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IMPACT OF DIETARY *LACTOBACILLUS PLANTARUM* POSTBIOTICS ON GUT HEALTH AND IMMUNITY OF LAYERS UNDER HEAT STRESS CONDITIONS

by
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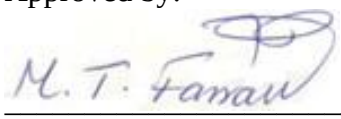

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ABSTRACT
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Title: Impact of Dietary *Lactobacillus Plantarum* Postbiotics on Gut Health and Immunity of Layers Under Heat Stress Conditions

The effect of feeding *lactobacillus plantarum* RS5 probiotic and its products on gut health and immune status in laying hens under heat stress were assessed in this study. A total of 192 twenty-week-old pullets of an Isa White strain were randomly assigned in cages in identical environmentally controlled chambers. During the starter period from 1 to 21 days, all the birds were fed the same basal diet. On day 22, the birds were weighed and randomly divided into six treatment groups of 32 birds each.

Half of the birds were reared under regular temperature conditions, while the other half was subjected to cyclic daily heat stress gradually reaching about 30°C. Layers were offered one of three different diets: 1) Control, or 2) Control + *Lactobacillus plantarum* RS5 probiotic, or 3) Control + *Lactobacillus plantarum* RS5 postbiotic products. The liquid probiotics (RS5 in De Man Rogosa and Sharpe (MRS) broth) and postbiotics (cell free supernatant in MRS broth) were mixed with 100 kg of the feed at a concentration of 200 ml and 300 ml of solution respectively and the feeding trial lasted for 5 months.

The study showed that heat stress had a negative impact on the blood profile, mainly on the concentration of red blood cells RBC (2.8×10^6 µl), hemoglobin HGB (11.34 g/dl), hematocrit HCT (27.32 %), mean corpuscular volume MCV (96.26 fl), platelets PLT (35.82×10^3/ml), mean platelet volume MPV (5.53 fl), and plateletcrit count PCT (0.02 ng/ml), where their means showed a significant ($p<0.05$) decrease compared to control groups at two and five months of the trial. The microscopic and macroscopic intestinal lesions highlighted the influence of probiotic/ postbiotic supplementation in the numerical improvement of the lesions that were caused by heat stress namely, mucosal cells degeneration, deciliation and the number of villi. Dietary probiotics numerically increased the humoral immunity response to Newcastle Disease vaccine at 2- and 4-weeks post vaccination, in comparison to the control group. In addition, the level of antibodies against the Fusion protein was not significantly different among various treatments.

In conclusion, both probiotics and postbiotics could be used as a potential alternative antibiotic health promoter and might alleviate the impact of heat stress in the poultry industry. New heat stress models can be evaluated while assessing various dietary probiotics and/or postbiotics in layers.

Keywords: *Lactobacillus plantarum*, layers, heat stress, postbiotic, probiotics, immunity, gut health.

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CHAPTER I

INTRODUCTION

The primary objective of the global poultry industry is to ensure a consistent supply of eggs and meat to consumers. However, the increasing environmental temperature worldwide poses a significant threat to the poultry industry in many countries (Rafiq Ahmad et al., 2022). When birds experience heat stress, they employ various strategies, including behavioral, hormonal, physiological, and biochemical adjustments, to maintain homeostasis (Bueno et al., 2017). The intestinal tract, being highly responsive to stress, undergoes changes during heat stress. The effective functioning of the intestinal tract is crucial for poultry production, impacting the overall health and performance of the birds (Marcos Rostagno, 2020).

Numerous studies have explored the effects of heat stress on chicken immune responses, yielding variable results (Mashaly et al., 2004). Heat stress has been reported to reduce antibody production in young chickens (Zulkifi et al., 2000). To counteract the negative impacts of heat stress on poultry gut health and immunity, a common practice involves incorporating feed additives, including antibiotics, into the diet at sub-therapeutic levels. Antibiotics in animal feeds have altered the intestinal flora in chickens and influenced chicken immunity, enhancing their ability to control diseases (Danladi, 2022). Antibiotics included as growth promoters in layer feeds have been shown to alleviate the effects of heat stress and improve performance (Loh et al., 2014).

However, the unregulated and indiscriminate use of antibiotics has led to antibiotic-resistant bacteria and increased residues in animal products, posing risks to animal and consumer

health (Danladi et al., 2022). Consequently, there is a pressing need for safer alternatives with comparable or superior effects on animal production, where probiotics and postbiotic metabolites are among the substitutes being explored.

Probiotics, defined as viable microorganisms that reach the intestine in an active state, exert positive health effects (Loh, 2017). Probiotics are natural microbial populations with antimicrobial activity (Danladi, 2022). Postbiotics, on the other hand, are non-viable bacterial products or metabolic by-products from probiotic microorganisms with biological activity in the host (Loh, 2017).

Probiotics colonize the gastrointestinal tract, enhancing the natural microbial environment and impeding the growth of disease-causing organisms. Molecular and genetic studies reveal mechanisms of probiotics that positively affect the host, including immunomodulation, inhibition of bacterial toxins, production of antimicrobial substances, and competition with pathogens for adhesion to epithelium and nutrients. Some mechanisms of action of probiotics have been reported. They include competitive exclusion, promoting gut maturation and integrity, regulating the immune system, preventing inflammation, improving growth, providing metabolism, improving the fatty acid profile, and oxidative stability in fresh meat. Despite their benefits, live bacteria probiotics carry antibiotic-resistant genes that may transfer between organisms, potentially discouraging their use over time. Postbiotics are alternatively better than probiotics due to several reasons. Firstly, postbiotics consist of microorganisms that lack the capability to replicate, making them less likely to induce bacteremia or fungemia compared to probiotics (Yelin et al., 2019). Additionally, postbiotics exhibit several appealing characteristics, including diverse molecular structures, extended shelf lives, and well-defined safety doses (Shigwedha, 2014).

Postbiotics offer probiotic effects without the presence of living cells, possessing most of the benefits of probiotics. They contribute to improved gut health, inhibit harmful bacteria growth, enhance nutrient utilization, and promote animal growth (Danladi et al., 2022). Reports indicate that postbiotics produced from L. plantarum exhibit broad antagonistic activities, showing potential in inhibiting pathogens of various species. Postbiotics have good metabolism, absorption, distribution, and excretion, which could affect many host organs and tissues and perform many biological tasks (Shenderov, 2013). Postbiotics have demonstrated improvement in broilers, laying hens, and pigs in terms of growth, meat quality, fecal lactic acid bacteria, villus height, and the ability to withstand heat stress (Danladi, 2022). Lactic acid and bacteriocins from lactic acid bacteria have antimicrobial activity and are considered alternatives to antibiotics (Zhong, 2022). Previous study showed that postbiotics obtained from Lactobacillus plantarum exhibit inhibitory action on various pathogenic bacteria, including Listeria monocytogenes, Salmonella typhimurium, Escherichia coli and vancomycin-resistant Enterococci (Kareem et al., 2014). In addition, postbiotics obtained from L. plantarum has been found to be particularly strong under heat-stress conditions (Loh et al., 2014). In heat-stressed broilers, postbiotics from L. plantarum are expected to provide similar benefits to those from probiotic bacteria (Loh et al., 2014).

This study seeks to assess the impact of dietary L. plantarum RS5 postbiotic preparations on the gut health and immunity parameters of laying hens, particularly under heat stress conditions. The evaluation will focus on several key aspects. Regarding gut health, the parameters to be examined include intestinal integrity, assessed through histopathological methods. As for immunity parameters, the study will encompass innate immunity, with a focus on complete blood count analysis, and humoral immunity, which will be evaluated through

hemagglutination inhibition tests and SDS-PAGE western blotting. It's important to note that the effectiveness of probiotic and postbiotic effects can vary based on the specific strain, host characteristics, and the immunological state of the host.

CHAPTER II

LITERATURE REVIEW

A. Impact of heat stress on poultry

Heat stress poses a significant environmental challenge in global poultry production and can be triggered by various conditions. Common factors include climatic conditions prevalent in specific regions, failures in ventilation and temperature control systems (whether manual or automatic), insufficient brooding conditions, high stocking density towards the end of the growth phase, and the adoption of new or alternative ("open") production systems such as free-range or organic, which present challenges in maintaining effective environmental controls and expose birds to the external environment regularly (Rostango, 2020).

Generally, stress signifies the biological response of an animal organism to external factors that disrupt its normal physiological equilibrium. Heat stress specifically arises from an imbalance between the net energy flow from the animal's body to its surrounding environment and the amount of heat energy generated by the animal (Lara and Rostagno, 2013). While the detrimental effects of heat stress are applicable to all species, commercial poultry strains appear to be particularly susceptible. Research indicates that poultry genotypes produce more body heat due to their sensitive metabolic activity, which diminishes their capacity to adapt to changes in environmental conditions (Deeb and Cahaner, 2022).

1. Thermoregulation of birds in response to heat stress

Poultry regulate and control their body temperature by managing the balance between metabolic heat production and dissipation, especially during shifts in environmental conditions.

