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BOOKLET

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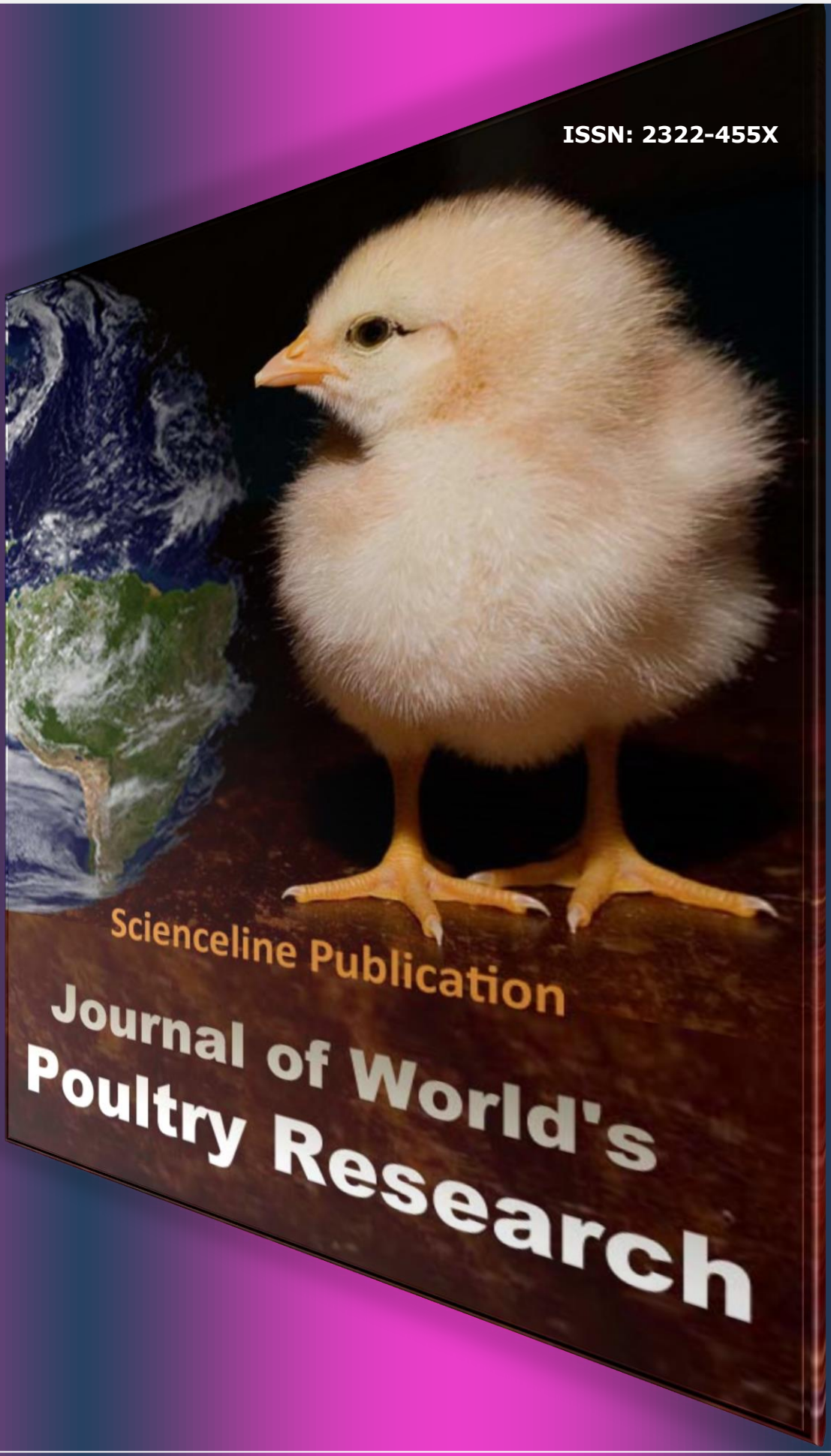

Volume 13, Issue 4, December 2023

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Review

The Importance and Challenges of Primary Chicken Embryo Liver Cells in Studies of Poultry Viral Diseases: A Review

Sohaimi NM and Clifford UC.

J. World Poult. Res. 13(4): 364-372, 2023; pii: S2322455X2300039-13

DOI: https://dx.doi.org/10.36380/jwpr.2023.39

ABSTRACT: Primary chicken embryo liver (CEL) cells are derived from the liver tissue of chicken embryonated eggs (CEE) using an aseptic isolation technique and growth under a controlled atmosphere in an artificial environment for cell attachment and proliferation. Although this primary cultured cell has been established for more than six decades, utilization of primary cells is still the preferable medium nowadays as the "gold standard" due to several advantages over other diagnostic techniques. Cells provide better adaptability of the viruses and easily mimic the natural host environment with high virus titration. The volume of virus suspension could be increased by applying an immortal chicken embryo liver-derived cell line. The current review aimed to highlight the importance and challenges of using primary chicken embryo liver cells in poultry virus studies. Primary CEL cells are widely used as an alternative host for diagnosis of infectious poultry viruses, cultivation and passaging of virus isolates, and vaccine production. Yet, there are some challenges and limitations in handling this primary cell, which requires appropriate facilities and environment to sustain the rapid growth of confluent monolayer cells, as highlighted in this paper. The availability of specific pathogen-free CEE is a major concern due to limited resources globally, thus creating a challenge for vaccine manufacturers to upscale the cultured cells. Future improvement of primary cell culture preparation necessitates new technology by applying cellular microcarrier in the bioreactor machine for efficient cell growth and subsequent routine virus cultivation. This study can help the researchers understand the advantages of primary CEL cells and their applications due to their significant impact on poultry viruses.

Keywords: Chicken embryonated eggs, Embryo liver cells, Poultry, Viruses, Vaccine

[Full text-PDF] [Crossref Metadata] [Scopus] [Export from ePrints]

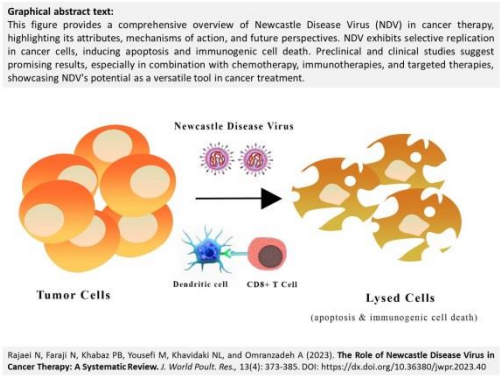
Review

The Role of Newcastle Disease Virus in Cancer Therapy: A Systematic Review

Rajaei N, Faraji N, Khabaz PB, Yousefi M, Khavidaki NL, and Omranzadeh A.

J. World Poult. Res. 13(4): 373-385, 2023; pii: S2322455X2300040-13

DOI: https://dx.doi.org/10.36380/jwpr.2023.40



ABSTRACT: Recently, immunotherapy has become a hopeful option for cancer therapy. Taking advantage of pathogens is a well-established method of cancer immunotherapy. It has been shown that the Newcastle disease virus (NDV), an avian virus with oncolytic properties, can be used to treat cancer. This study was designed to offer a comprehensive overview of the role of NDV in cancer treatment, focusing on its attributes, mechanisms of action, preclinical and clinical trials, and future perspectives. A systematic literature review was performed to gather relevant information about NDV in cancer therapy. The inclusion criteria of this study included studies conducted *in vitro* and *in vivo* as well as clinical trials to investigate the anti-cancer effects and mechanisms behind the action of NDV. A total of 34 out of 176 academic articles, preclinical studies, clinical trials, and review articles were analyzed to collect key findings. In addition to replicating selectively through invading cancerous cells, NDV has been shown to induce apoptosis in in vivo studies. There is evidence that it can induce apoptosis, induce oncolysis, and modulate immune function in preclinical research. Studies have demonstrated that combining this therapy with chemotherapy,

immunotherapies, and targeted therapies provides encouraging results regarding effectiveness and safety in animal models. As a result of NDV's ability to induce immunogenic cell death, the immune system is activated when it reacts to cancer cells. In addition, NDV infection promotes the recruitment and activation of immune cells, especially cytotoxic T cells, by releasing cytokines and chemokines. This dual mechanism triggers anti-cancer immune responses. An interesting new approach to cancer treatment is based on the selective replication of NDV, which can induce immunogenic cell death in tumor tissues and interfere with oncogenic signaling pathways. Research in preclinical models has yielded valuable information, as well as evidence of the effectiveness and safety of clinical trials. A synergistic effect has been demonstrated when chemotherapy, immunotherapies, and targeted therapies are used in conjunction.

Keywords: Cancer, Immune responses, Newcastle disease, Targeted therapy

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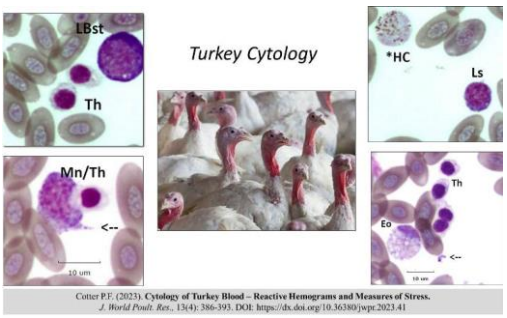
Research Paper

Cytology of Turkey Blood-Reactive Hemograms and Measures of Stress

Cotter PF.

J. World Poult. Res. 13(4): 386-393, 2023; pii: S2322455X2300041-13

DOI: https://dx.doi.org/10.36380/jwpr.2023.41



ABSTRACT: The current study was intended to offer a cytological counterbalance to published descriptions of how processing or other procedures affect turkey welfare. Cytology represents a detailed description of morphological atypia or unusual intracellular or intercellular behavior. The study aimed to describe the variation of blood cells of commercial turkeys. Blood films were collected from 4 turkeys at four different ages of 6, 12, 17, and 19 weeks at commercial farms by a qualified veterinarian. The slides, stained by Wright-Giemsa, were photographed and interpreted off-site. Normal cells of the lymphocyte (L) and heterophil (H) series were described first, followed by examples of atypical cells of other series. These were shown with descriptions of cellularity defined as the proportion of leukocytes in each microscopic field. The results indicated examples of cells whose presence in a standard differential count (SDC) was important enough to disqualify the simple H/L ratio as a stress measure. These cells were atypical members of the lymphoid series, plasmacytes, and other cell types. Atypical granulocytes were heterophils with irregular shapes and faint nuclear staining (hypochromia). An example of a representative total white count revealed how the H/L value could depend on where the cells were counted on the slide. In conclusion, the cytology clearly shows that the presence of atypical cells in a hemogram highlights the inadequacy of relying solely on the simple H/L ratio to estimate stress status.

Keywords: Atypical cytology, Heterophil/Lymphocyte ratio, Stress, Turkey

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Research Paper

Eucalyptus globulus as an Alternative to Antibiotics for *Isa brown* Laying Hens during the Starter Phase

Akue A, Lare L, and Talaki E.

J. World Poult. Res. 13(4): 394-405, 2023; pii: S2322455X2300042-13

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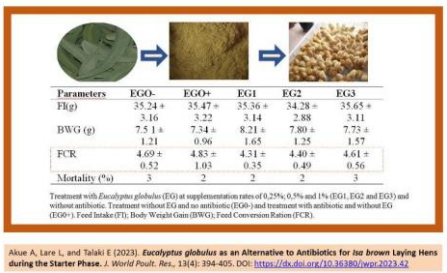

ABSTRACT: Identification of antibiotic residues in meat and eggs of laying hens in Togo and the ban in 2006 on using antibiotics growth promoter (AGP) in animal production by the World Health Organization induce the use of medicinal plants with antimicrobial effects, such as AGP alternatives in poultry production. For the same purpose, this study was conducted to contribute to studies using phytobiotics as alternatives to AGP in poultry production. Indeed, antibiotics have been substituted by *Eucalyptus globulus* leaf powder (ELP) during the starter phase. Polyphenolic compounds from ELP were determined, and the effects of different rates of ELP supplementation on growth performance, mortality, and hematological and biochemical parameters were evaluated. A total of 460 one-day-old laying chicks (*Isa brown*) were randomly allocated to 5 groups, each consisting of 4 replications, with 23 chicks in each replication. Treatments consisted of the basal diet (BD) without ELP and antibiotics, a negative control (group EGO-), BD with antibiotics and no ELP, a positive control (group EGO+), BD + 0.25% of ELP without antibiotic (group EG1), BD with 0.50% of ELP without antibiotic (group EG2), and BD with 1% ELP without antibiotic (group EG3). The rates of 0.25%, 0.5%, and 1% mean

0.25 kg, 0.5 kg, and 1 kg of ELP for 100 kg of BD, respectively. The study revealed that ELP contains flavonoids (4.85 µg QE/mg), tannins (30.34 µg CE/mg), and total phenols (165.2 µg AGE/mg). Supplementation did not affect feed intake (FI), body weight gain (BWG), feed conversion ratio, and mortality of *Isa brown* laying hens during the starter phase (8 weeks) in all treatment groups. However, the chicks that received ELP had the best FI and BWG, which was not significantly different from the control groups. The biochemical parameters such as total proteins, albumin, triglyceride, total cholesterol, and glycemia were not affected by ELP supplementation. Among the hematology parameters, the leukocyte decreased in the groups fed with ELP, while mortality was unaffected. The results of the present study indicated that ELP inclusion rate of 0.25% could serve as the best antibiotic replacement for *Isa brown* laying hens during the starter phase.

Keywords: *Eucalyptus globulus*, Growth parameters, *Isa brown*, Starter

[Full text-PDF] [Crossref Metadata] [Scopus] [Export from ePrints]

Research Paper

Effects of Microorganism Supplementation on Egg Quality and Production

Yitbarek MB.

J. World Poult. Res. 13(4): 406-418, 2023; pii: S2322455X2300043-13

DOI: https://dx.doi.org/10.36380/jwpr.2023.43

ABSTRACT: Effective microorganism (EM) is a combination of more than 80 different types of beneficial microorganisms contributing to a wide range of applications in medicine, environment, and agriculture (livestock sector, crop sector, and forestry). This experiment was conducted to evaluate the effects of EM supplementation on hen day egg production and egg quality traits of Bovans Brown laying hens. At the age of 16 weeks, 144 pullets were purchased from small-scale poultry farms in Debre Markos. The chickens were divided into four treatments, each of which was replicated three times and contained 12 chickens. The treatment groups were T1 (control, commercial ration only), T2 (supplemented 16 ml EM per liter of drinking water), T3 (supplemented 5% Bokashi in feed), and T4 (supplemented 16 ml EM per liter of drinking water and 5% Bokashi in feed). Prior to collecting the actual data, the layer chickens had 2 weeks of adaptation. The hen day egg production was evaluated, and laboratory analysis was conducted to detect the external and internal egg quality. The results showed no significant difference among treatments on hen day egg productions, which ranged from 74 to 80 percent. Among the external egg quality traits, T4 had the highest egg weight, compared to other treatment groups. The T3 and T4 treatments had the highest shell weight. The shell thickness ranged from 0.37 to 0.39 mm. The shape index ranged from 76.81 to 79.11%, with no difference among the groups. Moreover, T4 had a significantly higher egg mass than T1 and T2. The specific gravity of an egg ranged from 1.06 to 1.08 g/cm3. Among the internal egg quality traits, the albumin weights of T3 and T4 were significantly higher than those of T1. The highest and the lowest Hough units were observed in T4 and T1, respectively. The highest yolk weight was observed in T4 among the groups. The yolk index ranged from 0.45 to 0.49. The yolk color ranged from 5.27 to 7.33. Finally, overall egg quality parameters in T4 were better than in non-supplemented groups.

Keywords: Bovans Brown laying hen, Effective microorganism, Egg quality traits, Hen day egg production

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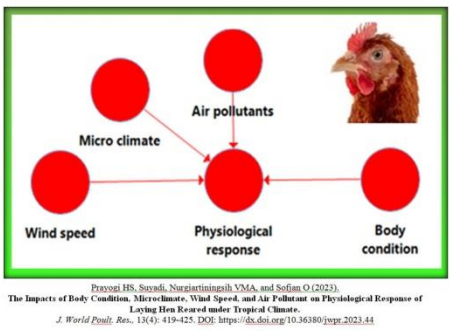
Research Paper

The Impacts of Body Condition, Microclimate, Wind Speed, and Air Pollutant on Physiological Response of Laying Hen Reared under Tropical Climate

Prayogi HS, Suyadi, Nurgiartiningsih VMA, and Sofjan O.

J. World Poult. Res. 13(4): 419-425, 2023; pii: S2322455X2300044-13

DOI: https://dx.doi.org/10.36380/jwpr.2023.44



ABSTRACT: The environmental changes in the animal's body status could manifest as a physiological response. The present study investigated the impact of body condition, microclimate, wind speed, and air pollutants on the physiological response of laying hens. Therefore, a total of 172 laying hens at 16 weeks of age from Isa Brown were investigated for 5 days. Data on body condition, microclimate, wind

speed, and physiological response were recorded and then analyzed using the SEM model by Partial Least Square-Structural Equation Modeling using smartPLS. The obtained result revealed that 59.71% of the physiological response of the chickens (respiratory rate and rectal temperature) reared at the open house system could be predicted by the independent. The microclimate (y = 0.465) was found to be more effective than body condition (y = 0.237), wind speed (y = -0.364), and air pollutant (y = 0.08). Moreover, it was found that as much as 83.1% of the air pollutants in the open house system could be predicted by the independent variables, and wind speed (y = -0.890) was more effective than microclimate (y = 0.074) variables.

Keywords: Laying hen, Microclimate, Physiological response, Tropical climate

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Research Paper

Quail Farming in Villages of Mogoditshane-Thamaga and Tlokweng Districts, Botswana

Bhawa S, Moreki JC, and Manyeula F.

J. World Poult. Res. 13(4): 426-439, 2023; pii: S2322455X2300045-13

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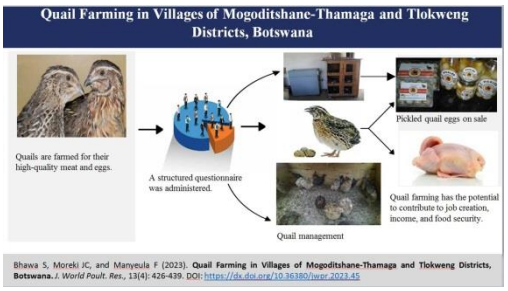

ABSTRACT: A The interest in quail farming has been increasing over the years due to the quail's many productive and financial benefits over other poultry species. Quail farming is still in its infancy in Botswana. This study investigated the current status, challenges, and prospects of Japanese quail farming in Mogoditshane, Gabane, and Tlokweng districts, Botswana. A total of 64 quail farmers were interviewed using a structured questionnaire from June 2022 to April 2023. Data were collected on the respondents' demographic characteristics (age, educational status, and sex), quail management aspects (feeding, housing, and health), ownership of quail, challenges in quail farming, and the use of quail products in the study area. Results showed that 67% of male respondents were involved in quail rearing. The youth (≤35 years) dominated the rearing of quails, followed by respondents aged 41-50 years (22%), 36-40 years (16%), and above 50 years (9%). In addition, 48% of the respondents reared ≤100 quails, followed by 39% and 13% who reared 101-500 and 500 quails, respectively. Furthermore, 81% of respondents reared quails in cages, 17% in conventional structures, and 2% in residential houses. Bobwhite, Jumbo, and Japanese quail were the three quail varieties reared in the study area. It was found that 55% of the respondents had less than one year of experience in quail farming. Moreover, 86% of the respondents used crushed maize or sorghum to feed quails, while 14% used commercial chicken diets. Finally, 92% of the respondents mentioned that quail eggs were used to treat various human diseases. Effective challenges in quail farming included external parasites (36%), diseases (30%), predation (13%), lack of commercial quail diets (12%), escaping (6%), and theft (3%). Quail farming should be considered for inclusion in government support programs as it has the potential to contribute to income generation and food and nutrition security.

Keywords: Food security, Job creation, Nutrition, Quail farming, Therapeutic properties

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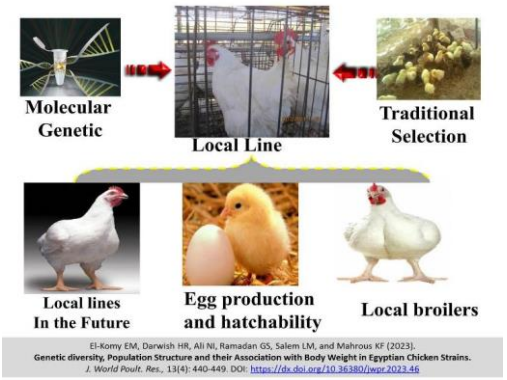
Research Paper

Genetic diversity, Population Structure and their Association with Body Weight in Egyptian Chicken Strains

El-Komy EM, Darwish HR, Ali NI, Ramadan GS, Salem LM, and Mahrous KF.

J. World Poult. Res. 13(4): 440-449, 2023; pii: S2322455X2300046-13

DOI: https://dx.doi.org/10.36380/jwpr.2023.46



ABSTRACT: A Genetic characteristics and population structure within and among Egyptian indigenous chicken strains are important for identifying some genetic resources. The present study aimed to use microsatellite markers to determine similarity and genetic distance among different genotypes and their association with growth and production traits in Egyptian indigenous chicken strains. The current study included 800 chickens and 100 genomic DNA samples obtained from four Egyptian local chicken strains of four different areas (Dokki-4, Mandarah, Anshas, and Al-Salam) in Egypt. Their genetic characteristics, population structure, phylogenetic relationships, and their association with body weight were analyzed using seven microsatellite markers. The performance of 200 chicks from each strain was assessed in terms of individual body weight

and growth rate. Al-Salam strain had a significantly higher body weight than the other strains up to 12 weeks of age among the four lines of Egyptian local chickens. Additionally, male chickens across all strains indicated significantly higher body weight than females from 2 weeks of age until the end of the experiment. The study revealed a total of 68 alleles from the 7 loci across 4 chicken strains, with an average of 9.71. The average of observed heterozygosity, expected heterozygosity, and polymorphism information content were 0.799, 0.358, and 0.707, respectively. The Mandarah strain had the highest observed allele number of 5.37; however, the lowest observed allele number was 3.12 for the Dokki strain. Analysis of population structure revealed that the four chicken strains should be divided into three clusters based on the highest log-likelihood values (ΔK value, 56.3). The results showed a degree of heterozygosity in the Mandara strain with 66.7% individual memberships, indicating a level of admixture. On the other hand, the Al-Salam strain indicated a high genetic diversity with 99% individual membership. The current study provides valuable insights for future genetic polymorphism studies, the advancement of breeding programs, and strategies for the conservation of the Egyptian local chicken strains.

Keywords: Body weight, Chicken, Genetic diversity, Microsatellite

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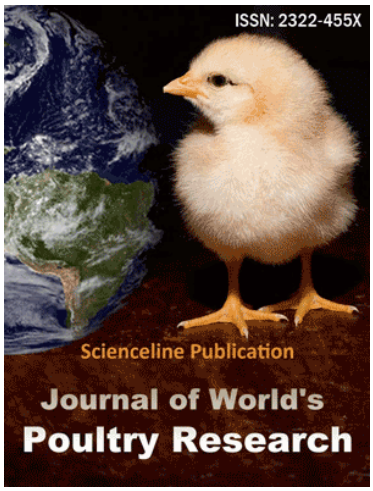

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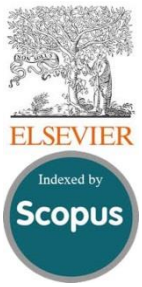

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The Importance and Challenges of Primary Chicken Embryo Liver Cells in Studies of Poultry Viral Diseases: A Review

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ABSTRACT

Primary chicken embryo liver (CEL) cells are derived from the liver tissue of chicken embryonated eggs (CEE) using an aseptic isolation technique and growth under a controlled atmosphere in an artificial environment for cell attachment and proliferation. Although this primary cultured cell has been established for more than six decades, utilization of primary cells is still the preferable medium nowadays as the "gold standard" due to several advantages over other diagnostic techniques. Cells provide better adaptability of the viruses and easily mimic the natural host environment with high virus titration. The volume of virus suspension could be increased by applying an immortal chicken embryo liver-derived cell line. The current review aimed to highlight the importance and challenges of using primary chicken embryo liver cells in poultry virus studies. Primary CEL cells are widely used as an alternative host for diagnosis of infectious poultry viruses, cultivation and passaging of virus isolates, and vaccine production. Yet, there are some challenges and limitations in handling this primary cell, which requires appropriate facilities and environment to sustain the rapid growth of confluent monolayer cells, as highlighted in this paper. The availability of specific pathogen-free CEE is a major concern due to limited resources globally, thus creating a challenge for vaccine manufacturers to upscale the cultured cells. Future improvement of primary cell culture preparation necessitates new technology by applying cellular microcarrier in the bioreactor machine for efficient cell growth and subsequent routine virus cultivation. This study can help the researchers understand the advantages of primary CEL cells and their applications due to their significant impact on poultry viruses.

Keywords: Chicken embryonated eggs, Embryo liver cells, Poultry, Viruses, Vaccine

INTRODUCTION

Cell culture consists of a complex process that is initiated by the isolation of cells from animal tissues (*in vivo*) and growth under a controlled atmosphere in an artificial (*in vitro*) environment (Verma et al., 2020; Dubovi and Rankin 2021; Zhao, 2023). Primary chicken embryo liver (CEL) cells are derived from liver tissue of chicken embryonated eggs (CEE), which involves explant and tissue dissociation under aseptic conditions prior to incubation into a controlled atmosphere for attachment, growth, and proliferation (Swain et al., 2014). Cell culture systems had been established for more than six decades by the early 1960s, mainly for isolating and detecting viruses (Leland and Ginocchio, 2007). Since then, the usage has largely expanded dramatically because commercial cell lines and highly purified reagents are readily available (Pandurangan and Hwang, 2014). Since cell culture systems essentially retain the same properties as the natural tissue, this virus isolation method is considered "gold standard".

As the availability of a live host, such as permissive cell cultures, is a requirement for the isolation of infectious viruses from chickens, monolayer culture of CEL cells is frequently used as a diagnostic and research tool, especially in the diagnosis of viral diseases affecting poultry (Li et al., 2018; Liebhart et al., 2023). In recent years, this cell culture has exhibited excellent substrate for the propagation of viruses needed for vaccine production

and manufacturing (Kim et al., 2014; Sohaimi et al., 2019; Ugwu et al., 2020). The liver in chicken embryos is comprised of hepatocytes and high glycogens, with less connective tissue, and it lacks a true lobular structure (Zaefarian et al., 2019; Bao et al., 2023). Upon dissociation, the liver cells yield monolayer CEL cells comprised of irregular epithelial islands, which consist of hepatocytes surrounded by a network of fibroblasts (Sohaimi et al., 2019). These cells are supported for effective proliferation by the provision of suitable growth media supplemented with a fetal bovine serum to provide nutrients and equipment to provide *in vivo*-like conditions *in vitro*, such as 37°C temperature, 5% CO_2, and 85 to 90% humidity (Barua and Rai, 2003; Soumyalekshmi et al., 2014).

Cell culture systems are more convenient and economical than eggs and animals, which can easily be examined microscopically for cytopathic effect (CPE) as an indicator of viral replication (Leland and Ginocchio, 2007). Additionally, there are benefits to cell culture, such as lower contamination, product purity, efficient use of wild-type viruses, decreased immunogenic changes, large-scale production, and rapid response to pandemics like the recent COVID-19 pandemic or influenza outbreaks (Whitford, 2010; Haredy et al., 2013; Yazawa et al., 2023). Primary CEL cells are the most susceptible cell culture for various poultry viruses, mainly fowl adenovirus (Kumar et al., 2003; Sohaimi et al., 2019). In primary cell culture, cells are taken from the organs of animals, insects, or plants, grown *in vitro* (Swain et al., 2014), and then kept in a medium to express therapeutics, enzymes, and antibodies. Additionally, viruses are grown to develop vaccines (Moreira, 2007; Marquis, 2019). Moreover, anchorage-dependent cells—such as liver cells—need surfaces to adhere to to stabilize and promote growth. Several viruses are routinely isolated using this monolayer cell, including reoviruses, infectious laryngotracheitis virus (ILTV), and Newcastle Disease Virus (NDV) strains from collected samples in the field outbreaks. Due to high sensitivity to these viruses, early CPE formation is recorded within 24 to 48 hours post-infection (pi) and indicated as a superior medium for virus isolation than other cell cultures (Barua and Rai, 2003; Mao et al., 2022).

Virus passaging and cultivation have been conducted globally to increase the virus suspension volume for further analysis (Leland and Ginocchio, 2007). To achieve this with less laborious processes than traditional methods, the use of microcarriers, especially for anchorage-dependent cells like liver cells, has been the major focus of interest for rapid cell proliferation with the purpose of large-volume production and upscaling of viruses for vaccine production (Ugwu et al., 2020) which can only be achieved by using cell cultures. An overview of primary CEL cells used in poultry virus research highlights the significant function that these cells perform for both virus propagation and research, which will help develop and produce vaccines. This could be useful since studying other viruses has been limited worldwide. This paper aims to review the literature on the significance of primary CEL cells, emphasizing their advantages and applications while highlighting the challenges of handling this cell culture.

THE IMPORTANCE OF PRIMARY CHICKEN EMBRYO LIVER CELLS

Advantages of chicken embryo liver cells over chicken embryonated eggs and other continuous cell lines

Primary CEL cells are routinely used as an alternative medium for cultivating poultry viruses and exhibit a significant impact in various studies due to several advantages over continuous cell lines. Cell culture methods are more economical and convenient than egg inoculation. Moreover, cell culture is superior to the egg-based method for the large number of viruses needed for adequate vaccines to meet the continually expanding animal population (Whitford and Fairbank, 2011). Further advantages of cell culture include less contamination, increased efficiency with the use of wild-type viruses, reduced immunogenic changes, fast pandemic response, product purity, higher doses produced in a shorter period of time, and a more reliable, flexible, and expandable process (Whitford, 2010). On the other hand, primary CEL cells could offer a better medium than continuous cell lines being a direct derivative of live chicken embryos which could provide better adaptability of the viruses and easily mimic the natural host environment (Verma et al., 2020).

Basically, primary cells originate from specific pathogen-free (SPF) CEE and are free from extraneous agents compared to commercial chicken eggs (Jungbäck and Motitschke, 2010). Both sterility and safety of virus inoculum are critically important for working on vaccine production (Ibrahim et al., 2019; Cahyani et al., 2020). Preparation of these cells under the aseptic environment with appropriate facilities is important to minimize contamination (Verma et al., 2020). The simple procedure involved harvesting and dissociation of liver tissue with larger size compared to other complex tissue, such as chicken embryo kidney (CEK) cells from the embryo`s

kidney (Soumyalekshmi et al., 2014; Styś-Fijoł et al., 2017). For virus passaging, viruses grown in every monolayer cell culture flask are uniformly maintained in an adequate volume of growth media, and the virus suspension is stored directly for further analysis (Leland and Ginocchio, 2007). In the egg inoculation procedure, the volume of the virus inoculum is variable depending on the size of the embryo's tissues and subsequently involves tissue processing, which is more laborious and time-consuming (Alemnesh et al., 2012).

Primary CEL cells exhibit rapid cell proliferation within 24 hours due to a high metabolic rate that causes a more rapid confluent period than other primary cells and cell lines (Ugwu et al., 2020). The selection of a growth medium is critical for cells to adapt and grow from the original host into an artificial condition (Yao and Asayama, 2017). L-glutamine-supplemented cells can function as a source of energy for cells that reproduce rapidly as well as those that utilize glucose inadequately (Yusof and Jainul, 2019).

Virus identification and detection were rapidly observed less than 7 days post-inoculation (pi) by cytopathic effect (CPE) formation compared to the egg incubation period. There is a possible superior sensitivity of CEL cells over chorioallantoic membrane (CAM) inoculation, as indicated by Sohaimi et al. (2019). Furthermore, in comparison with CK or CEK monolayers, the epithelial cells found in CEL monolayers are smaller and have a greater cell density per unit area, which increases the potential that the virus will invade susceptible cells and cause detectable CPE earlier (Nwajei et al., 1988).

High virus titration is normally achieved in this monolayer cell following virus passaging than in other cell culture systems due to high sensitivity for most poultry viruses (Sohaimi et al., 2019; Ugwu et al., 2020). Indeed, it is the major reason for selecting this medium specifically for vaccine production and is more appropriate than CEE (Wambura et al., 2006). Interestingly, the cell has a high potential to be used as a substrate for vaccine production (Ugwu et al., 2020). An upscaling technique like applying a complex bioreactor with microcarriers enhances cell proliferation within 24 hours for a confluent monolayer and improves virus titer. More than sixty different cell lines were cultivated in cell culture systems using the Cytodex™ 1 microcarrier, which was developed with all the features that contribute to a good microcarrier (Yang et al., 2019). But this technique, which is capable of increasing cell and virus yield for large volume production of vaccines, can only operate with cell cultures and is not adaptable to egg-based inoculation. This scale-up technology in vaccine production is more economical as it can be reused, is less laborious, and accommodates a high capacity of cell volume and virus suspension (Lawal et al., 2018). It was found that primary CEL cells are well adapted to Cytodex™ 1 microcarriers with rapid cell proliferation at 24 hours for confluent monolayer cells (Ugwu et al., 2020). Subsequently, FAdV strain UPM08136 from the 20th passage in primary CEL cells propagated in the microcarrier by stirring in a bioreactor, producing a virus titer of $10^{6.5}TCID_{50}$ /mL.

Routine applications of primary chicken embryo liver cells

Diagnosis of infectious avian viruses

Isolation and identification of viruses were routinely performed by inoculation into cell culture for confirmation of the aetiological agent from field outbreak (Leland and Ginocchio, 2007). This approach has been regarded as the "gold standard" to diagnose viral diseases for more than six decades, although extensive technical expertise is required. Indeed, primary CEL cell culture has proved to be an appropriate and sensitive substrate for various poultry virus isolation (Kumar et al., 2003)

Several poultry viruses can be isolated in CEL cells with clear CPE, while others failed to replicate due to differences in tissue tropism (Hofle et al., 2012). In earlier research, the isolation of adenoviruses from disease outbreaks in chicken farms occurred in primary CEL cells with CPE formation, followed by an electron microscopy examination (Gough et al., 1988). Recently, FAdV isolates from inclusion body hepatitis (IBH), hepatitis-hydropericardium syndrome (HHS), and gizzard erosion outbreaks have been routinely diagnosed by isolation in CEL due to it being highly sensitive, which took only 24 to 48 hours post-infection (pi) at the second passage to produce CPE (Soumyalekshmi et al., 2014; Radwan et al., 2019; Sohaimi et al., 2019). This could be attributed to the abundance of Coxsackievirus-adenovirus receptor (CAR) in the liver tissue, which aids virus attachment as the major reason for the cell's high susceptibility to FAdV replication (Wang et al., 2014).

In India, FAdV was confirmed in CEL cells from liver samples of dead chickens. Cells began to round, clump, and detach at the second passage, and within 48 hours pi, the CPE demonstrated cell swelling and rounding. The cells started to detach from the monolayer at 72 hours pi, as well as the cell monolayers completely detached at 96 hours pi (Soumyalekshmi et al., 2014). The type of CPE produced is compatible with human

adenoviruses (HAdV) in chicken embryo fibroblast (CEF) cells (Alameedy, 2016). The protein involved in the adenoviruses seems to utilize a similar virus epitope located at the penton base protein and induces cell rounding activity in the infected cells prior to lysis and monolayer detachment **(**Zhang and Bergelson, 2005; Russell, 2009**).**

On the other hand, avian reoviruses were successfully isolated into CEL cells as the medium for virus isolation. It seems that this primary cell exhibits excellent media quality which could be better than Vero cells based on virus titration produced after several passages (Zhang et al., 2019). Sample from hock joints obtained from arthritis and tenosynovitis in chickens were isolated into monolayer cells and produced the highest virus titer compared to other tested primary cells with early CPE formation within 24 to 72 hours pi (Zhang et al., 2019). A similar finding was also stated in earlier work (Barta et al., 1984), which indicates that the primary CEL cell is the most sensitive medium for reoviruses (Kort et al., 2013; Zhang et al., 2019).

Similarly, in cases of the ILT virus, the CEL cells are the most sensitive and rapid method for the isolation of the virus from the infected tracheal samples than other cell cultures and chicken embryos (Parra et al., 2016). CPE was detected as early as the first passage in the form of large syncytial or formation of the multinucleated giant cell due to fusion cell nuclei (Hughes and Jones, 1988). In addition, this monolayer culture cell is highly permissive to the Newcastle Disease Virus (NDV) as well (McGinnes et al., 2006; Bello et al., 2018). Isolation of astroviruses from chickens was conducted in CEL cells and produced a marked CPE after four to five passages (Baxendale and Mebatsion, 2004).

Avian influenza (AI), infectious bronchitis (IB), infectious bursal disease virus (IBDV), and Marek's disease are not very susceptible to CEL cells (Rekha et al., 2014; Han et al., 2017; Wu et al., 2020) regardless of the fact that these cells are highly susceptible for the isolation of FAdV, Reoviruses, ILT, NDV, and chicken astroviruses (CAstV). Some viruses target specific tissues, whereas others have a wide range of cell targets. Therefore, in CEL cells, only particular viruses are able to replicate and exhibit CPE. Nevertheless, the monolayer culture of CEL cells has high potential to be used for the diagnosis of other avian viruses since the CEL is rapidly growth due to a high metabolic rate compared to chicken embryo kidney (CEK) and chicken embryo lung (CELu) cells (Prasad et al., 2018).

To overcome this issue, the immortal CEL cell line developed by Lee et al. (2013) is capable of isolating some other viruses, such as avian metapneumovirus (AMPV) and Mareks`s disease virus serotype 1 (MDV-1). The cell culture has the potential to be used as an alternative host for primary CEL cells in the future.

