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Research Article

Effects of *Saccharomyces cerevisiae* and *Lactobacillus* Strains Supplementation on Immune Response and Intestinal Morphology in Broiler Chickens under Heat-Stressed Conditions

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ABSTRACT

Introduction: Global warming increases environmental temperature, threatening poultry production worldwide. The present study aimed to explore the effects of yeast and *Lactobacillus* strains on gut health and immune parameters in broiler chickens under heat-stressed condition.
Methods and materials: A total of 192 day-old Arbor Acre broiler chickens with average weight of 45 gr of both sexes were randomly divided into four treatment groups. Each group had three replicates of sixteen chickens in a completely randomized design, and intestinal morphology, microbial balance, immune response, and Temperature-Humidity Index (THI) were evaluated. Balanced basal diet were formulated and supplemented with no probiotic (control), *Saccharomyces (S.) cerevisiae* at 1g/kg (Sacc), *Lactobacillus* strains (10^{10} cfu/g) at 0.25 g/kg (Lac), and a combination of *Lactobacillus* and *S. cerevisiae* (Lac-Sacc) at 0.25g and 1g per kg, respectively. Feed and water were provided *ad libitum* in a deep litter system for 42 days.
Results: The THI data from the poultry house indicated that chickens were under severe heat stress during the experimental period. The results revealed that *S. cerevisiae*, *Lactobacillus* strains, and their combination significantly downregulated interleukin-6 (IL-6) expression and improved lymphocyte counts, with *S. cerevisiae* additionally upregulating IgM expression compared to control. Moreover, *Lactobacillus* supplementation positively affected villi height and crypt depth and lowered coliform bacteria compared to the control.
Conclusion: The inclusion of *S. cerevisiae* and *Lactobacillus* significantly enhanced gut health in broiler chickens under heat-stressed condition, with *Lactobacillus* being more effective at alleviating heat stress.

1. Introduction

Gut health relies on the coordinated action of several crucial components, including the gastrointestinal epithelium, the gut's immune defenses, and the complex community of intestinal microbes[1]. When animals are exposed to heat stress marked by lower oxygen levels and decreased nutrient intake, their digestive system can suffer several issues. This includes damage to the mucosal lining, structural changes, heightened oxidative damage, and increased inflammation. All of these factors can disrupt tight junction integrity and impair immune function[2]. In poultry, heat stress affects the gut barrier's integrity, resulting in heightened permeability and inflammation, especially in the small intestine's sections; the duodenum, jejunum, and ileum. This condition is characterized by a substantial influx of lymphoplasmacytic cells[3-5]. Heat stress disrupts the intestinal barrier, increasing permeability to endotoxins and facilitating the translocation of intestinal pathogens[6]. According to a study, increased crypt depth and decreased villus height led to a lower villus height-to-crypt depth ratio, characterized by broader villus bases and reduced epithelial cell surface area in the duodenal, jejunal, and ileal mucosa[7]. The poultry gut microbiome provides both health and productive benefits by influencing digestion, metabolism, as well as immunity and disease resistance.

The immune system is essential for maintaining balance and

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stability within the gut, as well as in protecting against pathogens by minimizing direct bacterial contact and quickly detecting and removing pathogens[8]. Over the past two decades, evidence has established that cytokines serve as key regulators of the immune system[9]. Cells release cytokine proteins and peptide messengers, which are essential in coordinating immune and inflammatory responses. They are critical for role in stimulating immune responses, particularly by promoting the production of proinflammatory mediators, such as IL-6 and IL-8[10].

In recent years, the poultry industry has increasingly adopted probiotics to enhance the health and performance of broiler chickens[11]. Among these, *Lactobacillus* strains have gained significant attention from their role in improving heat tolerance[12], villi height and crypt depth[13,14,] enhancing the colonization of beneficial intestinal bacteria[15] and their immune-modulatory effects[16]. The *Saccharomyces* (*S.) cerevisiae* has been shown to improve feed conversion ratios[17] and affect the populations of both *Salmonella* and *E. coli* in the gut[18,19]. The present study aimed to evaluate factors, including intestinal morphology, microbial balance, immune response, and overall gut function to evaluate the potential benefits of *S. cerevisiae* and *Lactobacillus* Strains in improving gut health and mitigating the negative consequences of stress in broiler chickens.