Heat stress in poultry can be triggered by various factors, including extreme heat, climate variations, temperature fluctuations, and elevated moisture levels (yahav, 2015). In response to heat stress, poultry undergo specific morphological, physiological, and behavioral adaptations to ensure the maintenance of their normal body temperature (farag, 2018). Poultry are comfortable and perform basic life processes from 23.9 °C to 26.7 °C (bell, 2002).

The primary indicator of heat stress development in a poultry flock is an increase in the conservation of energy costs. When the environmental temperature surpasses the comfort zone, the body responds by attempting to dissipate heat through radiation, particularly from areas such as the feet, comb, and wattles. Birds may also alter their behavior, engaging in activities like wing spreading and panting. Given that most of the poultry body is covered with feathers, the effectiveness of heat dissipation through the wattle, head, comb, and feet is limited. Panting becomes a crucial mechanism adopted by poultry birds to facilitate heat loss in conditions of heat stress.

Air sacs play a vital role in transferring body heat to the surrounding environment through a respiratory evaporative mechanism to reduce and maintain normal body temperature. In the panting mechanism, air sacs become more critical as they facilitate the dispersion of air across the body surface, minimizing heat dissipation through an evaporative process. However, excessive and uncontrolled panting during heat stress in poultry can lead to a reduction in calcium availability and carbon dioxide pressure. Consequently, blood pH levels increase, resulting in respiratory alkalosis, which can contribute to bone deterioration and lameness (rafiq ahmad, 2022).

2. Effect of heat stress on poultry production

Heat stress has a detrimental impact on animal welfare, resulting in lower feed intake and a negative energy balance in birds (De Rensis and Scaramuzzi, 2003). Research indicates that for every 1°C increase in the temperature range of 22-32°C, feed intake is reduced by 1.2%, and for a 1°C rise in the temperature range of 32-38°C, feed intake decreases by 5% (Ashish et al., 2019). In broilers, heat stress leads to a decline in growth rate due to reduced feed digestibility, including proteins, fats, and starch (Bonnet et al., 1997). In layer hens, egg production decreases due to a decline in the uptake of available nutrients and reduced digestibility of many components of the diet (Allahverdi et al., 2013).

For breeder hens, heat stress may impact reproductive efficiency by altering the acid-base balance and hormonal system (Mahmoud et al., 1996). Reduced feed intake can result in decreased body weight, feed efficiency, egg production, and quality (Deng et al., 2012). Additionally, heat stress is associated with reduced dietary digestibility and decreased plasma protein and calcium levels. Numerous studies have highlighted the harmful effects of heat stress on layer hen production, with significant impacts on egg quality and production. The extent of these impacts depends on factors such as age, genetic background, and the intensity and duration of the heat stress treatments applied (Lucas and Marcos, 2013).

3. Effects of heat stress on the immune response

Several studies indicate that heat stress exerts an immunosuppressive impact on both broilers and layers. Abundant evidence suggests that enteric neurons and intestinal immune cells share common regulatory mechanisms, and their coordinated responses to challenges can be disrupted by various stressors. This dysregulation of the immune response affects the interplay between these systems, increasing susceptibility to pathogens, influencing the severity of

infections and pathologies, and even negatively impacting responses to vaccines (Rostagno, 2020). Lymphocytes, monocytes or macrophages, and granulocytes have been shown to possess receptors for various neuroendocrine products of the hypothalamic pituitary adrenal axis HPA and Sympathetic-adrenal-medullary axes, such as corticosterone and catecholamines. These substances can affect cellular trafficking, proliferation, cytokine secretion, antibody production, and cytolytic activity (Quenteiro et al., 2010).

Previous investigations focusing on immune dysregulation in broiler chickens have reported significant decreases in the weights of immune organs such as the thymus, bursa of Fabricius, and spleen under heat stress conditions. The impact of heat stress on lymphoid tissues extends to mitochondrial function and the expression levels of inflammatory cytokines, including TNF-α, IFN-γ, IL-1, IL-2, IL-4, and IL-12 (Ohtsu et al., 2015). In a previous study, Ryota et al. (2020) concluded that the absolute number of T and B cells in lymphoid tissues decreased significantly under the HS condition due to their weight reduction with fewer CD45+ leukocytes.

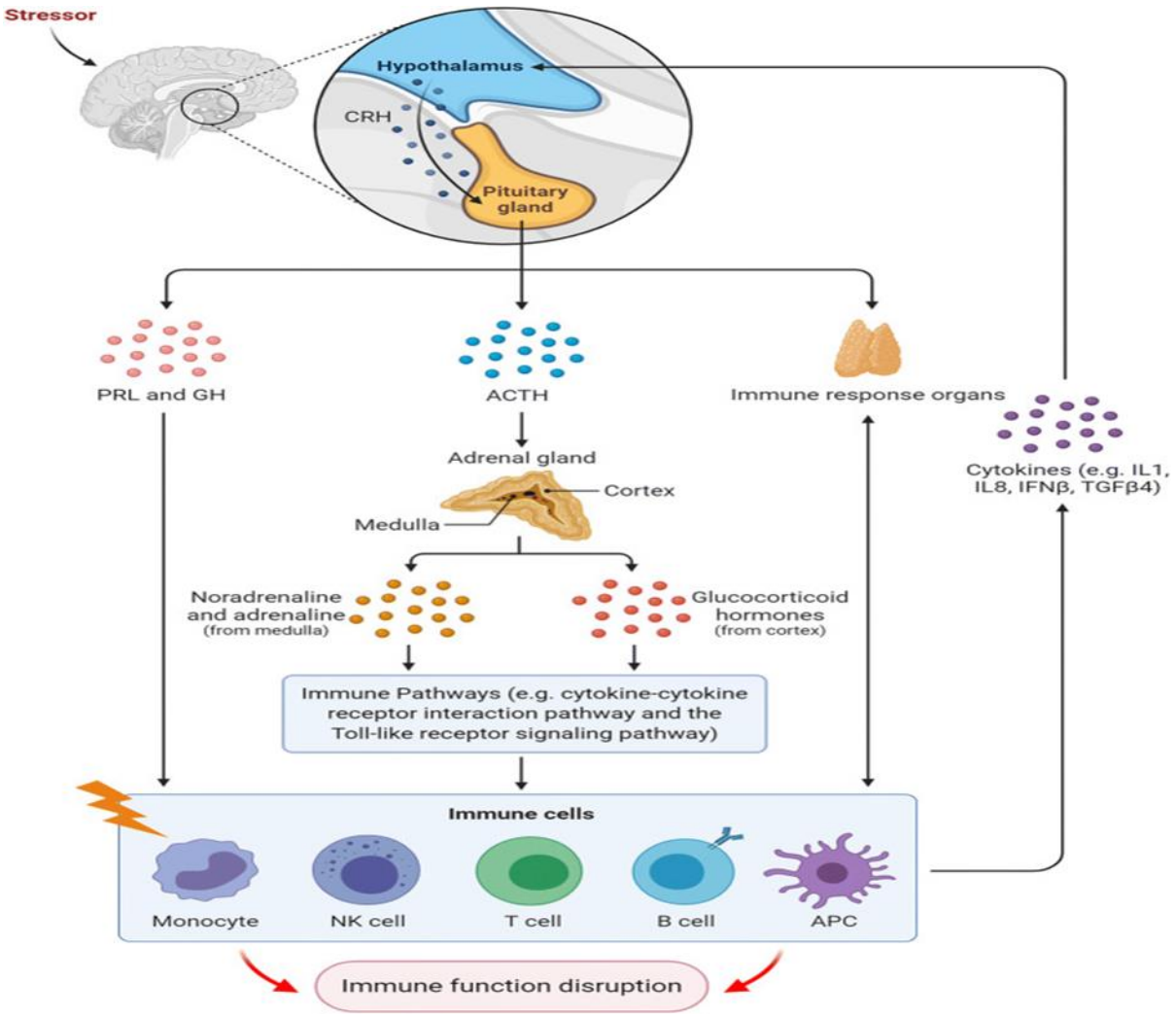

Figure 1 Effect of heat stress on immune system

Heat stress stimulates the hypothalamic-pituitary-adrenal (HPA) axis, which results in increases in corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) that lead to an increase of corticosteroid level, which in turn increases the fatty acid synthesis, fat accumulation, and protein catabolism and impairs the GIT functions, such as decreased jejunum villi height and increased permeability to microorganisms (Hangalapura 2006).

Poultry revealed a reduced number of intraepithelial lymphocytes and Immunoglobulin A-secreting cells along the intestinal tract, diminished antibody response, and lower phagocytic

activity of macrophages. Another study demonstrated that high temperature suppresses immune function by inhibiting total white blood cell counts and antibody level; thereby increasing mortality in laying hens (Mashaly et al., 2004). Some studies showed that plasma calcium and phosphorous concentrations were reduced by heat stress in laying hens.

Additionally, studies observed that broilers subjected to heat stress had lower levels of total circulating antibodies (Bartlett et Smith, 2003), and a reduced liver weight. In addition, broilers subjected to heat stress had lower levels of total circulating antibodies, as well as lower specific IgM and IgG levels, both during primary and secondary humoral responses. Moreover, they observed significantly reduced thymus, bursa, spleen, and liver weights (Bartlett and Smith, 2003).

