	63.2	62.7	64.7	63.5	0.262	0.309	0.145	0.969	0.362
	10	61.4	62.6	62.4	62.8	62.4	0.333	0.187	0.716	0.361	0.381
	20	60.4	60.7	62.2	62.2	60.2	0.412	0.346	0.392	0.715	0.089
	30	62.2	60.3	61.3	60.6	61.4	0.322	0.119	0.381	0.556	0.188
Shell, %	0	9.95ab	9.88ab	10.68a	9.47b	9.92ab	0.116	0.878	**0.010**	0.485	0.234
	10	10.4a	9.9ab	10.01ab	9.77b	10.02ab	0.074	**0.005**	**0.037**	0.056	0.050
	20	10.1	10.4	10.0	10.0	10.1	0.078	0.798	0.351	0.490	0.975
	30	10.2	10.1	10.3	9.9	10.2	0.073	0.734	0.643	0.778	0.894
Shell thicknees (mm)	0	40.7ab	40.3ab	42.4a	39.1b	39.0b	0.361	0.485	**0.010**	**0.041**	0.076
	10	41.1	39.7	39.9	39.0	40.0	0.311	0.063	0.301	0.187	0.149
	20	38.4b	40.3a	39.3ab	39.8a	40.2a	0.233	**0.008**	**0.044**	**0.037**	0.403
	30	41.4	39.9	40.8	39.8	40.8	0.242	0.078	0.199	0.445	0.129
MDA[3] (ng/g)	0	38.3a	27.5b	25.3bc	24.6bc	23.5c	0.148	**<.0001**	**<.0001**	**<.0001**	**<.0001**
	10	33.7a	23.8b	22.5b	22.8b	22.3b	0.118	**<.0001**	**<.0001**	**<.0001**	**<.0001**

20	32.5a	21.6b	22.3b	21.8b	20.7b	0.126	**<.0001**	**<.0001**	**<.0001**	**<.0001**
30	30.6a	19.3b	20.8b	20.7b	16.8b	0.138	**<.0001**	**<.0001**	**<.0001**	**0.035**

[ab]Means within a row with different superscripts differ significantly (P<0.05).

[1] Data are means for six replicates of five eggs/replicate.

[2] Contrast of the control treatment versus all purified lignin (PL) treatments.

[3]MDA - Malondialdehyd

DISCUSSION

The number of studies to determine the impact of purified lignin on poultry especially laying hens is limited. Throughout the experimental period, no differences were observed in the body weight of the hens between the treatments. Sozcu (2019) research indicated the PL supplementation in the amount of 10.0g/kg of feed stimulated body weight and feed conversion ratio on broilers, however, inclusion over 5.0g/kg may reduce feed intake.

The results of the present study show that laying hens fed supplementation of 2.5, 5.0 and 7.5g of PL/kg resulted in lower averages than the other treatments, in terms of overall productivity. In addition, when there was a difference, the treatment with 10.0g of PL/kg was statistically equal to the treatment without the inclusion of PL, except for the variable FCR, which presented a better value when compared to the control. These results indicate that 10.0g of PL/kg does not affect the productivity of laying hens during the 31-to-43-week period. No significant effects on the productivity of 36-week-old White Leghorn hens were observed when their feed was supplemented with saffron rhizome powder (*Curcuma longa Linn*), a substance that contains polyphenols, at concentrations of 10 and 30g/kg (Malekizadeh et al., 2012).

Assessing the quality of eggs is essential to determine their composition and structural integrity. No significant differences were observed in shell strength between treatments during the experiment. This result suggests the addition of PL to laying hen diets had no substantial effect on shell strength of eggs produced. Shell strength is a measure of the force required to break the eggshell. Shells with high strength is an indicator of quality, as it ensures the egg remains intact during handling, transport, and storage (Mertens et al., 2006).

When evaluating the use of different polyphenols in laying hen diets, studies have shown the inclusion of green tea or dried orange pulp resulted in a significant reduction in the shell strength (Zhu et al., 2020; Goliomytis et al., 2018). In contrast, El-Motaal et al. (2008) observed

by adding 3g of eucalyptus leaves/kg to the diet of laying hens, an improvement in the shell strength was obtained.

The percentage of yolk and shell are important parameters for egg composition and did not show statistically significant differences over the weeks. The percentage of yolk is related to the content of proteins, lipids, fat-soluble vitamins, and minerals, while the percentage of shell is related to the amount of minerals deposited in the eggshell and plays a key role in protecting the internal contents of the egg against damage and contaminations (Nys et al., 2011). The lack of significant differences in these variables indicates that treatments with the inclusion of PL did not significantly influence the composition of these egg parts.

In the literature, there are several studies investigating the effect of using polyphenols in the diet to verify the egg quality, especially in relation to the percentage of egg yolk and shell, and the results may vary depending on the specific source of polyphenols used. For example, Fathi et al. (2020) demonstrated that the inclusion of 1g of eucalyptus leaves/kg in the diet of Japanese quails resulted in better values of eggshell percentage. In contrast, studies that used garlic powder with and without copper in laying hens diets did not find significant differences in treatments regarding the percentage of shell (Aswal et al., 2017; Lim et al., 2006).

Conversely, the use of green tea polyphenols has been associated with significant negative effects on eggshell weight (Zhu et al., 2020). In addition, the inclusion of dried orange pulp in the diet, at a dosage of 90g/kg, resulted in a reduction in the percentage of yolk and shell, which implies negatively in the egg quality (Goliomytis et al., 2018). Showing different results to those found in this study, the effect of supplementation with green tea extract and curcumin in the diet of laying hens resulted in significant increases in the percentage of yolk (Ariana et al., 2011; Galli et al., 2018).

Albumen is a fraction of the egg composed mainly of proteins, such as ovalbumin, which have an important role in egg nutrition and texture (Nys et al., 2011). During the first two experimental cycles, a linear relationship was observed between the inclusion of PL in the laying hens diets and the percentage of albumen in the eggs produced. This means that as the amount of PL increased, the percentage of albumen improved proportionally. Despite that, this difference was not statistically significant for the following cycles, which indicates that the inclusion of PL did not have a substantial impact on the percentage of albumen in the eggs.

In a study conducted by Chen et al. (2021) in 50-week-old laying hens, the effect of supplementation with magnolol in diets was investigated. The results showed that addition at concentrations of 200 and 300mg of magnolol/kg of the diet resulted in significant increases in egg albumen content.

The specific gravity of eggs is a measure that assesses the density of the internal components of the egg, such as proteins, lipids, and minerals. This measure provides relevant information about the overall quality of the egg, its freshness, and the relative proportion of different components present. The inclusion of purified lignin in the diet of laying hens had an impact on the composition of these nutrients, resulting in statistically significant differences in the specific gravity of the eggs during the last two experimental cycles.

In a study conducted with 30-week-old laying hens, the effect of dietary supplementation with curcumin over a period of 21 days was investigated. The results obtained showed the inclusion of curcumin in the diet caused significant improvements in the specific gravity of eggs (Galli et al., 2018).

Regarding eggshell thickness, significant differences were also observed during the last experimental cycle. Thicker eggshell is generally considered stronger and less likely to break. The results indicate the inclusion of PL in the laying hen diet can have a negative effect on shell

thickness at the dose of 5.0 and 7.5g of PL/kg, but at the inclusion of 2.5 and 10.0g of PL/kg a difference was not indicated wih the treatment control.

However, it is important to note that the relationship between PL inclusion and shell thickness exhibited both a linear and a quadratic pattern, which suggests that different levels of inclusion may have varying effects. Negative impacts on eggshell thickness were also found when using polyphenol from green tea and drier orange pulp in the basal diet of laying hens (Fan et al., 2021; Zhu et al., 2020; Goliomytis et al., 2018).

Malondialdehyde is an indicator of oxidative stress and oxidative damage in lipids, being a marker of lipid quality and stability in eggs (Demirci-Çekiç et al., 2022). The treatment without inclusion of PL consistently showed the highest MDA value in all experimental cycles, differing statistically from all other treatments. This suggests that the absence of PL in the diet resulted in higher levels of oxidative stress and damage in egg lipids.

When supplemented with epigallocatechin-3-gallate (EGCG), a polyphenol, in laying hen diets, an increase in the antioxidant capacity of eggs was observed and the MAPK/Nrf2 signaling pathway was regulated (Wang et al., 2020), suggesting that polyphenols can interact with signaling pathways of oxidative stress and inflammation, leading to upregulation of antioxidant enzymes.

The linear and quadratic relationship between the treatments in this study suggests that the increase in the inclusion of purified lignin in the diets of laying hens is associated with a decrease in MDA levels in the analyzed eggs. This result indicates that the inclusion of PL in the diet can have a beneficial effect in reducing oxidative stress and preserving the lipid quality of eggs.

In the study by Grešáková et al. (2012), using the Isa Brown laying hen strain, it was observed that the addition of purified lignin in a diet contaminated with zearalenone had a

protective effect by preventing the increase in the activity of the enzyme glutathione peroxidase in the duodenal mucosa.

Thus, it was hypothesized that the dietary supplementation of purified lignin could affect the production of ROS and the antioxidant systems in the body of birds. This plays an important role in the antioxidant defense of cells, resulting in a decrease in oxidative stress in eggs, and consequently, improving the quality, stability, and nutritional value of eggs.

In an experiment with the objective of evaluating the effect of supplementation using eucalyptus leaves polyphenols in the diet of Yueqinhuang laying hens strain, effects were found in the reduction of MDA in the egg yolk when diets were supplemented with dosage of 0.8g/kg and 1.2g/kg (Chen et al., 2018). These results are in line with this study showing that polyphenols from the wood process are effective in reducing the concentration of MDA in egg yolks, indicating a reduction in lipid peroxidation.