Cultivation of poultry viruses

The aim of virus cultivation is to increase the volume of virus suspension, mainly for viruses originating from poultry farms for ultrastructural studies, development of vaccines, and as a reference strain for molecular work (Sohaimi et al., 2019; Ugwu et al., 2020). Therefore, virus propagation procedure necessitates a suitable alternative host for continuous growth and multiplication.

FAdV isolates obtained from hepatitis-hydropericardium syndrome (HHS) outbreaks in chickens were propagated in the CEL cells and produced CPE at first passage (Kumar et al., 2003). A similar finding was observed by Barua and Rai (2003), starting in the third passage onwards. It was demonstrated that CEL cells are highly susceptible to FAdV replication regardless of serotypes and strains from field outbreaks (Al Naguib et al., 2021). Interestingly, the CPE produced is identical to human adenoviruses (HAdV) in the form of refractile, rounding, clumping of cells, and detachment of monolayer cells from the flasks as terminal stage of viral infection (Adair et al., 1979; Barua and Rai, 2003). A similar finding was observed in FAdV serotype 8b from cases of IBH and gizzard erosion in commercial layer chickens, in which the virus isolate was propagated for 35th passages in primary CEL cells with early CPE formation within 24 to 48 hours pi from second passage onwards (Sohaimi et al., 2019).

The selection of CEL cells as an alternative host for the passaging of viruses was attempted in different works due to the high sensitivity of the cells for FAdV replication. In addition, the rapid formation of CPE within 24 to 48 hours pi is a major concern for virus adaptability for a high number of passages. The CPE produced is consistent, which is beneficial for virus propagation and attenuation for high virus titer production (Sohaimi et al., 2019). A large volume of viral suspension was produced compared to liver embryo tissues from SPF CEE, which is suitable for preparing virus seeds for vaccine production.

However, the susceptibility of CEL cells towards a wide range of poultry viruses is limited due to different tissue tropism for virus infectivity into host cells. Since hepatocytes are the major target site for FAdV replication, almost all pathogenic FAdV strains are propagated in this

cell (Ahmad et al., 2011; Shah et al., 2017). A study on Egg drop syndrome (EDS) disease from duck samples revealed varieties of replication activities of the *Atadenovirus* isolates in CEL cells (Kang et al., 2017). Although the virus is from a different group under the *Adenoviridae* family, the tropism toward CEL cells is similar to FAdV strains.

On the other hand, propagation of NDV in CEL cells produced CPE although the strain, namely, V4, is non-cytopathogenic to other conventional avian cell cultures (Nwajei et al., 1988; Uruakpa, 1997). There are similar findings for serial passages of infectious laryngotracheitis (ILT) from the UDCEOD1 strain caused by herpesvirus (Taylor, 2013). It shows that primary CEL cells are adaptable and highly permissive to various avian viruses from field outbreaks for extended passage level.

As an alternative option, the immortal CEL cells (CEL-im) served as a continuous cell line and were used for the passaging of avian viruses such as avian metapneumovirus (AMPV), Marek`s disease virus serotype 1 (MDV-1), and ILT virus. The virus titer was high for AMPV, which was more than 105pfu/mL; however, the lower titers were for the MDV-1 and ILT viruses. It suggests that the CEL-im could be tested for permissiveness to other avian viruses (Lee et al., 2013).

Application of chicken embryo liver cells in vaccine development

Production of vaccine viruses on a large scale is necessary in the poultry industry as a strategy for disease control and prevention (Gomez and Robinson, 2018; Sohaimi et al., 2019; Ugwu et al., 2020). Vaccines are important to stimulate antibody response to confer protection against the disease in commercial poultry farms (De Luca et al., 2020). Throughout the review on purposes of primary CEL cells, it is shown that they are useful for virus passaging for the production of a large volume of virus suspension and for further analysis in chickens for vaccine development. Furthermore, the virus attenuated in CEL cells produced high virus titer up to $10^{6.8}$ $TCID_{50}$/ml and was useful for the development of vaccine candidates in chickens (Sohaimi et al., 2019).

Nowadays, research on vaccine development in primary CEL cells has become a major focus of attempts due to high sensitivity for virus replication and virus titration determination. Previous reports mostly focused on FAdV instead of other viruses due to tissue tropism in the hepatocytes (Sohaimi et al., 2019; Ugwu et al., 2020).

Attenuation of FAdV isolate, UPM1137 was performed at 35th passages in primary CEL cells with virus titer of $10^{6.8}$ $TCID_{50}$/ml and induced antibody response in both SPF and commercial broiler chickens (Sohaimi et al., 2019; Sohaimi et al., 2021). The process exhibited several molecular changes in hexon and fiber gene proteins, which were crucial for the virus to continuously survive and replicate for serial passages in artificial conditions (Sohaimi et al., 2019). A recent study conducted by De Luca et al. (2020) successfully developed a fiber-based vaccine against IBH in chickens. Both FAdV-8a and 8b strains have been propagated into primary CEL cells according to the previous protocol by Schat and Sellers (2008), and the vaccine stimulates humoral immunity by type-specific virus neutralization associated with T and B cell responses.

High antibody response was induced by inactivated oil-emulsion vaccine from FAdV serotype 4 and effectively provided cross-protection against FAdV serotype 5, 8a, 8b, and 11 (Kim et al., 2014). The vaccine was developed by infecting the CEL cells with FAdV isolate and harvested prior to inactivation by formaldehyde (Kim et al., 2014). In previous work, the IBH vaccine oil-adjuvanted cell culture conferred high protection against IBH disease in chickens when compared to the autogenous vaccine (Shah et al., 2017). It seems that primary CEL cells have been used extensively worldwide for virus propagation and the production of inactivated vaccines (Kim et al., 2014; Junnu et al., 2015; Norfitriah et al., 2019).

The FAdV inactivated oil-emulsion vaccine consists of serotype two, which induces a high antibody response and confers full protection against the challenged FAdV strain. The vaccine was prepared from liver isolate passaged into primary CEL cells, and the infected CEL cell supernatant was inactivated by binary ethyleneimine (BEI) (Junnu et al., 2015). Those findings on the efficacy trial with a high protection rate were compatible with a recent study using FAdV serotype 8b strain from the 5th passage at titer $10^{7.5}$$TCID_{50}$/ml (Norfitriah et al., 2019).

In other poultry viruses, propagation and attenuation of the ILTV strain, UDCEOD1 was attempted in CEL cells for 29 passages. It was shown that it is necessary to continuously pass the strain approximately 100 times to achieve attenuation (Taylor, 2013). Based on previous research, there is still a lack of studies on other poultry viruses in primary CEL cells for poultry vaccine development. This monolayer cell could be very useful for the attenuation of other permissive viruses, such as avian reoviruses and NDV, for significant outcomes in the future.

CHALLENGES AND LIMITATIONS OF THE PRIMARY CHICKEN EMBRYO LIVER CELLS

Ongoing research revealed that the utilization of this primary cell has decreased due to some challenges and issues in handling primary cell culture. Literally, primary CEL cells have a finite lifespan with time-consuming and tedious preparation (Lee et al., 2013; Swain et al., 2014; Shittu et al., 2016). A specific age of embryos is needed prior to harvesting the liver tissues rather than the continuous cell lines, which are easier to propagate at an unlimited passage number (Swain et al., 2014). However, the sensitivity of cell lines towards various poultry viruses is limited compared to primary CEL cells (Lawal et al., 2018; Verma et al., 2020). To overcome this issue, the application of current technology could be considered using a bioreactor with less handling, cost-effectiveness, and improved volume yield. Growing cells in suspension facilitated by microcarrier in a bioreactor will increase FAdV titration (Ugwu et al., 2020). The study could be expanded for other viruses for vaccine manufacturing purposes.

Availability of the SPF eggs could be a major concern for CEL cell preparation since the primary cells rely on fresh liver tissues. Some countries have the capability to sustain an ongoing supply of eggs, yet since SPF eggs are pricey, the availability of eggs is limited in developing countries (Shittu et al., 2016). Thus, the intervention of these cells has been performed as an immortal cell line, as reported in previous literature, for the propagation of certain avian infectious viruses (Lee et al., 2013).

On the other hand, cell preparation needs appropriate facilities and environment to sustain the rapid growth of confluent monolayer cells (Coté, 2001; Swain et al., 2014). Some primary cells are reluctant to adapt and proliferate into cell culture flasks due to mishandling, shortage of electricity supply, or possibly inadequate CO_2 concentration in the cell incubator. Lack of experience for technical persons may contribute to this issue, and perhaps, it could be prevented by appropriate training under supervision with adequate arrangements. Furthermore, optimization of the right media should be attempted to obtain rapid confluent monolayer cells following 24 hours post-cultured.

Technically, the preparation of primary cells for vaccine production necessitates a sterile working area throughout the procedure until the product is reached. There are still high chances for microbial contamination in cell culture flasks or bioreactors due to expired media, improper techniques, unsterilized materials, or even through contaminated virus inoculum (Coté, 2001; Prasad et al., 2020). Although this technical concern could be prevented by adequate sterilization or treatment with antibiotic and antimycotic solutions, the quality control and adequate monitoring system of the working area and equipment should routinely be tested based on the standard operating procedure prior to the handling of cells and virus samples (Roth et al., 2020; Weiskirchen et al., 2023). Care and maintenance of the cell culture laboratory equipment in line with biosafety and biosecurity protocols are critical aspects of maintaining excellent primary cell culture procedures throughout the research process (Ochiai et al., 2021).

CONCLUSION

Primary CEL cells exhibit excellent performance for adaptation, passaging, and attenuation of poultry viruses. Perhaps the CEL cells have a high potential to be used for various poultry viruses in the future due to several advantages and significant impacts, as highlighted in this paper. For future recommendations, the immortal CEL cells should be tested for the adaptability of other avian viruses for diagnosis and vaccine development. It could be suggested that the price of SPF eggs could be slightly reduced globally by the supplier, mainly for vaccine producers and research institutes in developing countries such as Africa and Southeast Asia.

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Availability of materials and data

All data of the current study are available according to reasonable request.

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Authors' contribution

Norfitriah Mohamed Sohaimi was involved in the conception and design, carried out data collection, and drafted the manuscript. Ugwu Chidozie Clifford provided grammatical revisions and helped draft the manuscript. All authors read and approved the final manuscript.

Ethical considerations

The authors checked the manuscript for evidence of plagiarism, consent to publish, misconduct, data

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There are no conflicts of interest in accordance with the authors.

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The Role of Newcastle Disease Virus in Cancer Therapy: A Systematic Review

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ABSTRACT

Recently, immunotherapy has become a hopeful option for cancer therapy. Taking advantage of pathogens is a well-established method of cancer immunotherapy. It has been shown that the Newcastle disease virus (NDV), an avian virus with oncolytic properties, can be used to treat cancer. This study was designed to offer a comprehensive overview of the role of NDV in cancer treatment, focusing on its attributes, mechanisms of action, preclinical and clinical trials, and future perspectives. A systematic literature review was performed to gather relevant information about NDV in cancer therapy. The inclusion criteria of this study included studies conducted *in vitro* and *in vivo* as well as clinical trials to investigate the anti-cancer effects and mechanisms behind the action of NDV. A total of 34 out of 176 academic articles, preclinical studies, clinical trials, and review articles were analyzed to collect key findings.-In addition to replicating selectively through invading cancerous cells, NDV has been shown to induce apoptosis in *in vivo* studies. There is evidence that it can induce apoptosis, induce oncolysis, and modulate immune function in preclinical research. Studies have demonstrated that combining this therapy with chemotherapy, immunotherapies, and targeted therapies provides encouraging results regarding effectiveness and safety in animal models. As a result of NDV's ability to induce immunogenic cell death, the immune system is activated when it reacts to cancer cells. In addition, NDV infection promotes the recruitment and activation of immune cells, especially cytotoxic T cells, by releasing cytokines and chemokines. This dual mechanism triggers anti-cancer immune responses. An interesting new approach to cancer treatment is based on the selective replication of NDV, which can induce immunogenic cell death in tumor tissues and interfere with oncogenic signaling pathways. Research in preclinical models has yielded valuable information, as well as evidence of the effectiveness and safety of clinical trials. A synergistic effect has been demonstrated when chemotherapy, immunotherapies, and targeted therapies are used in conjunction.

Keywords: Cancer, Immune responses, Newcastle disease, Targeted therapy

INTRODUCTION

To treat cancer effectively, there is a constant demand for innovative and reliable treatment methods worldwide (Cheng et al., 2021; Asouli et al., 2023; Sadr et al., 2023a). A variety of cancer treatments, such as chemotherapy, surgery, and radiotherapy, have made substantial progress in recent years (Butler et al., 2021; Debela et al., 2021; Wang et al., 2021; Sadr et al., 2023b). The problem is that common treatments such as chemotherapy, surgery, and radiotherapy possess toxicities, resistance capabilities, and impermeability in the presence of cancer cells (Zhao et al., 2023). Therefore, it is imperative to develop novel approaches for detecting tumor cells and killing them with minimal damage to healthy tissues (Hajjafari et al., 2022; Saeed et al., 2022; Sadr et al., 2023c). A new field of investigation is the study of oncolytic virotherapy, in which viruses are used as cancer-fighting agents (Zheng et al., 2019; Nakao et al., 2020; Cheng et al., 2022). A virus is considered oncolytic if it is engineered or naturally

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occurs with the ability to selectively infect and destruct neoplastic cells, thereby regressing tumors (Lin et al., 2023). It is the inherent mechanisms of viruses that permit them to replicate in cancer cells selectively and lead to the death of the tumor cells without affecting healthy cells, providing a promising approach (Kooti et al., 2021; Ghasemi Darestani et al., 2023).

Known as a member of the Paramyxoviridae family, the Newcastle disease virus (NDV) has recently been considered an option for cancer therapy (Zamarin and Palese, 2012; Cheng et al., 2016). Various bird species are affected by NDV, leading to mild to severe symptoms of respiratory infections and neurological disorders. The NDV, however, can kill a wide range of human neoplastic cells, such as hematological malignancies and solid tumors (Russell, 2002; Cuadrado-Castano et al., 2015).

The NDV's distinctive features make it a suitable choice for oncolytic virotherapy (Garmaroudi et al., 2022). Within tumor cells, NDV replicates preferentially due to modifications of the signaling pathways within the cells and weakened antiviral defenses (Shao et al., 2019; Palanivelu et al., 2023). Furthermore, NDV can trigger immunogenic cell death, thereby increasing the efficacy of NDV by enhancing the immune system's ability to fight cancer (Buijs et al., 2015; Koks et al., 2015). NDV has received considerable attention due to these properties in terms of its efficacy as a cancer treatment approach (Apostolidis et al., 2007; Song et al., 2019).

The main objective of the current review is to summarize the clinical and preclinical research designed to assess the therapeutic potential of NDV in various cancer types. Through a thorough review of the existing data, the review looks at the oncolytic characteristics of NDV, underlying mechanisms, safety risks, and efficiency. Clinical and preclinical studies are both involved. Additionally, this study aimed to investigate the molecular processes behind NDV's anti-cancer activities, such as its interaction with neoplastic cells and the microenvironment surrounding tumors.

METHODOLOGY AND SEARCH STRATEGY

A contemporary perspective has been included in the review by taking into account studies that were published from 2000 to 2023. An extensive search strategy was employed across multiple databases, including Scopus, Embase, PubMed, and Web of Science, in order to initiate the review process. Several search terms were used, including "Newcastle Disease Virus," "NDV," "cancer therapy," "oncolytic virotherapy," and "oncolytic viruses." Boolean operators (AND, OR) were used with appropriate truncations to assure coverage of all relevant terms (Karimi et al., 2010).

Several inclusion criteria were established in order to include *in vitro* and *in vivo* studies along with a clinical trial, and this is aimed at assessing NDV's anti-cancer properties and unraveling its mechanisms of action. The current study was applied to the selection of studies specifically focusing on the therapeutic effects of NDV in cancer treatment. It is intended to limit the publication of *in vitro* and animal models, as well as clinical trials, to publications in English. Focusing on recent developments, a detailed description of study characteristics such as the authors, study year, study design, cancer type, NDV strains employed, participant populations, methodologies, major findings, and the outcome of cancer therapy were extracted. This systematic approach allows for the extraction of consistent data from multiple studies. The exclusion criteria for the study included any viruses other than NDV, and these viruses were specifically excluded from the analysis.

OVERVIEW OF NEWCASTLE DISEASE VIRUS

Characteristics of Newcastle disease virus

As one of the members of the Paramyxoviridae family, NDV belongs to the Avulavirus genus (Suarez et al., 2020). NDV is a single-stranded RNA virus, which is highly contagious among avian species (Getabalew et al., 2019; Behboudi and Hamidi Sofiani, 2021). Various hosts have been identified for NDV infection, including poultry, wild birds, and rarely mammals, like humans (Ashraf and Shah, 2014; Ul-Rahman et al., 2022). Electron microscopy indicates that the virus displays a pleomorphic morphology with filamentous or spherical particles (Rush et al., 2020). Oncolytic virotherapy presents a potential application of NDV because of its special capabilities, such as directly killing tumor cells and stopping angiogenesis (Bello et al., 2020). Mononegavirales viruses are non-segmented and have an envelope (Samal, 2021). NVD contains a negative-sense RNA genome encoding six main structural proteins, including matrix proteins (Ms), phosphoproteins (Ps), hemagglutinin-neuraminidase proteins (HNs), nucleoproteins (NPs), fusion proteins (Fs), and large polymerase proteins (Chen et al., 2015). Viruses and their hosts' replication, assembly, and interaction depend on these proteins.

Newcastle disease virus strains and oncolytic properties

Based on their virulence in poultry, several strains of NDV are categorized into distinct pathotypes (Choi et al.,

2013; Fentie et al., 2014; Dimitrov et al., 2016). The most common strain types are velogenic (highly virulent), mesogenic (moderately virulent), and lentogenic (avirulent) (Liu et al., 2007a). Several strains of NDV have demonstrated oncolytic properties, namely the ability to target and destroy cancerous cells (Davis and Fang, 2005; Everts and van der Poel, 2005). It has been extensively studied that a number of NDV strains exhibit the ability to treat cancer partly by selectively replicating only in tumor cells without causing harm to the normal cells (Howells et al., 2017). By exploiting the tumor microenvironment, NDV takes advantage of the unique properties of tumor cells to multiply and cause cell death, namely dysfunctional signaling pathways of interferons, impaired antiviral defenses, and altered surface proteins (Zhao et al., 2012).

Mechanisms of Newcastle disease virus-induced oncolysis

The entry of virus, reproduction, transmission, and triggering death of cancer cells are all steps in the oncolytic process of NDV. The following are some processes that contribute to oncolysis induced by NDV. As soon as NDV reaches the surface of the cancer cells, it binds to specific receptors on the cell surface (Meng et al., 2021). NDV uses sialic acid as its main receptor, which is commonly present in most cancer cells (Matveeva et al., 2015). NDV multiplies itself by injecting genomic RNA into the cytoplasm of its host (Fournier and Schirrmacher, 2013). When viral RNA is translated and replicated, it produces viral proteins and virus particles, which are the byproducts of viral RNA transcription and replication (Randall and Goodbourn, 2008). Via fusion between infected and non-infected cells, an NDV infection forms multinucleated giant cells known as syncytial cells (Krabbe and Altomonte, 2018; Dittmar et al., 2021). Apart from facilitating the virus' spread through tumors, syncytia also enhances the virus' oncolytic activity (Sasso et al., 2020).

The NDV infection stimulates a wide range of innate immune responses, including the release of cytokines and type I interferons (IFNs) (Fournier et al., 2012). These responses have a positive outcome, which is a greater immune reaction towards neoplastic cells since immune cells become activated and recruited. One of the most prominent mechanisms involved in NDV-induced oncolysis is apoptosis, which leads to cell membrane shrinkage, cellular DNA fragmentation, and cell membrane blebbing (Ali et al., 2011; Schirrmacher, 2022a). Tumor cells undergo apoptosis when NDV triggers cell death pathways. Moreover, NDV infection can also lead to necrotic cell death in some cases (Mohammed et al., 2019) (Figure 1).

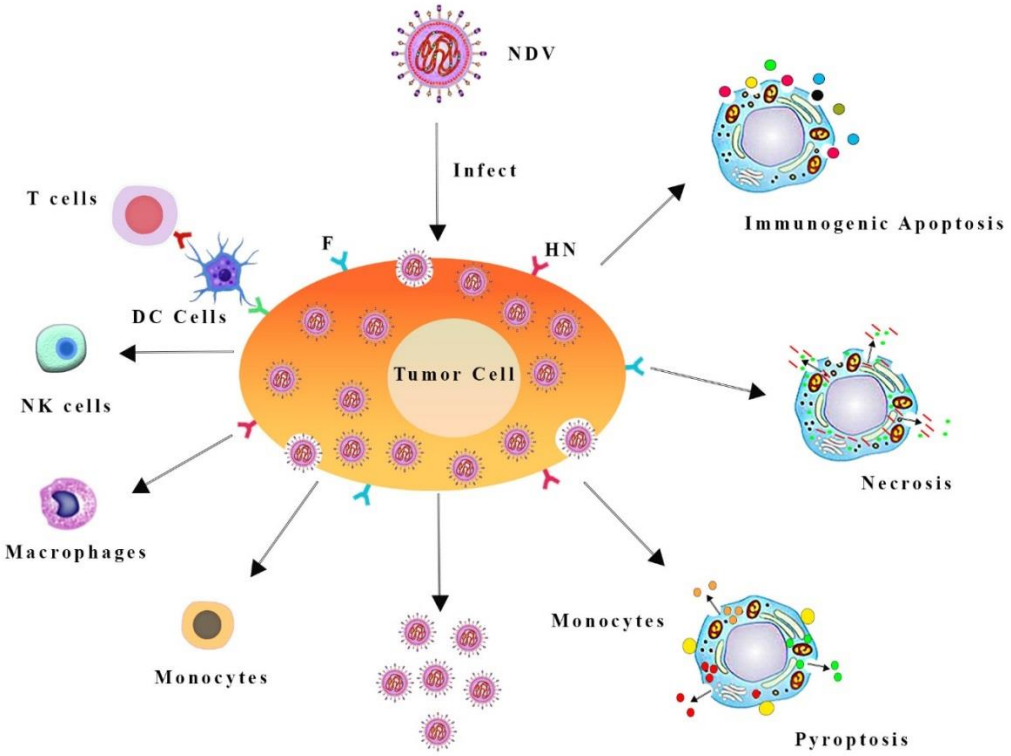

Figure 1. The various mechanisms by which the Newcastle Disease Virus (NDV) induces cell death in cancer cells upon binding to cancer cell receptors. It involves the activation of T cells, Natural Killer (NK) cells, and macrophages, as well as the initiation of apoptosis, necrosis, and pyroptosis pathways (Dendritic cells [DC] play crucial roles in this process).

PRECLINICAL STUDIES

In vitro studies evaluating Newcastle disease virus's anti-cancer effects

To better understand NDV mechanisms and their potential anti-cancer effects, *in vitro* experiments have been undertaken utilizing various cancer cell lines. In these investigations, various techniques are frequently employed to determine whether NDV is cytotoxic, if it replicates within cells, and how it influences cellular interaction (Sánchez et al., 2015; Yurchenko et al., 2018).

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) estimates the functions of mitochondria, whereas the trypan blue exclusion assay determines the integrity of the cell membrane (Ghorbankhani et al., 2023). MTT is commonly used for the assessment of cytotoxicity and viability of cells. These tests allow the quantification of NDV and its efficiency of viral replication via multiple cell lines to determine how it affects tumor cell viability (Jagtap et al., 2017).

Moreover, researchers have attempted to identify how NDV induces oncolysis. Changes in cell morphology and DNA fragmentation initiate apoptotic pathways (Elankumaran et al., 2006). A variety of methods are used to detect apoptosis markers, including Bcl-2 proteins, caspases, and DNA damage markers, utilizing different techniques, such as immunoblotting, flow cytometry, and immunofluorescence (Nirmala and Lopus, 2020; Kari et al., 2022).

Additionally, researchers have studied NDV's immunomodulatory abilities *in vitro* alongside its oncolytic properties (Yang et al., 2021; Hu et al., 2022). Cytokines and chemokines released during infection with NDV have been shown to induce immune cells' recruitment and activation when co-culturing with cancer cells (Tang et al., 2022). In many studies, it has been noted that NDV induces the expression of Major Histocompatibility Complex (MHC) molecules in addition to co-stimulatory molecules, which are involved in presenting antigens and activating immune cells (Burman et al., 2020; Schirrmacher et al., 2022b).

Animal models and their relevance to human cancers

A key part of evaluating NDV in cancer therapy is testing its performance and safety in animal models (bin Umair et al., 2022). Consequently, the oncolytic effects of the virus, its capability of inhibiting tumor growth and metastasis, as well as its tendency to interact with the immune system can be investigated by researchers (Li et al., 2022). Animal models need to be selected considering the purpose of the research, the type of cancer, and the available options (e.g., transgenic or xenograft models). Various animal models have been utilized to examine the effects of implanting human cancer cells into mice with immune deficiencies (Fang et al., 2023).

In addition to evaluating NDV's oncolytic effects *in vivo*, it is also possible to assess tumor regression and virus replication at tumor sites, along with effects on normal tissues (Rius-Rocabert et al., 2020; Kalafati et al., 2023; Sadri et al., 2023). Monitoring tumor growth involves calipers or non-invasive imaging methods like positron emission tomography (PET) or bioluminescence (O'farrell et al., 2013).

It is also possible to explore the therapeutic potential of NDV using genetically modified mice (GEMMs) with spontaneous tumor development (Chen et al., 2022; Tornesello et al., 2022). Several characteristics of these models are similar to those of human cancer, including tumor heterogeneity, stromal interactions, and immune responses. GEMMs offer a more physiologically appropriate way of investigating NDV's effects on cancer growth, metastasis, and immune reactions (Zeng et al., 2021).

Additionally, animal models have been employed to visualize tumor attributes and track treatment efficacy, and NDV biodistribution was assessed by Magnetic Resonance Imaging (MRI) and Positron Emission Tomography-Computed Tomography (PET-CT) (Pierce et al., 2021; Siafaka and Gündoğdu, 2023).

Although animal models cannot completely mimic the complexities of cancers in humans, adequately designed and carefully conducted studies can provide valuable preclinical information for advancements in NDV therapy and the development of clinical trials in humans (Prestwich et al., 2008; Wollmann et al., 2012).

Safety and toxicity profiles

It is paramount to assess the safety and toxicity profiles of NDV in preclinical studies before clinical trials to ensure patient safety. Several studies are being carried out on NDV in order to identify potential side effects, measure its maximum tolerated dose (MTD), and develop safety protocols for human application (Lorence et al., 2007; Koppers-Lalic and Hoeben, 2011; Abdullahi et al., 2018). The animals are closely monitored for signs of distress, behavioral changes, weight fluctuations, and any changes in vital signs. Hematological and

histopathological analyses and blood chemistry tests are carried out to assess NDV's toxicity (Kadhim et al., 2022).

Moreover, viral shedding and off-target effects are particularly assessed. The researchers may examine how long and how much NDV reproduces in non-tumor tissues if it can infect them (Everts and van der Poel, 2005). This type is evaluated to determine dosage and treatment schedules and identify potential risks associated with systemic administration (Yu et al., 2022). In addition to assessing potential drug interactions, preclinical studies are intended to determine whether NDV interacts with other medications. To enhance cancer treatment's success, chemotherapy, radiotherapy, and immunotherapy are often administered together (Yaghoubi et al., 2019). A comprehensive analysis of the safety and tolerability profiles of NDV-based therapies in preclinical studies allows for the safe translation of NDV-based therapies to the clinic (Al-Shammari et al., 2021; Al-Shammari et al., 2020). As a result, patients can be guided on the best dosing regimens and treatment strategies to suit their needs. This can be a safe and reliable method with minimal side effects (Figure 2).

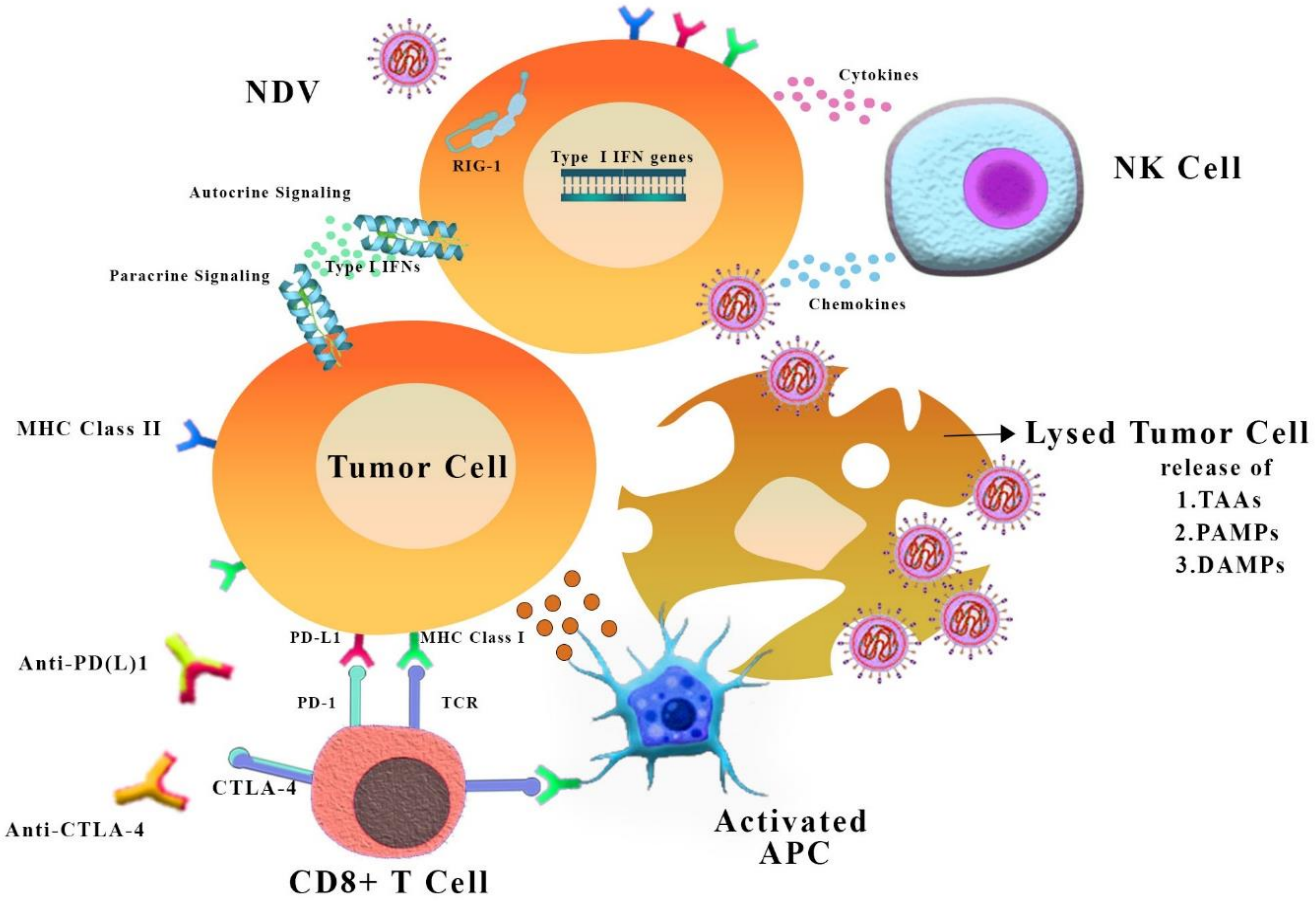

Figure 2. The activation of the immune system by Newcastle virus. The virus enters the cancer cell, triggering the activation of APC cells. Subsequently, CD8+ T cells become activated, and these immune cells lead to the cancer cell's death. APC: Antigen presenting cell, CTLA-4L: Cytotoxic T-lymphocyte associated protein 4, MHC: Major histocompatibility complex, IFN: Interferon, TAAs: Tumor associated antigens, PAMP: Pathogen associated molecular pattern, DAMP: Damage associated molecular pattern, PDL 1: Programmed death ligand-1

CLINICAL TRIAL PHASES

The use of the NDV for killing cancer cells has been successfully demonstrated in both *in vitro* and *in vivo* stages. Now, it needs to be investigated in clinical trials, and so far, a few clinical studies have been conducted. Freeman et al. (2006) and Lam et al. (2011) conducted phase I clinical trials as the first step in evaluating NDV for cancer therapy regarding safety, dosage, and effectiveness (Freeman et al., 2006; Lam et al., 2011). A few patients are involved in these studies, which mostly attempt to determine the MTD and identify adverse effects restricting its usage.

A Phase I study administers NDV via various routes, particularly intratumoral injection, intravenous injection, and intranasal injection, to identify its optimal delivery method and safety status (Malogolovkin et al., 2021). A high degree of homogeneity is guaranteed by setting criteria for patients' eligibility based on tumor type, stage of disease, and previous treatment history (Taguchi et al., 2017). Potential adverse events are identified in Phase I trials, the MTD is determined, and the recommended Phase II dose (Laurie et al., 2006). As secondary endpoints, NDV may also be evaluated for its pharmacokinetics and pharmacodynamics (PD), assessed for tumor responses with imaging techniques, and explored for preliminary anti-tumor activity (Liu et al., 2007b). Phase 1 of the study has yielded promising outcomes; however, it is imperative to underscore that these positive results constitute merely a preliminary step in a more extensive and intricate scientific investigation. Subsequent phases, notably Phases 2 and 3, demand rigorous scrutiny and thorough exploration to elucidate further insights, address potential limitations.

COMBINATION STRATEGIES

Combination with chemotherapy agents

Research indicates that NDV, combined with chemotherapy drugs, can enhance cancer treatment outcomes (Jiang et al., 2014). It is based on the potential synergistic effects of both oncolysis induced by NDV and cytotoxicity caused by chemotherapy that justify the use of these medications together (Al-Shammari et al., 2019).

Several mechanisms have been demonstrated in preclinical studies to sensitize cancer cells to chemotherapy by NDV (Zhu et al., 2021; Faranoush et al., 2023). When cancer cells become infected with NDV, they are more susceptible to chemotherapy agents that induce apoptosis, such as caspases, Bax, and Bak (Cuadrado-Castano et al., 2015). Additionally, NDV-induced immunogenic cell death can activate an immune response that enhances chemotherapy effectiveness through tumor-specific antigen release. Furthermore, NDV may be able to overcome chemotherapy resistance. Multiple drug resistance has been associated with its modulation of P-glycoprotein (P-gp) (Garg et al., 2015; Kadhim et al., 2022). The NDV counteracts chemotherapy drug resistance by inhibiting these transporters, which allows drug concentrations to increase inside the cell.

NDV, in conjunction with chemotherapy, appears to have promising results in clinical trials (Cross and Burmester, 2006; Ripp et al., 2022). Many of these trials require determining the most appropriate sequence, dosage, and treatment timing. These combinations improved the progression-free survival rate, overall survival rate, and response rates in several types of cancer, including pancreatic cancer, lung cancer, and ovarian cancer (Turnis et al., 2015).

Combination with immunotherapies

In order to enhance anti-tumor immune responses, NDV can be combined with immunotherapies, such as adoptive cell therapies or immune checkpoint inhibitors (Marchini et al., 2016). NDV is an ideal co-therapeutic agent for cancer standard medications because it induces immunogenic cell death and stimulates tumor antigen release. NDV combined with immune checkpoint inhibitors, such as anti-PD-1 and anti-CTLA-4 antibodies, strengthened the immune reaction against tumors in several preclinical experiments (Zamarin et al., 2014; Hwang et al., 2020). As a result of virus infection, immune checkpoint molecules are expressed more highly within cancer cells, increasing their susceptibility to immune checkpoint blockade. Further, NDV-induced release of tumor antigens can broaden the range of immune targets for immune checkpoint inhibitors, enhancing their effectiveness (Burman et al., 2020; Chiu et al., 2020).

It has also been shown that NDV can be used in conjunction with adoptive cell therapies, like Chimeric Antigen Receptor (CAR) T-cell (Bahmanyar et al., 2022). Infection with NDV can increase the expression of tumor-associated antigens on cancer cells, making them more recognizable and susceptible to CAR T-cell therapy (Mardi et al., 2022). Furthermore, NDV can induce immunogenic cell death, causing a pro-inflammatory microenvironment supporting adopting T cells' activation and persistence (Ajina and Maher, 2017; Rezaei et al., 2022).

Combination with targeted therapies

By combining NDV with targeted cancer treatments, such as monoclonal antibodies and tyrosine kinase inhibitors (TKIs), more effective treatment can be achieved (Zhu et al., 2021). Targeted therapies can specifically inhibit molecular targets or aberrant signaling pathways that contribute to tumor growth and survival.

Preclinical studies have demonstrated several mechanisms demonstrating how NDV can enhance targeted therapies' anti-tumor effects. Signaling pathways such as VEGF, HER2, or EGFR can be modulated by NDV infection (Howells et al., 2017; Ali et al., 2021). These pathways are commonly targeted by TKIs or monoclonal antibodies. With targeted therapies, NDV

inhibits tumor cell proliferation, induces apoptosis, and suppresses angiogenesis (Zhang and Cheng, 2020; Tian et al., 2022).

In combination with targeted therapies, NDV has demonstrated acceptable results in cancer types, including melanoma, breast cancer, and colorectal cancer (Markman and Shiao, 2015). It is essential to study the safety profiles of treatment options, determine the most beneficial treatments, and determine the most appropriate treatment schedule (Figure 3).