4. Effect of heat stress on intestinal integrity

The integrity of the intestinal barrier is critically important in poultry production. When the intestinal barrier is compromised, leading to intestinal barrier dysfunction, there is an increase in intestinal permeability. This condition is characterized by the non-mediated diffusion of large molecules, typically restricted in normal circumstances, from the intestinal lumen to the circulatory system (Lambert, 2009). High intestinal permeability often results in harmful local and possibly systemic inflammatory reactions.

In situations of heat stress, the reduced availability of oxygen and nutrients, caused by diminished blood supply and decreased feed intake, leads to morphological changes and mucosal damage due to oxidative stress and inflammation. Several studies, such as those conducted by Quinteiro-Filho et al. (2010), have demonstrated the impact of heat stress on the integrity of the intestinal barrier in poultry. This effect results in increased intestinal permeability and local

inflammation, characterized by an augmented lymphoplasmacytic infiltrate along the small intestine, including the duodenum, jejunum, and ileum. The presence of heterophils in the observed inflammatory infiltrate indicates bacterial invasion from the intestinal epithelia to the lamina propria. Studies have reported a higher prevalence of Salmonella spp. in the spleen and liver of heat-stressed birds compared to control groups (Al Henaky et al., 2017). The conclusion drawn was that heat stress disrupts the intestinal barrier, leading to increased permeability to endotoxin and the translocation of intestinal pathogens. Furthermore, heat stress-induced alterations included inflammatory responses and damage to tissues/cells, likely resulting from disruptions in the intestinal barrier and the infiltration of pathogens.

Numerous studies have documented changes in the morphology and histopathology of the poultry intestinal tract under heat stress conditions. Consistent findings across these studies include a decrease in villi height, an increase in crypt depth, and consequently, a reduced villi-to-crypt ratio (Song et al., 2014; Wu et al., 2018). Santos et al. (2015) also observed an expanded base width of villi and a decrease in epithelial cell area in the duodenal, jejunal, and ileal mucosa in broilers exposed to heat stress.

Environmental factors that can influence the dynamics of mucins have the potential to impact the viscosity and integrity of the mucus layer, consequently affecting nutrient transportation (Horn et al., 2009). As a result, disruptions in intestinal homeostasis bring about alterations in the mucus barrier, which serves to protect the enteric mucosa. The heightened permeability of the enteric mucosa, stemming from such interruptions, may lead to inflammatory processes and damage to mucosal cells (Dharmani et al., 2009).

A comprehensive morphometric analysis of intestinal segments, comparing control birds with those subjected to high temperatures, was conducted in a previous study. The findings revealed a

significant decrease ($P < 0.05$) in villus height, epithelial and total villus areas, and the villus-to-epithelial cell area ratio across all segments of the small intestine in the heat-stressed birds (Santos et al., 2014).

5. Effect of heat stress on blood parameters

Heat stress is known to affect the physiology of animals, often causing multiple organ dysfunction syndromes. System problems include blood, liver, and kidney malfunctioning and tissue damage. The extent of these problems is usually measured by blood profile tests such as CBC and liver and kidney function tests, urine analysis, and histological examination of different body tissues. Indeed, blood parameters serve as crucial indicators of the overall physiological state of the body.

Hematological indices, including values such as hematocrit, hemoglobin concentration, and red blood cell count, play a vital role in assessing the functional status of the blood's oxygen-carrying capacity (Maheswaran, 2008). These parameters are fundamental to understanding the health and well-being of an organism, providing valuable insights into aspects such as oxygen transport, immune function, and overall cardiovascular health. Monitoring changes in these blood parameters is a common practice in both clinical medicine and research to evaluate the impact of various factors, including nutritional interventions such as probiotics and postbiotics, on the physiological health of individuals, including poultry.

Several studies show that exposure to heat produced a profound effect on the blood. Differences in levels of RBC, HGB, HCT, MCV, and MCH were significant (salma hamid et al., 2021). Another study showed that exposure of Japanese quail to chronic heat stress decreased the number of WBCs, RBCs, PCV %, Hemoglobin concentration (g/dl), and lymphocyte% and increased the heterophils cells%, H/L ratio, eosinophil %, Monocyte %, basophile% (Usama

Mahmoud et al., 2013). Aengwanich (2008) provided evidence of reduced bursa weight in broilers exposed to heat stress, accompanied by decreased lymphocyte numbers in both the cortex and medulla regions of the bursa. More recent studies have further indicated that heat stress has an impact on the levels of circulating cells. Specifically, heat stress has been linked to an elevated heterophil-to-lymphocyte ratio, attributed to a decline in circulating lymphocyte numbers and an increase in heterophil numbers (Prieto and Campo, 2010).

6. Management approaches to reduce heat stress

Various management approaches can be employed to mitigate heat stress in poultry. Farmers can modify the environment by connecting ventilation systems, dropping bird density, adjusting feeding schedules to the evening, and managing nutrition (Dayyani and Bakhtiyari, 2013). Alterations in the energy-to-protein ratio in the diet can help minimize the adverse effects of heat stress. Decreasing protein in the diet can reduce heat increment and increase feed intake due to amino acid deficiency, but it is important to ensure that the low protein diet maintains a balanced critical amino acid profile, particularly methionine and lysine (Gous and Morris, 2005).

Additionally, studies have shown that supplementation with vitamins and minerals can be beneficial during periods of stress because heat stress increases the excretion of minerals from the body (Sahin et al., 2009). Specifically, vitamin A, D, E, C, and folic acid have been identified as effective supplements for animals under heat stress conditions (Sahin et al., 2002). Another effective feed supplementation strategy involves the use of probiotics, such as Lactobacillus strains. These probiotics contribute to enriching the diversity of Lactobacillus flora in the chicken's jejunum and caecum, thereby restoring microbial balance, and maintaining the natural stability of the jejunal and caecal microbiota in chickens experiencing heat stress (Loh et al., 2014).

B. Probiotics and heat stress

It is possible to use nutritional approaches to balance the negative effects of persistent heat stress on poultry. Feed additives particularly have been incorporated into poultry diets to mitigate these effects (Lara and Rostagno, 2013). Among the various feed additives, probiotics have gained significant attention from poultry nutritionists. Studies have shown that probiotics can improve the physiology, gut morphology, and structure, as well as the immune function of heat-stressed poultry, resulting in enhanced performance and overall health (Al-Fataftah and Abdelqader, 2010). The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have jointly defined probiotics as "live microorganisms that, when administered in adequate amounts, provide a health benefit to the host" (FAO, 2006).

The use of probiotics in poultry has steadily increased over the years due to the growing demand for antibiotic-free poultry and the well-documented benefits associated with their use. In 2018, the probiotic market reached $80 million, with a projected compound annual growth rate of 7.7%, which is expected to expand the global probiotics market to $125 million by 2025 (Ahuja et al., 2019).

Probiotic microorganisms widely employed in poultry consist of Bifidobacterium, Lactococcus, Lactobacillus, Bacillus, Streptococcus, and yeast like Candida. The established criteria for probiotic strain selection encompass tolerance to gastrointestinal conditions, adhesion to the gastrointestinal mucosa, and competitive exclusion of pathogens (Gadde et al., 2017). Furthermore, probiotics are chosen based on their resilience during manufacturing, transportation, storage, and application processes, ensuring viability and desirable traits. Research findings indicate that incorporating probiotics can yield several effects, including altering the intestinal microbiota, boosting the immune system, reducing inflammation,

preventing pathogen colonization, improving growth performance, modifying ileal digestibility, total tract apparent digestibility coefficient, and decreasing ammonia and urea excretion. Noteworthy risks associated with probiotics include genetic stability, infectivity, or the potential for in situ toxin production (Zhong et al., 2022).

Conversely, probiotics are commonly included in amounts exceeding the necessary dose to counteract potential losses of live microorganisms during production. The adhesion capability of probiotics also serves as a potential protective mechanism against pathogens by competing for binding sites on the epithelium.

1. Probiotics with gut health and immunity

Recent literature reveals that certain non-pathogenic species within the intestinal microbiota interact with the epithelium and immune system, influencing tissue physiology and the ability to combat infections. The alteration of intestinal environments is considered a notable impact of probiotics and serves as the foundation for various probiotic benefits. In the gut-associated lymphoid tissue, epithelial cells and dendritic cells act as mucosal sentinel cells (Jha et al., 2020). Probiotic bacteria play a role in enhancing intestinal barrier integrity by modulating mucin production. Mucins, the principal protein component coating the gastrointestinal tract, are vital for its protection. Probiotics contribute to the normalization of intestinal integrity by restoring the mucus layer, adjusting the composition of mucin monosaccharides, regulating mucus layer thickness, and influencing mucin gene expression. The structural and functional properties of mucins play a crucial role in bacterial adhesion to the mucosal surface (Aliakbarpour et al., 2012).

In a separate investigation (Song et al., 2014), it was demonstrated that a probiotic blend consisting of B. licheniformis, B. subtilis, and L. plantarum could mitigate the adverse effects of

heat stress on gut microflora, histomorphology, and barrier integrity in broilers. This supplementation led to alterations and increases in the populations of small intestinal Lactobacilli and Bifidobacterium, along with an elevation in jejunal villus height. The broilers experienced improved feed-to-gain ratios and a reduced burden of small intestinal coliforms.