2. Materials and Methods

2.1. Ethical approval

The current study was conducted under strict adherence to the ethical guidelines and principles established by the Department of Animal Production and Health, Faculty of Agriculture and Life Sciences, Federal University Wukari. It was approved by the Institutional Animal Care and Use Committee, with approval number FUW/ AGR/ APH/057.

2.2. Experimental design

The experiment was conducted at the poultry unit of the teaching and research farm of the Department of Animal Production and Health of the Federal University, Wukari, Taraba State, Nigeria. The farm is located at latitude 7º 50' 0 '' N, and longitude 9º 46' 0" E. Wukari Local Government has a total land area of 4,308 km^2 and 241,546 people.

192 day-old *Arbor Acre* broiler chickens with average weight of 45 gr were randomly distributed across four experimental treatment groups using a completely randomized design, with three replicates per treatment and 16 chickens per replicate. Broiler starter (days 1-21) and finisher (days 21-42) diets based on maize and soybean were formulated according to the NRC[20] nutrient requirements for broiler chickens. The details of the diets are indicated in Table 1. A commercially available yeast, (Pure™ *S. cerevisiae,* USA), and a mixed strain of *Lactobacillus*-based probiotic (NOW® Probiotic-10TM, USA) were used for the current study, containing a blend of 10 probiotic strains of probiotic bacteria (*L. acidophilus* (La-14), *L. plantarum* (Lp-115), *L. rhamnosus* (Lr-320), *L. salivarus* (Ls-330), *L. casei* (Lc-11), *L. paracasei* (Lpc-37), *Bifidobacterium (B.) breve* (Bb-03), *B. lactis* (Bl-04), *B. longum* (Bl-05), and *Streptococcus* thermophiles (St-21) of 10^{10} cfu/g[18]. Both products were purchased online from the United States of America. Four experimental diets were prepared, including a diet without *S. cerevisiae* and *Lactobacillus* (control), diet containing *S. cerevisiae* at the rate of 1g/Kg (Sacc), diet with *Lactobacillus* probiotic at a rate of 0.25g/kg (Lac), and a diet with a combination of *Lactobacillus* and *S. cerevisiae* (Lac-Sacc) at a rate of 0.25g and 1g/Kg respectively. The Chickens were raised on 2 by 8 meter deep litter with feed and water provided *ad libitum* for a period of 42 days.

Table 1. Ingredient and nutrient composition of experimental diets of broiler chickens for 42 days rearing

Ingredients	Starter (kg, days 1-21)	Finisher (kg, days 21-42)
Maize	51.00	56.00
Soybean meal	38.00	33.00
Maize offal	3.00	3.00
Fish meal	3.00	3.00
Bone meal	3.00	3.00
Limestone	1.00	1.00
Premix*	0.30	0.30
Methionine	0.20	0.20
Lysine	0.20	0.20
Salt	0.30	0.30
Total	**100**	**100**
Calculated Nutrient Composition		
ME (Kcal/Kg)	2834.00	2952.00
Crude protein (%)	22.79	19.14
Calcium	1.23	1.21
Phosphorus	0.79	0.79
Lysine	1.08	0.93
Methionine	0.58	0.53
Crude fibre	3.50	4.00
Ether extract	3.00	3.50

*Vitamin-mineral premix per kg as following: A: 1500 IU; D3: 3000 IU; E: 30 IU; K: 2.5mg; Thiamine B1: 3mg; Riboflavin B2: 6mg; Pyridoxine B6: 4mg; Niacin: 40mg; B12: 0.02mg; Pantothenic acid: 10mg, Folic acid: 1mg; Biotin: 0.08g; Chloride: 0.125g; Mn: 0.096g; Antioxidant: 0.125g; Zn: 0.06g; Fe: 0.024g; Cu: 0.006g; 10.0014g; Se: 0.24g; Co: 0.240g; ME: Metabolizable energy

Indoor temperature and relative humidity were measured using a digital thermometer twice a day at 8:00 a.m. and at 3:00 p.m. throughout the study. The temperature-humidity index (THI) was then calculated according to guidelines provided by Tao and Xin[21] using the dry-bulb temperature (Tdb, in °C) and wet-bulb temperature (Twb, in °C).