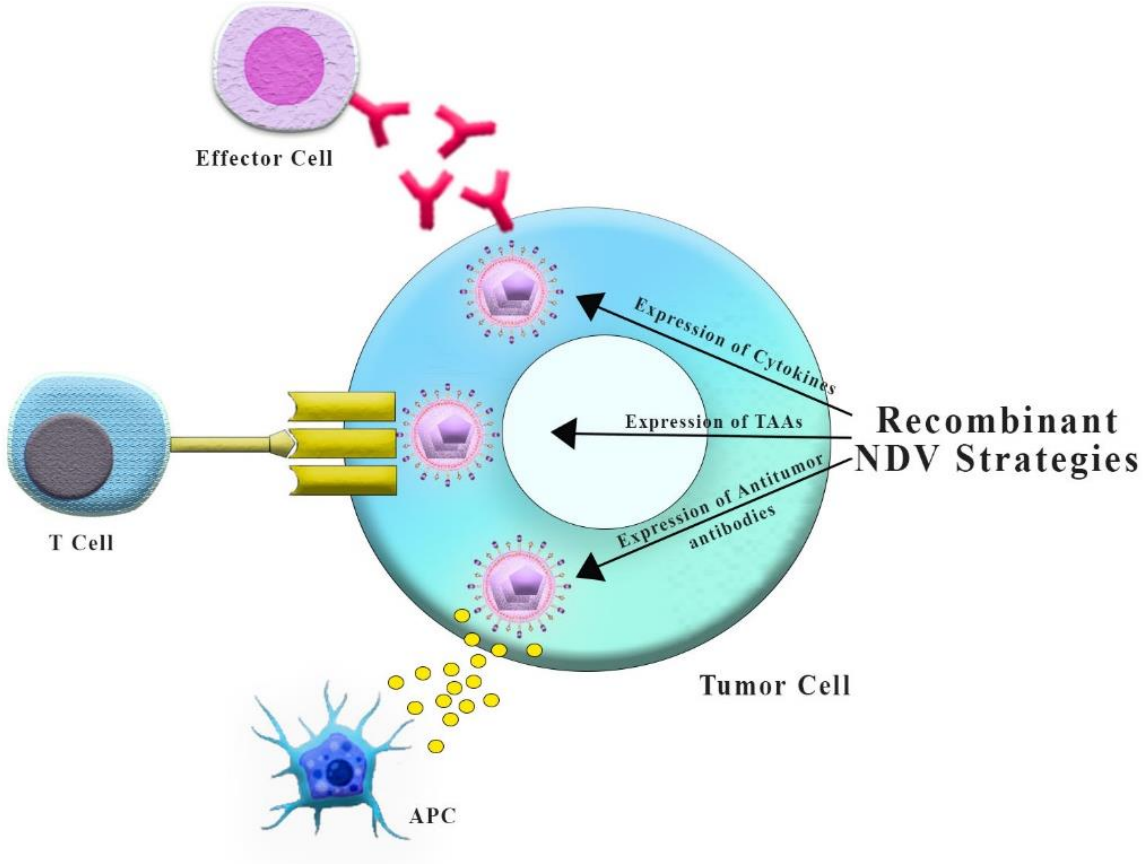

Figure 3. Recombinant Newcastle disease virus is a modified form of the Newcastle disease virus that has been engineered for various therapeutic and immunotherapeutic applications. It is known to activate cytotoxic T cells and induce the secretion of cytokines and chemokines from antigen-presenting cells, such as dendritic cells.

FUTURE DIRECTIONS AND CHALLENGES

Emerging research and novel applications

NDV investigation is being carried out to discover new applications for this virus and examine its potential in cancer therapy. Incorporating newly developed targeted therapies, radiotherapy, and epigenetic modulators into NDV is being studied (de Graaf et al., 2018). It is possible to enhance the anti-tumor effects of NDV through these combinations so as to circumvent potential resistance mechanisms and further improve its effectiveness (Oladejo et al., 2022). Using genetic engineering methods, NDVs with enhanced therapeutic properties are created (Everts and van der Poel, 2005; Kaufman et al., 2015). Therefore, it is possible to modify NDV so that tumor selectivity is improved, replication efficiency increases or therapeutic components are included to enhance anti-cancer activity (Howells et al., 2017; Zhu et al., 2021). It is modifying immune responses to enhance anti-tumor immunity triggered by NDV. It is necessary to boost the immune system in order to overcome immunosuppression caused by tumor microenvironment using cytokines, immune checkpoint inhibitors, or other immunomodulators in conjunction with NDV (Locy et al., 2018; Vijayakumar et al., 2020).

Facilitating the delivery of NDV directly to tumor cells while minimizing adverse effects (Raja et al., 2018; Scott et al., 2018). For instance, viral vectors, nanoparticles, and specific targeting ligands are capable of improving therapeutic performance and tumor targeting (Kontermann et al., 2021).

Addressing limitations

Achieving optimal results of NDV-based therapeutic plans calls for dealing with several limitations and challenges.÷ Optimizing NDV-based therapies with consideration of tumor heterogeneity as well as individualized patient profiles. Discovering and disabling immune evasion pathways cancer cells use to escape NDV-induced immune reactions (Twumasi-Boateng et al., 2018; Abdou et al., 2022). NDV can be combined with immune checkpoint inhibitors, immune stimulators, or innovative immunotherapeutic methods to increase the immune response against cancer (Moehler et al., 2016; Shaver et al., 2021). They establish the most appropriate dose, timetable, and delivery method for NDV-based treatments. Several factors are considered, such as the dose of the virus, the type and stage of the disease, the frequency with which the drug is administered, and the possibility of drug interactions (Farkona et al., 2016; Schirrmacher, 2020). They assess NDV's safety parameters across different patient groups and consider possible concerns like systemic toxicity, viral shedding, and long-term consequences (Vile et al., 2002). The monitoring and reporting of adverse reactions are essential to evaluating the safety of therapeutic interventions based on NDVs and complying with regulations and guidelines to receive approval for NDV-based medical applications (Jafari et al., 2022). Maintain compliance with regulatory requirements for NDV-based therapies, as well as facilitating their translation into standard clinical practice through awareness of the regulatory framework (Ricca et al., 2018; Burke et al., 2020; Svensson-Arvelund et al., 2022). Having a strategy for commercializing and an analysis of the economic feasibility is also imperative for NDV-based treatments to be widely adopted and accessible. Challenges such as these must be overcome, and research in these areas must progress in order for NDV-based therapies to become a mainstream treatment option for cancer and provide better results for patients. In order to overcome these challenges, collaboration is required among clinicians, scientists, regulatory authorities, and industry constituencies.

CONCLUSION

This review highlights the growing prominence of oncolytic virotherapy utilizing NDV in cancer treatment, underscoring its ability to replicate within tumor cells selectively, trigger immunogenic cell death, and influence oncogenic signaling pathways, as supported by various *in vivo* and *in vitro* studies.

Numerous clinical trials have been undertaken at the NDV site, assessing its safety, effectiveness, and use in combined strategies for cancer treatment. Although these advances have been made, several challenges remain, including immune escape mechanisms, tumor heterogeneity, and tailoring treatment options according to tumor traits. In addition, treatments based on NDV require careful consideration of both regulatory and commercialization issues in order for them to become widely available and accepted. The potential of NDV in cancer therapy is considerable. A combination of oncolytic virotherapy, cell death induced by immunogenic factors, and alteration of oncogenic signaling pathways is at the core of this innovative approach. However, it's crucial to acknowledge that the clinical translation of NDV-based oncolytic virotherapy may face challenges such as optimizing delivery methods, addressing potential off-target effects, and ensuring its safety and efficacy in human subjects, which require further research and development. NDV-based treatments are expected to revolutionize cancer treatment, improve patient outcomes, and allow for more individualized and precise cancer treatments by conducting further research, collaborating, and addressing challenges in the future.

DECLARATIONS

Authors' contribution

Alireza Omranzadeh conceptualized the study, while all authors contributed to the methodology, formal analysis, and investigation. The original draft was a collaborative effort, with all authors involved in writing, reviewing, and editing. All authors approved the final version of the manuscript for publication in the journal.

Ethical consideration

Ethical issues, such as data fabrication, double publication and submission, redundancy, plagiarism, consent to publish, and misconduct, have been checked by all the authors before publication in this journal.

Availability of data and materials

The datasets generated during the current study, on a reasonable request, are available from the corresponding author.

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Conflict of interests

All of the authors declare no conflict of interest.

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Cytology of Turkey Blood–Reactive Hemograms and Measures of Stress

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ABSTRACT

The current study was intended to offer a cytological counterbalance to published descriptions of how processing or other procedures affect turkey welfare. Cytology represents a detailed description of morphological atypia or unusual intracellular or intercellular behavior. The study aimed to describe the variation of blood cells of commercial turkeys. Blood films were collected from 4 turkeys at four different ages of 6, 12, 17, and 19 weeks at commercial farms by a qualified veterinarian. The slides, stained by Wright-Giemsa, were photographed and interpreted off-site. Normal cells of the lymphocyte (L) and heterophil (H) series were described first, followed by examples of atypical cells of other series. These were shown with descriptions of cellularity defined as the proportion of leukocytes in each microscopic field. The results indicated examples of cells whose presence in a standard differential count (SDC) was important enough to disqualify the simple H/L ratio as a stress measure. These cells were atypical members of the lymphoid series, plasmacytes, and other cell types. Atypical granulocytes were heterophils with irregular shapes and faint nuclear staining (hypochromia). An example of a representative total white count revealed how the H/L value could depend on where the cells were counted on the slide. In conclusion, the cytology clearly shows that the presence of atypical cells in a hemogram highlights the inadequacy of relying solely on the simple H/L ratio to estimate stress status.

Keywords: Atypical cytology, Heterophil/Lymphocyte ratio, Stress, Turkey

INTRODUCTION

The information on how production procedures and other stressors affect turkeys is limited to a few studies. For instance, Scanes et al. (2020) described the effects of shackling and transportation on both the heterophil/lymphocyte (H/L) ratio and blood cortisone. The H/L ranged from 0.25 (non-stress) to 3.25 (high-stress). Information on the blood cell morphology of turkeys is also limited, lacking detailed descriptions of atypical cells. The aim of the study was to describe some of the atypical blood cells of commercial turkeys. The study was intended to offer a cytological counterbalance to published descriptions of how processing or other procedures affect turkey welfare. The cells should provide diagnostic information on inflammation and blood infections on farms.

MATERIALS AND METHOD

Animal welfare

Animal welfare and ethical issues were followed according to standard commercial production procedures under the authority of a PAACO-certified auditor and licensed veterinarian and approved by Cotter Laboratory (Approval Number. 1945-015).

Blood collection and preparation of slides

Four blood samples from each farm were collected meticulously and sterilely from the ulnar vein by a licensed veterinarian at four different farm sites. Then, 1 mL of blood was drawn aseptically into a sterile 3 cc syringe. Immediately after, touch preparation smears were made by spreading one drop from the syringe (~ 3 µL) over a clean glass microscope slide. The slide was

dried in warm air. This method was used to avoid the effects of shipping and storage on hemogram quality and possible untoward effects on cytology by exposure to heparin or ethylenediaminetetraacetic acid (EDTA) anticoagulants (Campbell and Ellis, 2007). Slides were then sent to Cotter Laboratory, Arlington, MA, USA, for staining and analysis.

Turkeys

All samples in the current study came from male turkeys, commonly referred to as Toms. All were from commercial stocks, but two out of four were raised on standard conventional conditions, and two were raised on antibiotic-free (AB) facilities. They ranged in age from 6 to 19 weeks. The samples included in this manuscript were from two conventional and two antibiotic-free farms. To achieve this, blood films obtained from 4 turkeys each from flocks at 6, 12, 17, and 19 weeks of age were made directly on commercial farms by a qualified veterinarian. They were selected because of unusual cytology.

Light microscopy and photomicrographs

A light microscope (Olympus CX-41, Olympus, America) equipped with Plan N 40×, 0.65 numerical aperture dry, and Plan N, 1.25 numerical aperture 100× oil objectives was used. All images were captured at 100× with an Infinity-2 1.4-megapixel charge-coupled device Universal Serial Bus 2.0 Camera and were processed with Infinity Analyze software (Release 6.5; Lumenera, Inc., Ottawa, ON, Canada).

Staining

The staining process in this study was by an in-house procedure using Wright's method followed by brief exposure to Giemsa (W-G, Sigma Chemicals, St. Louis, Mo. USA, Procedure WSGD-128) done at Cotter Laboratory, Arlington MA, USA. A minimum of two counts of ~200 cells each were made at 40x magnification by sweeping across areas not thicker than monolayers. Edge-based counts were also performed when accumulations of reactive cells were noticed at low (20x) magnification. Photomicrographs of representative cells were at 100x (oil) magnification. All counts were performed by the author.

Total white blood counts

Total white blood counts (TWBC) were estimated from the standard differential counts (SDC) by applying the method of Campbell and Ellis (2007).

Heterophil lymphocyte ratios (H/L 1, H/L2, f(H/L)

H/L 1 = (HC + HT + HV) / Ls; H/L 2 = (HC + HT + HV) / (Ls + Lm); ΔH/L= H/L 1 − H/L 2; is given as real numbers. The field H/L f(H/L) is approximated by dividing the number of heterophils in a microscopic field by the number of lymphocytes and is given as a fraction.

Cellularity

Under normal quiescent (homeostatic) conditions, the composition of blood cells in mature turkeys follows specific parameters. Leukocytes (white blood cells) should make up approximately 1% of the total white blood cell count (TWBC) of mature turkeys (McGuire and Cavett, 1952; Lucas and Jamroz, 1961; Venkataratnam and Clarkson, 1962). In a thin microscopic field having space for 1-200 cells, 1 or 2 leukocytes can be expected to be found dispersed among erythrocytes (RBC). Most RBCs should be mature, fully hemoglobinized cells. Mature RBCs were recognized by their orange-yellow cytoplasm. Leukocytes should be predominated by small (D_C ~ 6 µm) lymphocytes (Ls). Thrombocytes (Th) can occupy field space, but these should not show signs of reactivity. Reactive and/or atypical cells and atypical behavior indicated that stress or frank pathology had already occurred. For the present purposes, field cellularity was given as leukocytes/number of RBC x 100. As a simplification, "cellularity" functioned as a cameo image of the entire hemogram.

Normal cells

Normal cells were defined as those not displaying atypical signs or those not participating in a behavior recognized as "reactive" or pathological. Clumping (leukergy), autophagocytosis, and zeiosis were considered examples of pathological behavior. Size was measured using both cell diameter (D_C) and nucleus diameter (D_N) to compute the respective areas (A_C and A_N). Nuclear cytoplasmic ratios (N/C) were computed by division (of A_N by A_C). In blood sampled during homeostasis, the cells of all series should display an appropriate Romanowsky-Giemsa effect or RGE; otherwise, they are considered reactive or pathologic (Wittekind, 1979).

RESULTS

Representative "normal" cells of commercial turkeys of given ages were illustrated first, followed by atypical cells. Atypia was recognized by abnormalities of cytology, staining characteristics (RGE), or behavior. The

hypothesis was that when atypical cells appeared in the SDC, their presence would alert the investigator to the likelihood that the simple H/L value would be disqualified as a stress determinator. Simply stated, the question of stress was rendered moot. The focus was on cells potentially entering the H/L calculus directly as a component of the numerator (heterophil/granulocyte series) or the denominator (lymphoid series). In other instances, atypical cells not used in the H/L computation were included since they were of sufficient importance to show that a complex hemogram exists. Reactive basophils and plasmacytes were examples of such cells.

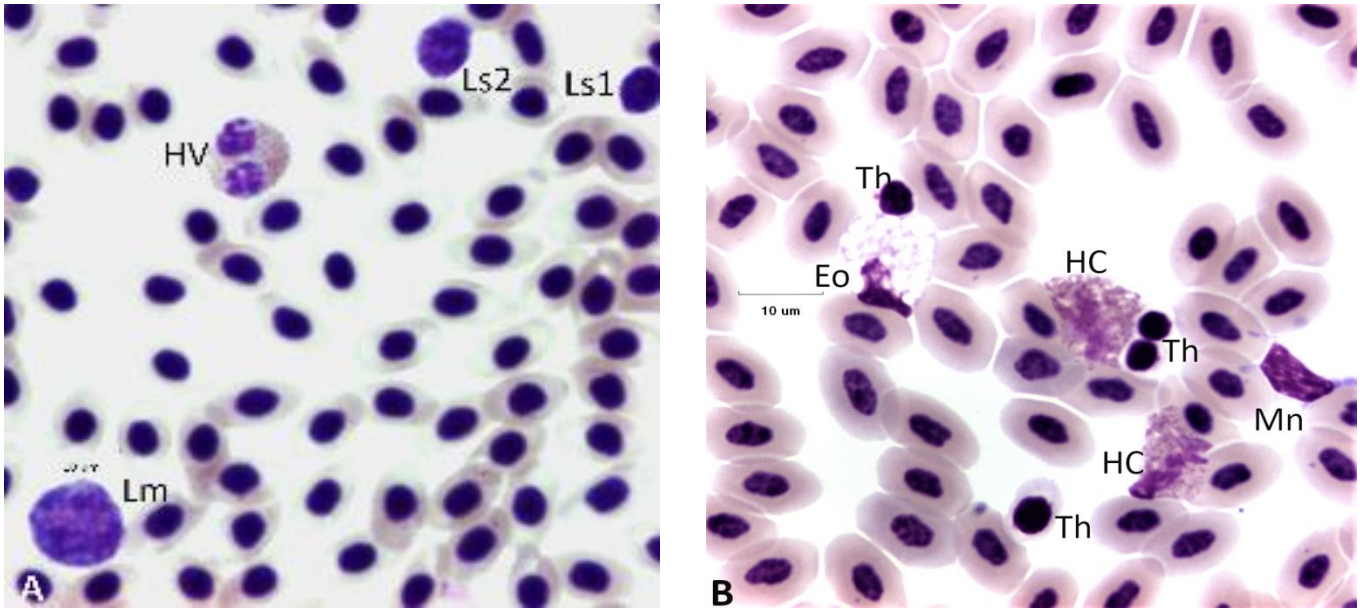

Figure 1. A: Normal cells of a 6-week Tom raised under standard conditions (HV) variant heterophil; Ls1 and 2 small resting lymphocytes, Lm medium/large lymphocyte. **B**: Reactive cells of a 19-week Tom raised on an antibiotic-free farm. Eo: Eosinophil, Th: thrombocyte; HC: Classic heterophil, Mn: Monocyte.

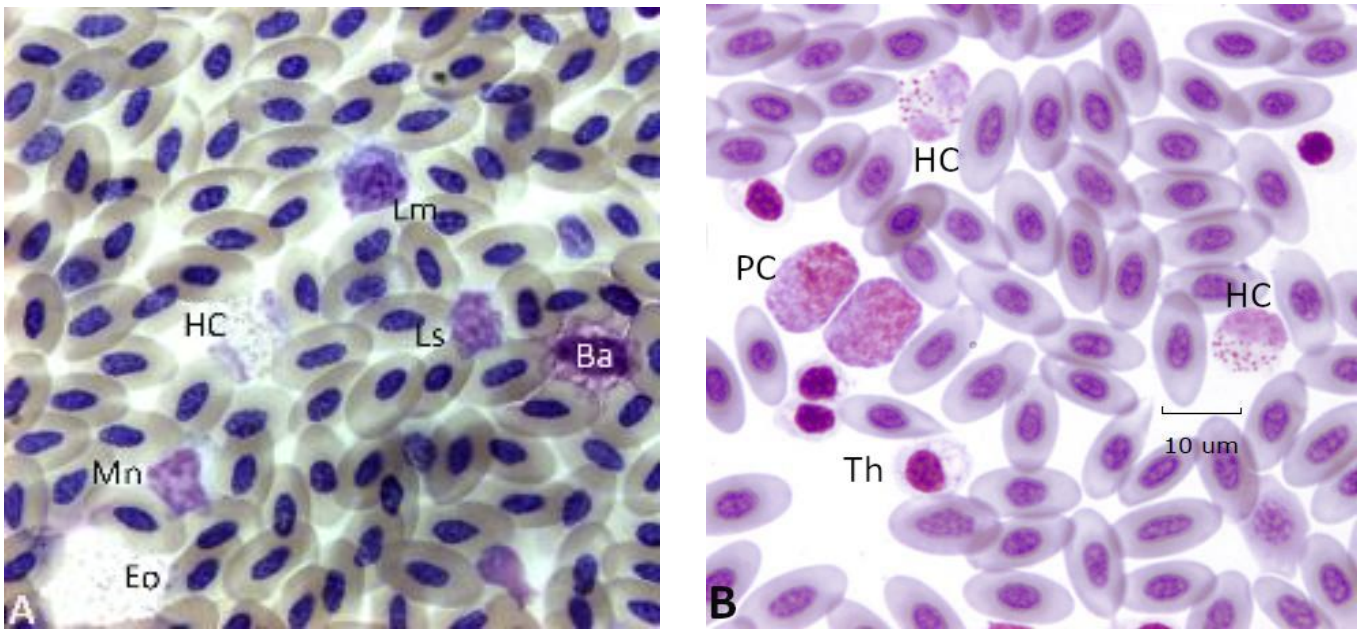

Figure 2. Cytology examples of a Tom turkey at 19-weeks of age raised on an antibiotic-free farm. **A:** A high cellularity (~6% WBC) field with small (Ls) and medium lymphocytes (Lm) a monocyte (Mn) a faint (hypochromic) heterophil (HC) a basophil (Ba) and an eosinophil (Eo). Background RBCs are unremarkable. **B**: Paired atypical plasmacytes (PC) with some Mott cell vacuole features. Reactive (swollen) thrombocytes (Th) and classic heterophils (HC) are also in the field.

Examples from a 6-week-old male (Tom) raised in a conventional facility are presented in Figure 1. Panel A. (100x) had four normal cells located in a single field (cellularity 7%; f(H/L) ~ 1/3): Ls1 and Ls2 were small "resting" lymphocytes (D_C Ls1, 6.8 μm; D_C Ls2, 8 μm; N/C's ~1), which were likely T-cells; and Lm (D_C 13.7 μm) was likely a B-cell. A variant heterophil (HV), sometimes called by mistake an eosinophil (D_C 11.6 μm) was standard-sized (A_C ~100 μm^2). Its orange cytoplasmic granules were spherical and smaller than those of turkey eosinophils, which often stained weakly with W-G alone. Figure 1B shows an (Eo) whose cytoplasmic granules are barely stained by W-G, normal for a turkey Eo, and is attached to a small "reactive" (Th). Classic heterophils were attached to Th, or RBCs were irregularly shaped. A resting Th was at the bottom middle of the field, and a monocyte (Mn), whose cytoplasm projected filopodia from a stretched nucleus (zeiosis), was in the middle right. Approximately 1/3 of the RBCs in this field were late polychromatic types (pRBC) clustered in the bottom hemisphere. Figure 1B (100x) shows two forms of atypia. First were HCs displaying distorted shapes, and an Mn whose cytoplasm was reduced to filopodia projecting from the nucleus, and an Eo whose cytoplasmic granules lack staining was normal and not an artifact. The behavioral issue of Th attachment to HCs and the Eo was superimposed on the atypical cytology. Moreover, the aggregation of pRBC was remarkable.

Examples from a 19-week-old male (Tom) raised in an AB-free facility are in Figure 2. Panel A (100x) had a high cellularity (~6% WBC) field with 2 two lymphocytes, and the topmost was large enough to be a B-cell (A_C 61 μm^2; N/C 0.53). The Lm may have transitioned into a plasmacyte as its cytoplasm appeared secretory. An Mn, an HC with a faintly stained nucleus (hypochromia), a reactive Ba, and an Eo with normal (weakly stained) cytoplasmic granules were on the lower left. The background RBCs were mature cells (full Hb) and otherwise unremarkable. Figure 2B (100x). An opposing pair of primitive (developmental) plasmacytes with Mott cytoplasmic features (Russell bodies, RB) were found (Cotter and Bakst, 2017). A polyploid (Th) was recognized by its ~2x nuclear size, and it also had a swollen cytoplasmic vacuole. Two classic heterophils (HC) were also present. All were in the blood of a 17-week-old Tom raised on an AB-free farm.

Figure 3 shows examples from a 17-week Tom raised in a conventional facility (100x). A field containing a normal small lymphocyte (Ls, D_C 6.2 μm, A_C ~ 30 μm^2; N/C ~ 1), a giant Mott plasmacyte (*Lm, D_C 11.6 μm, A_C ~ 106 μm^2; N/C ~ 0.4) and a thrombocyte were found. The cytoplasm of the plasmacyte was fenestrated by small vacuoles, Russell bodies (RB), with diameters < 0.5 μm. Some vacuoles displayed a faint pink tinge, and others were clear. The pink RB likely contained defective IgA, allowing this cell to be subcategorized as a "flame cell." A few Dutcher bodies could be seen in the nucleus (RB equivalents). The combination of pink and clear vacuoles suggests that this cell produces at least two (defective) Ig isotypes. Although plasmacytes are rare in normal circulation, a solitary cell, in the absence of other atypia, is insufficient for disqualification. However, not only was this plasmacyte a giant cell, but it appeared to be a very rare multiple isotype secretor. Defective plasmacytes (Mott cells) were more frequent under stress. The lysed RBC nuclear remnants and an encapsulated diplococcus (arrow) should not be left without notice. Additional examples of atypia from the same turkey are in Figure 3, Panel B. A large PC was accompanied by a giant amitotic RBC at the separation stage. After division, each of the daughter RBCs would be of unequal size. The smaller member of the pair will become a "pyrenocyte." A resting lymphocyte (Ls) was also in the field. Bacteria were scattered throughout the field, and a nuclear and the presence of a remnant added to evidence of the toxic environment.

Examples of cells from a second 19-week-old male, Tom, raised in an AB-free farm, can be seen in Figure 4 (100x). Panel A. A highly cellular field (cellularity, 8%) contained a reactive dendritic-type basophil (Ba), a pair of monocytes (Mn) whose large nuclei were at least tetraploid. Both Mn radiated pseudopods that extended to nearby RBCs. A thrombocyte, attached to the central Mn, was at an early reactive stage and partially phagocytized by the Mn. An HC and a Th were on a background of mature RBCs. Cell-associated bacteria (CAB) were located by arrows (Panel B). An atypical PC had an irregular cell membrane, and its cytoplasm differentiated into blue ectoplasm (external) and clear endoplasm regions. A (toxic) RBC was in the process of separation into an (anuclear) erythroplastid (ep) and a nucleated pyrenocyte (py).

A field displaying multiple atypia from a 12-week-old Tom is depicted in Figure 5. The PC, a large plasmacytoid cell (A_C ~222 μm^2; [perimeter] P_C ~ 53 μm; N/C ~ 0.58), had characteristic features. The eccentric nucleus with condensed chromatin was arranged as a cart-wheel, and a nucleolus was seen at 11 o'clock. The light

blue cytoplasm was fenestrated by many small diameter (~ 0.3 µm) clear vacuoles. A small cytoplasmic bleb was located at 12 o'clock and was likely to be shed as an apoptotic body. The large nuclei of RBCs 1 and 3 (A_N 44 μm^2 and 38 μm^2, respectively) were likely polyploid; the cytoplasm displayed an RGE appropriate for mature RBCs. These contrast with the faintly stained (diploid) nuclei of cell two types. The cytoplasm of RBCs 1 and 3 were not fully hemoglobinized. A giant (A_C 380 μm^2, P_C 69 µm) atypical cell (G) at the right has both granulocytic and histiocytic features. A nucleus was not obvious, and the cytoplasm contained a central pair of deep blue granules, not likely the products of phagocytosis. The remnants of a phagocytosed thrombocyte containing a phagocytosed bacterium are attached to (G) at 1 o'clock. This cell displayed a type of "second-order" phagocytosis, a term first coined to describe reactive bone marrow cells of lame ducklings. Panel B. An aseptate fungal hyphae, possibly a *Mucor sp*., had formed a coil with a pair of Th and RBCs, it was found in the same sample as in panel A.

To conserve space, a single representative standard differential count for 6 weeks. Tom was raised on a conventional farm as indicated in Table 1. Four individual counts of ~200 cells were used to compute values for the TWBC and the H/L statistics. Three of four cell counts were done in the mid (monolayer) regions of the slide (counts 1-3) and another in an area thinly populated with cells (count 4).

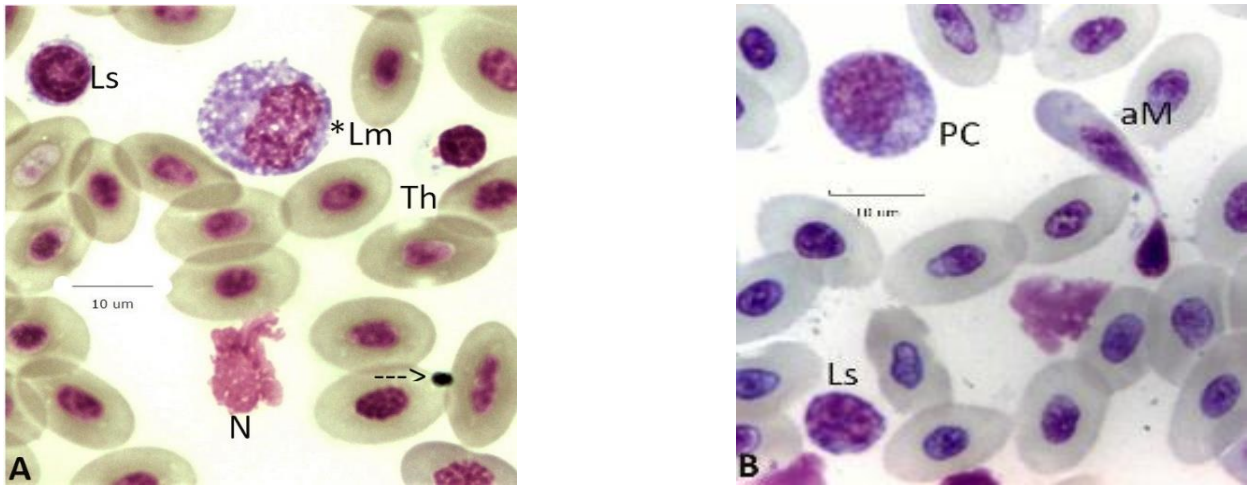

Figure 3. Reactive blood cells of a 17-week Tom raised at a conventional site located in Zeeland Mi, USA. **A:** Normal small lymphocyte (Ls) Mott plasmacyte (*Lm) and nuclear remnants of a lysed RBC (N) a thrombocyte (Th) and an encapsulated diplococcus is located by the arrow. **B:** Large plasmacyte (PC), a small lymphocyte (Ls), and a giant (tetraploid) amitotic RBC (aM) at the separation stage.

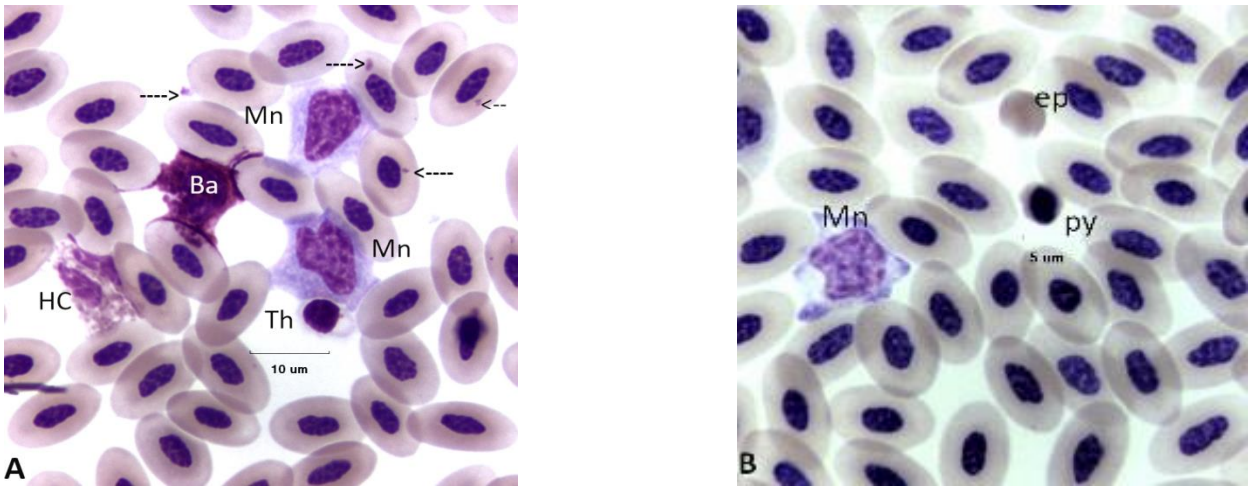

Figure 4. Cytology examples from a Tom turkey at 19 weeks of age raised on an antibiotic-free farm site located near Zeeland, MI, USA. **A:** The field contains a dendritic basophil (Ba), a pair of monocytes (Mn1 and Mn2), a toxic heterophil (*HC), and a partially phagocytized thrombocyte (Th). The background cells are mature RBCs. Surface bacteria are located by arrows. **B:** A large reactive monocyte (Mn), and an erythroplastid/pyrenocyte pair (ep/py) are nearby.

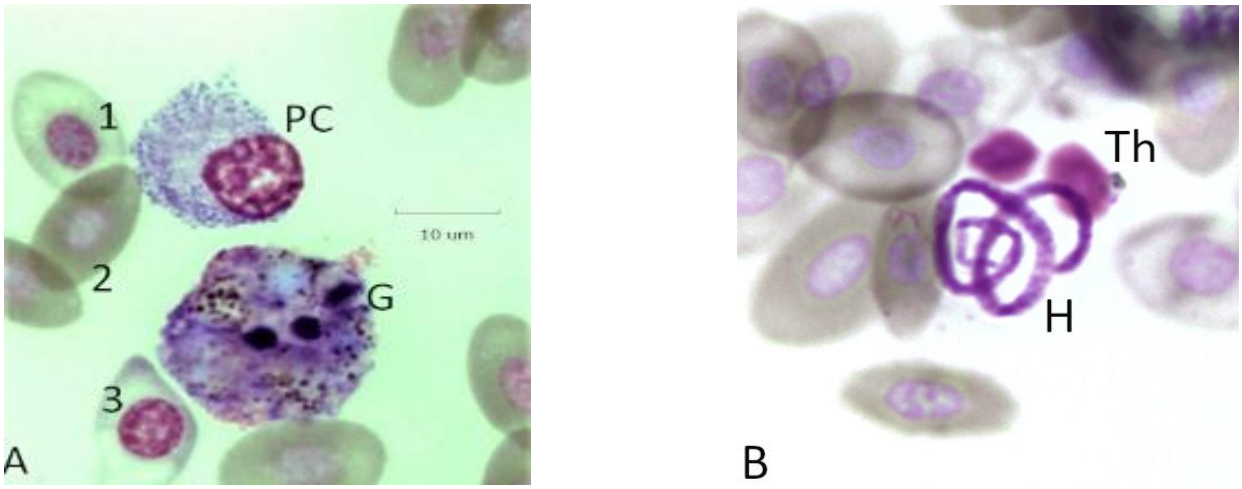

Figure 5. A: A field displaying multiple atypia from a 12-week Tom raised on a conventional farm, near Zeeland MI, USA. PC is a very large cell (A_C ~222 µm^2; P_C ~ 53µm; N/C ~ 0.58) with plasmacytoid features. The eccentric nucleus has condensed chromatin arranged as a cart-wheel and a nucleolus is seen at 11 o'clock. The light blue cytoplasm is fenestrated by fine vacuoles. The nuclei of RBCs 1 and 3 (A_N 44 and 38 µm^2, respectively) are polyploid, and RBC2 is diploid (A_N ~ 20 µm^2). A giant atypical cell (G; A_C 380 µm^2, P_C 69 µm) at the right has both granulocytic and histiocytic features. **B:** An aseptate fungal hyphae (H), possibly a *Mucor* sp. has formed a coil with a pair of thrombocytes (Th) and RBCs; all were found in the same sample as in Figure 1A.

Table 1. A representative standard differential count (%) and H/L 1, H/L 2 ratios based on four individual 2 × 200 cell counts from 3 mid-slide areas (with the average) and one thin slide area of a 6-week Tom raised on a conventional farm.

Ct	Area	HT	HV	HC	Ls	Lm	NK	Bst	Mn	Ba	Eo	TWBC (K)	H/L 1	H/L 2
1	Mid	75	0	0	79	39	0	0	0	10	0	50	0.95	0.64
2	Mid	75	0	0	62	46	0	3	1	18	2	50	1.21	0.69
3	Mid	76	0	0	62	46	0	3	1	18	2	50	1.23	0.7
Ave Ct 1-3 (%)	Mid	36.5	0.0	0.0	32.7	21.1	0.0	1.0	0.3	7.4	0.6	50	1.1	0.7
4	Thin	26.4	0.0	0.0	51.2	14.4	0.0	0.0	1.0	7.0	0.0	15	0.51	0.4

H: Heterophil (HC, classic, HV, variant, HT, typical), Ls: Small lymphocyte ~6 µm diameter, Lm: Medium, & Large, lymphocyte (diameter 8-10 µm), NK: Natural killer, Bst: Granulocyte blast, Mn: Monocyte, Ba: Basophil, Eo: Eosinophil, TWBC(K): Total white blood cells per cubic mL in thousands (K). H/L 1 = (HC + HT + HV) / Ls; H/L 2 = (HC + HT + HV) / (Ls + Lm); Average of Mid Counts 1-3 in (%)

DISCUSSION

The purpose of this manuscript was two-fold. First, it was designed to supplement scarce information on turkey hematology and (abnormal) cytology. Second, it draws attention to the theory and practice of using an SDC derivative statistic, the H/L alone, as a stress measure. The H/Ls are often used without reference to TWBCs or descriptions of abnormal cells. This aspect has been partially addressed by some of the observations already made on chickens, ducks, and turkeys (Cotter, 2023; Davis et al., 2008). Atypia described in some of those studies parallel the examples described here. Starting with examples of "normal" cells (Figure 1), the question of lymphocyte category is raised. How many Lm's (presumptive B-cells) can be used in the denominator before a hemogram is declared reactive? If a reactive hemogram exists, is there any merit remaining in a derivative statistic, the simple H/L? How many cells (TWBC) are allowed before leukocytosis or a leukemoid reaction is declared? The opposite also applies. The question of hemograms with too few white cells, panleukopenia, sorely needs to be addressed. In this regard, Girish et al. (2008) indicated turkeys (Commercial Strain H) exposed to *Fusarium* mycotoxin experienced a decline in TWBC during an 8-week experimental period. Examination of their data suggests that both the Fusarium exposed and control animals were suffering from panleukopenia as their TWBCs were less than 1/3 of turkeys' normal levels. Is this peculiar to Commercial

Strain H turkeys, or was there some systematic error in hematological procedures?