The results obtained indicate that different polyphenols can have different effects on egg quality, highlighting the need to investigate each compound individually in relation to each specific parameter considered.

After 12 weeks of experiment, no effects of the inclusion of PL were observed on the relative weight of the proventriculus, pancreas, liver, spleen, jejunum, and ileum, as well as the length of the small intestine of hens. On the other hand, results were found demonstrating an effect on the gizzard of laying hens that received PL supplementation in the diet compared to those that received only the basal diet.

Several studies have investigated the effect of dietary fiber on the development of the gizzard, small and large intestine in chickens (Jiménez-Moreno et al., 2009; Adewole D., 2020; Tejeda and Kim, 2021). In a study of hens fed diets containing 1% lignocellulose for a period of 10 weeks, an increase in the relative weight of the gizzard and small intestine was observed

(Yokhana et al., 2015). Röhe et al. (2020) found similar results, observing an increase in gizzard and small intestine weight when adding 10% of lignocellulose to the diet of laying hens between 32 and 42 weeks of age.

The 31-week-old laying hens that were fed diets containing 0.8% of lignocellulose for 12 weeks had heavier gizzards compared to hens fed the control diet (Yokhana et al., 2015). Nonetheless, there was no effect on gizzard weight development in younger hens fed these diets for shorter periods of 3, 6, and 9 weeks (Yokhana et al., 2015), suggesting that the duration of the period of feeding with lignocellulose may be a relevant factor for the development of effects in the digestive tract.

The weight of the gizzard and duodenum showed a linear effect, demonstrating that the increase in the PL dose is associated with the increase in the size of these organs. A more developed gizzard and duodenum are indicative of an increased ability to digest and absorb nutrients, which contributes to better utilization of feed consumed by hens. These results agree with the difference in FI between treatments with and without inclusion of PL. Considering that, even consuming less, hens supplemented with purified lignin were more efficient in the digestibility of ingested nutrients. These findings contribute to the understanding of a better FCR in laying hens supplemented with PL.

In this study, the effect of the purified lignin inclusion on the SCFA from the cecum of laying hens aged between 31 and 43 weeks was evaluated. The results showed a quadratic effect, indicating that the doses of 5.0 and 7.5g/kg resulted in lower means of all SCFA analyzed, when compared to the other treatments. Baurhoo et al. (2007), a study on the use of PL in the monogastric diet resulted in favoring the growth of beneficial bacteria and in the control of intestinal pathogens. Changes in the intestinal microbiota profile generate changes in SCFA

concentrations. Nonetheless, it is important to highlight that there is a lack of studies that specifically investigate the impact of feeding purified lignin on the microbiota of laying hens.

In a previous study carried out by Röhe et al. (2020), who used different levels of inclusion of lignocellulose in the diet of laying hens (8.0, 50.0, and 100g/kg), a reduction was observed in the averages of short-chain fatty acids analyzed as inclusion increases. These results suggest that the inclusion of purified lignin and lignocellulose in the diet of laying hens may have effects on cecal content and SCFA, however, the exact nature of these effects and their influence on the microbiota still require further investigation. Additional studies are needed to better understand these interactions and their physiological effects in laying hens.

At the end of the 30 days of storage, no significant effects were observed in specific gravity, shell strenght, shell thickness and percentage of albumen, yolk, and shell. Conversely, the MDA value in the egg yolk showed a linear and quadratic response in relation to the increase in levels of purified lignin in the diet. There was a significant difference between the treatments with the addition of PL and the treatment without addition on all evaluation days (0, 10, 20 and 30 days), and the treatment with the addition of 10.0g of PL/kg in the diet showed the lowest average compared to the other treatments with the addition of PL.

After specific supplementation periods, different phenolic compounds were evaluated for their effects on shelf life of stored products. Supplementation with 100mg of magnolol/kg derived from the bark of the magnolia plant for 12 weeks resulted in beneficial effects on eggs stored for 14 days (Chen et al., 2021). Similarly, supplementation with 50mg of curcumin/kg for 8 weeks has demonstrated positive effects on eggs stored for 21 days (Galli et al., 2018). Additionally, supplementation with 800mg of resveratrol/kg, a phenolic compound found in grapes, for 8 weeks has shown benefits on shelf life of eggs stored for 30 days (Zhang et al., 2019).

These results indicate that supplementation with specific phenolic compounds can be a promising strategy to extend the shelf life of stored eggs, ensuring their quality and safety. However, it is important to consider the appropriate concentrations, timing of supplementation, and specific product characteristics to obtain the best results.

CONCLUSION

The dose of 10.0g of purified lignin/kg was considered safe and equivalent to the control treatment, not affecting the performance, and had better feed conversion ratio of laying hens in the period from 31 to 43 weeks. This inclusion also proved to be beneficial in preventing the deterioration of lipids present in the eggs, resulting in greater oxidative stability and, consequently, contributing to better egg quality and shelf life.

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CAPÍTULO 3

"Effect of purified lignin on performance, egg quality, intestinal morphology, and histology of laying hens on *cage-free* system."

Effect of purified lignin on performance, egg quality, intestinal morphology, and histology of laying hens on *cage-free* system

A.B.S. Oliveira[1] *

[1]Dep School of Veterinary Medicine and Animal Science, São Paulo State University, Botucatu, SP, 18610-034, Brazil

Key words: phenolic, antioxidant, gut, short chain fatty acids, physiology

Primary audience: nutritionists, researchers, and feed formulators

* Corresponding author: ana.bs.oliveira@unesp.br

SUMMARY

The experiment objective was evaluating the effects of purified lignin (PL) on production, egg quality, morphology, and histology intestinal of laying hens in a *cage-free* system were analyzed. 300 Hy-Line W-36 hens aged between 51 and 63 weeks were used to compose two treatments, with three replications and 50 hens per pen. The two diets consisted of a control diet and a test diet supplemented with 1% of PL. The egg production and laid inside and outside the nests was measured per day, feed intake, egg weight, egg mass and feed conversion ratio were recorded at the end of 21 days. A total of five eggs/replication/day were collected for the last two days of each period to evaluate interior and exterior egg quality parameters. At the end of the experimental period the two hens per replicate were sacrificed to determine the weight of the internal organ, intestinal morphology and histology and short-chain fatty acids through the collect of cecal contents. In the results related to production and performance parameters, it was observed that the treatments exhibited $P<0.05$ throughout the experimental cycles, impacting the production, mass, and weight of eggs, as well as the proportion of eggs laid inside and outside the nests. In relation to egg quality parameters, treatments also showed $P<0.05$ throughout the experimental cycles, particularly regarding specific gravity and shell thickness. Notably, malondialdehyde concentration demonstrated $P<0.05$ in all experimental weeks. In analyzes related to intestinal morphology and histology, a $P<0.05$ were observed in the weight of the spleen and jejunum, in addition to the size of the villi present in the jejunum. In conclusion, the inclusion of 10.0g of PL/kg in the diet of hens raised in the *cage-free* system resulted in inferior performance during this period when compared with the treatment control. However, an improvement in the quality of the eggs was observed, due to its antioxidant effect, without impact on the intestinal morphology and histology of the hens.

DESCRIPTION OF PROBLEM

The modern bird has been genetically selected for high productivity, for this potential to be achieved, feeding and housing are needed to provide health and welfare (Mckay 2009; Underwood et al. 2021). Diets for laying hens are formulated to ensure maximum egg production, considering breed and housing conditions (Holt et al., 2011).

Most of the eggs sold on the market today come from laying hens raised in intensive breeding systems, in which the birds are kept in cages inside sheds throughout their life cycle (Elson and Croxall, 2006; Dikmen et al., 2016).

This mass production system enabled increased productivity, leading to more efficiency and ultimetly increased consumption of and affordable protein source (Vieira, 2000; Réhault-Godbert et al., 2019). However, this system of rearing in cages has been recognized as causing damage to the physical comfort and expression of natural behaviors of birds (Elson and Croxall, 2006; Matthews and Sumner, 2015).

Another factor is consumer demand on rearing systems, as they believe that hens need to be free for pecking and scratching areas, sand bath, wing flapping, laying eggs in nests and roaming freely to express their natural behavior, and are willing to pay a little more for a standout product (Parrott, 2004; Tauson, 2005).

The European Union in 2012 passed regulations that eliminate the caged housing system and only allow enriched cage systems or alternatives systems, such as *cage-free*, *free-range*, and organic production (EU Directive 1999/74/EC). Many companies in the feed industry have committed to gradually eliminating the use and sale of eggs produced in intensive systems.

However, there are some research reports negative impacts due the alternative system, including laying percentage, feed efficiency, cannibalism, mortality, and egg quality (weight, cracked eggs, nutritional composition, shell strength, cleanliness, microbial contamination, and conservation), causing economic losses (Elson and Croxall, 2006; Englmaierova et al., 2014; Jones et al., 2015; Karcher et al., 2015; Dong et al., 2017).

In order to mitigate the possible adverse impacts resulting from changes in the breeding system, several investigations have highlighted the importance of exploring alternative feed additives options that can contribute to improving the performance and health of hens (Alloui, et al., 2014; Hoste, et al., 2015).

The use of purified lignin in poultry diets has demonstrated a positive influence on the microbial population in the gastrointestinal tract of these animals (Baurhoo et al., 2007), which directly impacts the production of short chain fatty acids (SCFAs). These SCFA, play a vital role in maintaining the balance of the intestinal microbiota, in addition to supporting the integrity of the intestinal mucosa and contributing to the immune response and consequently improved productivity, egg quality and animal health. (Angelakis, E., 2017; Wang, et al., 2020; Li, et al., 2021).