In another study by Li et al., 192-day-old male Arbor Acre broiler chickens were utilized to assess immune functions and their responses. The findings indicated that B. amyloliquefaciens mitigated immunological stress in lipopolysaccharide-challenged broilers at an early age. Additionally, the supplementation increased lysozyme activity in plasma and elevated the white blood cell count. Li et al. (2015) concluded that B. amyloliquefaciens could partially alleviate compromised growth performance and immune status in broilers under immune stress at an early age (Li et al., 2015).

Sadeghi's investigation explored the impact of B. subtilis on antibody titers against Newcastle and infectious bursal viruses in 160 broiler chickens challenged with Salmonella enterica serotype Enteritidis. The findings revealed that B. subtilis did not significantly affect the immune parameters of chickens in uncontaminated environments but exhibited remarkable efficacy in environments contaminated with pathogens (Sadeghi et al., 2015). Similar outcomes were observed in the study by Teo and Tan, where a probiotic containing B. subtilis improved feed conversion, intestinal morphology, immune response, and inhibited colonization by C. jejuni, Escherichia coli, and Salmonella Minnesota in the gastrointestinal tract (Teo and Tan, 2007).

In a study on quails, heat stress was reported to reduce the number of mucus-producing goblet cells in the ileal villi (Sandicki et al., 2004). Lactobacillus-based probiotics were found to increase the number of goblet cells in the duodenum and jejunum of broilers exposed to heat stress. The reported mechanism through which probiotics increase goblet cells involves

modulating mucin mRNA expression and accelerating goblet cell differentiation (Smirnov et al., 2005).

Probiotics have been suggested to reduce circulating corticosterone levels, leading to a decreased heterophil-to-lymphocyte (H/L) ratio and improved immune responses (Hassan et al., 2007; Beski and Al-Sardary, 2015). Yang et al. (2015) proposed that corticosterone exerts immunosuppressive effects in poultry, implying that the reduction in corticosterone levels may be beneficial for restoring normal immune system function and development.

C. Concept of Postbiotic as Alternative

Despite the benefits of probiotics, non-viable microbial cells could offer safety advantages compared to probiotics. This is because they reduce the risk of microbial translocation, infection, or heightened inflammatory responses, particularly observed in individuals with imbalanced or compromised immune systems (Taverniti & Guglielmetti, 2011). Postbiotics are considered superior to probiotics because they consist of microorganisms that cannot replicate, reducing the likelihood of causing bacteremia or fungemia, a risk associated with probiotics (Yelin et al., 2019).

Postbiotics function similarly to probiotics, utilizing the same mechanism of action and effectiveness, mainly due to the presence of secondary metabolites from probiotics, but without the inclusion of living cells. One key advantage of postbiotics is their ability to maintain stability during industrial processes and storage, ensuring a longer shelf life. This characteristic enhances their potential applications compared to probiotics (Zhong et al., 2022).

Postbiotics are derived from probiotics through various methods. The inactivation of bacterial cells can be accomplished using physical methods such as mechanical disruption, heat treatment, γ- or UV irradiation, high hydrostatic pressure, freeze-drying, or sonication.

Alternatively, chemical methods like acid deactivation can also be employed. These processes may lead to alterations in microbial cell structures or their physiological functions, rendering the bacteria incapable of growth while retaining the health benefits associated with their viable form (de Almada, Almada, Martinez, & Sant'Ana, 2016).

These microbial products also called soluble factors are often produced either by live microorganisms or released after their inactivation or lysis (De Almada et al. 2016). These soluble factors synthesized by different microbial strains include enzymes, short chain fatty acids, peptides, organic acids (e.g. lactic acid, acetic acid), plasmalogens, endo- and exo-polysaccharides, ethanol, polyphosphates, teichoic acids, diacetyl, lactocepins, vitamins (e.g. B-group vitamins), cell surface proteins, muropeptides, hydrogen peroxide and teichoic acids (Rad et al. 2020).

1. Mechanism of postbiotic

The disruption of the gut microbiota, marked by the presence of pathogens and overgrowth of indigenous pathobionts, can lead to detrimental effects on gut health and contribute to various diseases. Postbiotics offer a therapeutic approach to combat pathogens by acting in five different ways:

a. Microbiota transformation:

Transforming the microbiota can be achieved through quorum quenching or by carrying quorum-sensing molecules (Grandclément et al., 2016). Additionally, the presence of lactic acid, utilized by certain microorganisms to produce beneficial butyrate and short-chain fatty acids (SCFAs), contributes positively to the microbiota (Laverde Gomez et al., 2019). Postbiotic adhesions, such

as fimbriae (Tytgat et al., 2016) and lectins (Petrova et al., 2016), compete for adhesion sites with resident microbes.

b. Intestinal barrier function improvement:

Adequate levels of short chain fatty acids SCFAs in a postbiotic preparation can protect against disruptions caused by lipopolysaccharide and alter the functions of epithelial barriers (Feng et al., 2018).

c. Modulation of immune response:

Postbiotics trigger immune-modulating activities at systemic and local levels through molecular patterns associated with microorganisms. Receptors like nucleotide-binding oligomerization domain receptors, C-type lectins, and Toll-like receptors regulate cytokines and immune responses (Lebeer et al., 2010).

d. Alteration of systemic metabolic response:

Enzymes and metabolites on and inside inactivated microorganisms' surfaces in postbiotics directly affect systemic metabolic responses. Bile acids, influenced by postbiotics, have downstream effects on host metabolic processes, including lipids, xenobiotics, glucose, and energy metabolism (Long et al., 2017).

e. Systemic signaling through the nervous system:

Adequate amounts of metabolites, such as SCFAs, in postbiotic preparations stimulate enterochromaffin cells, releasing serotonin into the bloodstream (Iwasaki et al., 2019). Fig. 2

illustrates the mechanism of action for postbiotics, showcasing some examples of microbial effector molecules mediating these systems. Preserving postbiotics' cellular structure, similar to vaccines, protects against rapid degradation by digestive enzymes and immune attacks while ensuring the continuation of effector molecules' activity.

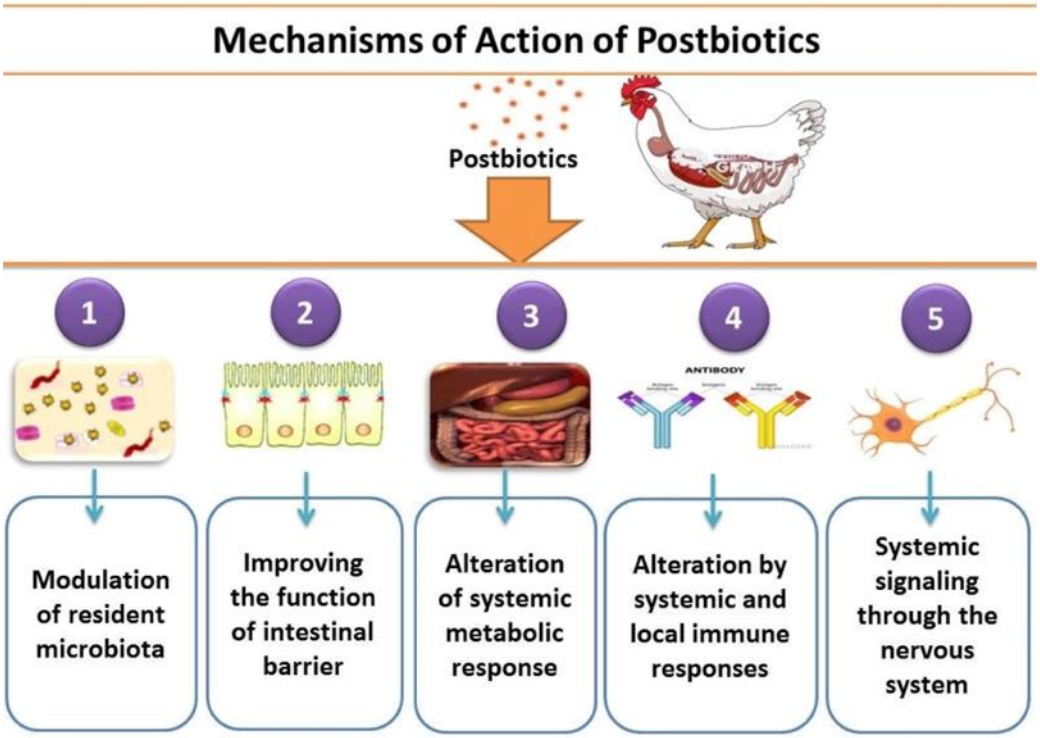

Figure 2 Mechanisms of action of postbiotics

2. Benefits of postbiotics

Under normal circumstances, supplementing broiler diets with postbiotics has shown positive associations with improved growth performance and health. This enhancement is attributed to the advancement of immune status and gut health, characterized by improved intestinal villus structure, increased lactic acid bacteria population, reduced Enterobacteriaceae population, and a decrease in fecal pH (Humam et al., 2019).