Additional examples of apparent leukopenia are in dos Santos Schmidt et al. (2009) and Azeez et al. (2011), who described highly unusual hemograms in Bronze and Nigerian turkeys, respectively. In both studies, normal hematocrits of ~ 40% were reported. This was accompanied by total RBC counts of ~ 1-2 x 10^6/µL, values well below what would be expected to support the reported hematocrits. A similar situation was reported by Chowdhury et al. (2005), indicating normal values for hemoglobin and hematocrit in control 4-week-old Commercial Strain H turkeys, coupled with a total RBC value ~ 1.5 x 10^6/µL, a value half of what is needed to support the reported hematocrit and hemoglobin values. None of these publications includes a single photo of cells. This lack of cell detail also extends to Scanes et al. (2020). It is clear from these examples that the available literature on the hematology of the turkey has insufficient cytologic detail.

One egregious example of poor hematology appears in Huff et al. (2010), where H/L ratios calculated using the data in Table 1 turn out negative.

The author acknowledges some limitations in the current study. The most important was the lack of consensus on how an H/L ratio should be computed. A partial solution was to recognize that atypical heterophils and lymphocytes should not be included in the computations. Finding these cells indicates the existence of a complex hemogram and implies stress exists. Moreover, heterophils were not a single series, a fact easily discerned by examination of figures available in Lucas and Jamroz (1961). A previous study indicated that there is as much variation in the turkey and duck heterophils as for chickens (Cotter, 2021). The same statements are true for lymphocytes, particularly members of the plasmacyte series.

Davison et al. (1983) used the granulocyte/lymphocyte (G/H) ratio as the stress measure in its original concept. This means basophils and developmental cells might have been included. An excess of basophils (basophiliosis) has been described in chickens. Basophiliosis may be a direct indication of bacteremia/fungemia. How could such a hemogram not indicate that microbiological stress is already in progress?

If stress activates the adrenal cortical axis and a demand is placed on the supply of heterophils, the new recruits to the circulating population should be "band" types (young cells), a circumstance rarely considered. If the circulating population is of the "right-shift" (aged) type (Lucas and Jamroz, 1961), then this circumstance alone challenges the utility of the H/L (Cotter, 2021). Lastly, many of the cells described here were found in the blood of turkeys whose ages are close to those described by Scanes et al. (2020). The turkeys of this study were raised either on conventional or antibiotic-free farms. Therefore, at least some of the atypical cells described here might be expected to have been in the samples described by Scanes et al. (2020) and presumably were ignored. Thus, their reported H/L values may be weak proxies for the true blood pictures of their turkeys.

CONCLUSION

In conclusion, the present observations add important information not widely available elsewhere concerning the cytological variation of blood cells in the turkey. They supply crucial observations missing in most publications on this species. Atypia complicates the interpretation of the simple H/L ratio as a stress measure. In the author's opinion, there is no good reason not to provide photos of representative cells and include the TWBC, along with the derivative H/L, in experiments on questions of stress in turkeys. This should be true where stress is either of direct interest or is included as a part of future studies of the physiology of this species.

DECLARATION

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Ethical consideration

The sole author has ensured that the research adheres to ethical principles such as avoiding plagiarism, obtaining consent to publish, avoiding misconduct, preventing data fabrication or falsification, refraining from double publication or submission, and avoiding redundancy.

Competing interests

The author declares that there is no competing interest with any financial organization regarding the materials discussed in the manuscript.

Availability of data and materials

The data for this study are carefully selected examples from a library of ~400 photomicrographs of turkey blood smears.

Authors' contributions

Paul F. Cotter, the sole author, conceived, wrote, and revised the manuscript.

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Eucalyptus globulus as an Alternative to Antibiotics for *Isa brown* Laying Hens during the Starter Phase

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ABSTRACT

Identification of antibiotic residues in meat and eggs of laying hens in Togo and the ban in 2006 on using antibiotics growth promoter (AGP) in animal production by the World Health Organization induce the use of medicinal plants with antimicrobial effects, such as AGP alternatives in poultry production. For the same purpose, this study was conducted to contribute to studies using phytobiotics as alternatives to AGP in poultry production. Indeed, antibiotics have been substituted by *Eucalyptus globulus* leaf powder (ELP) during the starter phase. Polyphenolic compounds from ELP were determined, and the effects of different rates of ELP supplementation on growth performance, mortality, and hematological and biochemical parameters were evaluated. A total of 460 one-day-old laying chicks (*Isa brown*) were randomly allocated to 5 groups, each consisting of 4 replications, with 23 chicks in each replication. Treatments consisted of the basal diet (BD) without ELP and antibiotics, a negative control (group EGO-), BD with antibiotics and no ELP, a positive control (group EGO+), BD + 0.25% of ELP without antibiotic (group EG1), BD with 0.50% of ELP without antibiotic (group EG2), and BD with 1% ELP without antibiotic (group EG3). The rates of 0.25%, 0.5%, and 1% mean 0.25 kg, 0.5 kg, and 1 kg of ELP for 100 kg of BD, respectively. The study revealed that ELP contains flavonoids (4.85 µg QE/mg), tannins (30.34 µg CE/mg), and total phenols (165.2 µg AGE/mg). Supplementation did not affect feed intake (FI), body weight gain (BWG), feed conversion ratio, and mortality of *Isa brown* laying hens during the starter phase (8 weeks) in all treatment groups. However, the chicks that received ELP had the best FI and BWG, which was not significantly different from the control groups. The biochemical parameters such as total proteins, albumin, triglyceride, total cholesterol, and glycemia were not affected by ELP supplementation. Among the hematology parameters, the leukocyte decreased in the groups fed with ELP, while mortality was unaffected. The results of the present study indicated that ELP inclusion rate of 0.25% could serve as the best antibiotic replacement for *Isa brown* laying hens during the starter phase.

Keywords: *Eucalyptus globulus*, Growth parameters, *Isa brown*, Starter

INTRODUCTION

In Togo, the poultry sector mainly focuses on egg consumption production and produced 30,668,872 eggs in 2005 (FAO, 2008). This level does not meet producers' expectations and national demand due to many avian diseases, which negatively impact productivity (Atakpama et al., 2016). Generally, the use of antibiotics is frequent and leads to the formation of residues in livestock products such as eggs and meat (Niyibizi, 2012). The World Health Organization banned the use of antibiotic growth promoters (AGP**)** in livestock production in 2006, although AGP improves animal performance and reduces the spread of disease (Mashayekhi et al., 2018). In Togo, one of the reasons is that AGPs are responsible for the presence of antibiotic residues in chicken meat and eggs from laying hens, which constitutes a food security problem (Gambogou et al., 2020). Furthermore, the use of antibiotics as growth promoters induces the dissemination of antibiotic-resistance genes (Bedkelabou et al., 2020). To limit the use of antibiotics in hen production and improve public health, nutritionists and animal health actors need to find alternatives that have the potential to mitigate the negative effects related to AGP (Lillehoj and Lee, 2012). In this dynamic, studies have been conducted to supplement plant products (*Moringa oleifera*, *Carica papaya*, *Eucalyptus*

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globulus) *in poultry feed as an alternative to antibiotic* growth promoters. Supplementation of *Moringa oleifera* leaves improved broilers' zootechnical performance (Teteh et al., 2013) and increased production performance in laying hens while causing a decrease in hematological parameters (Voemesse et al., 2018).

The studies about *Eucalyptus globulus* have related to the replacement of antibiotics by *Eucalyptus* leaves powder in broiler chicken production and the laying phase of hens (Mashayekhi et al., 2018; Abd El-Hack et al., 2023). *Eucalyptus globulus* is a medicinal plant belonging to the *Myrtaceae* family. It was discovered in Australia but is found worldwide, especially in tropical and subtropical regions (Salari et al., 2006). *Eucalyptus globulus* contains compounds such as cineol (60%), flavonoids (4.4%), and tannins (19.6%), which have antioxidant and antimicrobial properties that have led to improved appetite, health, and growth performance of broiler chickens (Mashayekhi et al., 2018). The metabolic and aqueous extracts from *Eucalyptus globulus* leaves showed antimicrobial activity in *Staphylococcus*, *Pseudomonas*, *Bacillus*, and *Escherichia coli* (Boukhalfoun, 2012). *Eucalyptus globulus* is effective against microorganisms that cause food intoxication (Takahashi et al., 2004). It can potentially improve the immune response of broiler chickens (Farhadi et al., 2017). The supplementation of 0.30% *Eucalyptus globulus* leaf powder in the laying hens' diet increased egg production and shell thickness (Kaur et al., 2022). However, there is little scientific data on *Eucalyptus globulus* usage in the starter phase of *Isa brown* laying hens although its use is widespread in Africa (Gaston and Parfait, 2017). This study aims to evaluate the effects of *Eucalyptus globulus* leaves on growth performance, and hematological, and biochemical parameters of *Isa brown* laying hens during the starter phase.

MATERIALS AND METHODS

Ethical approval

The animal care guidelines recommended by the Animal Ethics Committee of the University of Lome in Togo were followed (008/2021/BC-BPA/FDS-UL).

Study design

To begin, 6 kg of *Eucalyptus globulus* leaves were collected in the canton of Badja in Avé prefecture, Togo, and were dried and sheltered from light for 72 hours with natural ventilation (at a room temperature of 20-25°C and relative air humidity of 42-54%). The leaf samples were analyzed for their composition in flavonoids, tannins, and phenols. The dried leaves were ground into powder and added at different rates to the basal diet of *Isa brown* laying hens at the starter phase. The supplementation rates were 0.25%, 0.50%, and 1% per 100 kg of feed. The *Eucalyptus globulus* leaf powder was added to the feed, and the antibiotic was given through drinking water according to the described dosage (Bouassi et al., 2020). A total of 460-day-old chicks at 30.79 g average weight from CERSA-UL hatchery unity of Lomé, Togo, were used for the study. During the experiment, the chicks were raised 7 days and were randomly allocated to 5 treatments with 4 repetitions of 23 chicks, each housed in floor pens of identical size (3 × 2.5 m). Treatments consisted of a basal diet (BD) without ELP and antibiotics, a negative control (group EGO-), BD with antibiotics and no ELP, a positive control (group EGO+), BD + 0.25% of ELP, without antibiotic (group EG1), BD with 0.50% of ELP without antibiotic (group EG2) and BD with 1% ELP without antibiotic (group EG3). The rates of 0.25%, 0.5%, and 1% mean respectively 0.25 kg, 0.5 kg, and 1 kg of ELP for 100 kg of BD.

Table 1. Composition and characteristics of diets of *isa brown* chicks in the starter phase (8 weeks) per treatment

Ingredients (kg)	**EGO-**	**EGO+**	**EG1**	**EG2**	**EG3**
Maize	56	56	56	56	56
Soya roasted	12	12	12	12	12
Wheat bran	17	17	17	17	17
Beer grains	7.5	7.5	7.5	7.5	7.5
Fish meal	5	5	5	5	5
Lysine	0.3	0.3	0.3	0.3	0.3
Methionine	0.2	0.2	0.2	0.2	0.2
Oyster shell	2	2	2	2	2
ELP	0	0	0.25	0.50	1
Characteristics					
ME (Kcal/kg)	2841.04	2841.04	2841.49	2841.95	2842.87
CP (%)	17.05	17.05	17.06	17.07	17.09
Lysine (%)	0.95	0.95	0.95	0.95	0.95
Methionine	0.59	0.59	0.59	0.59	0.59
Methionine + cysteine (%)	0.81	0.81	0.81	0.81	0.81
Calcium (%)	0.97	0.97	0.97	0.97	0.97
Phosphorus (%)	0.65	0.65	0.65	0.65	0.65

ELP: *Eucalyptus globulus* leaf powder, ME: Metabolizable energy, CP: Crude proteins, EG0-: Groups fed basal diet (BD) without ELP and no antibiotics, EGO+: BD without Eucalyptus leaf powder (ELP) but with antibiotic, EG1: BD + 0.25% ELP and without antibiotic, EG2: BD+ 0.50% ELP and without antibiotic, EG3: BD+1% ELP without antibiotic.

The feed compositions and the nutritional values are presented in Table 1. The animals were raised on the floor, and feed and water were provided *ad libitum*. The lighting program consisted of 24 hours of light for the first day,

followed by 23 hours of light until day 7. The 21.30 hours of light was used in the second week and decreased by 1.30 hours weekly from the third week to 12 hours of light in week 8 (Voemesse et al., 2018). Vaccines against Marek, Newcastle, Gumboro, infectious bronchitis, and smallpox from the LAPROVET laboratory of France were administered to each group following the prophylaxis program (Table 2). The feed offered was weighed every day, and the feed not consumed was weighed at the end of the week. At 8 weeks, four animals were stunned and slaughtered per treatment for blood sampling and weighing of organs (heart, liver, gizzard, proventriculus, kidney, and pancreas). The amount of 1.5 ml blood samples was collected and analyzed to determine the hematological and biochemical parameters.

Table 2. Prophylaxis protocol of *Isa brown* laying hens during the starter phase

Treatments	Period
Marek, gumboro, Newcastle, and infectious bronchitis vaccines	Day 1
Newcastle, infectious bronchitis, and gumboro	Day 7
Newcastle, infectious bronchitis, and gumboro	Day 21
Newcastle, infectious bronchitis, and gumboro	Day 35
Smallpox+Newcastle	Week 7
Vitamins (Aminogrow ws, introchick oral, and Amin'total)	Weeks 1, 2, 4, 5, and 8
Anticoccidial (Amprolium 20 %)	Weeks 4 and 8
Deworming (Levalap)	Week 6
Antibiotics (Aliseryl ws) only for the positive control group (EGO+)	Weeks 3 and 8

Vitamin, anticoccidial, and deworming are produced by the LAPROVET laboratory of France, and aliseryl ws, aminogrow ws and introchick oral produced by Interchemie werken 'De Adelaar' BV Holland. The products were given according to the manufacturer's dosage.

Determination of total flavonoids

The flavonoid was evaluated with the colorimetric method based on the aluminum trichloride (AlCl3), using the protocol described by Mahmoudi et al. (2013). A volume of 200 µl of each extract of concentration 500 µg/mL, was added to 1,600 µL of distilled water, 120 µL of sodium nitrite (NaNO2 = 5%), then 80 µL of aluminum trichloride hexahydrated (AlCl3; 6H2O = 10%). After vortex stirring with each addition, the mixture was incubated at laboratory temperature in the dark for 5 min. The absorbance of the final solution obtained was read with the UV-5200PC spectrophotometer (METASH, China) against a blank at the wavelength of 510 nm. The tests were carried out in triplicate for each sample. A calibration range from 0 to 700 µg/mL was performed under the same conditions using quercetin as the reference standard. The total flavonoid contents of the samples were in mgEqQt/gES.

Determination of total condensed tannins

The rate of total tannins condensed in dry extracts was measured using vanillin and concentrated hydrochloric acid (HCl) by adopting the experimental protocol of Bahorun et al. (1996). A mixture was made using 400 µL of dry extract, 2000 µL of vanillin solution, 4% of methanol, and 750 µL of hydrochloric acid (37%). The mixture was thoroughly stirred and incubated at 18°C for 30 minutes. The absorbance of the final solution obtained was measured at the wavelength of 500 nm. The tests were performed in triplicate for each sample. A standard catechin solution was used for the calibration curve to measure tannin total condensed values, expressed in milligrams (mg) equivalent (Eq) of catechin (CA) per gram (g) of dry extract (ES), either mgEqCA/gES.

Determination of total polyphenols

The total polyphenols were determined through the method described by Suman et al. (2017). According to the experimental approach, a solution of an initial concentration equal to 1 mg/mL must be prepared for each sample to be determined, followed by a freshly prepared sodium carbonate solution (6%: w/v). On 500 µL of each previously prepared solution, a volume of 500 µL of Folin's reagent Ciocalteu (10%: v / v) was added. The mixture was stirred by a vortex and then stood for 5 minutes. A volume of 500 µL of the sodium carbonate solution (6%: w / v) was added to the mixture. The final mixture was left to rest for 30 minutes at room temperature and protected from light. The reading was performed at a wavelength of 725 nm against a blank, using the UV-5200 PC spectrophotometer (METASH, China). A calibration curve was plotted under the same operating conditions using gallic acid as standard in a concentration range from 0 to 300 µg/mL. The polyphenol total contents of the samples analyzed were expressed in mg EqAG/gES.

Physicochemical analysis

The moisture content of the leaves was determined by the NF EN ISO 665 (2000) method (Aziato et al., 2021). This determination is based on drying leaves at 103°C ± 2°C for 4 hours in the dry oven until practically constant mass was obtained. For this, 5 g of the *Eucalyptus globulus* (*EG*) leaves were weighed in a beaker and placed in the dry oven at 103°C ± 2°C for 4 hours.

The moisture content was determined by following Formula 1.

H = (m1 – m2)/ (m1 – m0) x 100 (Formula 1)

Where, m0 means mass of the empty beaker, m1 signifies the mass of the beaker with the test portion before drying, m2 denotes mass of the beaker with the test portion after drying.

The ash content was determined according to AOAC 923.03 (AOAC, 1999). It consisted of the incineration of *EG* leaves in the muffle oven at the temperature of 550°C until whitish ash was obtained. For this, 5 g of EG was placed in a dry porcelain crucible previously weighed. The whole (crucible and test portion) was then subjected to incineration in the furnace at 550°C for 4 hours. At the end of the incineration, the crucible containing the ash was cooled with a moisture analyzer for 30 minutes and then weighed. The ash content was determined according to Formula 2.

Ash content (%) = m1/m0 x 100, (Formula 2)

Where, m0 is the mass of the test portion, and m1 determines mass of the incineration residue.

The protein total content was determined by the Kjeldahl method according to NF V18-100 (1977) (Ayssiwede et al., 2011). The principle consists of mineralization of the material by concentrated sulfuric acid in the presence of a protein catalyst followed by alkalization of the reaction products. The ammonia released is distilled and collected in a boric acid solution and titrated with a solution of normal sulfuric acid (1 N). One gram of crushed EG leaves was weighed in a mineralization flask to which 25 ml of concentrated 96% sulfuric acid and 5 g of protein catalyst (1000 g of K2SO4 + 30 g of TiO2 + 30 g of Cu2SO4) were added. The final mixture was placed on a mineralization ramp and mineralized until the complete discoloration of the solution, which was initially black. After mineralization, the solution was cooled and then introduced into the nitrogen distiller (Lab Logistics Group of Germany). Then, it underwent neutralization with 40% soda. The released nitrogen was collected in an Erlenmeyer containing a boric acid solution at saturation, methylene blue, and methyl red. The distillate obtained was defined with 1 N sulphuric acid. The total protein content was determined by the Formula 3.

Tp = (V-V0) /P x T x ß x 0.014 x 100 (Formula 3)

Where, Tp is total protein content (%), V means the volume of sulphuric acid used for the test portion (ml), V0 signifies the volume of sulfuric acid used for the white (ml), T denotes the concentration of the sulphuric acid solution used for titration (1 N), ß refers to the nitrogen-to-total protein conversion factor (6.25), P is test portion (g), 0.014 determines conversion coefficient of the titer of the sulphuric acid solution used (normality) into mass titer.

Lipids were extracted from leaves using the Soxhlet method (gravimetric method) according to AOAC 960.39 (AOAC, 1999). A test portion of 3 g of *EG* sheets was fed into an extraction cartridge. The cartridge was corked with cotton wool and then placed in an extraction cup containing 100 ml of n-Hexane or light petroleum at 40-60°C. The entire extraction system was placed in the Soxhlet, and extraction was performed for 1 hour and 30 minutes. After extraction, the flask containing the lipids was placed in the oven for 1 H at 105°C and then in a moisture analyzer for cooling. A series of weighing, steaming, and drying of the flask was carried out alternately until a constant mass was obtained. The mass of lipids was obtained by the difference between the mass of the balloon containing the fat and the empty mass of the balloon. The lipid content (Tl) was determined by Formula 4.

Tl = (MF – M0) / ME x 100. (Formula 4)

Where, MF represents mass of fat matter + flask, M0 corresponds to mass of the vacuum balloon, ME indicates mass of the test portion.

The carbohydrate content of EG leaf was determined by differential calculation according to the method of Al-Hooti et al. (1998). It was obtained according to the following Formula 5.

Carbohydrates (%) = 100 -% (water + protein + fat + ash) (Formula 5)

The energy value was determined by the following Formula 6.

E = (protein %) × 4 + (carbohydrates %) × 4 + (Fat %) × 9 (Formula 6)

Growth performance

Feed intake (FI), body weight gain (BWG), and feed conversion ratio (FCR) were calculated per treatment. The offered and residual feed was weighed weekly. Each chick was weighed per treatment at the beginning and end of the week.

Data for FI and BWG were used to calculate the FCR. These parameters were determined by formulas 7, 8, and 9 (Guembo et al., 2021).

FI = (quantity of food distributed - quantity refused) / number of chicks (Formula 7)

BWG = (final weight - initial weight) / duration (Formula 8)

FCR = amount of feed consumed by the animal / (final weight - initial weight) (Formula 9)

Determination of hematological and biochemical parameters

At 8 weeks of age, 20 chicks were randomly selected at the rate of 4 chicks per treatment. Blood samples were taken between 09:00' and 10:00' in the Ethylenediaminetetraacetic acid (EDTA) tubes to determine hematological parameters and in dry tubes to determine biochemical parameters.

An average of 1.5 ml of blood was taken from each chicken and used within 3 hours of collection to determine hematological parameters using ABX Micros 60, a fully automated hematology analyzer from Sysmex Corporation Corporation Company (Japan). Biochemical parameters were determined using an enzymatic colorimetric method on 200 and 10 µl of serum according to the protocols provided by the reagent (Voemesse et al., 2018).

Statistical analysis

The collected data was saved in Excel software version 2019 and was processed in Software R version 3.6.0 (2019-04-26). The SHAPIRO TEST was used to check whether the data followed a normal distribution. The differences between mean values were evaluated using the ANOVA test followed by the Tukey test, and the significant level was set at $p < 0.05$. The results were presented as means ± standard error mean.

RESULTS

Phytochemicals compounds

The analysis showed the presence of flavonoids, tannins, and total phenols. The composition varied from one compound to another according to the reference standard or standard solution. Total flavonoids (4.85 µg/mg) were less represented than tannins (30.34 µg/mg) and total phenols (165.2 µg/mg, Table 3).

Table 3. Composition of *Eucalyptus globulus* leaves in polyphenols

Parameters	Concentration
Total flavonoids	4.85 µg QE/mg
Tannins condensed Totals	30.34 µg CE/mg
Total phenols	165.2 µg GAE/mg

QE: Quercetin equivalent, CE: Catechin equivalent, GAE: Gallic acid equivalent, µg: Microgram, mg: Milligram

Physicochemical compounds

The physicochemical composition of *EG* leaves is diverse. Table 4 shows water, total nitrogen, protein, fat, carbohydrate, and ash *EG* leaves content. The *Eucalyptus globulus* leaf contained carbohydrates (38.85%), lipids (1.12%), proteins (4.37%), nitrogen (0.70%), and ash (3.04%).

Table 4. Physicochemical composition of *Eucalyptus globulus* leaves

Physico-chemical characteristics	Result
Moisture content (% m/m)	52.60
Total Nitrogen content (% m/m)	0.70
Protein content (% m/m)	4.37
Fat content (% m/m)	1.12
Ash content (% m/m)	3.04
Carbohydrate content (% m/m)	38.85
Energy value (kcal)	183.04

Kcal: Kilocalorie

Growth performance

Feed intake

The chick's feed intake by treatment is presented in Table 5. The average consumption in grams (g) of chicks during the starter phase was 35.24 ± 3.16, 35.47 ± 3.22, 35.36 ± 3.14, 34.28 ± 2.88, and 35.65 ± 3.11 for *EG*O-, *EG*O+, *EG*1, *EG*2, and *EG*3 groups, respectively. Overall, the chick's consumption increased weekly during the starter phase. The weekly average feed intake of the chicks showed no significant difference across groups ($p > 0.05$). The group that received 1% of *Eucalyptus globulus* leaf powder had the highest level of feed consumption (35.65 g).

During the first 2 weeks, all chicks had a similar feed intake. The chicks of the EGO- and EG3 groups had the same feed intake in the last weeks. This similarity was related to the increase in feed intake from one week to another during the starter phase, but for groups that took the highest level of ELP (1%), the average individual feed intake decreased from 53.56 g to 49.14 g in the weeks 7 and 8, respectively. The feed intake evolution of *EG*O+, *EG*1, and *EG*2 groups was similar at the starter phase.

Weekly evaluation of chick's weight

The chick's weight at the starter phase is shown in Graph 1. The average weight per chick at the starter phase was 177.56 ± 24.21 g, 176.96 ± 23.46 g, 182.68 ± 24.09 g, 178.26 ± 23.87 g, and 167.88 ± 22.21 g for groups *EG*O-, *EG*O+, *EG*1, *EG*2 and *EG*3, respectively. The chicks that received 0.25% ELP had the average highest weight in all groups, followed by groups that received 0.50% ELP.

Chicks fed with 0.25% ELP had a higher weight (388.45 g), and the groups that received 1% of ELP had the lowest weight (337.15 g) at 8 weeks of age, but the difference between weights was not significant ($p > 0.05$).

Body weight gain

The BWG of the chicks during the starter phase is shown in Graph 2. The control groups *EG*0- and *EG*0+ indicated the BWG of 7.51 ± 1.21 and 7.34 ± 0.96 g/day/chick. For chicks who received *Eucalyptus globulus* leaf powder treatment *EG*1, *EG*2, and *EG*3, the BWG values were 8.21 ± 1.65, 7.80 ± 1.25, and 7.73 ± 1.57 g/day/chick, respectively. The differences between the values for the different groups were not significant at the starter phase ($p > 0.05$).

Feed conversion ratio and mortality rate

The FCR and mortality rate for each treatment is presented in Table 6. No significant differences were found between the FCR of the different groups ($p > 0.05$). The *EG*1 group had the lowest FCR (4.31 ± 0.35), followed by *EG*2 (4.40 ± 0.49) and *EG*3 (4.61 ± 0.56). The Control groups, EGO- and EGO+, had the highest FCR (4.69 ± 0.52 for *EG*0- and 4.83 ± 1.03 for *EG*0+). The Mortality rate values varied between the groups. The negative control group had the same mortality as the group that received 1% of ELP (3% of chick mortality). The chicks that received antibiotics and the groups that received 0.25% and 0.5% of ELP showed 2% mortality. The values were not significantly different among the groups ($p > 0.05$).

Table 5. Effect of *Eucalyptus globulus* leaves on feed intake of *Isa brown* laying hens during the starter phase

Age \ Treatments	EGO-		EGO+		EG1		EG2		EG3	
	$\bar{X}$	SEM	$\bar{X}$	SEM	$\bar{X}$	SEM	$\bar{X}$	SEM	$\bar{X}$	SEM
W1	9.91	0.04	9.93	0.04	9.93	0.04	9.93	0.04	9.93	0.04
W2	20.17	0.07	20.15	0.02	20.15	0.02	20.16	0.02	20.17	0.03
W3	27.68	1.73	31.62	1.95	31.76	1.49	33.58	1.39	31.30	4.55
W4	33.09	1.07	27.40	1.87	29.36	1.44	29.93	0.81	36.91	4.87
W5	44.52	2.29	50.85	1.72	43.28	1.67	39.11	1.23	37.05	5.42
W6	44.54	1.87	49.82	4.35	50.98	1.80	45.26	3.08	47.15	9.82
W7	48.31	5.50	46.95	3.29	48.50	1.66	48.01	1.82	53.56	6.28
W8	53.73	8.83	47.01	6.00	48.92	4.14	48.22	1.63	49.14	4.26
$\bar{X}$ (g)	35.24	3.16	35.47	3.22	35.36	3.14	34.28	2.88	35.65	3.11

EG0-: Groups received basal diet (BD) without ELP and no antibiotics, EGO+: BD without Eucalyptus leaf powder (ELP) but with antibiotic, EG1: BD + 0.25% ELP and without antibiotic, EG2: BD + 0.50% ELP and without antibiotic, EG3: BD + 1% ELP without antibiotic, W: Week, SEM: Standard error of Means, $\bar{X}$: Average in g

Table 6. Effect of *Eucalyptus globulus* leaves on feed conversion ratio and mortality of *Isa brown* laying hens during the starter phase

Parameters	EGO-	EGO+	EG1	EG2	EG3
FI(g)	35.24 ± 3.16	35.47 ± 3.22	35.36 ± 3.14	34.28 ± 2.88	35.65 ± 3.11
BWG (g)	7.5 1 ± 1.21	7.34 ± 0.96	8.21 ± 1.65	7.80 ± 1.25	7.73 ± 1.57
FCR	4.69 ± 0.52	4.83 ± 1.03	4.31 ± 0.35	4.40 ± 0.49	4.61 ± 0.56
Mortality (%)	3	2	2	2	3

FI: Feed intake, BWG: Body weight gain, FCR: Feed conversion ratio, g: Gram, EG0-: Groups were received basal diet (BD) without ELP and no antibiotics, EGO+: BD without Eucalyptus leaf powder (ELP) but with antibiotic, EG1: BD+0.25% ELP and without antibiotic, EG2: BD+ 0.50% ELP and without antibiotic, EG3: BD+1% ELP without antibiotic.

Weights vital organs

The weights of the organs, including the pancreas, kidney, and proventriculus, were identical ($p > 0.05$) among all treatment and control groups (Table 7).

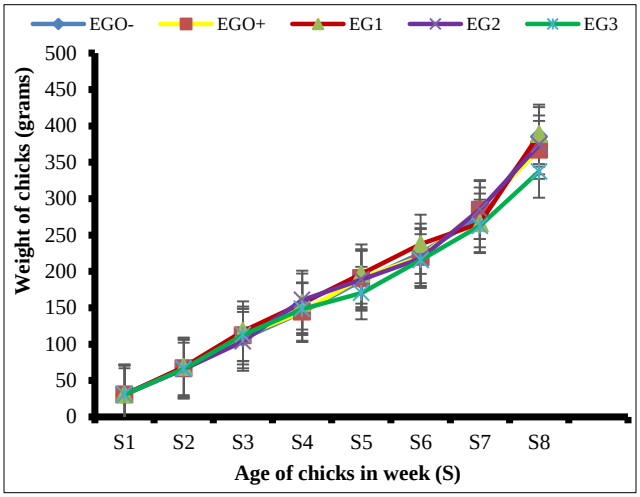
Chicks that received 0.50% ELP had the lowest liver weight, and chicks that received 1% ELP had the highest gizzard weight among the investigated groups ($p < 0.05$).

Hematological parameters

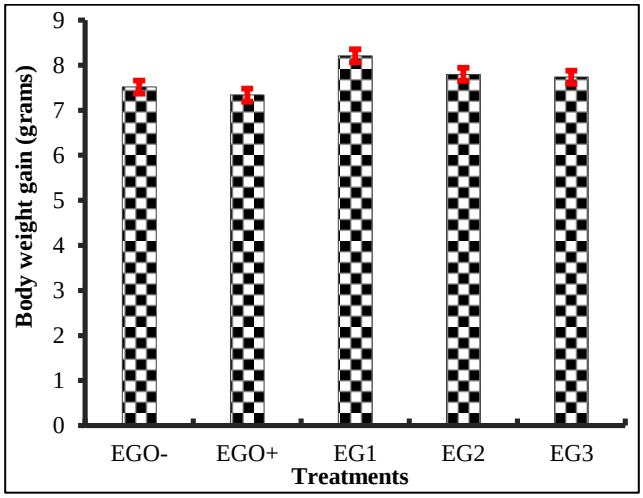
The hematological parameter is presented in Table 8. The study showed that the groups treated with *Eucalyptus globulus* leaf powder had the significantly lowest white blood cell count, compared to the control groups ($p < 0.05$). The value of red blood cell, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin content, mean corpuscular hemoglobin concentration, and platelet was similar for the groups treated, compared to the control ($p > 0.05$).

Effect of *Eucalyptus globulus* leaves powder on biochemical parameters

The biochemical parameters (total protein, albumin, triglycerides, total cholesterol, glycemia, alanine aminotransferase, aspartate aminotransferase) are presented in Table 9. The findings indicated that there was no significant difference in the biochemical parameters between the control and treatment groups ($p > 0.05$).



Graph 1. Effect of *Eucalyptus globulus* on the growth of *Isa brown* laying hens during the starter phase.
EG0-: Groups received basal diet (BD) without *Eucalyptus globulus* leaf powder (ELP) and no antibiotics, EGO+: BD without ELP but with antibiotic, EG1: BD + 0.25% ELP and without antibiotic, EG2: BD + 0.50% ELP and without antibiotic, EG3: BD + 1% ELP without antibiotic.



Graph 2. Effect of *Eucalyptus globulus* leaves on BWG of *Isa brown* laying hens during the starter phase.
EG0-: Groups received basal diet (BD) without *Eucalyptus globulus* leaf powder (ELP) and no antibiotics, EGO+: BD without ELP but with antibiotic, EG1: BD + 0.25% ELP and without antibiotic, EG2: BD + 0.50% ELP and without antibiotic, EG3: BD + 1% ELP without antibiotic.

Table 7. Effect of *Eucalyptus globulus* leaves on the vital organs of *Isa brown* leaves laying hens during the starter phase

Vital organs / Variable	Liver	Pancreas	Heart	Kidney	Proventriculus	Gizzard
EGO-	11 ± 0.11^{a}	1 ± 0.06	2 ± 0.18	1 ± 0.05	2 ± 0.12	21.75 ± 0.95^{b}
EGO+	10.25 ± 0.63^{a}	1.5 ± 0.29	2.25 ± 0.25	1.5 ± 0.29	2.75 ± 0.48	23.75 ± 1.55^{b}
EG1	10.25 ± 0.48^{a}	1.25 ± 0.25	1.75 ± 0.25	1.5 ± 0.29	2 ± 0.41	23 ± 2.04^{b}
EG2	8.75 ± 0.63^{b}	1.5 ± 0.29	2.25 ± 0.25	1.25 ± 0.25	2.75 ± 0.48	23.25 ± 1.65^{b}
EG3	10 ± 1.29^{a}	1.5 ± 0.28	1.95 ± 0.28	1.5 ± 0.28	2.75 ± 0.47	26.15 ± 1.52^{a}

EG0-: Groups received basal diet (BD) without ELP and no antibiotics; EGO+: BD without Eucalyptus leaf powder (ELP) but with antibiotic, EG1: BD+0.25% ELP and without antibiotic, EG2: BD+ 0.50% ELP and without antibiotic, EG3: BD+1% ELP without antibiotic. $^{a, b}$On the same column, the values assigned different letters are significantly different ($p < 0.05$).

Table 8. Effect of *Eucalyptus globulus* leaves powder on hematological parameters of *Isa brown* laying hens during the starter phase

Parameters / Variable	WBC (10^3/µL)	RBC (10^6/µL)	HB. (g/dl)	HCT (%)	CVD (FL.)	MCH (pg)	MCHC (g/dl)	PLT (10^3/µl)
EGO-	16.44[a]	2.53	6.20	29.10	117.55	26.53	21.33	2.00
EGO+	15.76[a]	3.09	7.30	29.73	118.90	25.73	20.00	3.33
EG1	7.76[b]	2.43	6.40	28.67	118.13	26.37	22.33	2.33
EG2	6.18[b]	2.38	6.30	28.47	118.93	26.63	22.23	2.33
EG3	5.82[b]	2.14	6.83	27.97	118.00	27.30	22.73	2.00

WBC: White blood cell, RBC: Red blood cell, HB: Hemoglobin, HCT: Hematocrit, CVD: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin content, MCHC: Mean corpuscular hemoglobin concentration, PLT: Platelet, EG0-: Groups received basal diet (BD) without *Eucalyptus globulus* leaf powder (ELP) and no antibiotics, EGO+: BD without ELP but with antibiotic, EG1: BD + 0.25% ELP and without antibiotic, EG2: BD + 0.50% ELP and without antibiotic, EG3: BD + 1% ELP without antibiotic. [a,b]: On the same column, the values assigned different letters are significantly different ($p < 0.05$). µL: Microliter, dl: Deciliter, FL: Femtoliter

Table 9. Effect of *Eucalyptus globulus* leaves on the biochemical parameters *of Isa brown* laying hens during the starter phase

Parameters / Variable	Total protein (g/l)	Albumin (g/l)	Triglycerides (g/l)	Total cholesterol (g/l)	Glycemia (g/l)	ALT (IU/L)	AST (IU/L)
EGO-	27.40	10.93	0.28	0.83	2.52	9.89	190.33
EGO+	29.77	11.47	1.00	0.91	2.79	9.17	189.70
EG1	28.67	10.50	0.30	0.69	3.03	8.86	191.00
EG2	29.13	10.43	0.44	0.77	2.60	9.08	190.40
EG3	28.50	11.37	0.77	0.85	2.94	9.60	191.01

EG0-: Groups received basal diet (BD) without *Eucalyptus globulus* leaf powder (ELP) and no antibiotics, EGO+: BD without ELP but with antibiotic, EG1: BD + 0.25% ELP and without antibiotic, EG2: BD + 0.50% ELP and without antibiotic, EG3: BD + 1% ELP without antibiotic, ALAT: Alanine aminotransferase, ASAT: Aspartate aminotransferase, g: Gram, l: Liter.