In addition to the potential effects on the intestinal microbiota, purified lignin is also recognized for containing polyphenolic fragments, which have been associated with antioxidant properties (Dizhbite et al., 2004). These components can play a significant role in reducing oxidative stress in birds, providing protection against cellular damage, and promoting a balanced physiological environment (Nimalaratne and Wu, 2015; Righi, et al., 2021).

The antioxidant compounds present in the laying hens diet have the ability to be transferred to the egg yolk, enabling the production of eggs enriched with antioxidant

properties (Nimalaratne and Wu, 2015; Obianwuna, et al., 2022). This transfer of these antioxidants to the egg yolk represents a significant opportunity to promote eggs that offer additional benefits to human health (Vlaicu, et al., 2021).

Therefore, aiming to meet the growing social demand for alternative breeding systems and mitigate possible impacts on production, the objective of this study was to evaluate the productive performance, intestinal histomorphology, SCFAs cecal and egg quality of hens supplemented or not with purified lignin in the *cage-free* system.

MATERIAL AND METHODS

The experimental procedures were approved by the Institutional Animal Care and Use Committee, of the School of Veterinary Medicine and Animal Sciences of the São Paulo State University (FMVZ/UNESP), Botucatu, SP, Brazil (protocol number: 0155/2020).

1. Animals, Diets, and Housing

The study was conducted at the facilities of the FMVZ/UNESP (geographical coordination: latitude 22° 49' 07" S and longitude 48° 24' 40" W). In total, 300 commercial Hy-Line W-36 laying hens at 51 weeks old with uniform body weight were used in the experiment. The experimental period was subdivided into 4 cycles of 21 days, totaling 84 experimental days. The hens were reared on the floor, without access to a pasture, with a galvanized steel nest (five birds per nest spot), with a density of seven hens/m², in a *cage-free* system.

The nutrition program consisted of one isonutritive diet formulated to meet the nutrients requirements for layer hens adapted according to the recommendations of the breeder (Hy-Line W-36, 2011) and Rostagno et al. (2017). Feed and water were provided *ad libitum* during the experiment.

The laying hens were assigned to two treatments with three replications of 50 hens per replicate. In the control group were given a corn-soybean meal-basal diet (Table 1) in mash form without purified lignin. The remaining group was given the same basal diet supplemented with an additional of 10g of purified lignin /kg.

The experiment was carried out between 51 to 63 weeks of age (12 weeks experimental period). The lighting regimen was 16 hours of continuous light per day from 06:00 to 22:00. The laying hens were kept in optimal and standard bio-climatic and welfare conditions.

Table 1 – Composition of basal diet.

Ingredient	Amount (g/kg)
Corn, grain	635.36
Soybean meal (45% CP)	243.81
Limestone	95.3
Di - Calcium phosphate	12.73
DL - Methionine	4.55
Sodium chloride	4.25
Vitamins-minerals premix[1]	4.00
Total	1000
Calculated chemical composition	
Dry Matter (%)	89.6
Crude Protein (%)	16.4
Methionine (%)	0.70
Lysine (%)	0.82
Ether Extract (%)	2.4
Calcium (%)	4.0
Phosphorus Total (%)	0.52
Metabolizable energy (MJ/kg)	11.4

[1] Provided per kilogram of diet: vitamin A, 8800 IU; vitamin D3, 2500 IU; vitamin E, 11 IU; vitamin K3, 2.2 IU; thiamin, 1.5 mg; riboflavin, 4 mg; nicotinic acid, 7.85 mg; pantothenic acid, 34.65 mg; pyridoxine, 2.46 mg; folic acid, 0.48 mg; vitamin B12, 4 mg; manganese 74.4 mg; zinc, 64.67 mg; iron, 75 mg; copper, 6 mg; iodine, 0.86 mg; selenium, 0.2 mg; choline chloride, 200 mg.

The purified lignin used in this study was extracted from *Eucalyptus urograndis* wood, being a by-product of the Kraft process of cellulose production (reaction of wood with sodium hydroxide (NaOH) + sodium sulfide (Na_2S)), in the form of a brown powder. The product was composed of lignin (> 95%), total phenolic compounds (4.6%): syringyl-OH

(2.3%), guaiacyl-OH (condensed) (1.11%), guaiacyl-OH (non-condensed) (1%) and hydroxyphenyl-OH (0.14%); pH 3 - 5, molar mass 1300 to 1400, moisture (1.92%), ash (2.42%), 61.1% total carbon, 31.3% oxygen, 5.5% hydrogen, 1.7% sulfur and 0.4% nitrogen and 0.99% carbohydrates. Purified lignin was added on top and to ensure that the diets remained isonutritive, sand was supplemented, as an ingredient with no nutritional value, to ensure uniform dilution of all diets used in the study.

2. Performance and Egg Quality

The laying hens were between 51st and 63rd week of age. Egg production (%) and egg laid out or in the nest were obtained by recording the number of eggs produced daily throughout the experimental period. The feed intake and feed conversion ratio were recorded in the end of each cycle (21 days). The feed conversion ratio (FCR) was expressed in grams of feed consumed per grams of egg produced. All production variables were determined on a replicate basis. All eggs collected in the last day of each period were weighed to the nearest 0.01 g. The average egg weight was multiplied by the total number of eggs produced during the experimental period, thus obtaining the total egg mass. This value was then divided by the total number of hens per replicate for each 21-day period.

A total of five eggs/replication/day were collected for the last two days of each period, 30 eggs in the total per treatment were analyzed to evaluate internal and external egg quality parameters. Specific gravity was measured by immersing eggs produced in the last two day of each period in saline solutions of 1.050 to 1.100g/ml (Moreng and Avens, 1990), at 0.005 intervals. The eggs were classified according to their specific gravity when they floated (Albino et al., 2014). Eggshells were washed and dried at 60°C for 48 hours, after they were weighed. Shell thickness was measured to the nearest 0.01mm in

tree points at the middle-transversal area of the shell, from which an average measure expressed in millimeters (mm) using a digital caliper. Shell strength, evaluated with the support of a texturometer TA. XT plus (Texture Analyzer TA-XT Plus, Surrey, England), equipped with a 75mm probe (p/2) and test speed of $1mm/s^1$, which will be expressed in kfg/cm^2. Percentage of yolk, albumen, and shell were calculated by the ratio between the mass of the respective constituents and the mass of the egg.

The contents of malondialdehyde (MDA) were assayed using colorimetric methods with a spectrophotometer (722N, Shanghai Precision and Scientific Instrument Co. Ltd. Shanghai, P.R. China). For this parameter, five eggs per replicate was collected at the end of each cycle and the egg yolk were analyzed using the assay kits purchased from Nanjing Jiancheng Institute of Bioengineering (Nanjing, Jiangsu, P.R. China) and the procedures accordingly. All samples were measured in triplicate, at appropriate dilutions, to give activities of the enzymes in the linear range of standard curves constructed with pure enzymes. The values are expressed in ng per g.

3. Internal Organs, Intestinal Morphological and Hitology Assessment

On the last experimental day, two hens per replicate (total of 6 birds per treatment), the hens were weighed, anesthetized, and euthanized by decapitation. The weight of the proventriculus, gizzard, pancreas, liver, and spleen were recorded, the values were expressed in relation to the body weight of each hen (percentage of live body weight). The duodenum, jejunum and ileum were removed to determine the relative weight and length, the latter expressed in relation to the total size of the intestine.

Tissue samples (Duodenum, jejunum and ileum sections) were then collected and processed according to the method described by Laika and Jahanian (2017). First, a 2cm segment of the middle part of the sections was removed from all euthanized birds; tissue

samples were washed in physiological saline and fixed in 10% buffered formalin and then dehydrated by consecutive inclusion in graded ethanol solutions. The fixed samples were then embedded in paraffin. Cross-sectional and longitudinal sections, 5μm thick, were prepared using a microtome and stained with hematoxylin-eosin. Finally, prepared tissue slices were used for three histological observations per slice.

4. Short Chain Fatty Acids (SCFA) from the Cecum

The cecum was also removed from the hens to collect of cecal contents from all the euthanized hens (total of six hens per treatment). The cecal contents were collected into plastic centrifuge tubes separately per birds and stored at -20°C until the analysis of short chain fatty acids. Approximately 0.1g of the cecal pool per treatment was subsequently squeezed out into a 10ml falcon, pipetted 1.6ml of the cecal content samples; 0.4ml of a 3:1 solution of 25% metaphosphoric acid (Vetec Química Fina Ltda., Rio de Janeiro-RJ) with 98-100% formic acid (Merck KGaA, Darmstadt, Germany) plus 0.2ml of a solution of 100mm 2-ethyl-butyric acid (internal standard; MW = 116.16; CAS 88-09-5; Sigma Chemie Gmbh, Steinheim, Germany) and mixed thoroughly to obtain a uniform pooled sample (Maia et al., 2012). The concentrations of short chain fatty acids (acetic, propionic, butyric, isobutryic, valeric and isovaleric acids) were determined by gas-liquid chromatography as described by Ferreira et al. (2015). Analysis was performed with an HP-FFAP capillary column chromatograph (19091F-112; 25m, 0.320mm, 0.50μm, J&W Agilent Technologies Inc., Palo Alto, CA, USA). Each sample peak profile was integrated and quantified against an internal placed in the same sample.

5. Statistical Analysis

Effects of treatment on productive performance, egg quality, intestinal morphology, and histology characteristics were analyzed using analysis of variance by Proc ANOVA

(SAS, 2019). The tests were carried out at the 5% level of significance ($\alpha = 0.05$) and by the Brown and Forsythe's method, the values presented homogeneity of variance ($P > 0.05$) and the Shapiro-Wilk test showed no normal distribution ($P < 0.05$).