Postbiotics derived from Lactobacillus plantarum exhibit inhibitory effects against various pathogenic bacteria, including Listeria monocytogenes, Salmonella typhimurium, Escherichia coli, and vancomycin-resistant Enterococci (Kareem et al., 2014). Additionally, these postbiotics exhibit a robust antioxidant capacity, particularly under heat-stress conditions. Lactobacillus, specifically L. plantarum cultures, have demonstrated high antioxidative activities (Ji et al., 2015).

Supplementation with postbiotics has been shown to reduce blood pressure, indicating their potential antihypertensive capacity. The protective effects on endothelial function are not fully understood, but they could be linked to changes in gut microbiota, restoration of gut barrier function, and effects on endotoxemia, inflammation, and renal sympathetic nerve activity (Robles-Vera et al., 2017).

Postbiotics also exhibit antiproliferative activity against colon cancer cells, likely through the activation of pro-apoptotic cell death pathways and regulation of immune responses (Tiptiri-Kourpeti et al., 2016). Reports suggest that postbiotics from Lactobacillus strains may decrease metalloproteinase-9 activity, inhibiting colon cancer invasion (Escamilla, Lane, & Maitin, 2012).

Furthermore, postbiotic administration from L. plantarum has shown positive effects on growth performance, protein digestibility, and a reduction in diarrhea incidence in laying hens. The alteration of mucosal architecture, longer villi, improved animal growth performance, and enhanced intestinal microbiota population, particularly protective bacteria like Lactobacillus and Bifidobacterium, contribute to the health benefits of postbiotics. These findings position postbiotics as potential feed additives to enhance productivity and improve layers health (Loh et al., 2014).

Another research about the effect of L. plantarum supplementation on the health of laying hens was done by Choe et al. (2012) showed that the supplementation of postbiotic metabolites gave the best result in improving the hen/day egg production, and showed positive effects such as reduced fecal pH, reduced plasma and yolk cholesterol, and increased small intestine villus height. This finding indicates that metabolites from locally isolated L. plantarum are a possible alternative feed additive in poultry production (Choe et al., 2012).

CHAPTER III

MATERIALS AND METHODS

A. Field evaluation of the preparation of dietary supplements

1. Lactobacillus plantarum strain

The Lactobacillus plantarum strain RS5 (NCIMB 701088) was obtained from the NCIMB laboratory in the United Kingdom. This specific strain was originally isolated from cheese by a researcher named A. A. Nichols. To cultivate the bacteria, it was suspended in a nutrient-rich solution called Man, Rogosa, and Sharpe (MRS) broth and then kept at a temperature of 37°C for 48 hours. To confirm the presence of the bacteria, a staining technique known as Gram staining was applied. This revealed the presence of Gram-positive, non-spore-forming rod-shaped cells when examined under a microscope. The bacterial suspension was then transferred to MRS agar growth medium and allowed to grow for 48 hours at the same temperature (37°C).

From the resulting colonies, a few white, circular ones were randomly selected. Some of these were further isolated by streaking on MRS agar to ensure pure cultures, while others were subjected to Gram staining for confirmation. Subsequently, these colonies were suspended in a sterile 0.85% saline solution. The optical density of this bacterial suspension was adjusted to 3% at a wavelength of 450 nm. After a series of dilutions, culturing on MRS agar, and counting the colonies, it was determined that this initial solution contained 10^15 colony-forming units (CFU) per milliliter. The original bacterial cultures were preserved for future use at a temperature of -80°C in MRS broth.

2. *Preparation of Postbiotics from L. plantarum Strains*

Cultures of Lactobacillus plantarum were prepared by introducing 10% (by volume) of active bacterial cells with a concentration of 10^9 colony-forming units per milliliter (CFU/mL) into MRS media. These cultures were then incubated at a temperature of 30°C for 10 hours. Following the incubation, the mixture underwent centrifugation at 10,000× g and 4°C for 15 minutes using an Eppendorf 5810 centrifuge from Eppendorf, Maryland, USA.

The resulting cell-free supernatant (CFS) was collected by filtering it through a cellulose acetate membrane with a pore size of 0.22 microns, as described by Loh et al. in 2014. This CFS was preserved at a temperature of -20°C until it was needed for the feeding trial. During the trial, the liquid postbiotics were combined with the animal feed using a horizontal feed mixer and a three-way mixing technique. Specifically, 300 ml of the CFS solution in MRS broth was added per 100 kg of feed.

3. *Preparation of Probiotics from L. plantarum Strains*

Bacterial growth was promoted using a culture medium known as MRS agar. The overnight culture of the Lactobacillus isolate was then introduced and allowed to incubate for a period ranging from 24 to 48 hours. Following this incubation, the bacterial colonies were collected and re-suspended in a solution of phosphate-buffered saline (PBS) with a pH level of 7.4. The bacterial count was fine-tuned to a concentration of 3.10^9 colony-forming units per milliliter (CFU/mL) with spectrophotometry.

Subsequently, this bacterial suspension was blended with the basal diet at a concentration of 200 ml of the RS5 solution in MRS broth for every 100 kg of feed. This mixing process was achieved by employing a three-way mixing technique within a horizontal mixer.

4. Birds' housings and treatments

The study took place at the American University of Beirut's research facilities (AREC) in the Beqaa region, utilizing four identical environmentally controlled poultry houses. The experiment extended over 6 months, comprising a one-month adaptation period followed by 5 months of the experimental phase. The initial live body weight was recorded individually for all birds at the end of the adaptation phase to allocate birds into different treatment homogeneously. Specifically, 192 twenty-week-old pullets of the Isa white strain were evenly distributed into six groups of 32 birds, each housed individually.

Within each group, birds were further divided between two houses, accommodating 16 birds in each pen. The first two houses maintained regular temperature conditions, while the other two exposed the birds to cyclic heat stress, reaching approximately 30°C for 4 consecutive hours daily. Temperature levels were monitored daily at 10am, 1pm, and 4pm, as well as weekly at 4am. Additionally, birds in each house were categorized into three diet groups: control, control with probiotic supplementation, and control with postbiotic supplementation. Throughout the experiment, birds had unrestricted access to water and feed, provided in accordance with the recommendations from the Manual (Institut de Sélection Animale BV, Villa 'de Körver', Boxmeer, Netherlands). The experimental design, detailed in Table 1 and Figure 3, encompassed six distinct treatments for the birds.

Table 1 control and experimental design

Treatment	Temperature	Diet	Cages	Replication
1	Regular	Control	32	32 birds
2	Regular	Probiotic	32	32 birds
3	Regular	Postbiotic	32	32 birds
4	Cyclic heat stress	Control	32	32 birds
5	Cyclic heat stress	Probiotic	32	32 birds
6	Cyclic heat stress	Postbiotic	32	32 birds

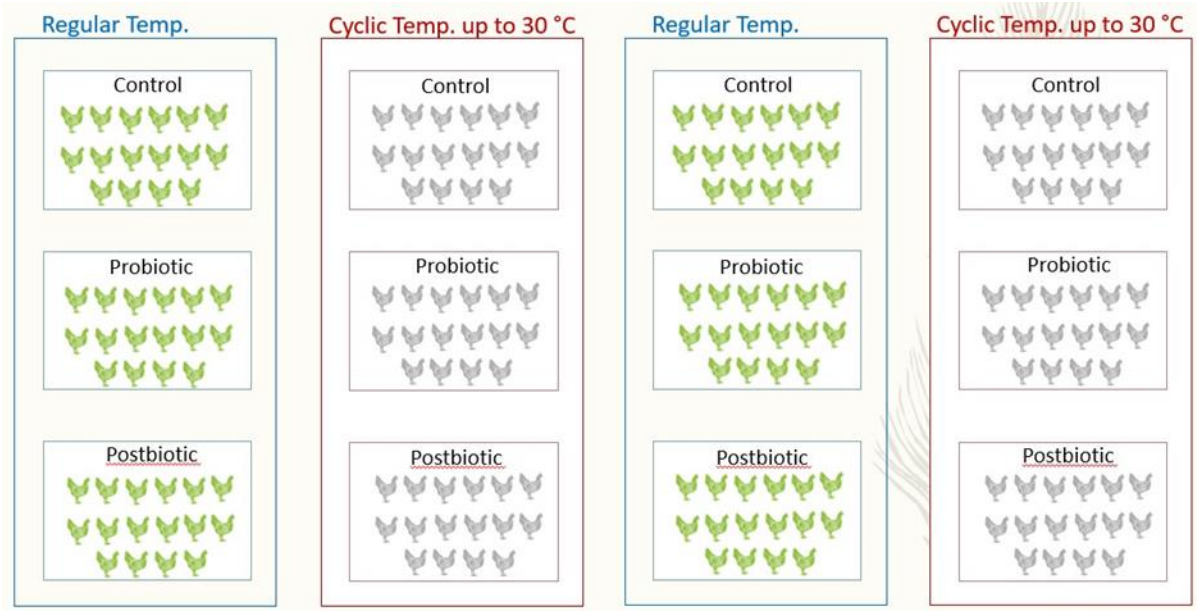

Figure 3 Experimental design

5. *Evaluation of hen's intestinal health*

a. Histopathology

Four birds were sacrificed from each treatment at the end of the experiment to evaluate intestinal health using Hematoxylin & Eosin staining (Shaib, 2004). Five cm of the mid-portion of the jejunum were excised and phosphate-buffered saline was used to wash the samples before storing the tissues in 10% formalin. Dehydration of the sample, embedding, and cutting of three