DISCUSSION

Eucalyptus globulus leaves contain flavonoids, tannins, and polyphenols (Amira and Amira, 2022; Drouiche and Haj Moussa, 2022). The present study confirmed the presence of flavonoids (4.85 µg QE/mg), tannins (30.34 µg CE/mg), and total phenols (165.2 µg GAE/mg) in *Eucalyptus globulus* leaves. The flavonoids were lower than the 7.57 mg QE/g flavonoids found by Amira and Amira (2022) and 20.28 mg QE/g flavonoids found by Drouiche and Haj Moussa (2022). The count of total phenols is higher than 71.98 mg GAE/g of phenols, as reported by Drouiche and Haj Moussa (2022). These differences would be due to the vegetative cycle of the plant, climate, season, and treatment methods (Dei et al., 2007; Boukhalfoun, 2012).

The feed intake of the layers chick in the starter phase was between 34 and 35 g per chick in the present study. Tayo et al. (2022) showed a value of 40.67 g/chick/day. This value is higher than the result in this study for the negative control, chicks that received only antibiotics, and chicks treated with ELP.

The BWG of chicks who received different rates of ELP is higher than those of the control groups. A similar study by Mohebodini et al. (2021) indicated a linear increase in BWG of broiler chickens that received 0.1 % of *Eucalyptus* essential oil. This finding confirms the results of Arise et al. (2009) that *Eucalyptus* leaves have multiple biochemical and physiological functions in the chick's body due to their antioxidant phytochemical active components. The highest BWG and lowest FCR of chicks that received ELP indicate that *Isa brown* laying hens in the starter phase have an enhanced performance with ELP. This corroborates with the findings of Mashayekhi et al. (2018), who indicated an improvement in the FCR of broilers supplemented with ELP by 0.50%. The improvement in growth performance in healthy chicks can be explained by the fact that the *Eucalyptus globulus* has a high ability to secrete digestive and pancreatic enzymes (Hashemipour et al., 2013). In addition, flavonoids have antimicrobial activity (Boukhalfoun, 2012) due to their

detrimental effect on the growth of harmful bacteria in the digestive tract (Gabriel et al., 2013), antioxidant activity, and the ability to scavenge free radicals (Ghedira, 2005). The role of appetite stimulators and the antimicrobial effect of ELP also explain the improvement of growth performance in the chicks treated during the study because bacteria compete with the chickens on the utilization of the feed (Windisch et al., 2008). The mean BWG obtained (7.72 g/day/chick) in this study is lower than the value of 9.34 g/day/chick found by Tossou et al. (2019). Indeed, the differences can be explained by some factors, including the age of the animals and the ingredients of diet (Halbouche et al., 2018; Khaber and Guermah, 2018). The average FCR of the different groups (4.31 to 4.83) at 8 weeks is higher than the result found by Ayodele et al. (2021); for which the FCR of *Isa brown* laying hens during the starter phase was 3.21 to 56 days of age.

The improvement in growth performance of the chick that received the *EG* leaves was not significantly different from the control groups. In the same line, Sedaghat and Karimi Torshizi (2017) found that supplementation of *EG* leaves in the quail diet did not significantly affect FI, BWG, and FCR. The studies of Kaur et al. (2022) revealed no significant differences between FI and FCR of laying hens who received *Eucalyptus* powder at different rates of 0.30%, 0.45%, and 0.60%. The same study showed that the lowest FCR was obtained in chicks that received 0.30% *Eucalyptus globulus* powder. Contrary to the result of these reports, the lowest FCR in chicks that received 0.50% ELP was obtained. This difference could be related to the fact that this study was done for the starter phase.

The mortality rate between the different treatments varied from 2 to 3%, with no significant difference between treatments over the starter phase of laying hens ($p > 0.05$). This value is considered acceptable in comparison with the rate of 5-8% indicated in hot countries (Ouedraogo et al., 2015).

The internal organs have the same weights, unlike the liver, which had a low weight for the treatment of 0.50% ELP. *Eucalyptus globulus* leaves would have improved macromolecule metabolism, which is reflected in the weight of organs that are similar to positive control treatments (Picard et al., 1999). According to Klasing (1998), the weight of the gizzard is more developed in poultry with more effort in grinding feed. In fact, during the experiment, chicks that received a high rate of ELP would have additional grinding efforts. The result is in agreement with the report of Kouadio et al. (2020) whose increase in the rate of supplementation of cassava peeling meal in broiler rations increased the gizzard muscle mass.

For hematological parameters, only the white blood cell concentrations of chicks who received the ELP decreased significantly, unlike the other parameters, which did not vary according to the rate of supplementation of *Eucalyptus globulus* leaf powder. The present findings can reflect the absence of bacterial infection in chicks during the study. The decrease in leukocytes during the experimentation would be due to the improvement of the effect of *Lactobacillus* on the infection by inhibiting the growth of harmful bacteria. *Lactobacillus* uses flavoproteins for terminal oxidation and forms hydrogen peroxide, which is a harmful bacterial inhibitor compound in the poultry digestive tract (Azieze et al., 2004). Supplementation of *Eucalyptus globulus* leaves did not affect the biochemical parameters of chicks, including total protein, albumin, triglycerides, total cholesterol, blood glucose, alanine aminotransferase, and aspartate aminotransferase , which did not vary with treatment. The values obtained for biochemical parameters are included in the ranges of the residual values for chicks published by Benlatreche and Lakehal (2013).

CONCLUSION

The present study showed that *Eucalyptus globulus* leaves and the antibiotic had the same effect on the growth performance of *Isa brown* laying hens during the starter phase. The results of the study elucidated that 0.25% of *Eucalyptus globulus* leaf powder may be a suitable substitute for antibiotics because its effects on growth performance, vital organs, and hematological and blood parameters were the same or even better than that of the antibiotic. *Eucalyptus globulus* leaf powder can be suggested as a phytobiotic for use in the diet of *Isa brown* laying hens during the starter phase. It is suggested that this study on the grower and the laying phase be pursued.

DECLARATIONS

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Authors' contribution

Aduayi Akue designed the experiment, collected and analyzed the data, and drafted the manuscript. Essodina Talaki, supervised the work and critically revised the manuscript. Lamboni Lare assisted in data collection and revised the manuscript. All authors read and approved the final version of the manuscript for publication in this journal.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article; supplementary information can be accessed at the reasonable request of the corresponding author.

Ethical consideration

This manuscript does not contain plagiarized sentences and has not been published or accepted for publication elsewhere or under editorial review elsewhere. The data are not fabricated or falsified; hence, the Regional Center of Excellence for Avian Sciences, University of Lomé representative is aware of this submission.

Competing interests

There is no conflict of interest with the authors in this study.

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Effects of Microorganism Supplementation on Egg Quality and Production

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ABSTRACT

Effective microorganism (EM) is a combination of more than 80 different types of beneficial microorganisms contributing to a wide range of applications in medicine, environment, and agriculture (livestock sector, crop sector, and forestry). This experiment was conducted to evaluate the effects of EM supplementation on hen day egg production and egg quality traits of Bovans Brown laying hens. At the age of 16 weeks, 144 pullets were purchased from small-scale poultry farms in Debre Markos. The chickens were divided into four treatments, each of which was replicated three times and contained 12 chickens. The treatment groups were T1 (control, commercial ration only), T2 (supplemented 16 ml EM per liter of drinking water), T3 (supplemented 5% Bokashi in feed), and T4 (supplemented 16 ml EM per liter of drinking water and 5% Bokashi in feed). Prior to collecting the actual data, the layer chickens had 2 weeks of adaptation. The hen day egg production was evaluated, and laboratory analysis was conducted to detect the external and internal egg quality. The results showed no significant difference among treatments on hen day egg productions, which ranged from 74 to 80 percent. Among the external egg quality traits, T4 had the highest egg weight, compared to other treatment groups. The T3 and T4 treatments had the highest shell weight. The shell thickness ranged from 0.37 to 0.39 mm. The shape index ranged from 76.81 to 79.11%, with no difference among the groups. Moreover, T4 had a significantly higher egg mass than T1 and T2. The specific gravity of an egg ranged from 1.06 to 1.08 g/cm^3. Among the internal egg quality traits, the albumin weights of T3 and T4 were significantly higher than those of T1. The highest and the lowest Hough units were observed in T4 and T1, respectively. The highest yolk weight was observed in T4 among the groups. The yolk index ranged from 0.45 to 0.49. The yolk color ranged from 5.27 to 7.33. Finally, overall egg quality parameters in T4 were better than in non-supplemented groups.

Keywords: Bovans Brown laying hen, Effective microorganism, Egg quality traits, Hen day egg production

INTRODUCTION

Ethiopia currently has an estimated 57 million chickens, the majority of which are laying hens (34.26%), followed by chicks (32.86%). According to breed, 78.85%, 12.02%, and 9.11% of the total poultry were reported to be native, hybrid, and exotic, respectively (CSA, 2020; 2021). Due to various limitations, poultry has a very small economic impact in Ethiopia. The cost and accessibility of ingredients for feed are the main limitations (Yitbarek, 2013). The low immune capacities of chickens and poor management techniques are responsible for the decreased performance of the bird as a whole (Arif et al., 2021). The production performance of native and exotic chicks is improved by the use of effective microorganisms (EMs), better feeding practices, and better health care (Atsbeha and Hailu, 2021).

The EM is a mixed cell culture made up of photosynthetic bacteria, actinomyces, yeast, *Lactobacillus*, and fungi (EMRO, 2010). The microorganisms used in EM are not genetically modified; rather, they are a combination of more than 80 different kinds of beneficial microorganisms that contribute to the broad range of applications. Pathogens cannot survive in EM because it is self-sterilizing, and the pH ranges from 3.4 to 3.7 (EMROSA, 2006).

In order to feed poultry without endangering the environment or the general public's health, EM is crucial

(Ferri et al., 2017). In a previous study, it was found that including 2% EM in a layer's diet increased egg production, reduced feed consumption, improved feed conversion ratio, increased egg specific gravity, and increased Haugh Units (Atsbeha and Hailu, 2021). The use of EM supplementation has the potential to enhance the performance of layers; however, there is limited research on its application for laying hens in Ethiopia. Moreover, EM is readily available in significant quantities at a low cost in various regions of the country. Therefore, the purpose of this study was to evaluate the effects of EM supplementation on hen day egg production and egg quality traits of Bovans Brown laying hens.

MATERIALS AND METHODS

Ethical approval

The study was carried out in accordance with the "Act of the protection of animals used for scientific purposes [and feed legislation if appropriate] of the Federal Democratic Republic of Ethiopian, which compiles with EU legislation for scientific purposes [and feed legislation if appropriate]". The university's ethical review board gave its approval to all procedures.

Study area

This study was conducted in Debre-Markos, East Gojjam Zone, Amharra Region, Ethiopia. The geographical coordinates of the town are 10°21' latitude North and 37°43' longitude East. The town is about 16000 hectares in size and is 2420 meters above sea level. The mean annual rainfall and temperature is 1308 mm and 16°C, respectively.

Ration and experimental supplementations

In this experiment, the commercial ration was supplemented with 16 ml of activated EM solution (lactic acid bacteria, yeasts, photosynthetic bacteria, actinomyces, enzymatically active fungi) added to a liter of drinking water, Additionally, 5% Bokashi was incorporated into the feed. The stock EM was purchased from Woljeejii Agricultural Industry PLC (WAI), Debre Zeit, Ethiopia. The stocked EM contained lactic acid bacteria (*Lactobacillus* and *Pedicoccus*) at 1 x 10^5 CFU/ml suspension, yeast (*Sacharomyces*) at 2 x 10^6 CFU/ml suspension, photosynthetic bacteria (*Rhodopseudomonas palustrus* and *Rhodobacter spaeroides*) at 1 x 10^3 CFU/ml suspension, actinomycetes (*Streptomyces albus*) at 3 x 10^3 CFU/ml suspension, fermenting fungi (*Aspergillus oryzae*) at 1.1 x 10^5 CFU/ml suspension. To prepare 10 kg of feed mash, 100 ml of EM solution, 100 ml of molasses, and 1000 ml of water were necessary (APNAN, 1995). The steps for making Bakashi were by dissolving the recommended amount of molasses in the recommended amount of water to make molasses solution, adding the recommended EM to the molasses solution, and then spraying the EM and molasses solution mixture onto the recommended amount of feed while thoroughly mixing it. The mixed feed should then be sealed in an airtight polyethylene bag and left for 8 days to ferment and develop a sweet smell (Atsbeha and Hailu, 2021). The proximate analysis of the commercial ration contained 90% dry matter, 16.5% crude protein, 9% crude fiber, 5% crude fat, and 2800 kcal ME/kg DM. The ration was formulated to contain 3.75% calcium, 0.7% phosphorus, 0.75% lysine, 0.36% methionine, and 0.25% vitamin premix in addition to the main feed ingredients. The layers were fed this commercial ration throughout the experimental period.

Management of layers, design, and treatments

Before the commencement of the experiment, the experimental pens, waterers, feeders, and laying nests were thoroughly cleaned and disinfected with two teaspoons of magic disinfectant per 4.5 litters of water, and sprayed 0.6 ml Diazinol per liter of water again. A total of 144 Bovans Brown grower chickens at the age of 16 weeks with an average body weight of 1280 g were purchased from small-scale commercial poultry farms in Debre Markos town. The chickens were kept in a deep litter experimental house and managed in the intensive production system. The average temperature and humidity of the house were 20^{O}C and 60%, respectively. The house was partitioned into 12 pens (each pen 2.4 m^2 per 12 chickens) by wire mesh. The pens were covered with a litter of dry teff straw, reaching a depth of approximately 8 cm. In each pen, 12 grower chickens were randomly distributed. The chickens were adapted to experimental supplementations for 2 weeks before recording the actual data. The chicken was vaccinated with the recommended vaccines based on vaccine programs. The chicken was vaccinated Lasota vaccine with drinking water at 17 weeks of age against Newcastle disease. At 40 weeks of age, the layer was vaccinated with the infectious bronchitis virus (IBV) vaccine (V-IBV) with drinking water for infectious bronchitis. All health precautions and disease control measures were carefully followed throughout the experimental period. The fluorescent lamp was suspended on the ceiling of the experimental house to provide 14 hours of light daily for laying hens. The

layers were fed a measured amount of feed in groups twice a day at 6:00 AM and 6:00 PM throughout the experimental period. A measured amount of clean tap water free from chlorine (expose the water for 24 hours to evaporate chlorine) was used for 336 days. The feed was offered on hanging feeders. The water was provided in plastic fountains, and the watering troughs were cleaned every morning.

A total of 144 layers were randomly distributed in 12 pens using a completely randomized design in four treatment groups and three replicates for each treatment. The treatment groups were control (commercial ration only, T1), supplemented 16 ml EM per liter of drinking water (T2), supplemented 5% Bokashi in feed (T3), and Supplemented 16 ml EM per liter of drinking water and 5% Bokashi in feed (T4).

Measurements

Feed intake

The feed offered and rejected for each replicate was recorded and multiplied by the respective DM content. The difference between the offered and rejected feed served as the basis for calculating the amount of feed consumed.

$$\text{Feed intake per layer} = \frac{\text{Feed offered- feed refusal}}{\text{Duration of experiment} \times \text{Number of layers}}$$

Body weight

In order to track changes in body weight, each layer's weight was recorded individually at the start of the experiment (initial body weight) and every two weeks till the end of the study period. By deducting the initial weight from the final weight and dividing it by the number of experimental days and layers. An average body weight gain was calculated for each replicate.

Egg production

At 9:00 AM, 11:00 AM, and 3:00 PM, eggs were recorded from each pen three times daily. The quantity of eggs laid that day was the sum of the three recorded numbers. It was also recorded how many birds were alive in each replicate on each day. The average percentage of hen-day egg production was used to represent the rate of lay for each replicate. According to Hunton (1995), hen-day egg production was calculated by multiplying the average daily egg production by 100 and dividing that result by the typical daily number of birds alive.

$$\text{HDEP} = \frac{\text{Total number of eggs produced on a day}}{\text{Total number of hens present on that day}} \times 100$$

Egg quality parameter measurements

For each replicate, parameters relating to egg quality were measured. Measurements were made of egg quality traits such as egg weight, shell weight, shell thickness, yolk height, yolk weight, yolk color, yolk index, yolk diameter, albumen height, albumen weight, and Haugh Unit Score (HUS). An electronic Digital Caliper (Mitutoyo, Japan) was used to measure the egg's length and width as well as its weight, which was measured in grams by digital balance.

The weight of the egg shell and its membrane were measured after the eggs were broken, and the egg shell thickness was then determined after the membrane had been taken off. Using a micrometer gauge, the egg's shell thickness was measured on three of its sides: the large end (the top or pointed part), the narrow end (the bottom or round part), and the middle portion. The thickness of the egg shell was determined by averaging the three sites.

The albumen was separated from the yolk, its height was measured using a tripod micrometer called a Spherometer, and its weight was determined using a sensitive balance. Egg weight and thickness of thick albumen were correlated using the Haugh Unit measurement technique. Following is the formula used to calculate the Haugh Unit (Haugh, 1937).

$$HU = 100 \log (AH - 1.7\, EW^{0.37} + 7.6)$$

Where, HU is Haugh Unit, AH signifies Albumen Height in millimeters, EW denotes Egg weight in grams.

The yolk's diameter and height were measured using a ruler and a tripod micrometer after it had been separated from the albumen. Sensitive balance was used to measure the yolk's weight. Using this formula, the yolk index was calculated.

$$\text{Yolk index} = \text{Yolk height} / \text{Yolk diameter}$$

The yolk color was assessed using a Roche fan, equipped with 1-15 strips representing a spectrum of hue from pale to orange yellow. The yolk membrane (vitelline membrane) was first removed, followed by a thorough mixing of the entire yolk, and then a yolk sample was taken on a piece of white paper and compared with Roche fan measurement strips (Vuilleumier, 1969). The shape index (percent) was calculated as 100 times egg width divided by egg length. Albumen percentage was determined by 100 times the albumen ratio to egg weight. Yolk percent was calculated as 100-time yolk weight divided by egg weight. Shell weight was calculated by egg weight minus (Albumen weight plus yolk weight). Shell percentage was determined by 100 times Shell weight divided by egg weight. Egg specific gravity (gm/cm3) was

determined by egg weight (gm)/egg volume (cm3), after determining egg volume. Egg volume (cm3) was obtained by 0.524 LB^2.

Where, 0.524 is a constant value, L stands for egg length (cm), B determines egg breadth (cm).

Statistical analysis

The Statistical Analysis Systems Software (SAS, 2008) Version 9.2 was employed to perform analysis of variance (ANOVA) on the collected data using the general linear model (GLM) procedure. Tukey HSD test was performed for mean separation when treatment effects were significant ($p < 0.05$).

RESULTS

Performance of Bovans Brown laying hen supplemented with EM in drinking water and Bokashi in feed is indicated in Table 1. A group supplemented in T4 and T1 had the highest and the lowest feed intake, respectively. The initial body weight, final body weight, the total body weight gains and the hen day egg production of laying hens did not significantly differ among the treatment groups ($p > 0.05$). The hen day egg production across different age groups on a weekly basis is presented in Graph 1.

There were no significant differences in the percent hen day egg production among all age groups ($p > 0.05$). At weeks 20-23 of age, the percent hen day egg production was very low. In the age of week 24-27, the percent hen day production indicated an increase. The maximum hen day egg production was registered in weeks 28-31, 32-35, 36-39, and 40-43. The percent hen day egg production revealed a diminishing order at 44-47 weeks of age and beyond this until the end of the experimental period.

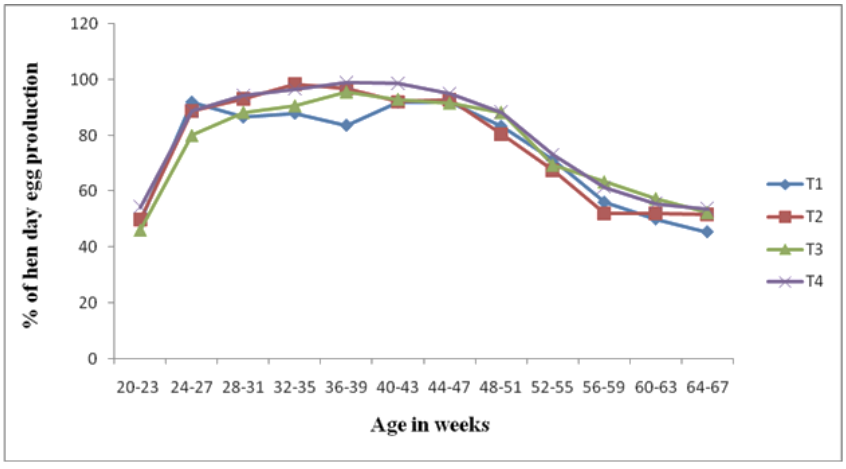
External and internal egg quality traits

The characteristics of the internal and external egg quality are shown in Table 2. The lowest egg weight among the external egg quality traits was observed in T1, compared to other treatment groups ($p < 0.05$). The T3 and T4 recorded the highest shell weights. However, there was no significant difference in the treatments for shell thickness, shape index, egg volume, or egg specific gravity ($p > 0.05$). The egg mass of T4 was significantly higher than T1 and T2 ($p < 0.05$). The albumin weight of T3 and T4 was significantly higher than T1 under the internal egg quality traits ($p < 0.05$). The highest ($p < 0.05$) and the lowest ($p < 0.05$) Hough Unit were seen in T4 and T1, respectively. Compared to the other treatment groups, T4 had the significantly highest yolk weight ($p < 0.05$). The treatment group of T1 had the significantly least amount of yolk color, compared to the other groups ($p < 0.05$).

Table 1. Performance of Bovans Brown laying hen supplemented with effective microorganisms in drinking water and Bokashi in feed from 20-67 weeks of age

Parameters	T1	T2	T3	T4	SEM	p-value
Average feed intake (g)	119.67^{c}	122.36^{b}	122.37^{b}	124.07^{a}	0.167	<0.001
Initial body weight (g)	1547.10	1545.53	1597.27	1592.63	13.265	0.395
Final body weight (g)	1775.80	1875.80	1909.47	1901.57	31.434	0.457
Total body weight gain(g)	228.70	330.30	312.20	308.97	35.030	0.746
Hen day egg production (%)	74.00	76.33	76.33	80.00	2.819	0.276

^{abc}Means with a different superscript in a row are significantly different ($p < 0.05$); T1: Control, T2: 16 ml EM/L of drinking water, T3: 5% Bokashi per quintal of feed, T4: 16 ml EM/L of drinking water + 5% Bokashi per quintal of feed, SEM: Standard error of the mean



Graph 1. The hen day egg production of Bovans Brown laying hens on a weekly basis. T1: Control, T2: 16 ml EM/L of drinking water, T3: 5% Bokashi per quintal of feed, T4: 16 ml EM/L of drinking water + 5% Bokashi per quintal of feed.

Table 2. External and internal egg quality traits of Bovans Brown laying hens supplemented with effective microorganisms from week 20 to week 67

Parameters	T1	T2	T3	T4	SEM	p-value
External egg quality traits						
Egg weight (g)	57.63^{b}	59.19^{ab}	61.44^{a}	62.47^{a}	1.356	0.003
Egg Breadth (mm)	43.33	43.13	43.87	44.42	0.513	0.067
Egg length (mm)	54.97^{b}	56.17^{a}	56.78^{a}	57.72^{a}	0.927	0.034
Shell weight (g)	5.25^{b}	5.39^{ab}	5.59^{a}	5.61^{a}	0.123	0.014
Shell thickness (mm)	0.38	0.37	0.39	0.39	0.016	0.527
Shell (%)	9.1	9.1	9.1	9.0	0.284	0.251
Shape index (%)	79.11	76.81	77.25	77.71	1.539	0.476
Egg volume (cm^3)	54.19	54.77	57.25	55.93	1.484	0.184
Egg specific gravity (g/cm^3)	1.07	1.08	1.07	1.06	0.019	0.793
Egg mass (g)	42.67^{b}	45.00^{b}	46.67^{ab}	50.00^{a}	1.509	0.008
Internal egg quality traits						
Albumen weight (g)	34.87^{b}	35.93^{ab}	37.35^{a}	38.04^{a}	0.825	0.001
Albumen height (mm)	6.49^{b}	6.98^{ab}	7.27^{a}	8.03^{a}	0.271	<0.001
Albumen length (mm)	60.66^{a}	60.13^{b}	63.05^{a}	63.43^{a}	1.275	0.024
Hough Unit	80.82^{b}	83.58^{b}	84.71^{b}	89.76^{a}	1.805	<0.001
Albumen (%)	60.5	60.7	60.8	60.9	1.016	0.253
Yolk weight (g)	17.51^{b}	17.87^{b}	18.51^{b}	18.81^{a}	0.413	0.011
Yolk height (mm)	18.13^{b}	19.00^{ab}	18.27^{ab}	19.13^{a}	0.398	0.026
Yolk length (mm)	40.04	39.37	40.81	41.16	0.626	0.027
Yolk color	5.27^{c}	7.33^{a}	6.80^{b}	6.27^{ab}	0.216	< 0.001
Yolk Index	0.46	0.49	0.45	0.47	0.017	0.217
Yolk (%)	30.4	30.2	30.1	30.1	0.604	0.241

^{abc}Means with a different superscript in a row are significantly different ($p < 0.05$); T1: Control, T2: 16 ml EM/L of drinking water, T3: 5% Bokashi per quintal of feed, T4: 16 ml EM/L of drinking water + 5% Bokashi per quintal of feed, SEM: Standard error of the mean

DISCUSSION

Egg production

At the age of week 20, both treatment groups in this study laid their first eggs. The first egg is laid by chickens when they are 21 weeks old (Anene, 2020). According to Abera et al. (2021), Brown hens start laying their first eggs between 18 and 20 weeks of age. In contrast to previous studies where the age of the first egg laying hen in control and EM-supplemented groups ranged between 179 and 186 days (Simeamlak et al., 2013), the current study yielded different results. The way chickens were treated during the experimental period might have made a difference. According to Atsbeha and Hailu (2021), chickens provided with EM in water and feed began laying eggs at 161 days, those given EM in water started at 166 days, control group chickens initiated laying at 168 days, and chickens receiving EM in feed commenced laying at 175 days. This suggests that EM supplementation positively influenced the age at first lay by promoting early maturity. The observed improvement in age at first lay may be attributed to the accelerated growth of chickens facilitated by the presence of beneficial microbes in the gut. This, in turn, enhances the rapid absorption of essential nutrients (Gnanadesigan et al., 2014). Both the health and the microscopic structure of the ileum and ceca were improved by dietary probiotic supplementation (Xiang et al., 2019).

Between 241 and 268 eggs were produced overall in this study during the experimental period (20-67 weeks). It was revealed that commercial egg type layers began laying eggs at the age of 20-21 weeks and produced 277 eggs till 72 weeks of their production cycle (Petek, 1999). Similarly, North (1984) reported that during the 52-week laying period, 266 eggs were produced. The average number of eggs laid per 100 hens per day in 2020 was 81, as reported by the USA (2020). Better nutrition, genetics, health and disease prevention, and flock management all contributed to the hens' higher productivity.

In the present study, the hen day egg production of the birds ranged from 74% to 80%. documented improved results in hen day egg production for layers treated with EM, reporting 88.28 %, 84.28 %, and 83.20 %, in three consecutive months of egg production, respectively. AL-Nasser et al. (2020) noted that the overall average hen-day egg production for brown hens was 85.6%, which was

higher than that of white hens (83.2%). The percentage of hen day egg production increased significantly up to 42 weeks of age and then gradually decreased until the end of the laying period (Sibanda et al.,2020). The findings of the present study contrast with those reported by Tanaka (1993), who documented a hen day egg production of 72.5%, and North (1984), who reported a rate of 73%.

External egg quality traits

Egg weight, egg breadth and egg length

At 72 weeks of age, the mean egg weight of a Bovans Brown laying hen is 63.2 g. At the end of the experimental period in the current study, the mean egg weight of Bovans Brown layers was 57.63-62.47 g. In all age groups, the eggs weighed better when EM was added to the drinking water and Bokashi to the feed. This result coincided with Gnanadesigan et al. (2014), reporting the maximum egg weight within the range of 61.6 g when layers were fed with activated EM (5 L/ton) treated commercial feed masses and activated EM treated (2 L/1000 L) drinking water. In the first 21-28 weeks of the egg-laying period, Atsbeha and Hailu (2021) reported an average egg weight of 59.1 g in chickens given EM in water, 58.3 g in chickens given EM in feed, 60.9 g in chickens given EM in water and feed, and 57.2 g in chickens given control feed. They also noted that during the peak egg-laying period (29-39 weeks), egg weight varied according to treatments. It was found that a group of EM-treated layers achieved a maximum egg weight of 61.6 g when provided with both EM-treated feed and drinking water (Gnanadesigan et al., 2014). In contrast, Ahan et al. (2020) found different results, noting that supplemented Brown laying birds produced eggs weighing 60.72 g, which were lighter than the control group's eggs at 61.34 g. It was found that the average weight of the eggs produced by the EM-treated chickens was comparable to that of the control groups (El-Deep et al., 2011). The variations in egg weight could be brought about by variations in breed, feed composition, environment, and other management factors.

The egg breadth of Bovans brown layer chicken in this experiment ranged from 43.13 to 44.12 mm. However, Gnanadesigan et al. (2014) reported that the egg breadth of EM-supplemented layers ranged from 45.79 to 46.10 mm. In the EM-supplemented groups, the egg width was 44 mm (Kinati et al., 2021). For chickens raised in deep litter, legume pasture, and grass pasture systems, the Isa Brown chicken egg width was 40.07 mm, 40.05 mm, and 40.13 mm, respectively (Oke et al., 2014). The findings of Abanikannda et al. (2007) revealed that there was a strong correlation between egg weight and egg width (r = 0.84). The highly significant positive correlation indicated that the egg width increases with increasing egg weight.

The Bovans Brown layer chicken used in this experiment had egg length that ranged from 54.97 mm to 57.72 mm. In comparison to the control group, layers supplemented with EM in drinking water and Bokesh were higher. In the same line, Abanikannda et al. (2007) found that the egg length varied from 53.86 to 57.43mm. In the study by Gnanadesigan et al. (2014), it was reported that the range of the mean egg length in the EM-supplemented groups was 54.1 to 55.8 mm. According to Ahan et al. (2020), the control group and EM-supplemented layers both had eggs that were 55.22 mm long. Abanikannda et al. (2007) reported a strong correlation between egg weight and length (r = 0.78). They also reported that as the age of the layer increased, there was an increase in egg weight, egg width, and egg length.

Shell weight, shell percentage, and shell thickness

In this experiment, the shell weights of the eggs ranged from 5.25 to 5.61 g. When layers received EM in drinking water, Bokashi in feed, and both of these supplements, there was a significant difference in shell weight ($p < 0.05$). Similar result was reported by Atsbeha and Hailu (2021). The shell weight of eggs supplemented with EM ranged from 5.57 to 6.18 g. However, Ahan et al. (2020) noted that there was no significant difference in the shell weight between the control group (6.96 g) and the EM-supplemented group (6.94 g). In addition, Congjiao et al. (2019) reported the shell weight of the egg in EM supplemented as 5.63 g, and Şahan et al. (2020) noted that the shell weight ranged from 5.8 to 6.2 g. According to John-Jaja et al. (2016), the weight of the egg shell increases steadily as the hen's age increases. Supporting this, Minelli et al. (2007) reported egg shell weights of 6.00 g at 28-32 weeks, 6.16 g at 47-50 weeks, and 6.29 g at 70-73 weeks. Rath et al. (2015) reported an egg shell weight of 6.00 g at 50 weeks, while Tumova et al. (2011) observed a range of 6.91-7.81 g in the New Black breed from 28 to 60 weeks of age, and 6.50-6.91 g for litter-raised Hisex Brown at 60 weeks. Ewa et al. (2005) obtained lower values of 5.05 g for the Black Olympia breed and 5.34 g for the H and N Brown Nick breed at 36 to 46 weeks of age. Additionally, Begli et al. (2010) reported an egg shell weight of 4.45 g for Iranian fowl at 30 weeks of age. Sreenivas et al. (2013) measured egg shell weights ranging from 4.32-5.12 g for four genetic groups in the White Leghorn breed at 40 weeks. According to John-Jaja et al. (2016), the average values of

egg quality traits indicate an increase in egg weight from 55 to 63 g, and egg shell weight increased from 6 to 7 g. The observed variations in these measurements may be attributed to breed differences, the age of the layers, environmental temperature, and management practices, especially the type of feed used (FAO, 1998). In the present experiment, the shell percentage fell within the range of 9.1%. Gupta (2008) suggested that the average egg shell weighs 5-6 g, constituting 10-11% of the total egg weight. John-Jaja et al. (2016) reported egg shell percentages of 8.39% at 25 weeks, 10.05% at 51 weeks, and 10.18% at 72 weeks of the hen. Other studies by Begli et al. (2010), Mube et al. (2014), and Zhang et al. (2010) reported egg shell percentages of 10.6%, 12.1%, and 10.90%, respectively. According to Mohammed and Eva (2018), the egg shell percentage ranged from 10.07 to 12.21%.

In the current study, the egg shell thickness ranged from 0.37 to 0.39 mm, a finding consistent with Atsbeha and Hailu (2021), who reported egg shell thickness of 0.35 to 0.40 mm for eggs supplemented with EM. On average, egg shell thickness is approximately 0.30 mm (Yalcinalp, 2018), while Ketta and Tůmová (2018) reported a range of 0.28 to 0.41 mm. Gnanadesigan et al. (2014) reported higher shell thicknesses ranging from 1.39 to 1.44 mm. Factors influencing shell thickness include the duration of the egg's stay in the uterus/shell gland and the rate of calcium deposition during shell formation. A shorter stay in the shell gland may result in less thickness. The time of day when the egg is laid also plays a role, with thicker shells occurring earlier in the day or during the light part of the photoperiod (Gupta, 2008). An egg shell, with a thickness of 0.03 millimeters, is composed of 7,000 to 17,000 tiny pores, ensuring strength while allowing the passage of oxygen, carbon dioxide, and moisture (Biggs, 2017). Larger pores and thinner shells diminish the protective capacity of eggs. The functional quality of the egg shell is influenced by various pre-laying factors, including strain, disease, management practices, moulting, age of the bird, medications, stress, environmental temperature, and nutrition (Gupta, 2008).

Shape index, egg mass, egg volume, and egg specific gravity

An important factor in assessing egg quality is the egg shape index, which is defined as the proportion of width to length of the egg (Narushin and Romanov, 2002). The shape index of the egg ranged from 76.81 to 79.11% in the current study. Similarly, Şahan et al. (2020) reported that the shape index of the control (80.63) and EM supplemented (80.09) group did not show any significant difference. According to Rath et al. (2015), the shape index of an egg was 73.53%. The shape index classifies eggs as sharp (<72), normal (standard, 72-76), and round >76 (Sarica and Erensayin, 2004). Normal chicken eggs have an oval shape. The hen eggs with unusual shapes, such as long and narrow, round, or flat-sided, cannot be graded AA (almost perfect) or A (slightly worse than AA), as eggs are typically oval in shape (72-76). When eggs are being packaged and transported, trays easily accommodate standard eggs. According to Sarica and Erensayin (2004), round eggs and eggs that are unusually long may not have an appealing appearance and may not fit well in egg cartons, making them more prone to breakage during shipping compared to eggs with a normal shape. According to Anderson et al. (2004), the shape index significantly influences the percentage of breakage strength variation. The risk of cracked eggs is influenced by egg characteristics such as shape index and shell thickness. As a result, the way the egg is handled after it is laid is also crucial, taking shape index and shell thickness into account (Galic et al., 2019).

Egg mass refers to the correlation between egg weight and production (Singh, 2000). In the present study, the egg mass of the treatments ranged from 42.67 g to 50 g, with the EM-supplemented groups in drinking water and feed exhibiting the highest values. Desirable egg masses are considered to be 50 g and higher. To attain the desired output for the farm, it is crucial to supplement EM in drinking water and include Bokashi for Bovans Brown laying hens (Kocevski et al., 2015).

Two crucial geometrical calculations, namely egg volume (V) and surface area (S), are required to predict chick weight, egg hatchability, shell quality traits, and egg interior parameters. These calculations are used in the poultry industry and biological studies. The volume of an egg can be assimilated into an ellipsoid (Stelzer, 2001). The volume of the eggs in this experiment ranged from 54.19 to 57.25 cm^3. The findings of the current study align with Narushin (2005), who reported minimum, maximum, and average chicken egg mass of 52 cm^3, 70.4 cm^3, and 60.19 cm^3, respectively. The current result is less than the egg volume reported as 63.0 cm^3 for a standard chicken egg (Zeidler, 2002).