Wet bulb temperature (Twb) was derived from ambient temperature and relative humidity using Stull's[22], empirical expression functions. The calculated THI was categorized into three classes, including < 26 (comfort limit), 26-29 (heat stress), and > 29 (severe heat stress) as described by Duduyemi and Oseni[23]. Using an infrared thermometer (ARTLIGHT ZK-YK1028, Greece), body temperature was measured[24], whereas respiratory rate was recorded by counting breaths per minute with a digital stopwatch. The treatments and vaccination were scheduled at day one, including Newcastle disease vaccine (ND, intraocular, India). Multivitamin and glucose was addministarted at day seven. Infectious bursal disease (IBD, India), Gumboro vaccine (India) were scheduled at day fourteen, as well ND vaccine, (Lasota, India) and multivitamins (1 ml/liter of water). The manual method was used for feeding and watering the chickens.

At the end of the 42-day feeding trial, two chickens from each replicate were selected. The chickens were slaughtered using manual slaughtering, cutting via the jugular vein with a sharp knife, and eviscerated. Tissue samples were collected for further lab analysis.

Tissue samples were taken just before Meckel's diverticulum (jejunum) and preserved in 4% paraformaldehyde to facilitate histological analysis[25]. The samples were transported to the Histology Laboratory, Central Diagnostic Division, National Veterinary Research Institute, Vom, Plateau State, Nigeria. Harvested tissues were initially fixed in 10% formalin for three days to ensure proper preservation, then dehydrated in graded ethyl alcohol (50 to 100%) and paraffin-embedded into action sections (Tissue-Tek, Sakura, Tokyo, Japan). Approximately 4 µm sections were cut using a Rotary Microtome (HM340E, Thermo Scientific, Germany). Sections were deparaffinized, hydrated, and stained with hematoxylin and eosin (H&E), and with Alcian blue to count villi, following the modified method of Winsor and Sluy[26]. Each tissue section was examined using a light microscope (Nikon, Eclipse i80, Tokyo, Japan). For each chicken, at least 10 villi per intestinal segment were measured to determine villus height, top and basal widths, crypt depth, as well as the areas of the epithelial cells were calculated; villi surface (Areas = 2 π [width/2] length) and villi height-to-crypt depth ratio according to Al-Garadi et al.[25]. The intestinal content of the jejunum was collected from two chickens per replicate. The samples were transported in an insulated ice bag to the Bacteriology Laboratory, Central Diagnostic Division, National Veterinary Research Institute, Vom, Plateau State, Nigeria. Samples were stored at 4°C until they were analyzed. The samples were freeze-thawed until they reached room temperature (25°C). De Man–Rogosa–Sharpe agar (MRS), Dextrose Agar (SDA, Merck KGaA, 64271 Darmstadt, EMD Millipore Corporation, Germany), and Mueller Hinton agar (MHA, M0203, FLINN SCIENTIFIC) were used for isolation and characterization of bacteria, respectively. The MRS was prepared, poured into Petri plates, and allowed to solidify[27]. A precise measurement of 10 g of the original sample (in 1000 mLs) and its third and fourth serial dilutions (10^3, 10^4) were poured on solidified agar plates. Plates were incubated at 37°C. Bacterial colonies obtained after 24 hours were enumerated to determine CFUs/ml using the following formula[28].

CFUs/ ml of Ringer's solution = (No of colonies × dilution factor)/volume of culture

The isolated bacteria were preserved as glycerol stocks for later use[28].