5 µm-thick sections was carried out to position them on glass slides and then heated to dry. The sections were stained using hematoxylin and eosin and viewed under a light microscope. Sections were examined under the microscope to determine the number of villi and mucosal cells in the intestinal lining, % of fields showing deciliation and % of fields showing mucosal cells degeneration.

b. Macroscopic lesions and pathology scoring of the intestine

Four birds from each treatment were sacrificed at two- and four-months post heat stress initiation to evaluate macroscopic lesions and pathology scores of various parts of the intestines. Lesions included the presence of the following: thin/ thick intestinal wall, intestinal hemorrhage, watery intestinal content, and undigested feed. Intestinal scores were recorded from 0 to 3 whereas a score of 3 indicated pronounced macroscopic lesions and a score of 0 was given to negative observations.

c. Blood collection and seroconversion studies

Blood samples were collected from 8 laying hens from each treatment at two, four and five months post the initiation of heat stress to test complete blood count. The hematological test measures: white blood cells (WBC, 10^9/L), red blood cells (RBC, 10^6/mm), hemoglobin (HGB, mg/dL), hematocrit (HCT%), mean corpuscular volume (MCV, fL), mean corpuscular hemoglobin (MCH, pg), mean corpuscular hemoglobin concentration (MCHC, %), red cell distribution width (RDW, %), platelets (PLT, 10^3/ml), mean platelet volume (MPV, fL), plateletcrit (PCT, ng/ml), and platelet distribution width (PDW%).

Blood samples were also collected from 8 laying hens from each treatment 2 weeks and 4 weeks post vaccination with live NDV to evaluate humoral immunity. For seroconversion studies, blood was centrifuged at 2000 rpm for 10 mins and sera were collected in 1.5 mL microcentrifuge tubes, followed by storage at −80 °C for subsequent assessments. These assessments included the determination of NDV hemagglutination inhibition (HI) titers and subsequent western immunoblotting to assess immunity at protective levels against the fusion protein.

d. Haemagglutination-Inhibition (HI) test

The HI test for antibody titers against Newcastle Disease Virus (NDV) was performed as per the OIE 2002 protocol. In brief, sera were serially diluted ½ in V-shaped wells and each well of the HI plates received four hemagglutination units (4 HAU) of NDV virus/antigen. The plate was then incubated at room temperature for 45 minutes and was later supplemented with 0.050 mL of 0.5% chicken red blood cells and incubated for an additional 30 minutes. Positive and negative sera samples were also used.

e. SDS PAGE western immunoblotting

The SDS-PAGE followed by western immunoblotting were applied on the sera of all birds to quantify the mean specific immune responses in the sera collected to trans-blotted fusion protein carried from the SDS resolving gel to the cellulose membrane of the western blotting. An amount of 100 µg of NDV virus were mixed with 16µl of Laemmli buffer and 1 µl β-mercaptoethanol (Bio-Rad, USA). Each sample was then subjected to 95℃ for 5 minutes and loaded onto 10-well polyacrylamide gels (Bio-Rad, USA). Gel electrophoresis was performed at

300V for 20 min, then the banded peptides were transferred directly onto nitrocellulose membranes (NCM) (Bio-Rad, Germany). NCMs were blocked with 5% gelatin in Tris-Buffered Saline (TBS) for 2 hours and then washed two times for 5 min with Tween 20-TBS (TTBS). The NCMs were incubated with the primary antibodies specific for the protein of interest for one hour in 1% gelatin in TTBS. Finally, after washing with TTBS (2 times for 5 min each), NCMs were incubated with secondary antibodies conjugated to horseradish peroxidase in 1% gelatin in TTBS for an hour. The detection of bands was performed using DAB reagents (Bio-Rad, CA, USA); and the intensity of the banded proteins was evaluated using Image Lab software (Bio-Rad, CA, USA).

d. Statistical design and analysis:

The experimental design followed a randomized block design with a factorial arrangement of treatments, specifically a 2x3 factorial design, resulting in a total of 6 treatments, with each treatment having 32 birds per replicate. Data analysis was carried out using multivariate methods, 2-way ANOVA, and mean comparisons were conducted at a 95% confidence level. The statistical analysis was performed using SPSS software (Statistical Package for the Social Sciences, Version 25).

CHAPTER IV

RESULTS AND DISCUSSION

A. Complete blood count

Blood samples were collected from the vein of the wing of bird in blood collection tubes containing anticoagulant EDTA to evaluate blood parameters, and levels of the CBC parameters were shown in Tables 2, 3, and 4. The normal ranges of the hematological parameters in chickens are RBC: 2.5-3.5 x10^6 µl, Hb: 7-13 g/dl and WBC: 12-30 x 10^3 µl (Osadcha, 2023; Bounous & Stedman, 2000; Jain, 1993). The MCV is used to calculate the average erythrocyte size, the MCH to measure haemoglobin amount per blood cell and the MCHC to know the amount of haemoglobin relative to the size of the cell per red blood cell. Their normal ranges are MCV: 90-140 fL, MCH: 33-47 pg/cell and MCHC: 26-35 g/dl (Osadcha, 2023; Bounous & Stedman, 2000; Jain, 1993).

Results showed that exposure to heat significantly decreased the concentration of red blood cells RBC, hemoglobin HGB, hematocrit HCT, mean corpuscular volume MCV, platelets PLT, mean platelet volume MPV, and plateletcrit PCT compared to control groups at two and five months post the initiation of heat stress. Remarkably, heat stress significantly increased platelet distribution width (PDW) at four months post the initiation of heat stress.

The decrease in RBC, ranging between 3.81× 10^6 µl and 2.8 × 10^6 µl was also reported by another study which showcased an indirect relation between the heat stress and the decrease in number of RBCs in exposed quail (Usama Mahmoud et al., 2013). The recorded Hematocrit values, ranging between 27.32 % and 36.11 %, also vary with the ambient temperature at which birds are reared. According to Kubana et al. and Vo et al., the exposure of chickens to high

temperatures causes a decrease in blood hematocrit values (Kubana et al., 1972, Vo et al., 1978). Besides, due to the positive relation between the RBCs number and hemoglobin concentration in blood, the HGB values also showed a significant decline (14.5 g/dl vs 11.34 g/dl for control and heat stress birds, respectively).

The result for hemoglobin was only the protein constituent of red blood cells. Hemoglobin serves as a carrier of oxygen for use in various processes in cells. These findings agree with Dinu et al. (2004), who reported that reduction in the level of hemoglobin and hematocrit values are the consequence of the heat stress, during thermal stress condition reduced value of hematocrit may be due to the decreased production of erythrocyte or the decreased erythrocytes number and size both (Altan et al., 2000). The mean corpuscular volume which indicates the size of the red blood cells also was affected negatively upon heat stress exposure. Research findings showed that the concentrations of blood HCT, RBC, MCV, MCH, MCHC, RDW, HGB, PLT, PCT, and MPV were lower in the heat stress groups compared with the control group in ducks (Byung et al., 2018).

The platelets profile was shown through the platelets count PLT, mean platelet volume MPV, plateletcrit PCT, and platelet distribution width PDW. The obtained results clearly showed that heat stress significantly reduced platelet parameters all over the experimental period except for PDW. Previous research supports our results, where heat-stressed ducks and other birds showed lower red blood cell and platelet counts and lower blood gas concentration, except PO_2 (Park et al., 2015). Although the platelets parameters were reduced due to heat stress, the PDW showed an opposite trend after 4 months of heat stress exposure. The PDW represents the heterogenicity in platelet morphology due to the presence of large platelets along with normal

sized platelets. Literature is not consistently reporting similar results. Abudabos et al. (2018a,b) and Köseman et al. (2021) reported a PDW reduction under heat stress in layers and broilers.

Platelets are cells in the blood that help stop bleeding. When heat stress conditions lead to a decrease in platelets, birds will be more prone to bruising, which affects healing process. This latter is highly correlated to enteric lesions mainly risks of hemorrhage (Saif and Fadly, 2008). A previous study confirmed a reduction in the number of platelets in the blood of broiler chickens in response to heat stress (Osadcha and Pavlovych, 2023).

Table 2 birds mean blood count of white blood cells (WBC, 10^9/L), red blood cells (RBC, 10^6/mm), hemoglobin (HGB, mg/dL), hematocrit (HCT%), mean corpuscular volume (MCV, fL), mean corpuscular hemoglobin (MCH, pg), mean corpuscular hemoglobin concentration (MCHC, %), red cell distribution width (RDW, %), platelets (PLT, 10^3/ml), mean platelet volume (MPV, fL), plateletcrit (PCT, ng/ml), and platelet distribution width (PDW%) under different fee and temperature parameters at 2 months post heat stress initiation.