RESULTS

1. Performance

Dietary supllementation of purified lignin did not alter egg weight, feed intake and feed conversion per gram of eggs in all evaluated periods and cycles (Table 2). In the first experimental cycle, which occurred between 51 and 54 weeks of age, differences were observed in both egg mass (P= 0.047) and productivity (P= 0.003). The treatment without adding PL showed higher values than the treatment with 10g of PL/kg for these variables.

In the second experimental cycle, from 54 to 57 weeks, a difference was observed between treatments in the percentage of eggs laid in the nest (P= 0.006). The control treatment showed a higher percentage of eggs laid in the nest compared to the treatment with inclusion of 10g of PL/kg.

At the third experimental cycle, which covered the period from 57 to 60 weeks of age of the hens, no differences were observed in any of the evaluated productivity parameters (P > 0.05). In the last experimental cycle, which occurred between 60 and 63 weeks of age, differences were observed in egg production (P= 0.027) and in the percentage of eggs laid inside and outside the nest (P= 0.001) between treatments. The control treatment showed better results than the treatment that included 10g of PL/kg.

When analyzing data from the complete experimental period, from 51 to 63 weeks of age, no differences were found in the percentage of eggs placed inside and outside the nest, egg weight, feed intake and feed conversion. However, a statistically difference was found in egg mass (P= 0.033) and egg production (P= 0.001). The control treatment showed higher values than the treatment that included 10g of PL/kg.

Table 2 – Effects of purified lignin supplementation on the performance parameters of laying hens[1]

Period	Item	Experimental diets		SEM	P-Value
		Purified Lignin (g/kg of diet)			
		0	10.0		
Weeks 51 to 54	Egg laid in the nest, %	95.2	95.0	0.214	0.561
	Egg laid out of the nest, %	4.78	5.04	0.214	0.561
	Egg production, %	92.6	88.8	0.704	**0.003**
	Egg weight (g)	63.3	63.6	0.229	0.539
	Egg mass (g/bird per day)	58.4	56.5	0.492	**0.047**
	Feed intake (g/bird per day)	127.1	124.1	1.312	0.261
	Feed conversion ratio	2.15	2.20	0.019	0.221
Weeks 54 to 57	Egg laid in the nest, %	94.9	94.0	0.189	**0.006**
	Egg laid out of the nest, %	6.65	7.80	0.558	0.325
	Egg production, %	87.0	87.1	1.743	0.973
	Egg weight (g)	63.5	63.0	0.453	0.565
	Egg mass (g/bird per day)	59.3	58.2	0.555	0.330
	Feed intake (g/bird per day)	126.2	126.0	1.555	0.957
	Feed conversion ratio	2.20	2.19	0.026	0.753
Weeks 57 to 60	Egg laid in the nest, %	89.5	90.1	0.227	0.193
	Egg laid out of the nest, %	10.54	9.94	0.227	0.193
	Egg production, %	94.6	93.3	0.383	0.101
	Egg weight (g)	64.4	64.5	0.170	0.764
	Egg mass (g/bird per day)	60.9	60.2	0.294	0.220
	Feed intake (g/bird per day)	128.4	127.7	1.131	0.782
	Feed conversion ratio	2.11	2.16	0.020	0.203
Weeks 60 to 63	Egg laid in the nest, %	92.6	89.9	0.439	**<.0001**
	Egg laid out of the nest, %	7.37	10.08	0.439	**<.0001**
	Egg production, %	94.1	91.3	0.647	**0.027**
	Egg weight (g)	65.1	65.9	0.256	0.125
	Egg mass (g/bird per day)	61.2	60.2	0.298	0.066
	Feed intake (g/bird per day)	135.0	137.4	1.651	0.490
	Feed conversion ratio	2.20	2.28	0.028	0.171
Weeks 51 to 63	Egg laid in the nest, %	92.9	92.1	0.313	0.195
	Egg laid out of the nest, %	7.37	8.21	0.302	0.166
	Egg production, %	93.5	90.9	0.350	**<.0001**
	Egg weight (g)	64.1	64.3	0.178	0.731
	Egg mass (g/bird per day)	60.1	59.1	0.243	**0.033**
	Feed intake (g/bird per day)	129.2	128.8	0.862	0.830
	Feed conversion ratio	2.16	2.21	0.013	0.071

[1] Data are means for three replicates of 50 laying hens/replicate.

2. Egg quality

The experiment lasted 12 weeks, with analyzes of egg quality every three weeks (Table 3). In all the analyzes carried out, no differences were found for the variables of thickness and strength egg, as well as for the percentage of albumen and yolk.

Conversely, in the third experimental cycle, a difference was observed in specific gravity (P=0.001) and eggshell percentage (P=0.007). In this case, the treatment with the inclusion of 10g of PL/kg showed higher means than the control treatment.

Table 3 – Effects of purified lignin supplementation on egg quality of laying hens[1]

Period	Egg quality	Experimental diets		SEM	P-Value
		Purified Lignin (g/kg of diet)			
		0	10.0		
Weeks 51 to 54	Egg specific gravity (g/ml)	1.089	1.090	0.524	0.273
	Shell thicknees (mm)	37.5	38.1	0.246	0.257
	Shell strength (kgf)	4.00	4.13	0.067	0.345
	Shell, %	9.51	9.69	0.076	0.257
	Yolk, %	28.0	27.9	0.230	0.841
	Albumen, %	62.5	62.4	0.256	0.883
Weeks 54 to 57	Egg specific gravity (g/ml)	1.088	1.087	0.463	0.544
	Shell thicknees (mm)	37.6	37.0	0.239	0.215
	Shell strength (kgf)	4.28	4.21	0.079	0.676
	Shell, %	9.36	9.22	0.077	0.386
	Yolk, %	27.7	27.5	0.242	0.684
	Albumen, %	62.9	63.3	0.263	0.529
Weeks 57 to 60	Egg specific gravity (g/ml)	1.087	1.090	0.393	**0.001**
	Shell thicknees (mm)	37.1	37.8	0.204	0.071
	Shell strength (kgf)	4.28	4.35	0.072	0.621
	Shell, %	9.42	9.79	0.071	**0.007**
	Yolk, %	26.7	26.4	0.234	0.583
	Albumen, %	63.9	63.8	0.260	0.848
Weeks 60 to 63	Egg specific gravity (g/ml)	1.087	1.087	0.469	0.921
	Shell thicknees (mm)	36.8	37.7	0.302	0.129
	Shell strength (kgf)	4.15	4.24	0.071	0.540
	Shell, %	9.35	9.50	0.067	0.272
	Yolk, %	26.9	27.3	0.254	0.385
	Albumen, %	63.9	63.2	0.275	0.217

[1]Data are means for three replicates of 12 eggs/replicate.

When analyzing the values of MDA in egg yolks along the experimental cycles (Table 4), differences were observed in all periods (P<0.005). Treatment with inclusion of PL showed lower values of MDA in eggs compared to treatment without PL.

Table 4 – Effects of purified lignin supplementation malondialdehyde (MDA) content in egg yolk of laying hens[1]

Egg quality	Period	Experimental diets		SEM	P-Value
		Purified Lignin (g/kg of diet)			
		0	10.0		
MDA[2] (ng/g)	Weeks 51 to 54	17.86	5.46	0.278	**<.0001**
	Weeks 54 to 57	6.71	4.50	0.050	**<.0001**
	Weeks 67 to 60	5.09	4.55	0.013	**0.016**
	Weeks 60 to 63	4.98	4.58	0.010	**0.018**

[1]Data are means for three replicates of six eggs/replicate.
[2]MDA - Malondialdehyde

3. Internal Organs, Intestinal Morphology and Histology

At the end of experimental period, the organs of the slaughtered hens were analyzed. In this analysis, not statistically differences were found in the length of the small intestine, as well as in the weight of the proventriculus, gizzard, pancreas, liver, duodenum, and ileum between the evaluated groups.

However, a statistically difference was observed in jejunum (P=0.031) and spleen (P=0.022) weight. The treatment with inclusion of purified lignin showed a higher weight in these organs compared to birds fed basal diet.

Table 5 – Effects of purified lignin supplementation on the internal organs and morphology parameters of laying hens[1].

Internal organs	Experimental diets		SEM	P-Value
	Purified Lignin (g/kg of diet)			
	0	10.0		
Length				
Duodenum, %	20.6	20.8	0.678	0.909
Jejunum, %	41.3	40.3	1.202	0.722
Ileum, %	38.6	41.5	1.077	0.188
Weigth				
Proventriculus, %	0.41	0.42	0.012	0.847
Gizzard, %	2.33	2.41	0.057	0.561
Pancreas, %	0.20	0.25	0.013	0.057
Liver, %	2.58	2.86	0.129	0.294
Spleen, %	0.08	0.11	0.007	**0.022**
Duodenum, %	0.71	0.76	0.022	0.232
Jejunum, %	1.21	1.52	0.075	**0.031**
Ileum, %	0.81	0.88	0.039	0.429

[1] Data are means for six replicates of two laying hens/replicate

During the analysis of the length of the villi and the depth of the crypts in the small intestine, measurements were taken and the relationships between these parameters were calculated (Table 6). Villus length values did not show statistically difference between the duodenum and ileum sections. Though, a significant difference was observed in the jejunum section.

As for the depth of the crypts and its relation to the length of the villi, no differences ($P>0.05$) were found. This indicates that, there were no significant variations in the structure of small intestine villi and crypts between the treatments analyzed.

Table 6 – Effects of purified lignin supplementation on intestine histology in laying hens[1].