Samples were collected from two centimeters (2 cm) of the jejunum of two chickens per replicate. These samples were placed in a test tube filled with normal saline and kept in an ice pack. They were then transported to the Animal Physiology Laboratory at the National Animal Production Research Institute (NAPRI) in Shika, Zaria, Kaduna State, Nigeria, for interleukin (IL-6) and immunological analysis. Tissue portions were homogenized. Interleukin-6 (IL-6) levels in the jejunum tissue were measured using a sandwich ELISA kit (Elabscience, USA), strictly following the manufacturer's protocols. In this method, IL-6 was bound to a capture antibody pre-coated on a microplate well, followed by the addition of an enzyme-conjugated detection antibody. The colorimetric reaction was read at 450 nm to determine IL-6 concentrations. Similarly, tissue IgM concentrations were measured using a chicken IgM ELISA kit (Eagle Biosciences, USA), also adhering strictly to the manufacturer's instructions. As with IL-6, a sandwich ELISA approach was used, where IgM was captured by a pre-coated antibody, and then an enzyme-linked detection antibody was added. The colourimetric reaction was measured at a wavelength of 450 nm to determine the concentrations of TgM. The ELISA kit used for lymphocytes detection was Chicken B lymphocyte antigen CD20 (MS4A1) ELISA kit (amsbio Cambige Street, USA). The manufacturer's protocol was followed, as described in the procedure above[10].

2.3. Statistical analysis

A one-way ANOVA was performed on all collected data using the Fit Y by X model according to JMP SAS (Version 15.2)[29]. Significant differences were determined using Tukey's HSD post-hoc test, applying a significance threshold of ($p < 0.05$). Graphical representations were prepared by the graph builder function of GraphPad Prism (Version 6.0).

3. Results

3.1. Villus height and bacterial count

The mean THI in the morning and afternoon was 27.1 and 30.5, respectively, compared to the control group. Table 2 indicates a significant effect on villus height (807.67µm) and crypt depth (218µm) of chickens supplemented with *Lactobacillus* ($p < 0.05$). The control group exhibited a significantly higher total bacterial count (5.12 cfu/g), coliform counts (4.00 cfu/g), *E. coli* counts (3.85 cfu/g), and *Lactobacillus* counts compared to the control group. (1.94 cfu/g). Notably, no *E. coli* was detected in the jejunal contents of chickens receiving *Lactobacillus*, as observed in both the Lac and Lac-Sacc groups.

3.2. Immune response

Table 3 indicates the jejunal immune response of heat-stressed broiler chickens fed diets supplemented with *S. cerevisiae* and *Lactobacillus* strains compared to control group. A significant difference was observed across the treatments, with the control group showing increased (120.30

µg/ml) expression of interleukin-6 ($p < 0.05$). Immunoglobulin IgM expression was significantly (139.24 µg/ml) higher in the *S. cerevisiae* group compared to control group, and lymphocyte counts 68.75%,69.25%, and 59.25% were also significantly increased in all probiotic-containing groups than in the control ($p < 0.05$).

Table 2. Jejunal villi morphology of broiler chickens under heat-stress fed with *Saccharomyces cerevisiae* and *Lactobacillus* strains

Parameters	Control	Sacc	Lac	Lac-sacc	SEM	P-Value
VH (µm)	706.5[b]	701.2[b]	807.7[a]	644.5[c]	6.72	0.0001
VW A (µm)	30.17	32.00	33.33	31.67	0.66	0.2567
VW B (µm)	110.9	111.3	111.5	111.5	1.79	0.9942
V A (μm^2)	6.82	6.75	7.23	6.37	0.23	0.4505
VCD (µm)	189.8[ab]	165[b]	216[a]	179.3[b]	5.49	0.0165
VH/C D	4.00	4.00	3.50	3.67	0.16	0.2584