TREATMENT	**COMPLETE BLOOD COUNT**											
	WBC	RBC	HGB	HCT	MCV	MCH	MCHC	RDW	PLT	MPV	PCT	PDW
FEED												
CONTROL	4.87	3.36	12.71	33.20	98.51	41.32	41.51	10.50	42.92	5.85	0.027	7.21
PROBIOTIC	13.41	3.15	12.81	30.98	97.84	40.62	41.36	10.56	45.50	5.78	0.026	7.05
POSTBIOTIC	8.39	3.18	13.09	32.03	97.45	41.22	41.32	10.68	44.00	5.84	0.027	7.52
TEMPERATURE												
CONTROL	91.87	3.47[a]	14.31[a]	34.40[a]	98.980[a]	41.25	41.64	10.63	52.26[a]	6.14[a]	0.032[a]	7.32
HEAT STRESS	91.23	2.96[b]	11.32[b]	29.36[b]	96.260[b]	40.77	41.09	10.53	35.82[b]	5.53[b]	0.020[b]	7.11
VARIABLES												
TEMPERATURE	0.988	0.050	<0.001	0.048	0.030	0.327	0.100	0.546	<0.001	<0.001	<0.001	0.461
FEED	0.240	0.800	0.936	0.791	0.802	0.444	0.902	0.707	0.896	0.899	0.882	0.370
T * F	0.108	0.840	0.802	0.692	0.176	0.686	0.049	0.651	0.693	0.941	0.718	0.183

a–b Means within a column in each comparison group with no common superscripts differ significantly (P<0.05).

Table 3 birds mean blood count of white blood cells (WBC, 10^9/L), red blood cells (RBC, 10^6/mm), hemoglobin (HGB, mg/dL), hematocrit (HCT%), mean corpuscular volume (MCV, fL), mean corpuscular hemoglobin (MCH, pg), mean corpuscular hemoglobin concentration (MCHC, %), red cell distribution width (RDW, %), platelets (PLT, 10^3/ml), mean platelet volume (MPV, fL), plateletcrit (PCT, ng/ml), and platelet distribution width (PDW%) under different fee and temperature parameters at 4 months post heat stress initiation.

TREATMENT	**COMPLETE BLOOD COUNT**										
	RBC	HGB	HCT	MCV	MCH	MCHC	RDW	PLT	MPV	PCT	PDW
FEED											
CONTROL	3.64	13.88	35.03	96.51	44.75	46.35	10.67	47.80	5.86	0.029	7.3
PROBIOTIC	3.05	13.49	28.66	94.04	44.08	46.86	10.60	47.13	5.86	0.029	7.5
POSTBIOTIC	3.41	12.76	32.24	95.08	42.85	44.93	10.73	48.00	5.99	0.030	7.5
TEMPERATURE											
CONTROL	3.81[a]	14.15	36.11[a]	94.87	43.47	45.86	10.77	48.21	5.88	0.029	7.18[a]
HEAT STRESS	2.87[b]	12.70	27.32[b]	95.53	44.30	46.33	10.70	47.00	5.93	0.028	7.75[b]
VARIABLES											
TEMPERATURE	0.005	0.108	0.006	0.535	0.444	0.615	0.750	0.767	0.576	0.584	0.038
FEED	0.364	0.621	0.277	0.152	0.374	0.221	0.438	0.983	0.415	0.967	0.084
T * F	0.390	0.228	0.403	0.766	0.254	0.194	0.431	0.310	0.207	0.279	0.802

a–b Means within a column in each comparison group with no common superscripts differ significantly ($P<0.05$).

Table 4 birds mean blood count of white blood cells (WBC, 10^9/L), red blood cells (RBC, 10^6/mm), hemoglobin (HGB, mg/dL), hematocrit (HCT%), mean corpuscular volume (MCV, fL), mean corpuscular hemoglobin (MCH, pg), mean corpuscular hemoglobin concentration (MCHC, %), red cell distribution width (RDW, %), platelets (PLT, 10^3/ml), mean platelet volume (MPV, fL), plateletcrit (PCT, ng/ml), and platelet distribution width (PDW%) under different fee and temperature parameters at 5 months post heat stress initiation.

TREATMENT	**COMPLETE BLOOD COUNT**											
	WBC	RBC	HGB	HCT	MCV	MCH	MCHC	RDW	PLT	MPV	PCT	PDW
FEED												
CONTROL	6.69	3.05	12.76	31.12	101.43	42.10	41.42	10.45	49.57	6.01	0.030	7.33
PROBIOTIC	13.23	3.03	12.28	29.89	98.44	40.39	41.05	10.58	40.13	5.68	0.023	6.90
POSTBIOTIC	7.54	3.17	13.45	32.26	99.42	41.12	41.75	10.69	42.60	5.87	0.160	7.51
TEMPERATURE												
CONTROL	9.31	3.42[a]	14.50[a]	34.74[a]	99.85	41.72	41.84[a]	10.6	52.9[a]	6.10[a]	0.0969	7.28
HEAT STRESS	9.28	2.80[b]	11.34[b]	27.70[b]	99.02	40.62	41.00[b]	10.6	36.0[b]	5.58[b]	0.0200	7.17
VARIABLES												
TEMPERATURE	0.994	0.003	<0.001	<0.001	0.557	0.062	0.015	0.876	<0.001	<0.001	0.198	0.666
FEED	0.333	0.849	0.539	0.652	0.172	0.062	0.250	0.610	0.207	0.111	0.129	0.137
T * F	0.417	0.524	0.563	0.507	0.729	0.923	0.258	0.981	0.160	0.872	0.587	0.571

a–b Means within a column in each comparison group with no common superscripts differ significantly (P<0.05).

B. Microscopic intestinal morphology (Histopathology)

The number of villi and mucosal cells are important indicators of gut function and animal health. The villi are the key components responsible for the absorbance of nutrients in the small intestine. Increasing villi number may result in higher nutrient absorption thereby improving growth performance. The results in Table 5 showed numerically high number of villi/ microscopic field in intestinal lining of birds fed with diet containing postbiotic supplementation 15.19 compared to the control group 14.16. Also, heat stress slightly affected the density of the villi number showing lower value than the control group ($P>0.05$). Deciliation areas in the intestines were the highest in birds of the control group 50.79. Probiotic and Postbiotic treatments numerically reduced the deciliation areas indicating an improvement of the mucosal and cilia structure of the Jejunum (Abd El Ghany et al., 2022). Another finding suggests positive impacts of both postbiotics and probiotics on the absorptive area of the jejunum. The enhancement in the morphological aspects of the intestinal lining contributes to increased absorption, likely arising from the competitive interactions between lactic acid bacteria (LAB) and harmful microorganisms, influencing resource utilization and ecological niches (Shojadoost et al., 2022).

It is worth noting that although Probiotic treatment significantly reduced the number of mucosal cells, it limited the percentage of degenerating mucosal cells (71.29/microscopic field) in comparison to the control (87.5) and postbiotic group (88.89) ($P>0.05$). These results, emphasizing the positive role of probiotics in supporting the mucosal layer integrity and function, are in agreement with the studies of Zhang et al., 2016, Gadde et al., 2017 and Wang et

al., 2018. Apparently, the adopted model was not stressful enough to induce significant changes at the level of the observed microscopic lesions (Rafiq et al., 2022, Fumika et al., 2020). This was reflected through the lack of significance between heat stressed and control birds regarding the said parameters.

Table 5 birds average number of villi, mucosal cells, and deciliation areas under microscopic fields and percentage of microscopic fields showing no mucosal cells degeneration under different temperature and feed parameters during 5 months of the experiment.

Treatment	***Histopathology***			
	Number of villi/microscopic field	**Number of mucosal cells/microscopic field**	**Deciliation areas/ microscopic fields**	**% of microscopic fields showing mucosal cells degeneration**
Feed				
Control	14.16	52.45[a]	50.79	87.50
Probiotic	14.41	44.22[b]	38.89	71.29
Postbiotic	15.19	52.50[a]	45.37	88.89
Temperature				
Control	14.70	50.02	47.41	87.66
Heat stress	14.61	48.51	40.97	77.47
Variables				
Temperature	0.428	0.201	0.197	0.170
Feed	0.232	<0.001	0.475	0.070
T*F	0.010	0.019	0.548	0.412

a–b

Means within a column in each comparison group with no common superscripts differ significantly (P<0.05).

C. Gross lesions (macroscopic)

The least observed macroscopic lesions were the presence of thick intestinal wall and undigested feed in the intestinal lumen (Table 6). Postbiotics exerted a positive impact in reducing major macroscopic lesions such as thinning of the intestinal wall (intestinal tissue integrity), hyperaemia (inflammation) and watery contents (against pathogens), specifically towards the end of the experiment. Results, shown in Table 6, are in agreement with other literature emphasizing the role of postbiotics/probiotics in relieving heat stress impact. Abd El-Ghany et al. illustrated in his research the efficacy probiotics, postbiotics and antibiotics on the enteric lesions where the histopathological effects were significantly improved for the treated groups (Abd El- Ghany et al., 2022).

The intestinal lesion evaluation reflected a mild impact of heat stress on intestinal health during the first half of the experiment. Although no significant differences in the second half of the experiment, as well, there is a major trend whereby heat stress increased the score of thin walls (75%), hyperaemia (100%), and watery content (50%). The postbiotics reduced the frequency of birds with watery intestinal contents to 25%; while the probiotic lessened the hyperaemia frequency to 25%. Recently published study by Xu, et al. (2020) observed that L. plantarum supplementation to layer diet not only enhances mucosal integrity, but also reduces intestinal inflammation (Xu et l., 2020).