Histology	Intestinal segment	Experimental diets		SEM	P-Value
		Purified Lignin (g/kg of diet)			
		0	10.0		
Villus height (μm)	Duodenum	1451	1476	15.036	0.426
	Jejunum	996	1198	38.710	**0.001**
	Ileum	623	559	26.944	0.255
Crypt depth (μm)	Duodenum	219	192	8.937	0.135
	Jejunum	175	162	7.090	0.392
	Ileum	105	94	3.988	0.170
Villus:crypt	Duodenum	6.91	7.77	0.244	0.075
	Jejunum	7.26	6.62	0.330	0.359
	Ileum	5.65	5.99	0.230	0.493

[1] Data are means for six replicates of two laying hens/replicate

4. Short Chain Fatty Acids (SCFA) from the Cecum

The analysis of the concentration of short chain fatty acids (Table 7) revealed that the inclusion of purified lignin did not have a statistically significant impact on the treatments evaluated. No statistically differences (P>0.05) were observed in the total concentration of SCFA and in acetic, propionic, butyrate, isobutyrate, valeric and isovaleric acids between the experimental groups.

Table 7 – Effects of purified lignin supplementation on the concentrations of short chain fatty acids (SCFAs) from the cecum of laying hens[1].

Concentrations of SCFAs	Experimental diets		SEM	P-Value
	Purified Lignin (g/kg of diet)			
	0	10.0		
SCFA (mmol/L of wet cecal content)				
Total SCFA	28.9	25.7	1.946	0.488
Acetic acid	21.3	18.7	1.360	0.393
Propionic acid	5.13	5.53	0.511	0.740
Butyric acid	0.94	0.96	0.176	0.962
Isobutyric acid	0.35	0.42	0.036	0.420
Valeric acid	0.52	0.66	0.065	0.335
Isovaleric acid	0.57	0.55	0.036	0.813

[1] Data are means for six replicates of two laying hens/replicate

DISCUSSION

Currently, there is a scarcity of studies that seek to investigate the effects of using polyphenols in the diet of laying hens in *cage-free* systems. Data from this study provide

an analysis of the impacts of supplementation or absence of purified lignin in laying hens aged between 51 and 63 weeks. Evaluating productivity, egg quality and intestinal morphology, and histology are the factors considered for this study.

Effective understanding of the performance parameters in laying hen production plays a crucial role in assessing economic impacts for the poultry industry. In the current study, a greater productivity and a egg mass were observed in the group that did not receive purified lignin supplementation during the first week. Nonetheless, this difference was not maintained in the subsequent weeks, but favored the maintenance of this result when considering the entire experimental period. These results indicate that purified lignin supplementation impaired the production of laying hens in the *cage-free* system.

However, when comparing the results obtained in this study with data from the strain manual, we observed that the expected productivity for the period from 51 to 63 in a conventional system under optimal conditions would be 87% to 82%, with an average of 84.5% (Commercial Hy Line W-36, 2020). Though, even with the inclusion of purified lignin and the finding of lower productivity, the average productivity obtained in this study was 90.9%. This indicates that, although purified lignin impairs productivity, it is still possible to achieve excellent chicken productivity in a *cage-free* system compared to conventional systems.

Roberson et al. (2005) conducted a study, using the Hy-Line W-36 strain, the control group received a diet composed of basic ingredients according to the nutritional recommendations of the manual for this strain for laying hens reared in a conventional system. Productivity values of 84.6%, 86.8%, 82.4%, 82.1% and 80.7% were recorded for weeks 50-51, 52-53, 54-56, 58-60 and 61-63, respectively. Regarding egg mass, the values were 54.7%, 55.9%, 52.6%, 53.9% and 53.7% for the weekly ages of 51, 53, 55,

60 and 63, respectively. These values were lower than those found in the present study carried out in the *cage-free* rearing system.

During weeks 54 to 57 and 60 to 63 of the hens age, statistically differences were observed in the percentages of eggs laid in and out of the nest. These results indicate that hens that received the addition of purified lignin in their diet had a lower rate of eggs laid in nests compared to the control group.

In the study by Abrahamsson and Tauson (1998), it was reported that the percentage of eggs laid outside the nest varies from 1% to 18%, depending on the type and attractiveness of the nest, as well as the material used as bedding. In the present study, the variation during the entire experimental period was 7.37% for the control treatment and 8.21% for the treatment with PL inclusion.

The laying of eggs outside the nest is a crucial factor that negatively impacts the profitability of the sector, since it is associated with problems such as a higher percentage of dirty eggs, bacterial contamination, an increase in the rate of broken eggs and work overload due to the need to manual collection of eggs (Tuyttens et al., 2013; Bovera et al., 2014; Englmaierova et al., 2014).

In the third experimental cycle there was a statistical difference for the variables of egg specific gravity and shell percentage, in which the treatment with inclusion of purified lignin had higher values compared to the control treatment. After supplementation with 50mg of curcumin/kg for 21 days, 30-week-old laying hens reared in a conventional system showed improvements in egg specific gravity and yolk index (Galli et al., 2018).

Shell quality is a feature of great economic importance related to the incidence of cracked eggs that can harm commercial earnings. Indeed, a desirable shell must be thick enough to withstand the shock of transport and handling. In the literature, many authors

have not detected differences in most shell characteristics in relation to housing systems (Shimmura et al., 2007 and 2010; Stojcic et al., 2012; Samiullah et al., 2017; Lordelo et al., 2016; Yilmaz-Dikmen et al., 2017).

During the entire experimental period, no differences were observed for the variables of shell thickness, shell strength, percentage of yolk and albumen in relation to the inclusion of purified lignin. These results indicate that the addition of purified lignin does not affect the physical and quantitative quality of eggs from hens reared in a *cage-free* system.

The addition of purified lignin showed, throughout the entire experimental period, a lower concentration of MDA compared to the control treatment. This suggests that there was a reduction in the amount of oxidized unsaturated fatty acids, indicating a lower occurrence of lipid oxidation processes and greater yolk stability. Such a reduction is considered desirable, since lipid oxidation can negatively affect egg quality and freshness, resulting in unpleasant flavors and odors (Meynier et al., 2014; Ren et al., 2013).

In the study conducted by Romero et al. (2022), the supplementation of 60g of Grape Pomace/kg, a product rich in polyphenols, was associated with a reduction in the concentration of MDA in the egg yolk of laying hens reared in a conventional system. The researchers suggest that gut bacteria metabolize these polyphenols, resulting in smaller-sized phenolic compounds that are more easily absorbed and more probable to reach animal tissues. Once in target tissues, these bioactive metabolites exert an antioxidant effect.

The inclusion of 10.0g of PL/kg in the diet of laying hens reared in a *cage-free* system resulted in a change in spleen weight. This lymphoid organ plays a stimulating role in the maturation of immune system cells, such as B and T lymphocytes, and in the production

of antibodies (Yahfoufi et al., 2018; Meng et al., 2023; Zhou et al., 2021). These results suggest that purified lignin may have a potential role in promoting the immune response of hens.

Fang Chen et al. (2021) have investigated the effect of magnolol supplementation in the diet of laying hens reared in a conventional system. The results showed that the addition of 100 and 200mg of magnolol/kg resulted in a significant increase in the relative weight of the spleen compared to the group without supplementation of this polyphenol. This increase in spleen weight suggests a possible more effective immune response in these birds.

In the present study, there was an increase in the relative weight and length of the jejunum villi when there was supplementation of purified lignin in the diet of laying hens. This greater relative weight is probably linked to the greater length of the villi. Increasing villus height can increase nutrient digestibility (Rattanawut et al., 2018). However, there was no greater crypt depth, which would be indicative of greater regenerative activity and cell turnover. This demonstrates there was a greater redirection of nutrients to mucosal growth, costing the energy needed for performance (Mehri et al., 2015).

When investigating the effects of resveratrol supplementation at dosages of 300 and 600mg/kg in broiler chickens, Mohebodini et al. (2019) observed a significant increase in the length of villi and crypts of the jejunum segment. This improvement in gut structure has resulted in better nutrient absorption and better gut health in birds. Nonetheless, this alteration was not found in the present study, indicating the type of polyphenol used can bring different results in terms of animal performance.

SCFAs are metabolites generated by bacterial fermentation in the hindgut, composed mainly of acetate, propionate, and butyrate (Frost et al., 2014; Ríos-Covián et al., 2016).

They play an important role in regulating intestinal health in birds, being absorbed, and used by intestine cells as a source of energy (Turner J.R., 2009; Kumar et al., 2020). Furthermore, SCFAs have antimicrobial properties, inhibiting the invasion and colonization of pathogens (Fukuda et al., 2011; Wen et al., 2012). In the present study, the supplementation of purified lignin in the diet of hens in a *cage-free* system showed no statistical difference in the production of SCFAs, indicating that purified lignin does not affect the bacterial fermentation in the hindgut.

CONCLUSION

The inclusion of 10.0g of purified lignin/kg in the diet of hens at the age between 51 and 63 weeks raised in the *cage-free* system resulted in inferior performance during this period when compared with the treatment control. However, an improvement in the quality of the eggs was observed, due to its antioxidant effect, without impact on the intestinal morphology, and histology of the hens.