By weighing an egg and dividing its weight by its volume, the specific gravity of an egg can be calculated (Iqbal et al., 2017). Egg shell thickness has a significant impact on specific gravity. The prevalence of cracks typically rises as specific gravity decreases. With increasing hen age, egg specific gravity typically

decreases (Roberts, 2004). There was no significant difference between the treatment groups in the current study despite the specific gravity of an egg ranging from 1.06 to 1.08 g/cm3 at 67 weeks of age in the treatment groups ($p > 0.05$). In most cases, 85-90% of the eggs fall into the 1.075, 1.080, and 1.085 categories (Gualhanone et al., 2012). According to Krist (2011), the specific gravity of sharp, standard, and round eggs was 1.088, 1.087, and 1.086, respectively. The specific gravity of the best-quality eggs is greater than 1.08 g/cm3, while normal eggs fall between 1.06 and 1.10 g/cm3 (Inoti, 2020). In a study comparing deep litter, legume pasture, and grass pasture production systems, the specific gravity of eggs was reported as 1.12, 1.21, and 1.12 g/ cm3, respectively, at 24 weeks old, 1.16, 1.13, and 1.10 g cm-3, respectively at 38 weeks old and 1.17, 1.12, and 1.25 g/ cm3, respectively at 60 weeks old (Oke et al., 2014). Sarica et al. (2012) noted that egg shape index and specific gravity had a positive non-significant correlation. Ozelik (2002) found a non-significant tendency to a negative correlation between egg shape index and specific gravity.

Internal egg quality characteristics

Albumen weight and albumen height

The egg white, or albumen, makes up two-thirds of an egg's weight (Sugino et al., 2018). In this study, the egg's albumen weight ranged from 34.87 to 38.04 g. Compared to the control group, layers that received EM in water and/or bokashi supplements in feed had higher albumin weights. The current result is better than the report of Gnanadesigan et al. (2014), indicating that the albumen weight of layers fed with standard commercial food masses with Activated Effective Microorganisms (AEM) solution (5 L/ton), and the layers fed with AEM (5 L/ton) treated commercial feed masses and AEM treated (2 L/1000 L) drinking water were 35.08 and 35.03 g, respectively. The study by Ahan et al. (2020) reported that the egg supplemented with EM had an albumen weight of 40.01g, which was higher than the results of the present investigation. In another study, layers supplemented with EM in drinking water, feed, and both drinking water and feed had albumen weights of 37.58 g, 37.97 g, and 38.53 g, respectively (Atsbeha and Hailu, 2021). In a different production system comparison, the albumen weight of eggs in deep litter, legume pasture, and grass pasture production systems were reported as 33.19 g, 32.20 g, and 33.98 g, respectively, at 24 weeks old; 36.81 g, 37.62 g, and 39.74 g, respectively, at 38 weeks old; and 39.36 g, 40.88 g, and 38.80 g, respectively, at 60 weeks old (Oke et al., 2014). According to Dottavio et al. (2005), the albumen weight of the egg for Fayoumi, White Leghorn, and Rhode Island Red layer chickens was 30.08, 35.07, and 40.01 g, respectively.

Albumen height indicates the sign of freshness of the egg. When all eggs have the same age, they can have the same number of albumen heights (Silversides and Budgell, 2004). Internal egg quality is determined by measuring albumen height and Haugh units (Roberts, 2004). However, because albumen height is a factor in determining Haugh units (Silversides and Villeneuve, 1994), thick albumen height is considered a more useful tool for gauging eggs' freshness level. The albumen heights in this study ranged from 6 mm to 8 mm on average. Atsbeha and Hailu (2021) found significant differences in albumen height when layer hens were supplemented with EM in drinking water (5.65 mm) and in feed and drinking water (6.15 mm). In contrast, Sahan et al. (2020) reported lower albumen height (9.6 mm) for laying hens with EM added to drinking water compared to the control group (10.55 mm). Kinati et al. (2021) found no statistically significant difference in albumen height between the EM-supplemented group (7.28 mm) and the control group (6.49 mm). Rath et al. (2015) reported an albumen height of 8.41 mm. The albumen height in this study falls within the acceptable range for superior quality, as mentioned by Zeidler (2002). The higher albumen height may be attributed to the freshness of eggs and the young age of the hens.

Albumen length, albumen percentage, and Hough unit

In this study, the albumen length for treatment groups ranged from 60.66 to 63.45 mm, with the control group having the lowest albumen length compared to other supplemented groups. This finding contrasts with the results reported by Sahan et al. (2020), where albumen length did not significantly differ between the control group (77.77 mm) and the treatment group (78.84 mm) receiving drinking water with a dose of 1000 ml EM/ton water. Rath et al. (2015) reported a higher albumen length of 92.37 mm. The albumen percentage of the egg in this study ranged from 60.5% to 60.9%. Sahan et al. (2020) reported no significant difference between the control group (65.87%) and the EM-supplemented group (66.09%) in terms of albumen percentage. Sapkota et al. (2020) reported that the average albumen percentage of Sakini chicken was 59.84%. Hanusova et al. (2015) reported lower albumen percentages for Oravka and Rhode Island Red chicken, at 57.26% and 56.74%, respectively. For Isa Brown laying hens, the albumen

percentage ranged from 64.14% to 65.93% (Koja Abbas et al., 2020).

Simeamelak et al. (2013) noted that using 4 to 12 ml of EM/liter of drinking water for laying hens had a significant improvement in egg quality (Haugh unit, yolk, and albumen height). Gnanadesigan et al. (2014) reported Haugh unit values for EM-supplemented groups ranging from 92.86 to 92.91, which were higher than the values observed in the current study. Atsbeha and Hailu (2021) noted that EM-supplemented groups had higher Haugh units in their drinking water (74.03) and feed and drinking water (77.25) compared to the control group (69.43). Koja Abbas et al. (2020) reported a range of Haugh unit values for Isa Brown laying hen eggs from 76.14 to 85.29. Kumar et al. (2014) also noted Haugh unit values of 82.15 for Bovans Brown breed and 83.67 for Rhode Island Red, both comparable to the results in the current study. The higher Haugh unit values in the current study indicate the freshness of the eggs. Assefa et al. (2019) highlighted that albumen height and egg weight have the greatest impact on the Haugh unit. The observed differences in EM supplementation amounts may be related to breed type, environment, diet composition, and other management techniques.

Yolk weight, yolk height, and yolk length

The yolk weight in this study was within the range of 17.51-18.81 g, and Bovans Brown laying hens supplemented with EM in drinking water and feed had the highest egg yolk weight among other treatment groups. According to Atsbeha and Hailu (2021), the yolk weight of the eggs ranged from 15.13 to 16.29 g and did not show any significant difference among the control group and EM-supplemented groups. Gnanadesigan et al. (2014) found that layers fed with commercial food masses treated with AEM (5 L/ton) had egg yolk weights of 15.83 g while layers fed with commercial food masses treated with AEM (5 L/ton) and AEM-treated drinking water (2 L/1000 L) had egg yolk weights of 15.95 g. According to Sahan et al. (2020), the yolk weight of the control group (13.84 g) and the EM-supplemented group (13.77 g) did not differ in any way, which was statistically significant. The higher yolk weight in this study could be attributed to various factors, including the breed of laying hen, the doses of EM supplementation, nutrient composition of the feed, environmental conditions, and other management factors. These variables can influence the overall egg quality, and understanding their interactions is essential for interpreting the study's results accurately.

The yolk height of the EM-supplemented groups with drinking water and in feed (19.13 mm) was higher than the control group (18.13mm). The findings in this report were different from those by Şahan et al. (2020), where the EM-supplemented groups exhibited lower yolk height (18.85 mm) than the control group (19.74 mm). Commercially available brown table eggs were reported to have a yolk height ranging from 20.1 to 24.5 mm (Hisasaga et al., 2020). In the current study, yolk length ranged from 40.04 to 41.16 mm, with no significant difference observed among the treatment groups ($p > 0.05$). Rath et al. (2015) reported a higher yolk length of 45.98 mm, which surpasses the findings in the current study. The discrepancies may arise from various factors, including differences in experimental conditions, breeds, and management practices across studies.

Yolk percentage, yolk index and yolk color

The yolk percentage of the eggs ranged from 30.1% to 30.4% for Bovans Brown egg layers, both in the control and EM-supplemented groups. In contrast, Sahan et al. (2020) observed that the yolk percentage between the control (22.56%) and EM-supplemented groups (22.68%) did not differ significantly. In a study involving Fayoumi, White Leghorn, and Rhode Island Red chickens in their last egg production cycle, the yolk percentages were reported as 33.3%, 30.2%, and 28.6%, respectively (Dottavio et al., 2005). Leeson (2006) stated that the components of a fresh egg are approximately 32% yolk, 58% albumen, and 10% shell. Zaheer (2015) noted that the egg's composition includes approximately 9-12% shell, 60% albumen, and 30%-32% yolk of the total volume. These variations in yolk percentage may be influenced by factors such as breed, nutritional conditions, and other environmental factors. The yolk index, calculated as the ratio of yolk height to yolk diameter, provides an indication of how recently an egg was laid. Eggs are considered extra fresh if their yolk index is higher than 0.38 (Yuceer and Caner, 2014). The yolk index for fresh eggs typically varies between 0.30 and 0.50, with a mean value of 0.42 (Ihekoronye and Ngoddy, 1985). In this study, the yolk index ranged from 0.45 to 0.49, which is higher than 0.38 and considered extra fresh; however, there was no significant difference among the treatment groups. Similarly, Gnanadesigan et al. (2014) reported a yolk index of 0.48 for layers fed with standard commercial ration and activated effective microorganisms (AEM) solution (5 L/ton), while layers fed with AEM-treated commercial ration and AEM-treated drinking water (2 L/1000 L) had a yolk index of 0.52. In contrast, Sahan et al. (2020) reported a higher yolk index for the control group (0.51), compared to the EM-supplemented group (0.48).

A hen's diet impacts the color of the yolk in her eggs, but the yolk color does not indicate the freshness of the egg. Egg yolks come in various colors, from pale yellow to deep orange. Carotenoids, organic pigments in some plants, lead to darker in color eggs (Zia-Ul-Haq, 2021). The yolk color of an egg in the treatment groups ranged from 5.27 to 7.33. The EM-supplemented groups had the highest yolk color than the control group. According to Atsbeha and Hailu (2021), the yolk color ranged from 6.2 to 6.8, which was in between the current study.

CONCLUSION

Layers supplemented with 16 ml EM in drinking water and 5% Bokashi in feed had significantly improved feed intake, hen day egg production percentage, external egg quality traits (egg weight, egg length, shell weight, and egg mass) and internal egg quality traits (albumen weight, albumen height, albumen length, Hough unit, yolk weight, yolk height, and yolk color). Therefore, it can be recommended that small-scale, medium-scale, and large-scall producers can supplement 16 ml EM per liter of drinking water and 5% Bokashi in feed to improve egg production and egg quality without any deleterious effect. Further investigation beyond the recommended level of this study is crucial.

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Authors' contributions

Melkamu Bezabih Yitbarek generated the idea, drafted the research protocol, developed the study design, analyzed the laboratory analysis, collected the data, performed the data analysis, and wrote and revised the manuscript. The author has read and approved the final data and manuscript.

Competing interests

The author declares that there is no financial or interpersonal competing interest.

Ethical considerations

This article has been checked by the author, and ethical issues such as plagiarism, publication consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy were not found.

Availability of data and materials

The prepared data of the present study will be sent by the corresponding author according to the reasonable requests.

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The Impacts of Body Condition, Microclimate, Wind Speed, and Air Pollutant on Physiological Response of Laying Hen Reared under Tropical Climate

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ABSTRACT

The environmental changes in the animal's body status could manifest as a physiological response. The present study investigated the impact of body condition, microclimate, wind speed, and air pollutants on the physiological response of laying hens. Therefore, a total of 172 laying hens at 16 weeks of age from Isa Brown were investigated for 5 days. Data on body condition, microclimate, wind speed, and physiological response were recorded and then analyzed using the SEM model by Partial Least Square- Structural Equation Modeling using smartPLS. The obtained result revealed that 59.71% of the physiological response of the chickens (respiratory rate and rectal temperature) reared at the open house system could be predicted by the independent. The microclimate (γ = 0.465) was found to be more effective than body condition (γ = 0.237), wind speed (γ = -0.364), and air pollutant (γ = 0.08). Moreover, it was found that as much as 83.1% of the air pollutants in the open house system could be predicted by the independent variables, and wind speed (γ = -0.890) was more effective than microclimate (γ = 0.074) variables.

Keywords: Laying hen, Microclimate, Physiological response, Tropical climate

INTRODUCTION

Indonesia is a tropical country with two rainy and dry seasons due to wind direction effects from two continents. Consequently, climate is a crucial aspect and becomes a limiting factor in livestock management. Temperature and humidity in Indonesia are relatively high. In East Java, in the year 2017, the temperature ranged from 20.7°C to 35.9°C, while the humidity ranged from 42% to 99% (Indonesia Statistical Bureau, 2017). This climate does not match the requirements to achieve a better poultry production performance. The comfort zone for chicken ranges between 18°C and 22°C and between 60 and 70% for temperature and humidity, respectively (Diaz et al., 2018; Kim et al., 2021; Mascarenhas et al., 2022). Temperature and humidity are manifested in the temperature and humidity index (THI). The best THI for poultry, especially laying hens, is below 70, (Olivares et al., 2013). Paliadi et al. (2015) revealed that the average THI in two regions at West Java (Kuningan and Cililin) was 86 and 72, respectively. Hence, the Indonesian environment is characterized by high temperature and relative humidity, which are higher than the requirement.

Most Indonesian farmers running businesses on laying hens have relied on a naturally ventilated open house system (Susanti et al., 2022). The microclimate involving temperature, humidity, and airflow in the open house system fluctuates. Thus, the microclimate inside the house relies on a region's macroclimate. On the other hand, chicken house produces harmful gas emissions resulting from raising poultry, considering that rearing of laying hens takes a very long time in one production

period, which is normally 100 weeks, even more in traditional farmers (Reddy et al., 2007; Hedrix Genetics, 2021). Pollutant gas concentrations, such as ammonia, carbon dioxide, and dust particles, are major factors to be concerned about (Al-Kerwi et al., 2022). The magnitude of these components inside the house that exceeds the threshold can disrupt livestock metabolism, reducing the productivity of laying hens (Li et al., 2020).

Laying hens are a commodity that has long been developed in Indonesia with such a climate. Although, climatically, this country has a big obstacle, the population of laying hens in Indonesia is increasing from year to year (Hartono et al., 2021), and Indonesia is the ninth producer of poultry products in the world (Fun and Wu, 2022). The main reason for this issue is that poultry needs certain requirements for better production, but their adaptive behavior allows them to adapt to environmental changes (Gerken et al., 2006). Therefore, this research aimed to study the physiological response of chicken rearing in hot and humid climates with open-house systems to all factors that contribute to their performance.

MATERIALS AND METHODS

Study location

This research was performed at Pojok Village, Wates Sub-district, Kediri Regency, East Java Province, Indonesia, from January to February 2022. The elevation of this region is 77 meters above sea level (MASL), -7.781 latitude and 112.071 longitude, with a rainfall rate of 1860 mm per year and an average daily temperature of 27°C (Indonesia Statistical Bureau, 2017).

Experimental animals and management

One hundred and seventy-two 16-weeks-old pullets were selected randomly from three hundred chickens of the population of chickens in a rearing house as Slovin's equation from litter floor housing (Ryan, 2013). This research was conducted at an open house system in dimensions of 15 m x 4 m x 3.5 m (length x depth x height) with a battery aligned in six rows with 60 chickens per row (three rows face to face/V-shaped liked). The battery was arranged 1.5 m above the floor. A lighting program of 14Light/10Dark was applied. Laying hens have *ad libitum* access to water. Feeding was confinity given as a feeding program prescribed from manual guidance of commercial laying hens for tropical countries (Hendrix Genetics, 2021). The commercial diet was from PT. Cargill Indonesia "Q MAX DEDEVOPER COMPLETE" (16% of crude protein and metabolizable energy of 2700-2970 Kcal/kg). The chickens were vaccinated during the rearing as a protocol from Medion company (Medion, 2018).

Measured parameters

Body weight was collected at the age of 16 weeks prior to the placement in the battery cage. The body weight of the chicken was measured in grams (g) using a digital scale weight. The fleshing score was determined according to Polley (2016) at once during weighing. Respiratory rate was counted first before data collection for rectal temperature. The abdominal region was observed to count respiratory movements within one minute with the aid of a stopwatch. Rectal temperature was obtained by introducing Kruuse digital thermometer Digi-Vet SC 12 (Zimbabwe) into the cloaca of chickens until the stability of reading. House temperature and humidity were recorded with mi-sol DS 102 data logger (China) at five different spots inside the house. Ammonia gas level was measured using ammonia detector AR8500 at five different spots inside the house. Number of Dust particles was measured using Fluke 985 Particle Counter (USA) at 5 different spots inside the house. Wind speed and direction were performed by Kestrel 3000 (USA). Data on microclimate, air pollutants, wind speed, and physiological response were collected simultaneously at four different times (2 am, 8 am, 2 pm, 9 pm) for 5 days.

Statistical analysis

Data analysis on this research was performed using the Partial Least Square-Structural Equation Model (PLS-SEM) using SmartPLS (v.3.3.9). The assessment of model results was made as recommended by Hair et al. (2021) and the significance level was considered at 0.05.

RESULTS AND DISCUSSION

Most studies evaluated the physiological responses of the chicken just based on certain variables. However, the chicken will respond physiologically to any environmental changes such as microclimate (Muharlien et al., 2020), harmful gases (Kristensen and Wathes, 2000), and the body weight of the chicken (Nascimento et al., 2017). The PLS-SEM is a statistical method that makes it possible to analyze observed and unobserved variables effectively in many fields, such as agriculture and livestock (Yalçin et al., 2021). Therefore, this study presented a four-structure with a nine-indicator model to evaluate the physiological responses of the chicken. The summary result of data analysis from SmartPLS is available in Figure 1. The

readout from SmartPLS analysis comprising indicator weights/loadings, composite reliability (CR), average variance extracted (AVE), path coefficients, cross-loading, variance Inflation Factor (VIF), t-value, and p-value are presented in tables 1, 2, and 3.

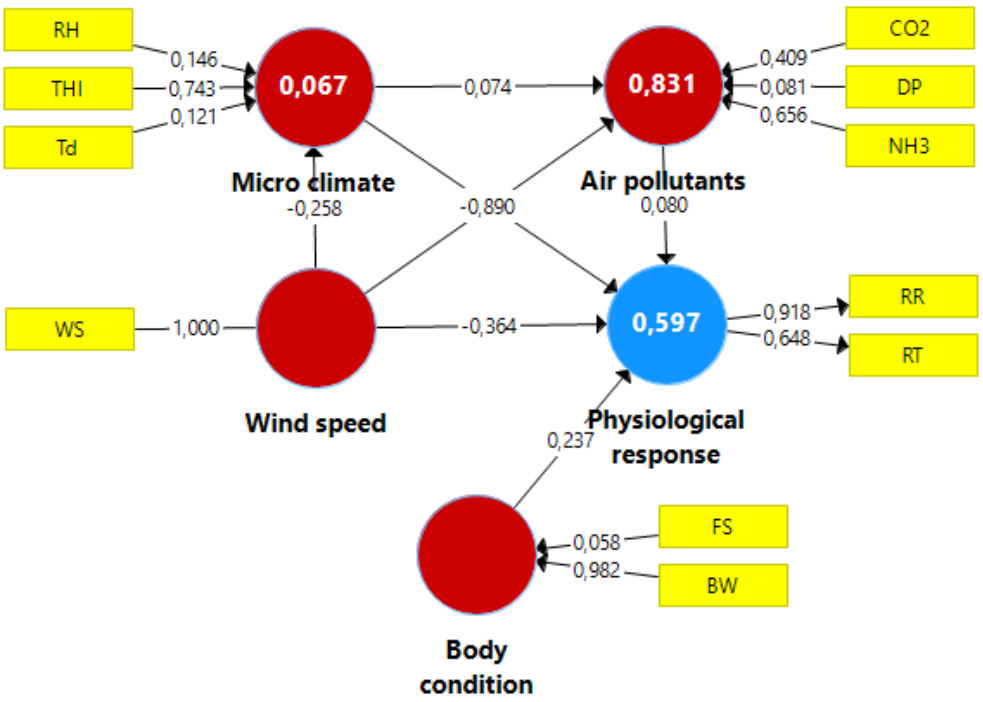

Figure 1. Modelling of the construct of body condition, microclimate, and wind speed analyzed by SmartPLS model of laying hen reared under tropical climate in Indonesia. BW: Body weight, FS: Fleshing score, Td: Dewpoint temperature, RH: Relative humidity, THI: Temperature and humidity index, NH3: Ammonia, CO2: Carbon dioxide, DP: Dust particle, RR: Respiratory rate, RT: Rectal temperature.

Table 1. Assessment of the physiological responses of laying hens for five days of observation (reflective measurement model)

	Reflective indicators	Convergent validity			Internal consistency reliability			Discriminant validity	t Value	p value
		Loadings	Indicator reliability	AVE	Composite reliability	Cronbach's Alpha	Rho_A			
		>0.5	>0.5	>0.5	0.70-0.95					
Physiological response	RR (beat/min)	0.918	0.797	0.631	0.768	0.451	0.574	Yes	24.18	p<0.05
	RT (^{o}C)	0.648	0.415						3.97	p<0.05

RR: Respiratory rate, RT: Rectal temperature, AVE: Average variance extracted, Loading: Correlation value between physiological response, respiration rate, and rectal temperature, Cronbach's Alpha: A measure of internal consistency of a test, Rho_A: Measurement the strength of association between two variables, less than 0.05 show significant.

Table 2. Assessments of body condition, microclimate, wind speed, and air pollutant (formative measurement models) of laying hen reared under tropical climate in Indonesia

Parameters	Formative indicators	Outer loadings	t Value	Significance	95% bootstrap confidence interval	Outer VIF
Body condition	BW (g)	0.982	5.220	Yes	[0.182, 1.000]	1.093
	FS	0.058	1.056	No	[-0.171, 0.985]	1.093
Microclimate	Td (^{o}C)	0.121	24.151	Yes	[0.908, 1.000]	889.88
	RH (%)	0.146	18.807	Yes	[0.831, 0.995]	143.79
	THI	0.743	33.771	Yes	[0.889, 0.999]	1639.0
Gas condition	NH3 (ppm)	0.656	52.255	Yes	[0.867, 0.991]	2.086
	CO2 (ppm)	0.409	26.853	Yes	[0.621, 0.922]	2.091
	DP (µg/m3)	0.081	1.666	No	[-0.996, -0.898]	1.005

BW: Body weight, FS: Fleshing score, Td: Dewpoint temperature, RH: Relative humidity, THI: Temperature and humidity index, NH3: Ammonia, CO2: Carbon dioxide, DP: Dust particle, VIF: Variance inflation factor, ($p < 0.05$).

Table 3. The result from the Partial Least Square-Structural Equation Model cross-loadings test for discriminant validity, air pollutant, body condition, microclimate, and physiological response of laying hen reared under tropical climate in Indonesia

Factors	Air pollutant	Body condition	Microclimate	Physiological response
BW	0.042	0.998	0.057	0.285
FS	0.077	0.345	0.085	0.098
Td	0.295	0.065	0.984	0.591
RH	0.297	0.044	0.944	0.554
THI	0.302	0.063	0.999	0.599
NH3	0.954	0.001	0.287	0.559
CO2	0.888	0.095	0.260	0.470
DP	0.140	0.074	0.109	0.041
RR	0.691	0.105	0.562	0.918
RT	0.029	0.485	0.361	0.648

BW: Body weight, FS: Fleshing score, Td: Dewpoint temperature, RH: Relative humidity, THI: Temperature and humidity index, NH3: Ammonia, CO2: Carbon dioxide, DP: Dust particle, RR: Respiratory rate, RT: Rectal temperature.

Assessment of outer model (reflective and formative measurement models)

According to the measurement model, physiological response contained two factors with loading of 0.918 and 0.648 for respiratory rate (RR) and rectal temperature (RT), respectively. This result indicated that respiratory rate contributes more to the construct than rectal temperature. Respiration is the first step for birds in maintaining body temperature which assists in the evaporation of water from the moist linings of the respiratory system instead of by radiation, convection, and conduction (Chandrasekar, 2011). Richards (1970) reported that respiratory rate increases linearly with body temperature. Basuony (2011) also wrote that Birds have no sweat glands, and under heat stress, they rely upon increased evaporation from the respiratory system as a major avenue for heat dissipation. Respiratory rate and rectal temperature are two variables related to each other in regulating body temperature (Bianca and Kunz, 1978; Son et al., 2022). Nurmeiliasari et al. (2020) explained that respiratory symptoms and stress are observable parameters. Therefore, the indicator of respiratory rate has higher reliability (0.918) compared with the rectal temperature indicator (0.648).

The value of AVE for the physiological response construct was 0.631, which exceeded the cut of 0.5 (Table 1). This means that the latent variable of physiological response could explain 63.1% of indicators on average. Based on the value of loading and the AVE, the outer model of reflective measurement from physiological response met the criteria for convergent validity. For the discriminant validity, the construct of physiological response appeared to meet the criteria since the internal consistency reliability (using composite reliability-CR) was 0.768, Table 1). Furthermore, based on the cross-loading rate of each indicator in the latent variable (Table 3), it has produced greater cross-loading rates. Therefore, the latent variable of physiological response was valid as discriminant validity. All the two indicators of the physiological response (respiratory rate and rectal

temperature) were found to be significant based on the bootstrapping procedure ($p < 0.05$). All indicators used in the assessment of the outer model seem to be appropriate to measure the latent variables (physiological response). Dewpoint temperature, RH, and THI posed multicollinearity with a score exceeding 5, but the rest showed no multicollinearity. This is due to the equation of THI, which includes two indicators within the same latent variable. However, since THI is an indicator of the thermal regime that an environment presents, it is still carried out from the assessment of the measurement model.

Wind speed or air velocity is a very important component in the management of poultry rearing because it can affect the microclimate inside the house, air pollutants, and the chickens' physiological response, which in turn can reduce the production performance of the poultry. Sohsuebngarm et al. (2019) reported a significant association between air velocity and body temperature of broiler chickens. Purswell et al. (2013) reported that air velocity impacts hen day production, feed consumption, and feed consumption per dozen eggs. However, there was no significant difference in feed conversion ratio and egg weight for laying hen production from 24 to 27 weeks ($p > 0.05$). Therefore, the current study put wind speed as a single indicator in the model to find out the interaction between wind speed and microclimate, air pollutants, and the physiological response of chickens.

Assessment of structural model

Based on the proposed model in Figure 1, five constructs and their seven path coefficients represent the relationships between constructs. The assessment of path coefficients of the structural model is available in Table 4. Body condition, microclimate, wind speed, and air pollutants gave a moderate contribution of 59.71% ($R^2 = 0.597$) of the total variability in the physiological response of the chicken. Among those four latent variables, microclimate had the most key role in the physiological response of the chicken (γ=0.465, $p < 0.05$, and $f^2 = 0.486$). These findings agree with the work of Sohsuebngarm et al. (2019), who reported that microclimate (involving ambient temperature, relative humidity, and heat index) significantly affected the chicken's body temperature. Nascimento et al. (2012) reported that broilers under different microclimate stress conditions (temperature and humidity) significantly affected the physiological parameters of the chicken at the time of exposure between the third and sixth weeks of age. However, broilers were able to significantly decrease their respiratory breaths after exposure to the comfort condition. This indicates that the chicken will respond physiologically to the changes in the microclimate condition. Thomas et al. (2021) wrote that microclimate is the climate directly surrounding the chickens, which is the only important parameter for the chickens.

Wind speed and microclimate substantially contributed to 83.1% ($R^2 = 0.831$) of the total variability in air pollutants. Wind speed had a more key role in air pollutants (γ = -0.890, $p < 0.05$, and $f^2 = 4.832$) than microclimate (γ = 0.074, $p < 0.05$, and $f^2 = 0.030$). These findings agree with the work of Huda et al. (2021), who reported that the higher the air velocity in different zones inside the closed house, the lower the ammonia detected. This is in line with the fact that the increase in wind speed could decrease or eliminate the presence of hazardous gases, such as NH_3 and CO_2 (Kilic and Yaslioglu, 2014; Longley, 2014).

Table 4. Assessment of path coefficients of the structural model on all latent variables of laying hen reared under tropical climate in Indonesia

Constructs	Path coefficients	t values	Sig. ($p < 0.05$)	95% bootstrap confidence interval	f^2 Value (Effect size)
AQ → PR	0.08	0.524	<0.05	[-0.176, 0.422]	0.003 (small)
BC → PR	0.237	2.882	<0.05	[0.036, 0.352]	0.136 (medium)
MC → AQ	0.074	2.196	<0.05	[0.011, 0.139]	0.030 (small)
MC → PR	0.465	5.426	<0.05	[0.273, 0.596]	0.486 (large)
WS → AQ	-0.890	55.295	<0.05	[-0.916, -0.857]	4.832 (large)
WS → MC	-0.258	3.800	<0.05	[-0.400, -0.141]	0.071 (medium)
WS → PR	-0.364	3.581	<0.05	[-0.543, -0.135]	0.057 (medium)

AQ: Air pollutant, PR: Physiological response, BC: Body condition, MC: Microclimate, WS: Wind speed

CONCLUSION

The results clarify the open house system in Kediri regency, the wind speed predisposes to many components such as respiratory rate, microclimate, and air pollutants. The microclimate and wind speed of the house contribute significantly to the air pollutants of the house in terms of ammonia, carbon dioxide, and dust particles. Body condition, microclimate inside the house, and wind speed contribute significantly to the physiological response of the chicken in terms of respiratory rate and body temperature. It is suggested to perform the same study with more houses in different elevations.

DECLARATIONS

Availability of data and materials

The data of this study are available from the corresponding author upon reasonable request.

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Authors' contributions

Heni Setyo Prayogi contributed to the conceptualization, investigation, data curation, writing, review, and editing of the manuscript. Suyadi, V.M. Ani Nugiartiningsih, and Osfar Sofjan were involved in the conceptualization of the study, data curation, analysis, resource provision, and supervision. All authors checked and approved the final version of the manuscript for publishing in the present journal.

Competing interests

The authors have declared that there are no competing interests.

Ethical considerations

All the authors checked for ethical issues such as plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy.

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Quail Farming in Villages of Mogoditshane-Thamaga and Tlokweng Districts, Botswana

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ABSTRACT

The interest in quail farming has been increasing over the years due to the quail's many productive and financial benefits over other poultry species. Quail farming is still in its infancy in Botswana. This study investigated the current status, challenges, and prospects of Japanese quail farming in Mogoditshane, Gabane, and Tlokweng districts, Botswana. A total of 64 quail farmers were interviewed using a structured questionnaire from June 2022 to April 2023. Data were collected on the respondents' demographic characteristics (age, educational status, and sex), quail management aspects (feeding, housing, and health), ownership of quail, challenges in quail farming, and the use of quail products in the study area. Results showed that 67% of male respondents were involved in quail rearing. The youth (≤35 years) dominated the rearing of quails, followed by respondents aged 41-50 years (22%), 36-40 years (16%), and above 50 years (9%). In addition, 48% of the respondents reared ≤100 quails, followed by 39% and 13% who reared 101-500 and 500 quails, respectively. Furthermore, 81% of respondents reared quails in cages, 17% in conventional structures, and 2% in residential houses. Bobwhite, Jumbo, and Japanese quail were the three quail varieties reared in the study area. It was found that 55% of the respondents had less than one year of experience in quail farming. Moreover, 86% of the respondents used crushed maize or sorghum to feed quails, while 14% used commercial chicken diets. Finally, 92% of the respondents mentioned that quail eggs were used to treat various human diseases. Effective challenges in quail farming included external parasites (36%), diseases (30%), predation (13%), lack of commercial quail diets (12%), escaping (6%), and theft (3%). Quail farming should be considered for inclusion in government support programs as it has the potential to contribute to income generation and food and nutrition security.

Keywords: Food security, Job creation, Nutrition, Quail farming, Therapeutic properties

INTRODUCTION

The Japanese quail (*Coturnix cortunis japonica*) was domesticated over 700 years ago and is now the most frequently farmed species for its egg production and meat (Mondry, 2016). Recently, quail production has gained relevance due to its usage as a laboratory bird for poultry and biomedical research, as well as its commercial use in the production of meat and eggs (Berterchini, 2012). As quails have many productive and financial benefits over other bird species, quail breeding has recently been effectively developed in many African countries, including Botswana, Ghana, Nigeria, South Africa, and Zimbabwe (Minvielle, 2009). Japanese quails are tiny birds of the Galliformes order that are widely distributed throughout the Palaearctic (Khaleel et al., 2021). Japanese quails have a strong genetic capacity for productivity; they may produce 310 eggs annually, with 12.5 g as the average egg weight (Katerynych and Pankova, 2020), and the bird's life expectancy is 2-2½ years (Bakoji et al., 2013).

Despite their small size, quails are not inferior to chickens when it comes to vitamin content and other beneficial components, including calcium, iron, zinc, and protein. Quails generally represent a distinctive protein-vitamin-mineral complex (Priti and Satish, 2014; Ali and Abd El-Aziz, 2019). For example, one gram of quail egg contains 2.5 times as much vitamin A, 2.8 times as much vitamin B1, and 2.2 times as much vitamin B2 as a

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chicken egg. Five quail eggs, weighing the same as one chicken egg, have five times the amount of phosphorus and potassium and 4½ times the amount of iron (Nepomuceno et al., 2014). Quail eggs are extremely beneficial not just as foodstuffs but also as a wonderful therapeutic agent due to the high amount of vital nutrients such as protein, calcium, and vitamins A, B, K, and D (Mnisi et al., 2021), that medical professionals across the world strongly advise using (Arthur and Bejaei, 2017). In addition, quail eggs are richer than chicken eggs in essential amino acids such as tyrosine, threonine, lysine, glycine, and histidine (Genchev, 2012). These essential amino acids give the quail egg its antibacterial, immune-modulating, anticancer, and normalizing effects on the cardiovascular, gastrointestinal, and other systems. In Japan, the egg is known to eliminate radionuclides from the body (Katerynych and Pankova, 2020).

Quail meat outperforms all other types of farm fowl in terms of nutritional value and flavor. It is also succulent, savory, and tender (Mnisi et al., 2021). Along with treating chronic conditions and disorders of the heart, stomach, liver, lungs, and kidneys, quail meat also strengthens bones and enhances tone (Costăchescu et al., 2018). Quail meat has a hypotensive effect due to the large amount of potassium, which is necessary for the brain's function (Katerynych and Pankova, 2020). Additionally, quail meat is a great way to prevent gout because it contains vitamin PP (nicotinamide), which helps to improve blood microcirculation, as well as being a source of sulfur and phosphorus that are essential for restoring normal metabolism (Katerynych and Pankova, 2020). Quail meat also contains significantly more vitamins A, B1, and B2 than chicken, along with vitamin D, making it an effective way to prevent rickets (Santhi and Kalaikannan, 2017).

As Africans consume the least protein daily per person compared to other continents (Illgner and Nel, 2000), the use of quail meat as an alternative protein source will undoubtedly increase in the near future, especially for those in the developing World (Mnisi et al., 2021). Considering the numerous uses of quail as food and medicine, the quail industry's global expansion is likely to be determined by these uses. In Botswana, quail farming is a relatively uncommon form of agricultural activity, but those who have adopted it are not only benefiting financially from it but are also reaping its nutritional and health benefits. Data on quail farming in Botswana is limited. Therefore, this study endeavors to investigate the status, challenges, and prospects of Japanese quail farming in the selected villages of Mogoditshane-Thamaga, and Tlokweng Districts of Botswana.

MATERIALS AND METHODS

Study area

The study was conducted in three villages (Gabane, Mogoditshane, and Tlokweng) of Mogoditshane-Thamaga and Tlokweng districts of Botswana (Figure 1). The geographical positions of the study sites are Gabane (24.6641^{o} S, 25.7836^{o} E), Mogoditshane (24.6072^{o} S, 25.8540^{o} E), and Tlokweng (24.6680^{o} S, 25.9764^{o} E). The human populations of Gabane, Tlokweng, and Mogoditshane are estimated to be 20010, 55508, and 88006, respectively (Statistics Botswana, 2022). Gabane and Mogoditshane are located west of Gaborone, and Tlokweng is in the eastern part of Gaborone (the capital city of Botswana). Mogoditshane and Tlokweng share a boundary with Gaborone, while Gabane is approximately 15 km away from Gaborone.

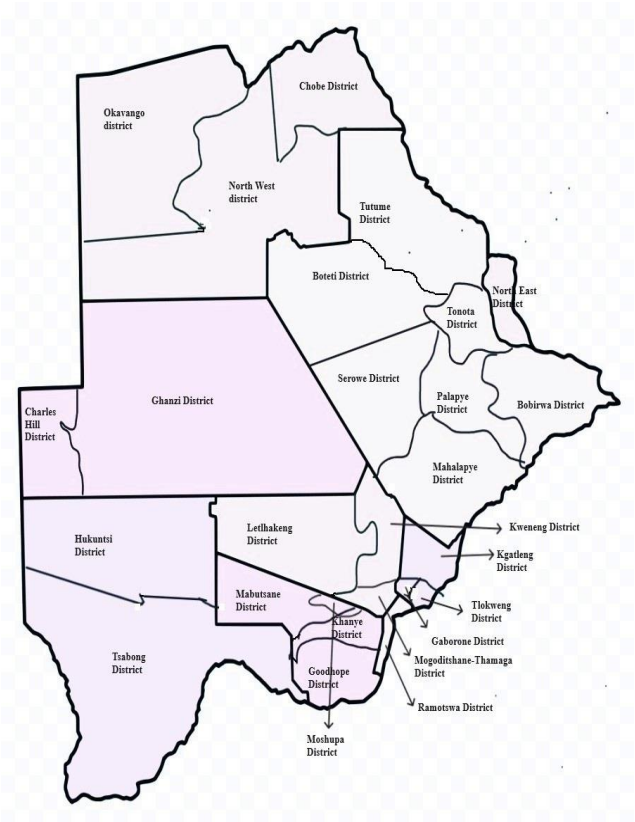

Figure 1. A map of Botswana showing Mogoditshane-Thamaga and Tlokweng districts of *Botswana* among others. Source: Ministry of Local Government and Rural Development (2023)

Selection of the study sites and sampling techniques

In this study, the sample size was determined by the human population in the villages and the availability of rearers. A total of 64 quail rearers were identified in the Mogoditshane-Thamaga and Tlokweng districts utilizing a cluster random selection technique described by Gabanakgosi et al. (2014). A total of 24 respondents were identified in Mogoditshane, followed by 21 respondents in Tlokweng, and 19 respondents were from Gabane village.