Adding to that, the duodenum, jejunum, and ileum intestinal sections were given scores for lesions determination in support with the above results. The jejunum part was affected mostly scoring 1 in the control group where the probiotic enhanced its integrity to reduce the scoring to 0.375. As for the ileum, postbiotic also revealed its positive impact, scoring 0.125 in comparison to the control (0.75). Nevertheless, heat stress affected all the intestinal parts negatively showing

lower scoring compared to the control. This emphasizes the negative role of heat stress on the integrity of the mucosal tissue of the intestine reflected in the reduction of mucosal cells per microscopic fields and the positive impact of pro/postbiotic on decreasing deciliation areas.

Previous studies reported that the alterations in the intestinal epithelium integrity and microbiota, which could disturb the homeostasis of the intestinal ecosystem and lead to enteritis, have been linked to heat stressed birds (Burkholder et al., 2008; Awad et al., 2018).

The inflammation of intestinal mucosa can directly affect the intestinal barrier function, impairing the absorption of nutrients and slowing down the growth of birds. It has been indicated that the exposure to acute heat stress causes a substantial impairment in the gut microbiota, intestinal integrity, and villus morphology (Burkholder et al., 2008).

Table 6 percentages of intestinal thin wall, thick wall, hemorrhage, watery content and undigested feed under six different treatments taken at the mid and the end of the 5-month experimental trial.

Intestinal integrity	***Thin wall***	***Thick wall***	***Hyperaemia***	***Watery content***	***Undigested feed***	***Thin wall***	***Thick wall***	***Hyperaemia***	***Watery content***	***Undigested feed***
	Mid					**End**				
Scoring percentage										
Control/control	0%	0%	25%	75%	0%	75%	0%	75%	100%	0%
Probiotic/control	50%	0%	0%	50%	0%	75%	25%	50%	75%	0%
Postbiotic/control	75%	0%	0%	75%	0%	50%	0%	50%	75%	0%
Control/ heat stress	50%	0%	25%	25%	0%	75%	0%	100%	50%	0%
Probiotic/ heat stress	75%	0%	25%	25%	0%	50%	0%	25%	50%	0%
Postbiotic/ heat stress	50%	0%	25%	75%	0%	25%	0%	50%	25%	0%
Significance	0.306	-	0.791	0.432	-	0.634	0.390	0.360	0.319	-

Table 7 Average scoring of the duodenum, jejunum, and ileum parts of the intestine under different feed and temperature parameters at the mid and end of the 5-month experimental period

Treatment	***Intestinal scoring***					
Time	**Mid**			**End**		
	Duodenum	**jejunum**	**ileum**	**duodenum**	**jejunum**	**ileum**
Feed						
Control	0.125	0.125	0.125	0.750	1.000	0.750
Probiotic	0.125	0.000	0.000	0.375	0.375	0.250
Postbiotic	0.000	0.125	0.250	0.625	0.625	0.125
Temperature						
Control	0.000	0.083	0.167	0.420	0.580	0.420
Heat stress	0.167	0.083	0.083	0.750	0.750	0.330
Variables						
Temperature	0.152	1.000	0.557	0.303	0.534	0.731
Feed	0.614	0.614	0.350	0.636	0.142	0.065
T * F	0.615	0.250	0.721	0.298	0.487	0.879

D. Hemagglutination inhibition test

To determine the effect of probiotic and postbiotic supplementation on Humoral immunity of layers under heat stress conditions, serum samples were tested using HI (Hemagglutination Inhibition) method to depict antibody production in response to live Newcastle disease vaccine. The immune response results at 2- and 4-weeks post vaccination against NDV vaccine are shown in Table 8. No significant differences were observed in all the results of the HI titers against NDV vaccine. Although the results are not significant at 2- and 4-weeks post vaccination with NDV, the HI titer of probiotic and postbiotic groups were numerically higher than that of the control group. The results at week two post vaccination ranged between 192.59 and 312 titer and decreased after four weeks post vaccination to range between 31.89 and 36. This decline of HI titer after 4 weeks post vaccination agrees with previous references reporting a maximal antibody titer obtained at two weeks post vaccination (Saif and Fadly, 2008). The magnitude of humoral response depends largely on the vaccine/infecting strain, bird's age and breed, and the environmental conditions.

The probiotics and postbiotics clearly enhanced the HI titer compared to the control group. It's worth noting that all birds showed Positive HI titers at two- and four-weeks post vaccination (greater than 16). This highlights the efficacy of probiotics and postbiotics in reducing the immunosuppression effect of heat stress in poultry (Humam et al., 2019). The same authors reported an enhanced efficacy in utilizing *L. plantarum* RI11 postbiotics in comparison to RS5 metabolites, used in this study, to improve IgG and IgM antibody levels in heat stressed broilers.

On the other hand, impact of specific pro-and postbiotic was not significant on the humoral immunity of heat stressed birds as reported by Rahimi and Khaksefidi (2006) and Humam et al.,

2019. This clearly shows that the role of dietary probiotics and postbiotics as immunopotentiators is still debatable.

E. SDS PAGE: western immunoblotting protective level

In Western blots, the Signal-to-Noise (S/N) ratio is determined by comparing the signal intensity to the noise in the local background for each band present on the blot. When a blot exhibits bands of relatively low intensity, a higher S/N ratio indicates that these bands are more likely to be discernible and, consequently, potentially measurable, in contrast to a scenario with a low S/N ratio.

The results showed no significant difference between the control and treatment groups at 2- and 4-weeks post vaccination. As for the value of the postbiotic group, it showed higher S/N (1.1) than the control group (1.08) 2 weeks post vaccination, whereas the probiotic group showed greater (1.17) S/N ratio compared to control group (1.07) at 4 weeks post vaccination.

The statistical analysis of the quantitative seroconversion's antigen F of NDV, revealed complete failure of seroconversion to the protective F-protein of NDV in birds ($p>0.05$). High HI titers against the Haemagglutinin protein coupled with the low antibody titers against the fusion protein raise the question on the protective efficacy of the used vaccine against velogenic NDV strains. The importance of obtaining a significant seroconversion to the F-protein is due to its role in the pathogenesis of ND, in which the F-protein assists the virus to be involved in cell fusion leading to penetration of the host cell, a prerequisite for the multiplication mechanism (Saif and Fadly, 2008).

These results open the door towards future investigation and evaluation of specific NDV vaccines that should be used in Lebanese farms to provide protective immune response against

highly velogenic NDV strains circulating in the country. This evaluation should not be only based on ELISA or HI tests, but should include WB analysis as well.

Table 8 Average Hemagglutination inhibition titers and average S/N ratios of western blotting of birds' sera under different temperature and feed parameters during 5 months of experimental period

Treatment	*Hemagglutination inhibition test and western blot*			
Time	Week 2 post vaccination		Week 4 post vaccination	
	HI	S/N	HI	S/N
Feed				
Control	204.89	1.08	31.89	1.07
Probiotic	312.00	1.08	36.00	1.17
Postbiotic	216.00	1.10	32.00	1.04
Temperature				
Control	192.59	1.10	33.26	1.08
Heat stress	296.00	1.07	33.33	1.10
Variables				
Temperature	0.066	0.596	0.986	0.732
Feed	0.229	0.938	0.660	0.138
*T*F*	0.018	0.434	0.414	0.017

[a–b] Means within a column in each comparison group with no common superscripts differ significantly (P<0.05).

CHAPTER V

SUMMARY, CONCLUSION AND RECOMMENDATION

The present research revealed that heat stress adversely affected the blood parameters, particularly the levels of red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), platelets (PLT), mean platelet volume (MPV), and plateletcrit (PCT) in layers subjected to cyclic heat stress. These parameters exhibited a significant ($p<0.05$) decrease compared to the control groups after two and five months of the trial.

Additionally, the examination of intestinal integrity, both at microscopic and macroscopic levels, underscored the positive impact of probiotic/postbiotic supplementation in this regard. This was evident in the numerical improvement of scored lesions attributed to heat stress, manifested by the mitigation of mucosal cell degeneration and deciliation in both probiotic and postbiotic groups, along with an increase in the number of villi. The impact of *L. plantarum* probiotics and postbiotics was not significant in the enhancement of humoral immunity, specifically against the fusion protein of NewCastle Disease Virus.

Protbiotic metabolites can be an alternative feed additive to achieve healthy gut and strong immunity while reducing the use of conventional chemotherapeutic agents such as in-feed antimicrobials under heat stress conditions. Further research is needed to study the impact of different strains and/or mixtures of dietary probiotics and postbiotics upon heat stress conditions. Adding to that, experimental adjustments might be included by increasing the incubation hours while preparing the postbiotic solution to ensure that the whole process of producing the metabolites is completed. This would give a better insight into the role of such products in mitigating heat stress impact.

In addition, the paucity of significant differences in various immunity parameters between heat stressed and control birds, suggests evaluating new heat stress models adopting various checkerboard designs that include different temperature, cycling hours and layer breeds.

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