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CAPÍTULO 4

"Effect of purified lignin on performance, egg quality, gut morphology, and histology of laying hens at *cage-free* system under heat stress"

Effect of purified lignin on performance, egg quality, gut morphology, and histology of laying hens at *cage-free* system under heat stress

A.B.S. Oliveira[1]

[1] School of Veterinary Medicine and Animal Science, São Paulo State University, Botucatu, SP, 18610-034, Brazil

Key words: phenolic, antioxidant, gut, short chain fatty acids, physiology

Primary audience: nutritionists, researchers, and feed formulators

* Corresponding author: ana.bs.oliveira@unesp.br

SUMMARY

The experiment objective was to evaluate the effects of purified lignin (PL) on productivity, egg quality and intestinal morphology and histology of layers in a *cage-free* system under high temperatures. 300 Hy-Line W-36 hens aged between 63 to 66 weeks were used to compose two treatments, with three replications and 50 birds per pen. The two diets consisted of a control diet and a test diet supplemented with 1% of PL. The egg production of laying hens was measured per day, feed intake, egg weight, egg mass and feed conversion ratio were recorded at the end of 21 days. A total of five eggs/replication/day were collected for the last two days of each period, total of 30 eggs per treatment to evaluate interior and exterior egg quality parameters. The malondialdehyde (MDA) content was measured in the egg yolk. At the end of the experimental period the two hens per replicate were sacrificed to determine the weight of the internal organ, intestinal morphology and histology and short-chain fatty acids through the collect of cecal contents. In productive and performance parameters, the treatments showed $P<0.05$. PL supplementation had negative impacts on feed conversion ratio and the proportion of eggs laid inside and outside the nests. Regarding egg quality parameters, there were $P<0.05$, PL supplementation increased the percentage of shell but reduced the percentage of yolk. MDA concentration showed $P<0.05$ during acute heat stress. Though no effect was observed ($P>0.05$) when exposure to high temperatures was prolonged. In the analyzes regarding intestinal histology, $P<0.05$ were noted in the length of the Duodenum villi. In conclusion, the supplementation of 10.0g of PL/kg in the diet of hens in a *cage-free* system under heat stress did not affect the performance and intestinal morphology of the laying hens. However, a beneficial effect on egg yolk lipid peroxidation was observed during acute stress.

DESCRIPTION OF PROBLEM

Stress occurs when an animal experiences a change that can be physical, environmental, emotional, or social, in which it stimulates the body's responses to stressful stimuli, through physiological processes to reestablish homeostatic conditions. (Freeman, 1976; Lara and Rostagno, 2013). Heat stress caused by high ambient temperature is a serious welfare problem and causes economic losses due to compromised health and productive status of animals (Siegel, 1980; Downing and Bryden, 1999; Mashaly et al., 2004).

Reducing feed intake is one of the causes when there is a high environmental temperature, leading to a reduction in nutrient bioavailability (Kilic and Simsek, 2013; Azis et al., 2012). Studies have found that heat stress can cause morphological changes in the intestinal epithelium, negatively affecting digestion and nutrient absorption, in addition to affecting intestinal epithelial integrity, which may facilitate the invasion of pathogens (Sun et al., 2015; Mishra and Jha et al., 2019).

Among all animals production, poultry is the most sensitive to heat stress due to their low ability to dissipate body heat (Lara and Rostagno, 2013). Tumova and Gous (2012) have shown that the ideal environmental temperature for laying hens is around 20°C to 25°C. The classification of heat stress in birds typically includes the following types: acute, which refers to temperatures ranging from 27 to 38°C for a duration of 1 to 24 hours; moderate, which encompasses temperatures between 27 and 38°C for up to 7 days; and chronic or severe, which involves temperatures ranging from 38 to 50°C for 7 days or more (Mujahid et al., 2007; Sohail et al., 2012; Zmrhal et al., 2018).

In the thermoregulation process, there is an increase in mitochondrial energy generation, which is intrinsically associated with an increase in the production of free

radicals (Mujahid et al., 2007; Akbarian et al. 2016). The overproduction of free radicals subsequently causes disruption of the antioxidant system, impairing the control of various physiological processes, the main process being the maintenance of homeostasis (Lin et al., 2006; Dröge, 2002; Surai et al., 2019).

Therefore, many aspects of hen physiology have been explored in relation to the heat stress response. Some nutritional strategies aim to reduce the negative effects of heat stress through the supplementation of natural compounds with antioxidant and antimicrobial potential, which has become a common practice in the poultry industry (Zhao, et al., 2011; Liu et al., 2020).

Polyphenols from purified lignin are antioxidant compounds that are isolated during the purification process of lignin, whith is a polymer present in plants that confers rigidity and resistance to cell walls (Boudet and Grima-Pettenati, 1996; Dizhbite et al., 2004). These polyphenols have free radical scavenging properties, protection from oxidative damage, and may have anti-inflammatory and antimicrobial effects (Baurhoo et al., 2007). Therefore, they have been studied and used as functional additives in animal feed.

Given the above, the premise of this study was to evaluate the effects of purified lignin and its antioxidant potential in the diet of laying hens in a *cage-free* system, under thermal stress, through productive, physiological and egg quality parameters.

MATERIAL AND METHODS

The experimental procedures were approved by the Institutional Animal Care and Use Committee, of the School of Veterinary Medicine and Animal Sciences of the São Paulo State University (FMVZ/UNESP), Botucatu, SP, Brazil (protocol number: 0155/2020).

1. Animals, Diets, and Housing

The study was conducted at the facilities of the FMVZ/UNESP (geographical coordination: latitude 22° 49' 07" S and longitude 48° 24' 40" W). In total, 300 commercial Hy-Line W-36 laying hens with uniform body weight were used in the experiment. The experimental period was 21 days, with high temperature (33 ± 2 °C) for 8h/day, from 08:00 to 17:00h (heat stress, HS), followed by 22°C for 16h. In order to validate the presence of heat stress in the animals, thermographic images were taken, rectal temperature and respiratory rate were measured - data not shown in this work. The hens were reared on the floor, without access to a picket, with a galvanized steel nest (five birds per nest spot), with a density of seven birds/m², in a *cage-free* system.

The laying hens were assigned to two treatments with three replications of 50 hens. In the control group were given a corn-soybean meal-basal diet (Table 1) in mash form without purified lignin. The remaining group was given the same basal diet supplemented with an additional of 10g of purified lignin/kg.

The nutrition program consisted of one isonutritive diet formulated to meet the nutrients requirements for layer hens adapted according to the recommendations of the breeder (Hy-Line W-36, 2011) and Rostagno et al. (2017). Feed and water were provided *ad libitum* during the experiment. The experiment was carried out between 63 to 66 weeks of age (three weeks experimental period). The lighting regimen was 16 hours of continuous light per day from 06:00 to 22:00.

Table 1 – Composition of basal diet.

Ingredient	Amount (g/kg)
Corn, grain	635.36
Soybean meal (45% CP)	243.81
Limestone	95.3
Di - Calcium phosphate	12.73
DL - Methionine	4.55
Sodium chloride	4.25
Vitamins-minerals premix[1]	4.00
Total	1000
Calculated chemical composition	
Dry Matter (%)	89.6
Crude Protein (%)	16.4
Methionine (%)	0.70
Lysine (%)	0.82
Ether Extract (%)	2.4
Calcium (%)	4.0
Phosphorus Total (%)	0.52
Metabolizable energy (MJ/kg)	11.4

[1] Provided per kilogram of diet: vitamin A, 8800 IU; vitamin D3, 2500 IU; vitamin E, 11 IU; vitamin K3, 2.2 IU; thiamin, 1.5 mg; riboflavin, 4 mg; nicotinic acid, 7.85 mg; pantothenic acid, 34.65 mg; pyridoxine, 2.46 mg; folic acid, 0.48 mg; vitamin B12, 4 mg; manganese 74.4 mg; zinc, 64.67 mg; iron, 75 mg; copper, 6 mg; iodine, 0.86 mg; selenium, 0.2 mg; choline chloride, 200 mg.

The purified lignin used in this study was extracted from *Eucalyptus urograndis* wood, being a by-product of the Kraft process of cellulose production (reaction of wood with sodium hydroxide (NaOH) + sodium sulfide (Na_2S)), in the form of a brown powder. The product was composed of lignin (> 95%), total phenolic compounds (4.6%): syringyl-OH (2.3%), guaiacyl-OH (condensed) (1.11%), guaiacyl-OH (non-condensed) (1%) and hydroxyphenyl-OH (0.14%); pH 3 - 5, molar mass 1300 to 1400, moisture (1.92%), ash (2.42%), 61.1% total carbon, 31.3% oxygen, 5.5% hydrogen, 1.7% sulfur and 0.4% nitrogen and 0.99% carbohydrates. Purified lignin was added on top and to ensure that the diets remained isonutritive, sand was supplemented, as an ingredient with no nutritional value, to ensure uniform dilution of all diets used in the study.

2. Performance and Egg Quality

For this experimental procedure the hens were at 63rd and 66th week of age. Egg production (%) was obtained by recording the number of eggs produced daily throughout the experimental period. The feed intake and feed conversion ratio were recorded in the end of 21 days. The feed conversion ratio (FCR) was expressed in grams of feed consumed per grams of egg produced. All production variables were determined on a replicate basis. All eggs collected in the last day of period were weighed to the nearest 0.01g. The average egg weight was multiplied by the total number of eggs produced during the experimental period, thus obtaining the total egg mass. This value was then divided by the total number of hens per replicate for 21-day period.

A total of five eggs/replication/day were collected for two days after the first day with heat stress and two days after the end of the experimental period, a total of 30 eggs per treatment, were analyzed to evaluate internal and external egg quality parameters. Specific gravity was measured by immersing eggs in saline solutions of 1.050 to 1.100g/ml (Moreng and Avens, 1990), at 0.005 intervals. The eggs were classified according to their specific gravity when they floated (Albino et al., 2014). Eggshells were washed and dried at 60°C for 48 hours, after which they were weighed. Shell thickness was measured to the nearest 0.01mm in tree points at the middle-transversal area of the shell, from which an average measure expressed in millimeters (mm) using a digital caliper. Shell strength, evaluated with the support of a texturometer TA. XT plus (Texture Analyzer TA-XT Plus, Surrey, England), equipped with a 75mm probe (p/2) and test speed of $1mm/s^1$, which will be expressed in kfg/cm^2. Percentage of yolk, albumen, and shell, calculated by the ratio between the mass of the respective constituents and the mass of the egg.