[a,b,c] Different superscript letters means significant different on the same row ($p < 0.05$). SEM: Standard error of the mean, VH: Villi height, VWA: Villi widths A, VWB: Villi widths B, VA: Villi area, Sacc: *Saccharomyces cerevisiae*, Lac: *Lactobacillus*

Table 3. Jejunal immune responses of broiler chickens under heat-stress fed with *Saccharomyces cerevisiae* and *Lactobacillus* strains

Parameters	Control	*Sacc*	*Lac*	*Lac-sacc*	SEM	P-Value
IL-6 (pg/ml)	120.30[a]	73.04[b]	57.53[b]	71.06[b]	3.99	0.0009
IgM (µg/ml)	53.02[b]	139.24[a]	75.07[b]	81.59[b]	3.95	0.0091
Lymphocyte (%)	35.02[b]	68.75[a]	69.25[a]	59.25[a]	7.68	0.0003

[a,b] Different superscript letters means significant different on the same row ($p < 0.05$), SEM: Standard error of the mean, Sacc: *Saccharomyces cerevisiae* , Lac: *Lactobacillus*

4. Discussion

Broiler chicken production is highly sensitive to thermal conditions, as its thermoneutral zone ranges between 18 to 25°C. Under or below this temperature, broilers may show physiological changes that could compromise welfare, health, and production[30]. According to the THI scale described by Duduyemi and Oseni[23], the broiler chickens were under a very severe heat stress in the afternoon during the present study. The THI serves as an effective indicator for evaluating how climatic conditions influence livestock productivity[31,32]. Basically, it represents an empirical calculation of all external forces that may cause an animal's body temperature to stray from its ideal set point[31]. Additionally, Ademu et al.[33] noted that broiler chickens raised under THI conditions up to 30.0 experienced severe stress according to an increase in rectal temperature. Habeeb et al.[34] reported a value of 30.0 for THI as a severe heat stress in small animals. In the current study, the chickens were observed while moving away from their feeding troughs, scatter around in their pen, spread their wings, and pant. Chickens exposed to high temperatures often detected the signals of interia by squatting near the ground, resting more, and deduction in feeding and walking[35].

The obtained data demonstrated that broiler chickens reared under heat-stressed condition feeding with probiotics, *S. cerevisiae* and *Lactobacillus* strains, improved epithelial integrity characteristics, such as jejunal villi height and villi crypt depth compared to control group. Hernandez-Coronado et al.[36] revealed similar results where *Bacillus subtilis* at 2×10^9 cfu/g was given to heat-stressed broiler chickens. A higher villi height and villi height/crypt depth ratio indicated superior intestine growth and improved capacity for nutrition absorption[37]. An increase in the villus height and intestinal epithelial cell count suggested an improve in absorbing nutrients potential. The small intestine's secretory activity is reflected in the depth of the crypt[38]. A previous study by Ledezm-Torres et al.[39], noted an increase in villi height and crypt depth when probiotics were supplemented on heat-stress broiler diets. An increase in villus height indicates a larger surface area for absorbing more available nutrients. Similarly, higher villus heights may enhance the activity of the enzymes produced from the villi's tips, leading to better digestibility. A healthy gut is indicated by improved gut shape, which is crucial for the chickens' best performance[40]. The obtained results are in line with the findings of Awad et al.[41], who found that the chickens' villus height increased in the ileum and duodenum when *Lactobacillus* sp. was added to their diet. Multi-microbe probiotic pills were found to increase villus height to crypt depth[42]. Increase in enzymatic activities and epithelial enterocytes turnover could be one of the reasons for increased villi height. Different *Bacillus* species show distinct impact on promoting intestinal epithelial cell differentiation and proliferation as well as enhancing nutrient uptake[43].