Data collection

Data on management techniques, consumption, and contribution of quail towards income and nutrition in the selected villages were acquired through a structured questionnaire and direct observation from June 2022 to April 2023. Data were collected on socio-economic characteristics (sex, age, and education level of the respondents), the economic value of quail, management practices, production systems, and production challenges. Participants were asked, among others, to give reasons for keeping quails. Secondary sources of data were also reviewed. Only one member of the household was interviewed by reading and interpreting the questions in the local language, while responses were recorded in English.

Statistical analysis

Quantitative and qualitative data were analyzed using IBM SPSS version 25. The means were separated using the Chi-square (X^2) mean separation test was used to determine the statistically significant differences and the significance was declared at $P < 0.05$.

RESULTS AND DISCUSSIONS

Socio-economic characteristics

Males operated 67% of farms, and quail farming was the primary occupation for 42.2% of farm holders. The results revealed that there was no significant difference (p > 0.05) in respondents' sex (Table 1). Similarly, Nasar et al. (2016) and Ekpo et al. (2020) reported that the quail farming business was mainly operated by males in Bangladesh and Benin, respectively. About seven percent of women in this study were involved in quail rearing compared to 55.6% for Uganda (Nasaka et al., 2017) and 68% for Zimbabwe (Majoni et al., 2018).

Fifty-three percent of the respondents in the present study were under the age of 35 years, 16% were between the ages of 36 and 40 years, 22% were between the ages of 41 and 50 years, and 9% were adults between the ages of 51 and 60 years (Table 1). The results showed no significant difference (p > 0.05) in age of respondents. Results show no significant differences in the age of the respondents, as reported by Muthoni (2014) and Ekpo et al. (2020) in Kenya and Benin, respectively. The results of the current study indicated that the respondents aged 40 years and below were more active in quail farming than other age groups. This might be attributable to the fact that young and middle-aged people are more likely to take risks in investing in a new venture such as quail farming than the elderly, who are risk averse. Additionally, this age group is active and better able to handle the physical strain associated with quail production.

Only 8% of the respondents were illiterate, 17% dropped out at the primary level, 44% completed their secondary education, and 31% attended tertiary education. This finding indicates that the majority of the respondents were likely to understand extension messages and try new technologies. The results revealed that there was no significant difference in the educational level of respondents (P > 0.05). The respondents who never attended school were above 60 years old. The current results supported the findings of Aliyu (2016) and Muhammad-Lawal et al. (2017) but differed with those of Oksana (2016), who reported that 59% of small-scale quail farmers in the Kaduna State of Nigeria lacked formal education. The scenario in Botswana is encouraging since education is essential for adopting novel ideas, experiencing new things, and managing some of the difficulties that may occur from quail production to marketing. Heads of households were mostly females (47%).

Fifty-two percent of the respondents relied on livestock sales, 16% operated family tuck shops, 13% depended on proceeds from vegetable and crop sales, 11% depended on children who were working, and 2% depended on pension (Table 2). The present study revealed that 23.4% of respondents were formally employed compared to 34.4% who were informally employed. Setlalekgomo (2012) found that 90.7% of women who reared chickens in Lentsweletau village in the Kweneng District of Botswana were unemployed.

Table 1. Socio-economic characteristics of respondents in Gabane, Mogoditshane, and Tlokweng of Botswana from 2022 to 2023

Respondent number (%) / Variable (n = 64)	Category	Gabane	Mogoditshane	Tlokweng	Overall	X^2	P value
Gender	Male	13(62)	16(73)	14(67)	43(67)	0.959	0.619
	Female	8(38)	6(27)	7(33)	21(33)		
Age (years)	≤ 40	18(82)	11(50)	15(71)	44(69)	11.734	0.068
	41-50	2(10)	7(32)	5(24)	14(22)		
	51-60	1(8)	4(18)	1(5)	6(9)		
	≥ 60	0	0	0	0		
Educational level	Illiterate	1(5)	3(14)	1(5)	5(8)	3.828	0.700
	Primary	3(14)	3(14)	5(24)	11(17)		
	Secondary	8(38)	10(45)	10(48)	28(44)		
	Tertiary	9(43)	6(27)	5(24)	20(31)		
Marital status	Married	7(33)	4(14)	2(10)	12(19)	9.402	0.052
	Single	9(43)	16(73)	12(57)	37(58)		
	Widowed	5(24)	2(13)	7(33)	14(22)		
Head of household	Husband	3(17)	7(32)	2(9)	12(19)	4.257	0.372
	Single father	7(33)	6(27)	9(43)	22(34)		
	Single mother	11(50)	9(41)	10(48)	30(47)		
Position in household	Father	3(14)	7(32)	8(38)	18(28)	9.669	0.139
	Mother	8(38)	6(27)	6(29)	20(31)		
	Son	6(29)	5(23)	4(19)	15(24)		
	Daughter	4(19)	4(18)	3(14)	11(17)		
Household size by sex	Male	26	52	25	103		
	Female	30	68	55	153		
Occupation	Formal	4(19)	7(32)	5(24)	15(23.4)	5.544	0.244
	Informal	8(38)	4(18)	9(43)	21(34.4)		
	Unemployed	9(43)	11(50)	7(33)	27(42.2)		

Table 2. The sources of income in selected villages without considering the quail farming in Botswana from 2022 to 2023

Respondent number (%) / Source of income	Gabane	Mogoditshane	Tlokweng	Overall
Livestock sales	10(48)	12(55)	11(52)	33(52)
Children working	3(14)	2(9)	2(10)	7(11)
Relative working	0	1(5)	0	0
Pension	1(5)	0	0	1(2)
Vegetable and crop sales	3(14)	2(9)	3(14)	8(13)
Tuck shop	2(10)	4(18)	4(19)	24(16)
Renting a house in Gaborone	2(9)	1(5)	1(5)	4(6)

Training and record-keeping

Eighty-three percent of the respondents across the villages did not have poultry management training or experience in quail farming. Gabane had 90% of quail farmers who were not trained in quail farming, followed by Tlokweng (86%) and Mogoditshane (73%, Table 3). The results revealed no significant difference ($p > 0.05$) in poultry training. The current results are in line with Nasar et al. (2016), who found that 67.3% of farmholders did not receive any training in poultry farming. All respondents in the current study agreed that training is critical for the long-term viability of quail production. This information might explain why farmers in Botswana are not actively involved in quail farming. The current results point to the inadequacy of technical support from the government extension services in supporting farmers who are involved in small-scale quail farming. Similarly, Siddique and

Mandal (1996) reported that quail farmers in Dhaka lack training in quail husbandry.

Eighty-six percent of the respondents did not keep quail business records (Table 3). Ninety-five percent of the respondents in Gabane mentioned that they did not keep records, followed by Tlokweng (86%) and Mogoditshane (77%). The results showed no significant difference ($p > 0.05$) in record keeping among respondents, indicating that most respondents did not keep records. A minority of the respondents who kept records were those who had tertiary education. This finding indicates that extension support to quail rearers is lacking. The majority of farmers who kept farm records had tertiary education. On the contrary, Akarikiya (2021) reported that more than half of respondents retained records in Ghana. The present study revealed that 41% of the respondents considered monitoring production as a reason for keeping records, implying that many farmers need training in record keeping. Thirty-six percent of the respondents mentioned that they monitored profit and loss using financial records that they keep, 17% mentioned that they used records to check the flock's health status, whereas 4% did not see any usefulness in keeping records. The results revealed no significant difference ($p > 0.05$) in the reasons for record keeping.

Livestock species reared in the study area

Livestock species reared in the study area are summarised in Table 4. The main livestock species reared include goats (35.3%), indigenous chickens (also referred to as traditional or family chickens, 21.8%), cattle (16%), sheep (12.6%), pigs (2.5%), and others (donkeys, ducks, and guinea fowl) that accounted for 11.8% (Table 4). A previous study by Gabanakgosi et al. (2013) in four areas of Botswana (Lobatse, Mokubelo, Khudumelapye, and Serowe) reported that the main livestock species reared were indigenous chickens (42%), followed by goats (32%), and cattle (16%). Furthermore, Simainga et al. (2011) in Zambia reported that chickens (50.7%) were the most reared livestock species, followed by cattle (35.4%), pigs (7.76%), and goats (6.08%).

About 45% of the respondents had ≤1 year of experience in quail farming, 31.3% had two years, 11% had three years, and 13% had ≥4 years of experience. Mogoditshane had a greater percentage of new farmers (55%) who had ≤1 year of experience in quail business, followed by Gabane (43%) and Tlokweng (38%). This means that quail farming is gaining recognition among poultry farmers in the surveyed villages. A minority of farmers with more than four years of experience in the quail business were found to be 14% in Mogoditshane, 10% in Tlokweng, and 5% in Gabane. This finding validates the past study that indicated quail farming is new in Africa (Akarikiya, 2021). Respondents in the present study stated the main reasons for starting quail farming were lower production costs, the nutritional benefits of quail eggs and meat, and the quails' tolerance to diseases. In another study, Ojo et al. (2014) in Nigeria reported that farmers enter the quail business due to the bird's hardiness, short generation intervals, and lower production costs of the quail enterprise.

Table 3. Training and record keeping of quail production in Gabane, Mogoditshane, and Tlokweng of Botswana from 2022 to 2023.

Variable n = 64	Category	Respondents number (%)			Overall	X^2	P-*value*
		Gabane	Mogoditshane	Tlokweng			
Poultry training	Trained	2(10)	6(27)	3(14)	11(17)	4.664	0.097
	Not trained	19(90)	16(73)	18(86)	53(83)		
Training useful	Useful	21(100)	22(100)	21(100)	64(100)		
	Not useful	0	0	0	0		
Do you keep records	Yes	1(5)	5(23)	3(14)	9(14)	3.399	0.183
	No	20(95)	17(77)	18(86)	55(86)		
Reasons for record-keeping	Monitor profit and loss	2(10)	6(27)	15(71)	23(36)	0.774	0.679
	Monitor production	2(10)	12(55)	12(57)	26(41)	0.889	0.641
	Identify health status	3(14)	12(55)	13(62)	11(17)	1.588	0.452
	Not useful	3(14)	1(5)	0	4(6)	2.087	0.352

Table 4. Ownership of livestock by respondents in three selected villages of Mogoditshane-Thamaga and Tlokweng districts Botswana from 2022 to 2023

Variable \ Number of respondents	Animal	Gabane	Mogoditshane	Tlokweng	Overall
Species of livestock	Cattle	8	5	6	19
	Goats	10	19	15	44
	Chickens	8	8	9	25
	Sheep	7	5	3	15
	Pigs	1	0	2	3
	Others	11	1	0	12

Table 5. Status of quail farming in selected villages of Botswana from 2022 to 2023

Variable n=64	Breed	Respondents number (%)			Overall
		Gabane	Mogoditshane	Tlokweng	
Farm type	Layer	2(10)	3(14)	1(5)	6(9)
	Broiler or meat type	1(5)	4(18)	2(10)	7(11)
	Mixed type	18(85)	15(68)	18(85)	51(80)
Farm size	<100	12(57)	11(50)	8(38)	31(48)
	101-500	7(33)	9(41)	9(43)	25(39)
	>500	2(10)	2(9)	4(19)	8(13)
Number of breeds	One variety	1(5)	2(9)	3(14)	6(9)
	Two varieties	11(52)	7(32)	8(38)	26(41)
	Three varieties	9(43)	13(59)	10(48)	32(50)
Reared with other poultry	Yes	1(5)	4(18)	0	5(8)
	No	20(95)	18(82)	21(100)	59(92)

Status of quail farming

Eighty percent of the respondents were involved in mixed-type quail farming (eggs and meat), 11% reared quails for meat only, while 9% reared quails for egg production only. In Gabane, 85% of respondents were involved in mixed-type quail farming, laying quail (eggs) production (10%), and quail broiler production (5%). In Tlokweng, 85% of the respondents participated in mixed-type quail farming, 10% in broiler quail production, and 5% in layer quail production. Similarly, the majority of farmers in Mogoditshane (85%) were involved in mixed-type quail farming, followed by quail broiler farming (10%), and layer quail farming (5%, Table 5). The current results are consistent with Nasar et al. (2016), who found that 63.4% of the respondents in Bangladesh practiced mixed-type quail farming. Egbeyale et al. (2013) mentioned that Japanese quails are well suited for commercial egg and meat production under intensive management and that mixed-type quail farming is used globally. This is due to their resilience and capacity to live in small cages (Odunsi et al., 2007); the relatively short generation interval, and lower production costs (Ojo et al., 2014).

Approximately 92% of quail farmers in this study rear quail alone. Most of the farmers (48%) had ≤100 birds, 39% reared between 101-500 quails, while only 13% reared over 500 quails with 2-3 quail varieties reared in cages separately from other poultry species (Table 5). El-Sheikh et al. (2016) found that egg production for quails raised in battery cages was higher than that for quails reared in deep litter. However, Arumugam et al. (2014) observed that the fertility level of Japanese quails was unaffected by the rearing techniques.

Housing and management

All respondents in the study area provided shelter to quails during the day and at night. Eighty-one percent of the respondents kept quails in cages as they indicated that quails can easily fly away, followed by 17% that used open-sided houses, and one percent used the owner's house (Table 6). Monika et al. (2018) argued that as quails are small birds, they may readily be kept in restricted spaces inside multitier colony cages. Eighty-four percent of the respondents in this study said they cleaned quail shelters weekly, followed by 14% that cleaned monthly, while 2% only cleaned the shelters when there was too much feces (Table 6). The results revealed no significant

difference ($p > 0.05$) in the type of housing and the frequency of cleaning quail shelters. The quail shelters were swept by family members using locally purchased brooms, and no disinfectants were used, indicating lack of biosecurity. Similarly, Gabanakgosi et al. (2014) reported that family chicken houses were cleaned using locally-made brooms without applying disinfectants. All the respondents mentioned that they used quail droppings to fertilize vegetable gardens. In agreement with the current results, Dikinya and Mufwanzala (2010) reported that quail manure was used to fertilize the gardens, as it is a potential source of plant nutrients and chemical conditioner.

Nutrition and water provision

Feeds such as crushed yellow maize and drinking water were provided mainly by family members. Quails in the current study were given portable water. The respondents also mentioned that field crops were the major sources of feed for quail. Similarly, Gabanakgosi et al. (2014) reported field crops as the major feed resources available to poultry. Akarikiya (2021) asserted that in backyard quail farming, the birds could be fed any available agro-by-products, household leftover food grains, and commercial chicken feed. Eighty-six percent of the respondents in this study used crushed maize or sorghum to feed quails, while 14% used commercial chicken diets predominantly laying chicken mash as there are no quail feeds locally. The results revealed no significant difference ($p > 0.05$) in feed type (Table 7). Sixty-three percent of the respondents provided feed and water to quail at *ad libitum*. The results showed a significant difference ($p < 0.05$) in the frequency of water and feed provision among the villages. About 28% of the respondents provided feed and water only in the morning (once a day), followed by 9% that provided feed twice a day (before going to work in the morning and upon return from work in the evening).

Fifty-five percent of the respondents stated they provided quail feed *ad libitum*, while 34% mentioned they gave limited feed for survival only, 6% of the respondents had no idea of how much feed they offered to quail daily while 5% provided kitchen wastes (bread crumbs, rice, and maize meal (Table 7). Similarly, Gabanakgosi et al. (2014) in Botswana and Akarikiya (2021) in Ghana reported that respondents provided poultry with kitchen leftovers. The results revealed a significant difference ($P < 0.05$) in the feed type used by quail farmers.

Table 6. Quail housing by the respondents in three selected villages of Mogoditshane-Thamaga and Tlokweng districts of Botswana from 2022 to 2023

Respondents number (%) / Variable (n=64)	Category	Gabane	Mogoditshane	Tlokweng	Overall	X^2	P-value
Type of housing	Owner's house	1(5)	0	0	1(2)	2.324	0.676
	Cage	16(76)	18(82)	18(86)	52(81)		
	Conventional structure	4(19)	4(18)	3(14)	11(17)		
Frequency of cleaning	Daily	0	0	0	0	2.871	0.238
	Weekly	19(90)	15(68)	20(95)	54(84)		
	Monthly	2(10)	6(27)	1(5)	9(14)		
	When droppings accumulate	0	1(5)	0	4(2)		

Table 7. Feed and water provision of quail by respondents in the selected villages of Botswana (2022 to 2023)

Respondents number (%) / Variable n=64	Category	Gabane	Mogoditshane	Tlokweng	Overall	X^2	P value
Feed type	Crushed maize/sorghum	19(90)	19(86)	17(81)	55(86)	0.793	0.693
	Commercial feeds	2(10)	3(14)	4(9)	9(14)		
	Others	0	0	0	0		
Quantity	Do not know	0	1(4)	3(14)	4(6)	7.074	0.314
	Just a little	0	2(9)	1(5)	3(5)		
	Just on average	4(19)	12(55)	6(29)	22(34)		
	Ad libitum	17(81)	7(32)	11(52)	35(55)		
Frequency of feeding water provision	Morning	3(14)	6(27)	9(43)	18(28)	11.901	0.018
	Morning and afternoon	1(5)	4(18)	1(5)	6(9)		
	Ad libitum	17(81)	12(55)	11(52)	40(63)		

Meat and eggs

Quail eggs were used mainly for hatching, consumption, and sale (61%); hatching only (58%), consumption (53%); and hatching and sales (31%, Figure 2). Respondents who did not consume quail eggs (6%) stated that they wanted to allow their flocks to increase as consuming eggs would affect their flock sizes. Egg consumption happens when egg production is very high or in summer when low hatchability is experienced due to heat stress. The respondents also mentioned they did not consume eggs because they wanted to make a living out of the sale of quail eggs, which are claimed to have health benefits. In contrast to the current findings, Ogunwole et al. (2015) in Oyo state, Nigeria, reported that the majority of respondents (55%) suggested eating more quail eggs because they thought the eggs were a very healthy and rich source of protein.

Seventy-seven percent of the respondents mentioned that they slaughtered quail for family consumption and to honor guests (23%). This finding agrees with Moreki (2006), who reported that family chickens were usually slaughtered to honor guests. Quail meat is recommended as a low-fat meat as it contains low fat and cholesterol contents due to its thin skin and low-fat accumulation between its tissues (Faraq et al., 2021). Quails are valuable for the high nutritional content of their eggs and meat (Wen et al., 2017).

Quail eggs provide essential nutrients for maintaining human health (Jeke et al., 2018). The present results revealed that 94% of respondents consumed quail eggs and that 92% of respondents in this study agreed that quail eggs have nutritional benefits. The present results appear to support Akarikiya (2021), who reported that less than half of the survey participants stated that their primary motivation for quail breeding was to take advantage of the alleged nutritional and therapeutic benefits of quail meat and eggs.

It has been proven that quail meat and eggs contain high-quality protein of high biological importance, little fat content, and less low-density lipoprotein (Tolik et al., 2014). To supplement their diets, many of the respondents in this study acknowledged that they shared part of the quail meat and eggs with their family members and friends. Mnisi et al. (2021) posited that regular consumption of quail eggs aids in the prevention of numerous diseases and acts as a natural remedy for conditions of the digestive tract, such as stomach ulcers. According to Tunsaringkarn et al. (2013), quail eggs boost the immune system, support healthy memory, stimulate the brain, and calm the nervous system by boosting the body's hemoglobin levels and eliminating toxins and heavy metals. Quail eggs also aid in the treatment of anemia.

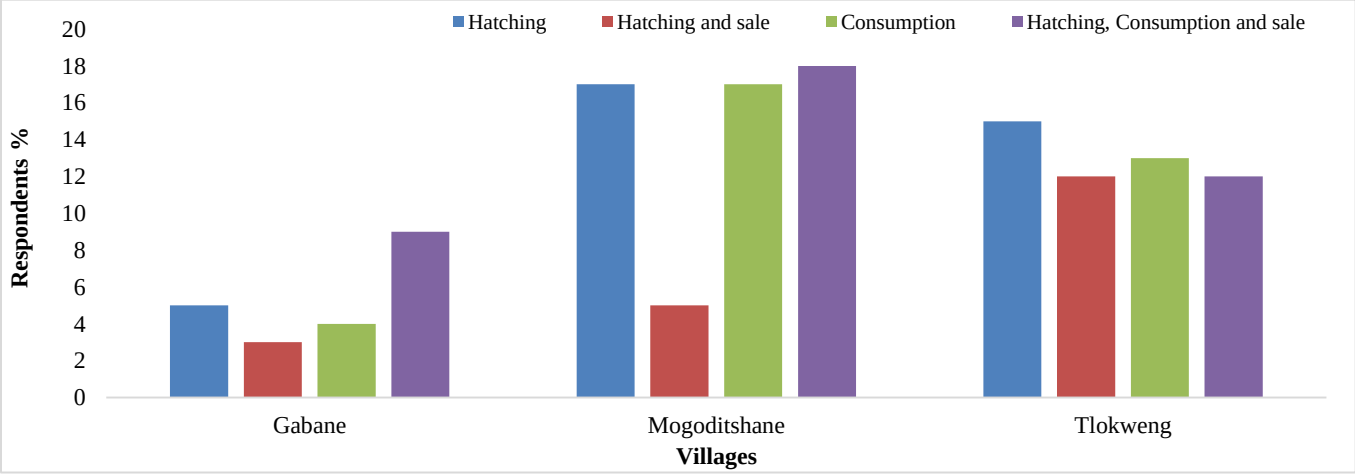

Figure 2. Uses of quail eggs in Mogoditshane-Thamaga and Tlokweng districts of Botswana, from 2022 to 2023

Marketing

Ninety-five percent of respondents mentioned that they sell quails to meet immediate family needs, while the remainder stated that they did not sell quail as they were still building their flock sizes (Table 8). Fifty-six percent of the respondents marketed quail at 28-35 days of age followed by 17% (above 35 days old), 14% (14-28 days), 10% (7-14 days), and 3% (below 7 days of age). The average price for a quail chick at 2 weeks and 5 weeks of age was P20.00 (USD 1.49) and P40.00 (USD 2.90),

respectively. Quail meat was sold at P150.00 (USD 10.90)/pack of 5 carcasses. According to Nasar et al. (2016), domestic quail grows quickly and reaches sexual maturity at 5-6 weeks of age. Modern meat-type (broiler) quail strains undergo slaughter at 5 weeks of age and weigh 160–250 g (Ophir et al., 2005; Jatoi et al., 2013). Seventy percent of respondents in the current study stated that they sell quail often, 16% rarely sold quail, whereas 14% sold quail only when the need arose to meet their immediate family needs. Ninety-seven percent of the respondents mentioned that they sell quail when they need money while only 3% sold quails to avoid overcrowding. All the respondents in Gabane village indicated that they sold quail eggs when they needed money to cover household expenses.

Seventy-five percent of the respondents mentioned that the market for quails was satisfactory as quails could be sold throughout the year, while the remainder stated it was unsatisfactory. The main buyers of quails were individuals (95%), followed by retailers (3%) and some organizations such as schools (2%). The average price of an egg was P3.00 (USD 0.23). Eggs were sold as fresh eggs and some as pickled eggs. The pickled eggs were sold at P70.00 (USD 5.09) per bottle. In addition, respondents stated eggs were sold as per customer preferences, as some wanted them cooked and others uncooked.

Table 8. Quail marketing in selected villages of the two districts Mogoditshane –Thamaga and Tlokweng districts of Botswana from 2022 to 2023

Variable n=64	Category	Respondents number (%)			Overall	X^2	P - value
		Gabane	Mogoditshane	Tlokweng			
Do you sell quail?	Yes	21(100)	20(91)	20(95)	61(95)	2.133	0.344
	No	0	1(9)	2(5)	3(5)		
Marketing age (days)	<7	1(5)	1(4)	0	2(3)		
	7-14	3(14)	2(9)	2(10)	7(10)		
	14-28	1(5)	4(18)	3(14)	8(14)		
	28-35	13(62)	12(55)	11(48)	36(56)		
	>35	3(14)	3(14)	5(24)	11(17)		
Frequency of selling quail	Rarely	2(10)	3(14)	5(24)	10(16)	4.625	0.328
	Often	18(86)	15(68)	12(57)	44(70)		
	At times	1(4)	4(18)	4(19)	10(14)		
Reason for selling	Limited housing	0	1(5)	2(10)	2(3)	3.256	0.516
	Money	21(100)	21(95)	19(90)	62(97)		
Quail market satisfactory	Yes	14(67)	19(86)	15(71)	39(75)	1.600	0.449
	No	7(33)	3(14)	6(29)	16(25)		
Main buyers	Individuals	21(100)	21(95)	19(90)	61(95)	2.087	0.352
	Retailers	0	1(5)	1(5)0	2(3)		
	Organisations	0	0	1(5)	1(2)		

Challenges in quail farming

Figure 3 illustrates that parasites were a major challenge (36%), followed by diseases (31%), predation (13%), lack of quail diets (11%), escaping (6%), and theft (3%). Parasites were the major cause of losses in Tlokweng, followed by Mogoditshane and Gabane, respectively. This could be attributable to the failure of farmers to clean and disinfect the cages. Figure 3 also shows that Tlokweng, Mogoditshane, and Gabane had the highest disease incidences, indicating a lack of health management by the rearers. Siddique and Mandal (1996) reported that high feed expense, inadequate institutional credit, lack of veterinary services and medicine, lack of training on quail husbandry, and inadequate product marketing facilities are major challenges in quail farming in Dhaka, Bangladesh.

Figure 4 shows the common diseases of quail in the study area. In order of prevalence, the three major diseases that affected the productivity of quail were sudden death, fowl pox, and bumble foot. Across the villages, predation ranked second after diseases. The respondents stated that predation occurred in the first week of age and that they suspected rodents could be responsible for killing quail chicks. In addition, the respondents claim that high chick mortalities occurred during the brooding phase due to inadequacy of heat. The study by El-Demerdash et al. (2013) found that respiratory diseases may be to blame for the high mortality rate among day-old quail chicks during the first week of age. However, this problem may be resolved after the first week with adequate management techniques.

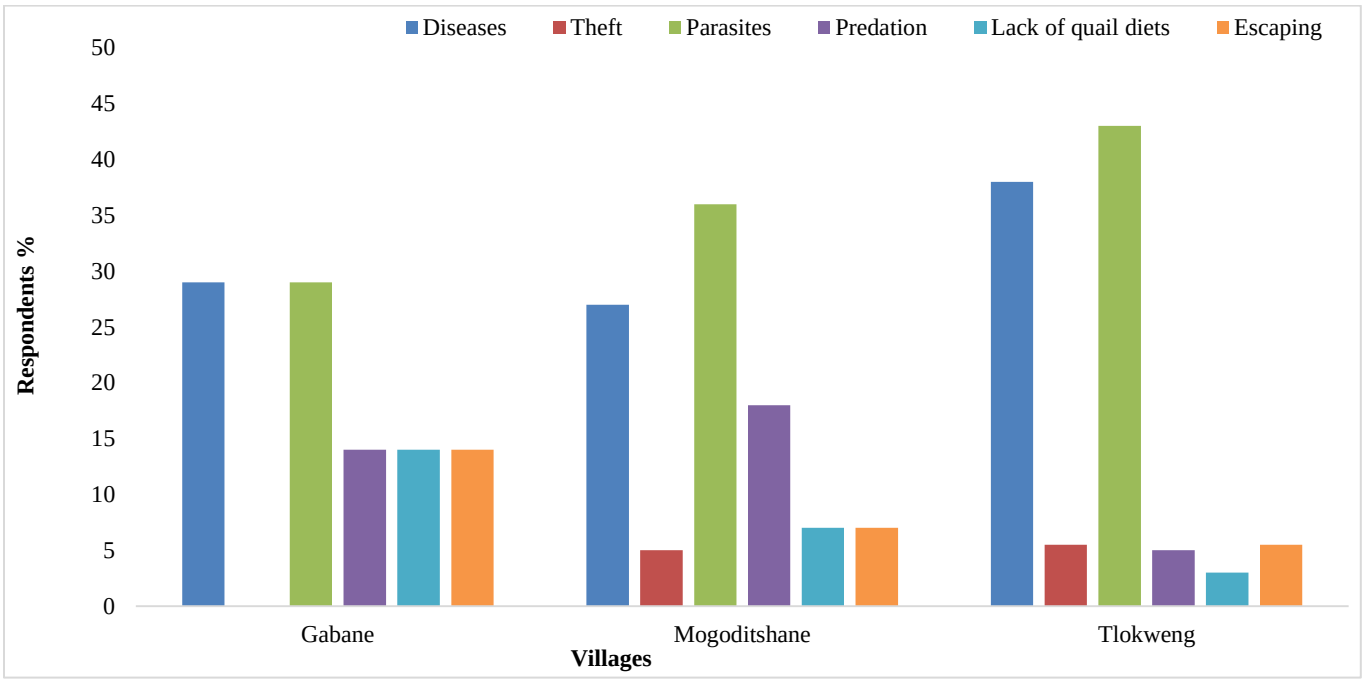

Figure 3. Challenges in quail production faced by respondents in the selected villages of Botswana, from 2022 to 2023

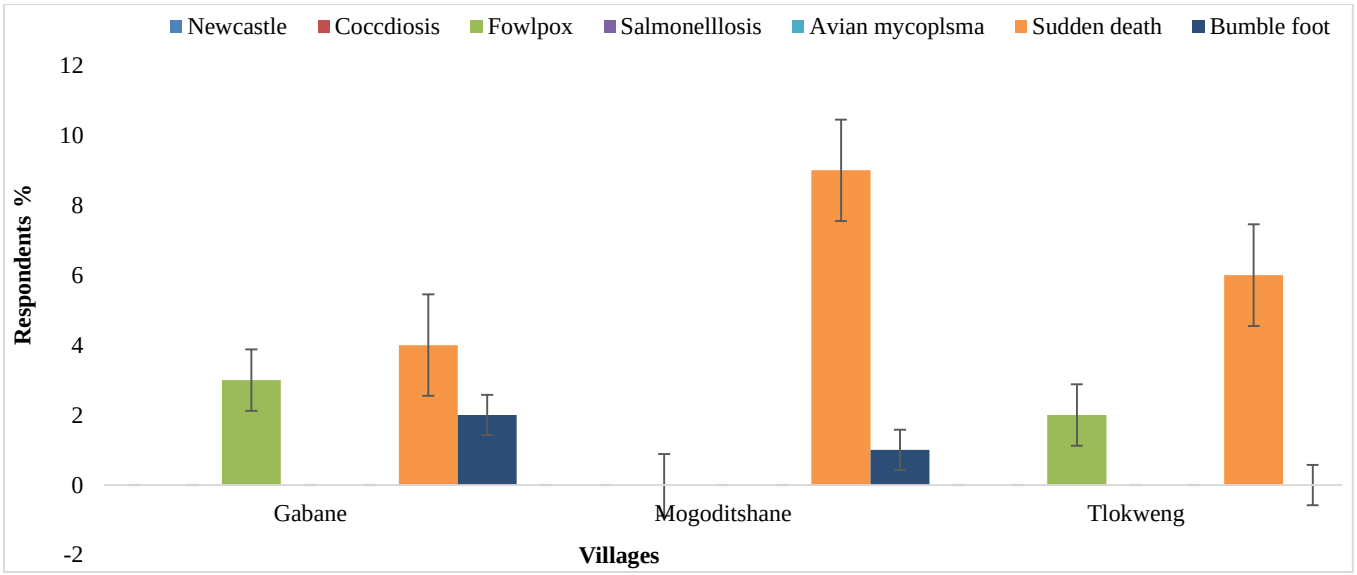

Figure 4. Disease prevalence in the selected villages of Botswana from 2022 to 2023.

Table 9. Veterinary or extension service used by respondents in three villages of Botswana from 2022 to 2023

Variable / Respondents number (%)	Gabane	Mogoditshane	Tlokweng	Overall
Rarely	5(24)	6(27)	5(24)	16(25)
Often	6(29)	10(45)	5(24)	21(33)
At times	10(47)	6(28)	11(52)	27(42)
No access	0	0	0	0

None of the farmers in this study recorded any diseases of economic importance. This finding is in line with Nasar et al. (2016) and Akarikiya (2021), who reported that most of the farmers did not experience any form of disease or parasite on their farms. Across the villages, sudden death (30%) was a major problem, especially during brooding, followed by fowl pox (8%), and bumble foot condition (5%, Figure 4). This study supported the claims made by Chakrabarti et al. (2014) that quails are less susceptible to most poultry diseases and parasites. Contrary to a study by Nasar et al. (2016) in Bangladesh, diarrhea (21.4%) was found to be the most common problem, followed by pneumonia (19.4%), infectious coryza (16.5%), Newcastle disease (15.5%), dysentery (5.8%), and avian influenza (4.9%). Most respondents in the present study were concerned about the inadequacy of biosecurity measures which could easily expose their flocks to diseases. Forty-two percent of the respondents received advice from animal health experts, 33% visited the veterinary office to seek help, while 25% rarely engaged veterinary technicians (Table 9). Similarly, Nasar et al. (2016) reported that 52% of the respondents received veterinary advice when needed. Forty-two percent of the respondents in this study obtained veterinary knowledge online, explaining a high percentage of the respondents engaged in self-medication for the treatment of diseases and parasites. The present results support Akarikiya (2021), who found that respondents sourced veterinary information from the Internet to medicate their quails without the assistance of trained animal health experts.

Across the villages, the respondents used modern and traditional medicines to control ectoparasites. On average, 28% of the respondents used traditional medicine alone to control diseases and parasites, 20% used modern medicine alone, whereas 52% used a combination of modern medicine and traditional medicine. The use of modern medicine was high in Mogoditshane (22%) compared to Gabane (19%) and Tlokweng (19%). Tlokweng had the highest percentage of respondents who used traditional medicine (33%), followed by Gabane and Mogoditshane, with 29% and 22%, respectively. On the other hand, Mogoditshane had the highest percentage of respondents (56%) who used modern and traditional medicines, followed by Gabane (52%) and Tlokweng (48%). Similarly, Gabanakgosi et al. (2014) reported that 65% of rearers of family poultry used modern medicines, 10% used traditional medicines, and 25% used traditional medicine and modern medicine. The high use of modern and traditional medicine is attributable to the fact that farmers are using internet solutions to poultry problems in places close to the city, such as Mogoditshane and Tlokweng. In Namibia, Petrus et al. (2011) observed that ethnoveterinary medicine was culturally acceptable and economically viable. In another study, Sadr et al. (2022) in Iran reported that a mixture of three herbal plants (*Quercus infectoria*, *Allium sativum,* and *Artemisia annua*) was useful in the reduction of the pathogenic effects of *Trichomonas* spp. The authors concluded that the mixture can be used as an alternative to chemotherapeutic drugs in trichomoniasis treatment.

External parasites

One of the main factors that might cause the poultry business to suffer significant economic losses is parasitism (Hassan et al., 2020). Twenty-eight percent of the respondents in the present study cited mosquitoes as a major problem, followed by fleas, mites, and fowl ticks (22%), fleas and fowl ticks (19%), mites (16%), fleas and mites (8%), and fowl ticks (7%). In another study, Ranwedzi (2002) found that mites (77%) and fleas (9.3%) were the major parasites in family chicken production in South Africa. Similarly, Moreki and Radikara (2013) found that the most problematic parasites in chickens in Botswana were tampans, mites, fowl lice, and ticks. Since family chickens and quail are kept in the backyards and usually in the same shelter, it is possible that chicken mites and lice can easily migrate to quails. As indicated earlier, mosquitoes were the most prevalent parasites in this study, perhaps explaining the high incidences of fowl pox in Gabane and Tlokweng. The study by Mazyad et al. (1999) showed that 31 species of mites were recovered from quail. Studies by Monte et al. (2018) and Yu et al. (2022) showed that quails that suffer from parasitism experience stunted growth, poor productivity, increased susceptibility to various infections, and eventually high mortalities.

Thirty-eight percent of the respondents used Karba dust (Carbaryl) and Blue death powder (Permethrin and Carbaryl), 17% Blue death powder (Permethrin and Carbaryl), 14% Jeyes fluid (Tar Acids), 13% wood ash, and 13% wood ash and Karba dust, whereas only 5% used paraffin to control external parasites (Table 10). Similarly, Ranwedzi (2002) in Port Elizabeth (South Africa) reported that respondents used wood ashes (19.4%), Jeyes fluid (0.9%), Blue death powder (0.9%), hot water (6.5%) and paraffin (6.5%) to control external parasites. Moyo (2009) in the Eastern Cape of South Africa also reported that wood ash (28%), Jeyes fluid (10%), paraffin (8.4%), used

engine oil (2.8%) and Karba dust (4.2%) were used to control ectoparasites. According to the respondents, these chemicals and remedies are cheap to buy or access, and do not need any prescription; hence their wide use.

Table 10. Control of external parasites of quails by respondents in the selected villages of Botswana from 2022 to 2023

Control method	Gabane	Mogoditshane	Tlokweng	Overall
Wood ash	3	4	1	13
Karba dust and wood ash	2	4	2	13
Jeyes fluid	1	3	5	14
Blue death	3	6	2	17
Karba dust and blue death	8	7	9	38
Use of paraffin	0	1	2	5

CONCLUSION

Quail farming in the study area is practiced only at a small-scale level, with 55% of farmers having less than one year of experience in the business. Most of the farmers (48%) reared ≤100 quails and 39% reared between 101-500 quails. External parasites were a major challenge (36%), followed by diseases (30%), predation (13%), escaping (6%), and theft (3%). Twenty-eight percent of the respondents used traditional medicine alone to control diseases and parasites, 20% used modern medicine alone, whereas 52% used a combination of modern medicine and traditional medicine. Forty-two percent of the respondents received advice from animal health experts, indicating that technical support for quail rearers was lacking. Quail farming has the potential to contribute to job creation and additional revenue. It is recommended that quail farmers establish active farmers' associations to enable them to support one another in marketing, advertising, and raising awareness of their products. The Government should consider incorporating quail production in support programs such as Livestock Management and Infrastructure Development since quails are highly prolific and can contribute to food and nutrition security.