The contents of malondialdehyde (MDA) were assayed using colorimetric methods with a spectrophotometer (722N, Shanghai Precision and Scientific Instrument Co. Ltd. Shanghai, P.R. China). For this parameter, five eggs per replicate was collected two days after the first day with heat stress and the end of the experimental period and the egg yolk were analyzed using the assay kits purchased from Nanjing Jiancheng Institute of Bioengineering (Nanjing, Jiangsu, P.R. China) and the procedures accordingly. All samples were measured in triplicate, at appropriate dilutions, to give activities of the enzymes in the linear range of standard curves constructed with pure enzymes. The values are expressed in ng per g.

3. Internal Organs, Morphological and Histology Assessment

On the first and last experimental day, two hens per replicate (total of 6 birds per treatment), the hens were weighed, anesthetized, and euthanized by decapitation. The weight of the proventriculus, gizzard, pancreas, liver, and spleen were recorded, the values were expressed in relation to the body weight of each hen (percentage of live body weight). The duodenum, jejunum and ileum were removed to determine the relative weight and length, the latter expressed in relation to the total size of the intestine.

Tissue samples (Duodenum and jejunum sections) were then collected and processed according to the method described by Laika and Jahanian (2017). First, a 2cm segment of the middle part of the duodenum and jejunum was removed from all euthanized birds; tissue samples were washed in physiological saline and fixed in 10% buffered formalin and then dehydrated by consecutive inclusion in graded ethanol solutions. The fixed samples were then embedded in paraffin. Cross-sectional and longitudinal sections, 5μm thick, were prepared using a microtome and stained with hematoxylin-eosin. Finally, prepared tissue slices were used for three histological observations per slice.

4. Short Chain Fatty Acid (SCFA) from the Cecum

The cecum was also removed from the hens to collect of cecal contents from all the euthanized birds (total of six birds per treatament). The cecal contents were collected into plastic centrifuge tubes separately per birds and stored at -20°C until the analysis of short chain fatty acids. Approximately 0.1g of the cecal pool per treatement was subsequently squeezed out into an falcon 10ml, pipetted 1.6ml of the cecal contents sample; 0.4ml of a 3:1 solution of 25% metaphosphoric acid (Vetec Química Fina Ltda., Rio de Janeiro-RJ) with 98-100% formic acid (Merck KGaA, Darmstadt, Germany) plus 0.2ml of a solution of 100 mm 2-ethyl-butyric acid (internal standard; MW = 116.16; CAS 88-09-5; Sigma Chemie Gmbh, Steinheim, Germany) and mixed thoroughly to obtain a uniform pooled sample (Maia et al., 2012). The concentrations of short chain fatty acids (acetic, propionic, butyric, isobutryic, valeric and isovaleric acids) were determined by gas-liquid chromatography as described by Ferreira et al. (2015). Analysis was performed with an HP-FFAP capillary column chromatograph (19091F-112; 25m, 0.320mm, 0.50μm, J&W Agilent Technologies Inc., Palo Alto, CA, USA). Each sample peak profile was integrated and quantified against an internal placed in the same sample.

5. Statistical Analysis

Effects of treatment on productive performance, egg quality, intestinal morphology, and histology characteristics were analyzed using analysis of variance by Proc ANOVA (SAS, 2019). The tests were carried out at the 5% level of significance ($\alpha = 0.05$) and by the Brown and Forsythe's method, the values presented homogeneity of variance ($P > 0.05$) and the Shapiro-Wilk test showed no normal distribution ($P < 0.05$).

RESULTS

The results obtained in this study demonstrate the influence of tropical temperature on the productivity of laying hens over a period of three weeks. Table 2 presents the data related to this productivity. It is evident that there are statistically differences in relation to the number of eggs laid inside (P<0.0001) and outside the nest (P<0.0001), as well as in the feed conversion ration of the hens, when comparing diets supplemented with and without purified lignin.

Table 2 – Effects of purified lignin supplementation on the performance parameters of laying hens with 63 to 66 weeks of age at *cage-free* system under heat stress[1]

Item	Experimental diets		SEM	P-Value
	Purified Lignin (g/kg of diet)			
	0	10.0		
Egg laid in the nest, %	92.3	89.4	0.460	**<.0001**
Egg laid out of the nest, %	8.33	11.21	0.423	**<.0001**
Egg production, %	97.1	93.8	1.109	0.145
Egg weight (g)	66.0	66.3	0.189	0.520
Egg mass (g/bird per day)	64.1	62.3	0.728	0.224
Feed intake (g/bird per day)	136.3	140.3	2.197	0.380
Feed conversion ratio	2.12	2.25	0.035	**0.047**

[1] Data are means for three replicates of 50 laying hens/replicate.

In the treatment without the inclusion of purified lignin, a greater number of eggs laid inside the nests and a smaller number of eggs laid outside were observed, in comparison with the treatment with the inclusion of 10.0g of PL/kg in the diet. In addition, it was found that the treatment with the inclusion of PL resulted in a higher feed conversion ratio per gram of eggs laid (P<0.047), compared to the treatment without purified lignin. However, not statistically differences (P>0.05) were found for the variables production, weight, and mass of the eggs, as well as for feed intake.

Tables 3 and 4 provide comprehensive information on egg quality, addressing physical characteristics and the presence of MDA, allowing a complete analysis of the effects of

acute and prolonged stress, as well as supplementation with purified lignin, on egg quality.

Table 3 – Effects of purified lignin supplementation on egg quality of laying under heat stress[1]

Egg quality	Experimental diets		SEM	P-Value
	Purified Lignin (g/kg of diet)			
	0	10.0		
Acute stress				
Egg specific gravity (g/ml)	1.085	1.087	0.626	0.070
Shell thicknees (mm)	38.2	38.8	0.297	0.295
Shell strength (kgf)	4.13	4.20	0.078	0.680
Shell, %	9.49	9.75	0.064	**0.038**
Yolk, %	27.8	26.9	0.227	**0.048**
Albumen, %	62.8	63.5	0.257	0.194
Chronic stress - weeks 63 to 66				
Egg specific gravity (g/ml)	1.088	1.089	0.395	0.622
Shell thicknees (mm)	38.3	38.8	0.310	0.506
Shell strength (kgf)	4.49	4.30	0.094	0.322
Shell, %	9.55	9.64	0.078	0.561
Yolk, %	27.0	27.1	0.235	0.794
Albumen, %	63.5	63.3	0.231	0.641

[1]Data are means for three replicates of 12 eggs/replicate.

Two days after the onset of high temperatures, egg quality was evaluated to verify whether acute stress affected the quality of eggs from hens supplemented or not with purified lignin. The results obtained revealed that there was not statistically difference ($P>0.05$) in the variables of egg specific gravity, shell thickness, shell strength and albumen percentage. However, there was a statistically difference in the percentage of shell ($P<0.038$) and yolk ($P<0.048$), and the treatment without inclusion of purified lignin showed a higher percentage in these two variables.

When investigating the effect of lipid peroxidation during acute stress, a statistically difference ($P<0.001$) was found in the amount of MDA found in eggs shell. Eggs from hens supplemented with purified lignin proved to be more efficient in maintaining lower MDA values.

Table 4 – Effects of purified lignin supplementation malondialdehyde (MDA) content in egg yolk of laying hens under heat stress[1]

Egg quality	Period	Experimental diets Purified Lignin (g/kg of diet) 0	10.0	SEM	P-Value
MDA[2] (ng/g)	Acute stress	50.7	42.9	0.018	**0.001**
	Chronic stress - weeks 63 to 66	52.4	46.9	0.043	0.582

[1]Data are means for three replicates of six eggs/replicate.
[2]MDA - Malondialdehyde

There was no effect of prolonged heat stress on the quality of eggs from hens supplemented or not with purified lignin. Even when examining the effect of lipid peroxidation on the yolk of these eggs, not statistically differences (P>0.05) were observed in the amount of MDA formed in the yolk.

Digestive organs weight values and small intestine length, as shown in Table 5, did not show statistically differences (P>0.05) between treatments with addition or not of PL.

Table 5 – Effects of purified lignin supplementation on the internal organs and morphology parameters of laying hens with 66 weeks old under heat stress[1]

Internal organs	Experimental diets Purified Lignin (g/kg of diet) 0	10.0	SEM	P-Value
Length				
Duodenum, %	22.7	22.0	0.647	0.570
Jejunum, %	48.5	43.9	1.425	0.112
Ileum, %	46.7	45.1	1.688	0.651
Weigth				
Proventriculus, %	0.39	0.39	0.014	0.955
Gizzard, %	2.37	2.70	0.106	0.117
Pancreas, %	0.23	0.22	0.009	0.522
Liver, %	2.51	2.48	0.091	0.858
Spleen, %	0.10	0.08	0.006	0.277
Duodenum, %	0.75	0.84	0.051	0.414
Jejunum, %	1.49	1.55	0.098	0.771
Ileum, %	1.17	1.12	0.102	0.802

[1] Data are means for six replicates of two laying hens/replicate

Examining the length of the villi and crypts, as well as the relationship between them in the different portions of the small intestine, as shown in Table 6, a difference was observed only for the average length of the villi in the Duodenum portion. However, not

statistically differences (P>0.05) were found in the other portions of the small intestine or in the relationship between villi and crypts in any of the evaluated portions.

Table 6 – Effects of purified lignin supplementation on intestine histology in laying hens with 66 weeks old under heat stress[1]

Histology	Intestinal segment	Experimental diets		SEM	P-Value
		Purified Lignin (g/kg of diet)			
		0	10.0		
Villus height (μm)	Duodenum	1661	1458	39.50	**0.003**
	Jejunum	1124	953	80.83	0.316
	Ileum	820	759	42.68	0.515
Crypt depth (μm)	Duodenum	173	181	4.619	0.427
	Jejunum	132	141	9.405	0.646
	Ileum	125	133	6.423	0.590
Villus: crypt	Duodenum	9.30	8.11	0.312	0.051
	Jejunum	7.73	7.74	0.097	0.964
	Ileum	6.53	6.06	0.289	0.442

[1] Data are means for six replicates of two laying hens/replicate.