Probiotics, including *S. cerevisiae* and *Lactobacillus* strains promote the growth of beneficial microorganisms while suppressing harmful bacteria from the present study. Probiotics, by leveraging competitive exclusion, effectively prevent harmful bacteria such as *Clostridium perfringens* and *Salmonella* spp. from colonizing the gut[44]. The inclusion of *Lactobacillus* strains in poultry diets has the benefit of reducing the population of coliform bacteria. In the present study, *E. coli* was completely eliminated from the chickens' jejunum. *Lactobacilli* (a heterogeneous group of Gram-positive, non-spore-forming, catalase-negative bacteria) thrive in a wide range of environments. *Lactobacilli* are notably acid-tolerant and rapidly convert carbohydrates into lactic acid, even under the challenging conditions present within the gut[45]. The current study agrees with the findings of Li et al.[46], who concluded that probiotics primarily improve gut microbiota modulation, improve enterocyte turnover, with competitive exclusion of pathogens. A well-balanced gut microbiota is crucial for the proper functioning of the HPA and Hypothalamus-Pituitary-Thyroid (HPT) axes[47,48]. Gut microbiota modulation is emerging as an innovative strategy to enhance host health and well-being across a variety of conditions[49].

The potential of yeast products to enhance growth and modulate immune responses in poultry production has been well documented[50]. The pro-inflammatory cytokine IL-6 is transiently produced in reaction to infections and tissue injury, contributing to host protection by initiating acute phase responses, promoting haematopoiesis, and engaging key immune mechanisms[51]. According to Wang et al.[52] yeast treatment reduced jejunal IL-6 gene expression and transcription levels. Furthermore, IL-6 is one of the necessary cytokines to start inflammatory reactions[51]. Ashraf et al.[14] announced that *Lactobacillus*-based probiotics reduce the excessive inflammatory response throughout all intestinal segments in broiler chickens under heat-stressed condition. They increase the number of goblet cells that produce mucins,

a critical component of innate immunity in the duodenum and jejunum. The increase in IgM and lymphocyte levels in the probiotic groups could be due to the low expression of IL-6 in those groups. This is in agreement with Xu et al.[38] that noted an increase in IgM and lymphocyte levels in the poultry gut with the inclusion of probiotics in poultry diets. Heat stress raises the ratio of heterophils to lymphocytes, indicating a state of immune suppression[53]. This was not observed in the probiotic-containing groups, indicating the immuno-modulatory function of both *S. cerevisiae* and *Lactobacillus* strains.

5. Conclusion

The present study demonstrates that the supplementation of *S. cerevisiae* and *Lactobacillus* strains in the diet of heat-stressed broiler chickens significantly enhances their physiological and gut health. The probiotics can played a crucial role in modulating immune responses, as evidenced by the lower expression of IL-6 and the improved lymphocyte count. The *S. cerevisiae* supplementation significantly increases IgM expression, underscoring its immunostimulatory effect. Additionally, the probiotics contributed to better gut health by reducing jejunal microbial populations and enhancing villi height and crypt depth. The *Lactobacillus* strains were particularly effective in reducing coliform bacteria populations in the jejunum. Poultry producers are advised to incorporate *Lactobacillus* strains into broiler feed at a concentration of 0.25g/kg to combat the detrimental effects of heat stress. More studies need to be conduct with different age chickens and breed to clarify the effects of yeast and *Lactobasili* starin on chickens health and performance.

Declarations

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Authors' contributions

In this study, the conception of the study was carried out by Adum Alor and Danladi Tsokwa; methodology and data collection were performed by Danladi Tsokwa and Rapheal James Wafar ; data preparation and validation were handled by Danladi Tsokwa, Jivini Aji Nwuku, and Lawrence Anebi Ademu; data analysis was conducted by Adum Aluor; the original draft and subsequent drafts were written by Danladi Tsokwa, Jivini Aji Nwuku, and Lawrence Anebi Ademu; supervision was provided by Adum Aluor and Rapheal James Wafar; and all authors reviewed and approved the final draft.

Availability of data and materials

The datasets used and analyzed in the study are available upon reasonable request from the corresponding author.

Competing interests

The authors declare no conflict of interest.

Ethical considerations

The authors confirm that this work is original and has not been published previously, nor is it under consideration for publication elsewhere. No AI tools was used to conduct this study.

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