DECLARATIONS

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Authors' contribution

Shame Bhawa conducted the survey, analyzed data, and wrote the manuscript. John C. Moreki conceptualized the study, and reviewed and edited the manuscript. Freddy Manyeula reviewed and edited the manuscript. All authors have reviewed and agreed with the final version of the manuscript for publishing in the Journal of World's Poultry Research.

Competing interests

The authors declare no conflicting interests.

Ethical consideration

Each author has reviewed this work for ethical problems, such as plagiarism, consent for publication, misconduct, data manipulation and/or deceit, and duplication of work.

Availability of data and materials

Data will be available upon request to the corresponding author.

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Genetic Diversity, Population Structure and their Association with Body Weight in Egyptian Chicken Strains

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ABSTRACT

Genetic characteristics and population structure within and among Egyptian indigenous chicken strains are important for identifying some genetic resources. The present study aimed to use microsatellite markers to determine similarity and genetic distance among different genotypes and their association with growth and production traits in Egyptian indigenous chicken strains. The current study included 800 chickens and 100 genomic DNA samples obtained from four Egyptian local chicken strains of four different areas (Dokki-4, Mandarah, Anshas, and Al-Salam) in Egypt. Their genetic characteristics, population structure, phylogenetic relationships, and their association with body weight were analyzed using seven microsatellite markers. The performance of 200 chicks from each strain was assessed in terms of individual body weight and growth rate. Al-Salam strain had a significantly higher body weight than the other strains up to 12 weeks of age among the four lines of Egyptian local chickens. Additionally, male chickens across all strains indicated significantly higher body weight than females from 2 weeks of age until the end of the experiment. The study revealed a total of 68 alleles from the 7 loci across 4 chicken strains, with an average of 9.71. The average of observed heterozygosity, expected heterozygosity, and polymorphism information content were 0.799, 0.358, and 0.707, respectively. The Mandarah strain had the highest observed allele number of 5.37; however, the lowest observed allele number was 3.12 for the Dokki strain. Analysis of population structure revealed that the four chicken strains should be divided into three clusters based on the highest log-likelihood values (ΔK value, 56.3). The results showed a degree of heterozygosity in the Mandara strain with 66.7% individual memberships, indicating a level of admixture. On the other hand, the Al-Salam strain indicated a high genetic diversity with 99% individual membership. The current study provides valuable insights for future genetic polymorphism studies, the advancement of breeding programs, and strategies for the conservation of the Egyptian local chicken strains.

Keywords: Body weight, Chicken, Genetic diversity, Microsatellite

INTRODUCTION

Poultry production is considered an integral part of agriculture all over the world, with particular emphasis on native poultry due to their meat quality and production potential (Bennett et al., 2018). Chickens play an essential role in providing economically feasible and nutritionally essential human resources (Fontanesi, 2009). A lot of poor rural societies keep native chickens for numerous reasons, such as white meat and egg production (FAO, 2016). In Egypt, native chicken production accounts for 55% of white meat production (Hassanane et al., 2018). Therefore, chicken genetic resources are a necessary part of the biological basis for world diet safety. In recent years, Egyptian local chicken breeds have received insufficient attention on a commercial scale since breeders focus on the use of highly productive commercial broiler chickens (Ramdan et al., 2014a; Nassar et al., 2019). Moreover, native chicken breeds indicate greater disease resistance and environmental adaptability, compared to commercial strains (Padhi, 2016; Rashid et al., 2020). The Egyptian chicken strains exhibit tolerance to heat stress conditions, greater resistance to diseases, and are well-adapted to challenging environments (El-Gendy et al., 2011). Egyptian local chickens have a series of necessary meat

qualities containing superior tenderness and favored tastes that are often preferred by consumers. However, their growth rate and egg production rates are lower than commercial chicken breeds (Nassar et al., 2019). Therefore, crossbreeding among native strains and foreign strains has been used as a strategy to produce some Egyptian strains, such as Dokki-4, El-Salam, Anshas, and Mandarah (Kosba and El-Halim, 2008; Heba et al., 2017).

The importance of preserving chicken biodiversity has received increasing attention in recent years. Although there are a lot of efforts to characterize chicken breeds using morphological measurements and performance traits, molecular methods for characterizing chicken breeds have not been extensively explored (Ramadan et al., 2014b; 2018). Utilizing molecular genetic tools for selection offers a valuable method to enhance the quality of chicken meat and carcasses (Zhou et al., 2006). According to Sahu et al. (2022), ADL0273 has significant effects on some economic traits in chickens. Understanding the genetic characteristics and identifying similarities and differences within and among different breeds is crucial for genetic improvement programs in farm animals (Mirhoseinie et al., 2005). Microsatellite markers are invaluable tools in assessing genetic variation and similarities among strains and species. These markers display a high polymorphism rate and are codominant, making them particularly useful in genetic studies (Yang et al., 2013). The implementation of marker-assisted selection has significantly expedited the breeding process for enhancing chicken growth. Notably, this approach has yielded substantial advancements in genetic improvement while also reducing costs and time requirements (Boichard et al., 2016). Microsatellite markers for studying biodiversity within and among breeds are now identified, with each marker sequence located on loci associated with performance traits (FAO, 2016). Microsatellites have become optimal markers for an assortment of molecular investigations due to their adaptability, operational flexibility, and minor price, compared to other marker methods (Kantartzi, 2013; Zimmerman et al., 2020). Given this, the present study aimed to use microsatellite markers to determine similarity and genetic distance among different genotypes and their association with growth and production traits in Egyptian indigenous chicken strains.

MATERIALS AND METHODS

Ethical approval

The experimental procedure used in this investigation was approved by the Animal Care and Use Committee (Medical Research and Ethics Committee) of the National Research Centre in Egypt, with certificate number 1484052023.

This study was executed at Nobaria Research Station, Animal Production Biotechnology Lab, Central Laboratory Network, National Research Centre, and Department of Cell Biology, Institute of Biotechnology Research, National Research Centre, Giza, Egypt.

Populations and management

Four Egyptian chicken strains (Dokki-4, Mandarah, Anshas, and Al-Salam) were utilized in this study. A total of 200 chicks for each strain were brought from Fayoum Poultry Station, Egypt. Three replicates were used. The one-day-old chicks were wing-banded, intermingled, and brooded (10 chicks/m^2) in floor pens. This process was carried out in a conventional-type house, placed in floor-rearing pens in a conventional-type house until reaching 12 weeks of age.

Incubation and ration

Chicks were incubated at 35°C from hatch to 3 days of age, with a gradual reduction to 32°C by day 7. Subsequently, the temperature was decreased by 2°C per week until reaching 24-25°C by week 5. Humidity was at 45-55% during the experimental period. The chicks were provided with a diet containing 22-23% crude protein (CP) and 2800 Kcal metabolizable energy (ME)/ kg from hatch to 4 weeks. From week 4 to week 12, the diet included 19-20% CP and 3100 Kcal ME/ kg. Water and feed were available *ad libitum*. The lighting period was 24 hours/day. All chickens were vaccinated according to the vaccination program described by Nassar et al. (2019). The chicks were vaccinated against Newcastle disease at day 7 (eye drop, Hitchner, Nobilis®, The Netherlands), day 10 (sub-cutaneous injection with Newcastle inactivated vaccine, Nobilis®, USA), and day 21 (eye drop, La Sota strain, Nobilis®, The Netherlands). Additionally, vaccinations against infectious bursal disease occurred on days 14 and 24 (eye drops) using a Gumboro D-78 strain (Nobilis®, The Netherlands) and against the avian influenza virus using the sub-cutaneous injection of H5N2 (Nobilis®, The Netherlands) inactivated vaccine at the first week of age.

Phenotypic measurements

The parental and progeny chicken populations were weighed at hatch and then biweekly up to 12 weeks of age individually. Biweekly body weight gains and growth

rates were then calculated. The growth rate was calculated using the Formula 1.

(Formula 1)
Growth rate = Body weight gain / Average of both weights at first and end period × 100.

Sampling and DNA processing

At 8 weeks of age, blood samples (3 ml/ chicken) were randomly collected from a total of 100 chickens representing four local Egyptian chicken strains, with 25 from each strain. The blood was collected in tubes containing ethylene diamine tetra acetic acid (EDTA) as an anticoagulant and kept at -20°C. Genomic DNA was extracted using 200 µl of each sample according to Thermo Scientific Gene JET Whole Blood DNA Purification Kit (Paisley PA4 9RF, UK). The DNA quality and quantity were determined using UV-spectrophotometer, the UV spectrum at 260 nm and 280 nm (FLUOstar OPTIMA F-Microplate Fluorimeter, Germany) using the Formula 2 described by Sambrook et al. (1989).

(Formula 2)
DNA concentration (µg / µl) = A260 x 400 x 0.05

Microsatellites and polymerase chain reaction program

Seven Microsatellite markers related to chicken performance traits were used based on information available in the gene bank database. Details of microsatellite markers are shown in Table 1. The conditions and program of PCR were described by Ramadan et al. (2018).

The PCR products were electrophoresed at 100 V on a 2% Agarose gel and visualized by staining with ethidium bromide. Sambrook et al. (1989) used the procedure for the allele separation using 8% acrylamide gel.

Table 1. Microsatellite primer codes, sequences, and distribution in chicken chromosomes

Marker	Chromosome number	Forward primer	Reverse primer	References
ADL0158	10	TGG CAT GGT TGA GGA ATA CA	TAG GTG CTG CAC TGG AAA TC	(Das et al., 2015)
ADL0273	Z	GCC ATA CAT GAC AAT AGA GG	TGG TAG ATG CTG AGA GGT GT	(Goto et al., 2016)
ADL0292	5	CCA AAT CAG GCA AAA CTT CT	AAA TGG CCT AAG GAT GAG GA	(Choi et al., 2015)
LEI0079	1	AGGCTCCTGAATGAATGCATC	TCATTATCCTTGTGTGAAACTG	(Podis et al., 2013)
LEI0094	4	GATCTCACCAGTATGAGCTGC	TCTCACACTGTAACACAGTGC	(Cho et al., 2020)
MCW0064	8	CTTCAAGAGCCATAGGTGGTCT	TCTCAGCACTACAAAATACACAGG	(Zhou et al., 2006)
MCW0300	27	CAGAGAAACGTGCATGTGGAC	TGTGCACATTTCTCTGCTGAC	(Ambo et al., 2009)

ADL: Avian Disease and Oncology Laboratory, Michigan State University Lansing, LEI: University of Leicester, Leicester, UK, East, MCW: Microsatellite chicken Wageningen, Netherlands

Genotyping

The genotype of chickens was described by Ramadan et al. (2018). Convert version 1.3.1 was used to prepare input files compatible with various genetic software packages, as suggested by Glaubitz (2004). Heterozygosity (H) was estimated using POPGENE 3.2 software package, while PIC was determined using CERVUS 3.0 software. Sysat 7.0 software was employed to draw the dendrogram presentations (Yeh et al., 1999).

Statistical analysis

The Xlstat software, a general linear model XLSTAT 2017, was used for data analysis as a two-way analysis of variance. The main effects were line and sex. The traits analyzed included body weights at hatch, 2, 4, 6, 8, and 12 weeks of age using the following model.

$Yijk= \mu + Li + Sj + LSij + eijk$

Where, Yijk is the k_{th} observation of the j^{th} sex within the i^{th} line, µ denotes the overall mean, Li accounts for the effect of the i^{th} line, Sj determines the effect of the j^{th} sex, Sj refers to the effect of the j^{th} sex, LSij signifies the interaction between the i^{th} line and the j^{th} sex, and Eijk is the random error.

All data are presented as least square means ± standard deviations. Mean values were separated when significance existed, using Duncan (1955). P value less than 0.05 was considered statistically significant.

Genetic structure and the admixture degree are defined using the Baysian algorithm implanted by the STRUCTURE software v.2.3.4 (Pritchard et al., 2000). To infer the ancestral population number, 10 independent runs were achieved for each K value from 2 to 6. For all runs, the admixture models had a burn-in period of 50,000 repeats, followed by 100,000 repeats of the Markov Chain

Monte Carlo algorithm. The Structure Harvester website implementing the Evanno method was used to identify the K value that fits the maximal value of L(k) of the given data. Structure software is a tool that uses a systematic Bayesian clustering approach aiming at defining the cluster number of individuals on their genotypes at multiple loci using Markov Chain Monte Carlo (MCMC) estimation. The MCMC begins by randomly assigning individuals to a pre-determined number of groups. Variant frequencies are estimated in each group, and individuals are re-assigned based on those frequency estimates (Earl and vonHoldt, 2012).

RESULTS AND DISCUSSION

Lest square Means and standard deviation of body weight for hatch, 2, 4, 6, 8, and 12 weeks of age for the four chicken lines (males and females) are shown in Table 2. Al-Salam strain had a significantly higher body weight than the other strains until 12 weeks of age among the four lines of Egyptian local chickens (Table 2, $p \leq 0.05$). Al-Salam chickens were shown to have the highest body weight at hatch, 2 and 12 weeks of age (35.95, 110.80, and 906.60 g, respectively), followed by the Mandarah strain indicating an increase in body weight at 12 weeks of age (879 g), followed by Anshas chickens (826.52 g). On the other hand, Dokki-4 chickens recorded the lowest body weight at 4, 6, 8, 10, and 12 weeks of age (33.45, 89.26, 154.75, 244.45, 458.05, and 806.26 g, $p \leq 0.05$). These findings align with similar results reported in various studies, emphasizing the impact of breed variations on body weight (Ajayi and Ejiofor, 2009; Taha et al., 2012).

The findings indicated significant sexual dimorphism in body weight at all ages studied, except at hatch. The body weights of males were significantly higher than females from 2 to 12 weeks of age ($p \leq 0.05$).

At 12 weeks of age, males had higher body weight (884.82 g) than females (814.73 g), and the differences were statistically significant (Table 2, $p \leq 0.05$). This aligns with findings by Rashed (2012), who reported significant sexual dimorphism in body weights at all ages, excluding hatch time. The body weight of males from 2 to 19 weeks of age was significantly higher than that of females. These results agree with previous reports by Ramadan et al. (2014a; 2019). Compared to females, the data analysis indicated that males had higher weights in both CairoB-2 selected chickens and the control group.

Table 3 shows the genetic variability using seven microsatellite markers in four chicken strains in terms of the observed allele numbers, observed heterozygosity (Ho), expected heterozygosity (He), and polymorphic information content (PIC). Microsatellite markers are used as valuable tools to improve the chickens' performance, biodiversity within and among breeds, and breeding plan programs (FAO, 2016). Association among microsatellite markers and body weight traits were observed with age in the four Egyptian chickens. Previous studies demonstrated similar results, indicating the selected chicken strain had more alleles than the control line (Nassar et al., 2013; Ramadan et al., 2014a,b). Similarly, EL-Gendy and Hela (2014) stated that a genetically improved strain (CE1) had a higher number of alleles (5.72) than the control line (2.35).

According to Table 3, a total of 68 alleles were observed from 7 loci in four chicken strains, with a mean of 9.71. The mean values for Ho, He, and PIC were 0.799, 0.358, and 0.707, respectively. The highest number of alleles was 14 alleles at locus MCW006, followed by 13 alleles at locus LEI0094, 10 alleles at loci ADL0158 and ADL0292, and 8 alleles at loci LEI0079. However, the lowest number of alleles was 5 at locus ADL0273.

The Ho values were generally less than the expected ones in the four Egyptian chicken strains for 7 loci. The highest Ho value was 0.799, whereas the average He was 0.358. The greatest Ho value was 0.838 at locus MCW006 and 0.508 at locus MCW030. The lowest values of both Ho and He were The locus with the maximum Ho was LEI0094 (0.816), followed by ADL0292 (0.734), LEI0079 and MCW0300(both at 0.701), MCW0064 (0.700), ADL0158 (0.683). The average polymorphic information content was 0.707, and the minimal polymorphic information content was 0.614 at locus ADL0273. Comparing the genetic diversity observed in Egyptian chickens in this experiment, similar patterns were found in Korean chickens by Seo et al. (2017), where Ho ranged from 0.709 to 0.882, He ranged from 0.466 to 0.852, and PIC ranged from 0.648 to 0.865. While in 3 breeds reared in Egypt, El-sayed et al. (2021) found that the Ho ranged from 0.1 to 0.85, He ranged from 0.42 to 0.71, and PIC ranged from 0.4 to 0.69. Rashid et al. (2020) detected 171 alleles using 16 microsatellite markers and the average of alleles was reported 10.7, where the mean of Ho was 0.669, He was 0.71, and PIC was 0.749.

The sample size, number of observed alleles, Ho, He, and PIC content for the four Egyptian local chickens using seven microsatellites are shown in Table 4. The Mandarah strain had the highest observed number of alleles (5.37), followed by the Al-Salam strain (3.48 alleles), Anshas strain (3.35 alleles), and Dokki strain (3.12 alleles). The same trend was observed for He. The Mandarah strain had the highest He

(0.809), followed by the Al-Salam strain (0.724), Anshas strain (0.708), and Dokki strain (0.604). The He values were generally higher than Ho values across the four chicken strains and the seven loes. The Mandarah strain had the highest Ho (0.435), followed by the Anshas strain (0.361), Dokki strain (0.285), and Al-Salam strain (0.265).

Table 2. Live body weight (g) least square mean and standard error at different ages of four lines of local chickens

Trait / S.O.V / Strain	Hatch	2	4	6	8	12
Mandarah	33.10^{b}	100.05^{b}	212.85^{b}	295.40^{c}	580.85^{c}	879.00^{b}
Al-Salam	35.95^{a}	110.80^{a}	225.55^{a}	359.02^{a}	599.15^{a}	906.60^{a}
Dokki-4	33.45^{b}	89.26^{b}	154.75^{d}	244.45^{d}	458.05^{d}	806.80^{d}
Anshas	35.34^{a}	110.38^{a}	161.35^{c}	349.20^{b}	590.99^{b}	826.52^{c}
SE	0.38	0.36	0.41	0.41	0.40	0.40
Sex						
Male	34.89^{a}	107.28^{a}	209.94^{a}	341.05^{a}	416.72^{a}	889.52^{a}
Female	34.58^{a}	97.96^{b}	194.75^{b}	283.08^{b}	500.80^{b}	814.73^{b}
Standard error	0.27	0.25	0.3	0.29	0.3	0.28
Strain*Sex						
Mandarah♂	34.30^{b}	102.40^{c}	221.00^{b}	335.90^{c}	592.60^{c}	889.50^{c}
Mandarah♀	34.10b^{c}	97.70^{d}	204.70^{f}	254.90^{f}	569.10^{e}	868.50^{d}
El-Salam ♂	36.10^{a}	119.70^{a}	235.58^{a}	359.02^{b}	639.40^{a}	914.70^{a}
El-Salam ♀	35.60^{ab}	108.90^{b}	215.52^{c}	359.03^{b}	558.90^{f}	898.50^{b}
Dokki-4 ♂	34.10^{bc}	102.30^{c}	169.50^{g}	263.40^{e}	576.70^{d}	864.90^{e}
Dokki-4 ♀	32.80^{c}	76.23^{e}	150.00^{h}	225.50^{g}	339.40^{g}	748.70^{g}
Anshas ♂	34.86^{ab}	111.74^{a}	213.70^{d}	405.90^{a}	622.19^{b}	889.01^{c}
Anshas ♀	35.83^{ab}	109.02^{b}	208.80^{e}	301.90^{d}	559.80^{f}	764.03^{f}
Standard error	0.54	0.51	0.58	0.59	0.6	0.54
Probability						
Strain	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Sex	0.432	0.0001	0.0001	0.0001	0.0001	0.0001
Strain*Sex	0.023	0.0001	0.0001	0.0001	0.0001	0.0001

N: 150 per sex within the line; ^{abcd}: Means, within age and different chicken strains (S.O.V), with different superscripts are significantly different ($p \leq 0.05$).

Table 3. The statistics for a microsatellite profile, observed allele, the observed and expected heterozygosity, polymorphic information content for seven microsatellites in Egyptian local chicken

Locus	NA	H_e	H_o	PIC
ADL0273	5	0.701	0	0.614
ADL0158	10	0.761	0.459	0.683
LEI0094	13	0.837	0.491	0.816
MCW0300	8	0.816	0.508	0.701
ADL0292	10	0.832	0.344	0.734
LEI0079	8	0.806	0.311	0.701
MCW0064	14	0.838	0.393	0.700
Total	68	5.591	2.506	4.949
Mean	9.71	0.799	0.358	0.707
SD	3.09	0.05	0.17	0.056

Na: Observed number of alleles content; Ho: Observed heterozygosity; He: Expected heterozygosity; PIC: Polymorphic information; ADL: Avian Disease and Oncology Laboratory, Michigan State University Lansing, USA; LEI: University of Leicester, Leicester, UK, East; MCW: Microsatellite chicken Wageningen, The Netherlands.

Table 4. The sample size, number of observed alleles, the observed (Ho) and expected (He) heterozygosity, polymorphic information content, mean (SD) for four Egyptian local chickens using seven microsatellites

Population	Sample size	Na	He	Ho	PIC
Mandarah	42	5.37 ± 2.26	0.809 ± 0.07	0.435 ± 0.22	0.722 ± 0.05
Al-Salam	42	3.48 ± 1.06	0.724 ± 0.10	0.265 ± 0.21	0.644 ± 0.07
Dokki	42	3.12 ± 1.66	0.604 ± 0.28	0.285 ± 0.29	0.559 ± 0.24
Anshas	42	3.35 ± 0.79	0.708 ± 0.07	0.361 ± 0.23	0.602 ± 0.09

Na: Observed number of alleles, Ho: Observed heterozygosity, He: Expected heterozygosity, PIC: Polymorphic information content, SD: Standard deviation

Table 5. The genetic differentiation among different chicken strains.

Strains	Dokki-4	Anshas	El-salam	Manarah
Dokki-4	****	0.3944	0.2236	0.0481
Anshas	0.9303	****	0.3579	0.0305
El-salam	1.4977	1.0275	****	0.2929
Mandarah	3.0338	3.4894	1.2279	****

Note: The genetic differentiation among different chicken strains. Nei's genetic identity (above diagonal) and genetic distance (below diagonal). ****: Not evaluated.

Table 6. Correlation matrix (Pearson) between the seven microsatellite markers and different ages of four local Egyptian chickens

Variables	Hatch	2-week	4-week	6-week	8-week	12-week	ADL0273	ADL0158	LEI0094	MCW0300	ADL0292	LEI0079	MCW0064
hatch	1	0.74	0.55	0.67	0.49	0.34	-0.28	-0.22	-0.21	-0.22	-0.28	-0.19	-0.18
2-week	0.74	1	0.81	0.79	0.87	0.64	-0.08	-0.09	-0.02	0.06	-0.11	-0.09	0.06
4-week	0.55	0.81	1	0.77	0.81	0.55	0.26	0.37	0.19	0.40	0.35	0.38	0.36
6-week	0.67	0.79	0.77	1	0.64	0.40	-0.30	-0.21	-0.31	-0.14	-0.24	-0.17	-0.15
8-week	0.49	0.87	0.81	0.64	1	0.76	0.19	0.19	0.22	0.35	0.16	0.10	0.26
12-week	0.34	0.64	0.55	0.40	0.76	1	-0.03	-0.01	-0.08	0.01	0.02	-0.18	0.04
ADL0273	-0.28	-0.08	0.26	-0.30	0.19	-0.03	1	0.92	0.95	0.93	0.95	0.84	0.96
ADL0158	-0.22	-0.09	0.37	-0.21	0.19	-0.01	0.92	1	0.8363	0.87	0.97	0.94	0.81
LEI0094	-0.21	-0.02	0.19	-0.31	0.22	-0.08	0.95	0.84	1	0.86	0.83	0.73	0.90
MCW0300	-0.22	0.06	0.40	-0.14	0.35	0.01	0.93	0.87	0.86	1	0.91	0.86	0.89
ADL0292	-0.28	-0.11	0.35	-0.24	0.16	0.02	0.95	0.97	0.83	0.91	1	0.91	0.90
LEI0079	-0.19	-0.09	0.38	-0.17	0.10	-0.18	0.84	0.94	0.73	0.86	0.91	1	0.73
MCW0064	-0.18	0.06	0.36	-0.15	0.26	0.04	0.96	0.81	0.90	0.89	0.89	0.73	1

ADL: Avian Disease and Oncology Laboratory, Michigan State University Lansing, LEI: University of Leicester, Leicester, UK, East, MCW: Microsatellite chicken Wageningen, The Netherlands

The highest PIC value was 0.722 for the Mandarah strain, followed by 0.644 for the Al-Salam strain, and 0.602 for the Anshas strain. Meanwhile, the lowest PIC value was 0.559 for the Dokki strain. Mandarah strain had the highest values of allele numbers, He, Ho, and PIC (5.37, 0.809, 0.435, and 0.722, respectively). Meanwhile, the Dokki strain had the lowest values of the observed alleles, the He and PIC (3.12, 0.604, and 0.559, respectively). Al-Salam strain had the lowest Ho value (0.265). The findings of the current study on allelic numbers, Ho, and He are in line with values reported by Pirany et al. (2007) and Dorji et al. (2012), who found that the mean of allele numbers was 10.33 and 14.17 in Indian and Bhutanese, respectively. On the other hand, Van Marle-Koster and Nel (2000) indicated a moderately lower mean of allele numbers than the present results, ranging

from 2.3 to 4.3 in five African chicken populations. The estimated means of the allele numbers, Ho, He, and PIC of the four Egyptian strains might be linked to the results in diverse chicken lines (Rajkumar et al., 2007). The variances in genetic polymorphism may be due to diversity in genetic base, breed, line, and strain, likewise using different microsatellite markers.

The genetic differentiation among different chicken populations was analyzed by the molecular procedure based on the identity and distance matrix (Table 5). The closest genetic makeup was observed between Dokki-4 and Anshas, which had an identity score of 0.3944 and a genetic distance of 0.9303. The same trend was observed between the Al-Salam and Mandarah chickens, who had an identity score of 0.0481 and a genetic distance of 1.2279. Phylogenies were constructed using the neighbor-joining procedure and genetic distance DA (Figure 1). The multiple alignments of the concatenated 7 loci aggregated the Dokki-4 and Anshas chickens closely in one cluster, whereas Al-Salam and Mandarah chickens joined in a different close cluster branch. Both were supported by 100% bootstrap confidence.

The genetic structure analysis was performed to identify the uniformity of the four breeds and investigate their admixture and genetic differentiation. The structure analysis showed that the most potential number of derived clusters was K = 3. The delta k value was 56.3, where the highest average of link polt posterior probability (Ln Pr) of K, termed X. Ln Pr (x/k) was shown at K = 3, and then it dropped subsequently (Figure 2). Therefore, it is expected that K = 3 is the most probable number of ancestral stains, contributing to the observed genetic diversity in the four given strains (Figure 3). Based on the individual q values, Dokki and Mandara chickens were assigned to an independent cluster 1, and the average genetic distance between their chickens and other clusters was 2.28, while the average genetic distance between them was 0.10 (Figure 4). Dokki chickens had an individual genotype membership (76.9%) within cluster 1, while the Mandara breed was a heterogeneous breed, where the percentage of its assigned chickens had a low proportion membership (66.7%). This might happen because they have multiple origins or can reflect gene pool dilution due to the inbreeding or crossbreeding of Egyptian chickens. On the other hand, Anshas chickens could be in a second independent cluster 2 with 82% of its membership and the mean genetic distance of 1.87 among its chickens and other clusters. Al-Salam breed chickens were designated to cluster 3 and the mean genetic distance among its chickens and other clusters was 1.29. Al-Salam breed was highly homogenous, showing very high proportions of individual chicken memberships (99%). Al-Salam strain chickens are heritably diverse and retain high genetic diversity. The seven microsatellite markers studied had a significantly positive association with the body weight of different strains at 8 weeks of age (Table 6, $p < 0.05$). Studies indicated that microsatellites markers MCW0010 (Liu et al., 2007; Zhang et al., 2008), MCW0018 (Sewalem et al., 2002; Navarro et al., 2005), LEI0079 (Liu et al., 2007), c3-77696549 and c5-105790 (Uemoto et al., 2009) were associated with chicken body weight at 6 weeks of age. These findings harmonize with the outcomes of Uemoto et al. (2009), who found significant positive correlation coefficients among MS c3-77696549 and c5-105790 and practically all their studied traits (body, carcass, breast, and leg meat weights).

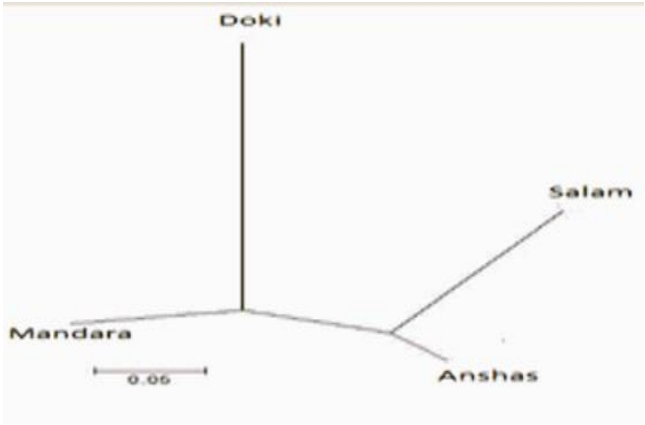

Figure 1. Dendrogram trees between four chicken genotypes using the nearest neighbor hierarchical cluster method

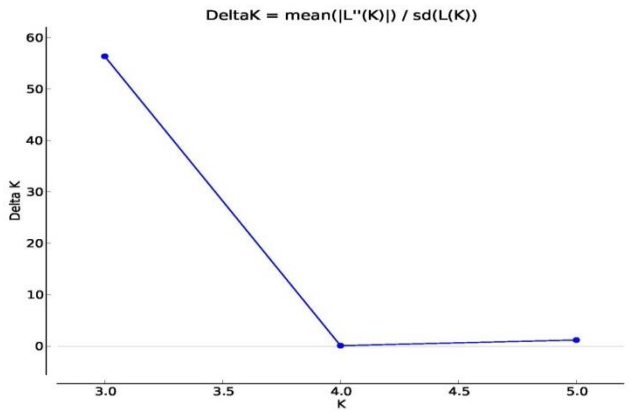

Figure 2. The genetic structure analysis to identify the uniformity and genetic differentiation of the breeds. The modal value of this distribution is the true K (=3), the uppermost level of the structure.

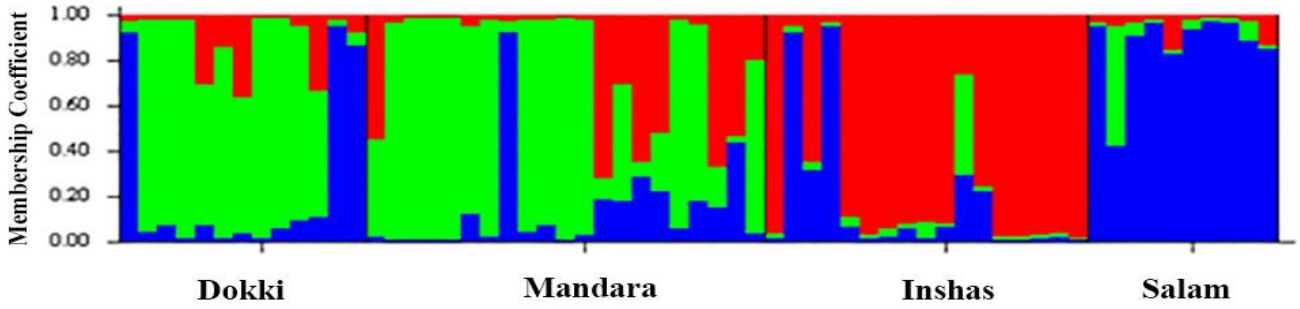

Figure 3. Clustering assignment of the four Egyptian breeds defined by STRUCTURE analyses. Each chicken is represented as a vertical rectangle that is divided into segments whose color and size correspond to the relative proportion of the chicken genome of a particular cluster. The inferred clusters were Dokki (green), Mandara (blue), and Anshas (red).

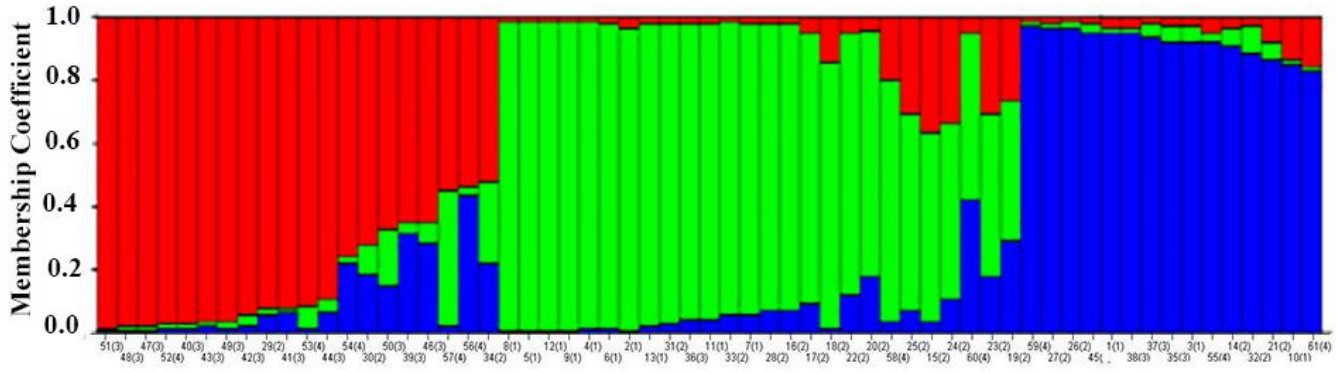

Figure 4. Graphical representation of individual genotype membership coefficients (q) in each of 3 the clusters: Anshas cluster (red), Dokki cluster (green), and Mandara (blue). Anshas and half of Al-Salam's chickens are in a group and Dokki and half of Al-Salam chickens lie in the group.

CONCLUSION

From all results of the current study, it is recommended to use the 7-microsatellite marker to study genetic biodiversity and selection and crossing program for the four local Egyptian chickens. These seven markers showed the highest significant correlation coefficients with body weight at 8 weeks of age. The findings of the present study offer valuable insights for identifying superior local chickens based on genotype characteristics. These insights provide new clues for further studies on breeding programs in local Egyptian chicken strains.

DECLARATIONS

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Author's contribution

This study was done in collaboration with all authors. Karima Fathy Mahrous[2] and Esteftah Mohamed El-Komy conceived the idea, designed the experiments and supervised the research. NIA, HRD, EME and GSR, performed the experiments and co-wrote the paper. LMS performed the experiments. HRD, EME and GSR analyzed the data. KFM critically revised the manuscript. All authors read and approved the final manuscript.

Competing interests

There is no competing interest to declare.

Availability of data and materials

All data generated or analyzed during the current study are included in this published article.

Ethical considerations

This article has been checked by all authors for ethical issues such as plagiarism, publication consent, misconduct,

data fabrication and/or falsification, double publication and/or submission, and redundancy aspects before submission.

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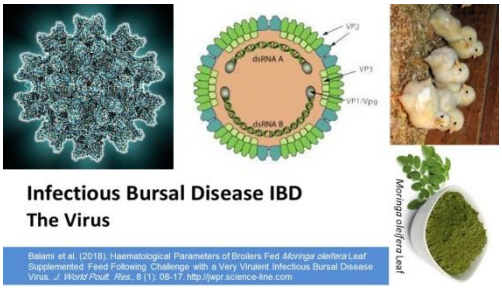

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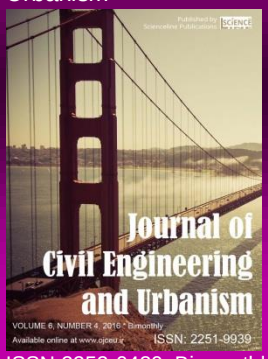

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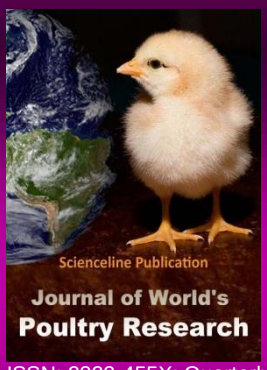

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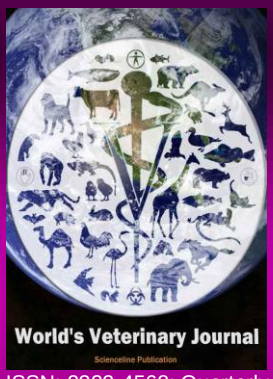

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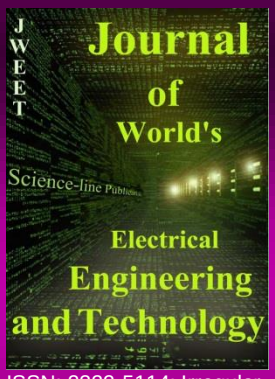

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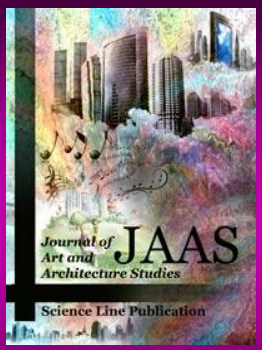

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