The results of the total concentration of SCFA and acetic, propionic, butyric, isobutyric, valeric and isovaleric acids, as presented in Table 7, did not show statistical difference (P>0.05) when comparing the diets with and without PL supplementation.

Table 7 – Effects of purified lignin supplementation on the concentrations of short chain fatty acids (SCFAs) from the cecum of laying hens with 66 weeks old under heat stress[1].

Concentrations of SCFAs	Experimental diets		SEM	P-Value
	Purified Lignin (g/kg of diet)			
	0	10.0		
SCFA (mmol/L of wet cecal content)				
Total SCFA	32.4	31.4	4.670	0.928
Acetic acid	22.5	22.0	2.808	0.933
Propionic acid	4.66	6.67	0.802	0.248
Butyric acid	1.42	1.72	0.466	0.785
Isobutyric acid	0.11	0.12	0.018	0.938
Valeric acid	0.39	0.38	0.048	0.884
Isovaleric acid	0.30	0.33	0.076	0.867

[1] Data are means for six replicates of two laying hens/replicate

DISCUSSION

Laying eggs in the nest is an essential priority for hens and plays a significant role in their welfair (Lay et al., 2011; Cronin et al., 2012; Widowski et al., 2013). This study investigated the performance of laying hens between 63 and 66 weeks of age under stressful temperature conditions and revealed that the addition of purified lignin to the diet of these hens affects laying location and feed efficiency.

The inclusion of PL in the diet resulted in an increase in out-of-nest egg laying, indicating that the hens were subject to greater environmental stress. This stressor also led to a higher feed intake per gram of eggs produced. However, despite the higher feed intake, hens were less efficient in laying eggs.

A broiler study conducted by Zhang et al. (2018) analyzed the use of curcumin in a stressful environment and its effects on bird performance. It was observed that this polyphenol improves the performance of broiler chickens. However, there was a higher feed conversion in the group receiving the additive compared to the control group.

No statistically differences were found between the groups that supplemented or not with PL in terms of feed intake and production, mass, and weight of eggs, in this study. This indicates that the addition of PL to the hens' diet did not have a statistically impact on egg production.

To combat heat stress and regulate body temperature, birds resort to panting as a thermoregulation mechanism, increasing the respiratory rate, and promoting evaporative cooling of the respiratory tract. Compared to birds reared in thermoneutral conditions, blood pH increases significantly due to high ambient temperature and panting. This increase in plasma pH, together with respiratory alkalosis, results in decreased levels of

ionized calcium in the blood, which is the form used in eggshell formation (Teete et al., 1985; Mahmoud et al., 1996; Barrett et al., 2019; Boukherroub and Noll, 2022).

In this study, it was observed that the percentage of eggshells was lower in the group that did not receive PL inclusion. This suggests that the addition of PL in the diet may contribute to maintaining eggshell quality. However, during the period of acute stress caused by elevated temperatures, the inclusion of PL did not have a statistically impact on egg specific gravity, shell thickness, shell strength and albumen percentage.

Ebeid et al. (2012) conducted a study on laying hens exposed to high temperature conditions for one month. A reduction was observed in egg quality parameters, including weight, density, and shell thickness. These results obtained collaborate with what was found in this study, indicating that thermal stress negatively affects the eggshell quality of laying hens.

A statistically difference was also observed in the percentage of egg yolk. This variation may be related to the low feed efficiency observed in eggs from hens that received diets supplemented with purified lignin. It is well established that a lower amount of yolk is associated with a lower deposition of nutrients in the eggs, which may reflect the lower efficiency of nutrient utilization by hens fed these diets.

The results of this study indicated that, over time under high temperature conditions, there was an improvement in the averages of the quality parameters analyzed in the hens. These improvements were observed similarly in all treatments, indicating an absence of significant differences between the groups in relation to the quality variables evaluated in this study. This suggests that, under these specific conditions, the different treatments did not significantly influence the quality parameters examined.

The effects of PL inclusion in the diet revealed a significant reduction in egg yolk lipid peroxidation when exposed to high temperatures. When collecting the eggs two days after the onset of heat stress, it was observed that the presence of purified lignin acted as an antioxidant, accumulating in the egg yolk, and inhibiting the peroxidation chain reaction. This is particularly important as lipid peroxidation can lead to a rancid taste in the egg, resulting in decreased sensory and nutritional quality (Belitz et al., 2009).

It is important to highlight that the antioxidant properties of PL may be related to the phenolic compounds present in it. These compounds could scavenge free radicals, form complexes with metal ions and prevent or reduce the formation of singlet oxygen. Previous studies, such as those by Rice-Evans (1995), Surai (2014) and Olszowy, M. (2019), corroborate this association between phenolic compounds and their antioxidant properties. Thus, it is likely that the antioxidant effects of PL are related to the presence of these phenolic compounds in its composition.

In the present study, no statistical differences were found between treatments for the level of MDA when we had prolonged heat stress. In contrast to this study, Dosoky et al. (2021) used a supplementation of 1000 mg/kg of licorice root powder in the diet of laying quails for eight weeks under stressful temperature conditions, found a lower concentration of MDA in the blood of the birds, indicating a lower lipid oxidation of the yolk egg. These results suggest that the use of polyphenols may have varied effects in terms of reducing oxidative stress and preserving egg quality under heat stress conditions.

The relative weight of lymphoid organs such as the spleen, thymus and bursa of Fabricius tend to increase in response to heat stress due to increased immune system activity (Liu et al., 2014; Khafar et al., 2019). However, in this study, not statistically differences were observed in the weight of organs of the digestive system and in the length

of the small intestine with supplementation or absence of purified lignin. This indicates that the PL inclusion did not have a direct impact on the development of these organs related to the digestive system.

When analyzing the histology of the small intestine, a statistically difference was observed in the length of the Duodenum villi. The addition of purified lignin in the diet affected the length of Duodenum villi, resulting in smaller villi in size compared to chickens that did not receive supplemental purified lignin in their diet.

These findings suggest that purified lignin may have a selective influence on villous growth and development in the duodenum, without affecting other regions of the small intestine. Villus height has been associated with increased absortion area (Boleli and Morita, 2017). Therefore, this difference in villous length may be one of the factors responsible for the observed effect on feed efficiency. Then the hens fed with the diet containing PL showed a worse efficiency of consumption per gram of eggs and a smaller percentage of yolk.

Exposure of broiler chickens to chronic cyclic heat stress, with a temperature of 33°C for 10 hours a day, for 20 days, between days 22 and 42 of age, resulted in changes in the height of the villi and the depth of the intestinal crypts (Wu et al., 2018). These changes indicate a possible adaptive response of the small intestine of broilers to heat stress, which may affect the function and absorption of nutrients in this region. Understanding these changes is important to improve the management and performance of birds under heat stress conditions.

In the *cage-free* system the hens are constantly exposed to their excreta, which can alter the intestinal microbiota (Hannah et al., 2011). Heat stress also alters the intestinal microflora population; however, studies have reported that phytochemicals such as

phenolics, carotenoids and dietary fibers could modulate the intestinal microflora population and optimize the balance of the microbiota (Yin et al., 2019).

Polyphenols can move to the large intestine and colon, where they can increase microbial activity or decrease the number of toxic microorganisms through antimicrobial activity (Kim et al., 2018). In the present study, the results showed that the polyphenols present in PL have no effect on intestinal fermentation in laying hens, highlighting the absence of statistically differences in the analyzed parameters related to short-chain fatty acids. However, more research is needed to fully elucidate the underlying mechanisms of the polyphenols present in the PL for *cage-free* hens under heat stress.

CONCLUSION

The supplementation of 10.0g of purified lignin/kg in the diet of hens in a *cage-free* system under heat stress did not affect the performance and intestinal morphology of the laying hens. However, a beneficial effect on egg yolk lipid peroxidation was observed during acute stress. These findings suggest that the inclusion of purified lignin could be a promising strategy to improve the lipid stability of eggs under heat stress conditions.

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IMPLICATIONS

In the conventional system, supplementation of 10.0g of purified lignin/kg did not demonstrate any impact on the performance of layers during the period between 31 and 46 weeks. On the other hand, in the *cage-free* system, the inclusion of this same dose in the diet of hens aged between 51 and 63 weeks resulted in lower performance compared to the treatment control. However, in the *cage-free* system under high temperatures, supplementation with 10.0g of purified lignin/kg did not demonstrate any adverse effect on the performance of layers aged between 63 and 66 weeks.

Nonetheless, in both systems and different environmental conditions exhibited an improvement in egg quality due to the antioxidant properties of purified lignin, without impact on the intestinal morphology, and histology of the hens. This phenomenon highlights the relevance of this component as a potential beneficial additive to produce quality eggs and shelf life.

One point of attention is the handling of purified lignin, as it is a very fine-textured powder, its handling has proven to be challenging, especially in relation to cleaning equipment in feed production facilities. This challenge gains magnitude when considering the industrial scale or factories that deal with multiple species, given the significant possibility of cross-contamination of the ingredient to other feed formulations. Furthermore, the animals exhibited a dirty appearance after ingesting the purified lignin.

To better clarify the effects of purified lignin in the diet of laying hens, new experiments are necessary to evaluate its physicochemical properties, including its combination with other zootechnical additives. This research must be conducted to better understand the modes of action and effects on hens, both in breeding systems and under thermal stress conditions.