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MASTER OF SCIENCE IN VETERINARY EPIDEMIOLOGY

ASSESSING HERD IMMUNITY POTENTIALS AGAINST INFECTIOUS BURSAL DISEASE VIRUS BEFORE AND AFTER VACCINATION, VITAMIN AND PROTEIN TREATMENTS IN BROILER CHICKEN

MVSc. THESIS

BY
HELEN AKLILU REDIE

JUNE, 2023
BISHOFTU, ETHIOPIA

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BY

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A Thesis Submitted to Addis Ababa University College of Veterinary Medicine and Agriculture in Partial Fulfillment of The Requirements for The Degree of Master of Veterinary Science in Veterinary Epidemiology.

JUNE, 2023

BISHOFTU, ETHIOPIA

Addis Ababa University College of Veterinary Medicine and Agriculture

Department of Veterinary Clinical Studies

As MVSc research advisors, we here certify that we have read and evaluated the Thesis prepared under my guidance by Helen Aklilu entitled: "**Assessing Herd Immunity Potentials against Infectious Bursal Disease Virus before and after Vaccination, Vitamin and Protein Treatments in Broiler Chicken**. We recommend that it can be submitted as fulfilling the MVSc Thesis requirement.

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STATEMENT OF AUTHOR

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LIST OF ABBREVIATIONS

BALT	Bronchial Associated Lymphoid Tissue
BF	Bursa of Fabricius
CALT	Conjunctiva Associated Lymphoid Tissue
CSA	Center of Statistical Authority
dsRNA	Double-stranded Ribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FAO	Food and Agriculture Organization
FAOSTAT	FAO Statistical databases
GALT	Gut Associated Lymphoid Tissue
IBD	Infectious Bursal Disease
IBDV	Infectious Bursal Disease Virus
OIE	Office International Des Epizooties
PCR	Polymerase Chain Reaction
SNNPR	Southern Nations, Nationalities and Peoples Region
USDA	United States Department of Agriculture

ABSTRACT

The poultry sector of Ethiopia is at infant stage and constrained by lack of feed and emerging infectious diseases. Completely randomized design was used for the current study and it was conducted on 135 Cobb 500 broiler chicks with the aims of to measure maternally derived infectious bursal disease virus antibody transfer, to evaluate infectious bursal disease antibody titer level before and after vaccination and to determine level of humoral and cellular immunity response to vitamin and protein based feed supplements. A total of 90 sera samples were collected in three consecutive bleeding times at ten day interval and the sera samples were examined using commercial proFLOK IBD ELISA kit for antibody detection. Among those samples, 35 (38.8%) reacted positive to antibody against infectious bursal disease virus. There was difference in detection of antibody level across the sampling dates. Higher number of reactors were noted from day eight samples (77.78%), followed by samples from day 28th (36%) and the least reactors were recorded during the second bleeding on 18th day (13.16%). The mean titer level at the three bleeding times had statistically significant difference (P=0.000). The main factor contributing for this variation of antibody titer in the experimental units was sampling date, which determined to be statistically significant predictive variable. The mean antibody titer among chickens received supplement A and supplement B were higher as compared to the mean titer of the control group. The mean titer level recorded in group that was treated with supplement A was significantly higher compared to group 2 and the control. This may imply supplements with amino acids might have a better immune boosting effect. Regarding the effect of the supplements on cellular immunity, the mean count of lymphocyte (P= 0.047) and monocyte (P= 0.033) had statistically significant difference among the experimental groups. Though, the overall effect of supplements on humoral and cellular response noted in present study was positive, bleeding time (age) remained key factor for herd immunity. Improving herd immunity potential of young poultry population through effective immunization program and proper nutrition could help in prevention and mitigation the impact of IBD in poultry farm. Therefore, timely vaccination of parent and chicks is recommended. Moreover, provision of multivitamin and amino acid based supplements can help to reduce impact of infectious bursal disease outbreak.

Keywords: Antibody Titer; Chicken; ELISA Test; Infectious Bursal Disease; Supplement

1. INTRODUCTION

Poultry production is one segment of livestock production and an important agricultural activity in almost all developing communities in Africa and elsewhere, and also it is the fastest growing components of global agricultural demands because it has a peculiar privilege to contribute to the sector as well as to improve the living standards of the poor livestock keepers (Fisseha, 2009; Moyo and Swanepoel, 2010). Ethiopia has about 59.5 million chicken populations (CSA, 2017), from this population of chicken, 99% are reared under the management of traditional backyard system while 1% is under intensive management system (CSA, 2009).

The poultry sector of Ethiopia is at infant stage and constrained by lack of feed and emerging infectious diseases (Dessie *et al*., 2013; Getu and Birhan, 2014). Infectious bursal disease (IBD) is one of the emerging disease following with the poultry farming intensification and introduction of exotic breeds which have less tolerance to heat stress and disease challenge (Zeleke *et al*., 2005). IBD is the one that become to cause frequent outbreaks and a serious threat and a challenge to the infantile poultry industry (Mazengia, 2012).

Infectious bursal disease also called Gumboro disease is highly contagious viral disease of young chickens with a characteristics of destruction of cells of bursa of fabricius with severe immunosuppression (<3 weeks) and impaired growth of young chickens (Chakman, 2015; Beenish *et al*., 2016). Primary target organ of the virus is bursa of fabricius which is functional and well developed in young chickens. In clinical cases, it is associated with mortalities, hemorrhages and bursal damage as well (Minalu *et al*., 2015). Lymphoid depletion and the final destruction of the bursa results in sever immunosuppression hence increase susceptibility to other infectious diseases. IBDV cause heavy mortality and morbidity and the emergence of antigenic variant as well as very virulent strains in vaccinated flocks considerably encouraged research efforts on IBD and IBDV (Yao and Shijun, 2017). IBDV has two serotypes (serotypes 1 and 2). Virus strains in serotype 1 are pathogenic for chickens, and causes clinical disease (Akinaw, 2021).

Researchers conducted in different parts of Ethiopia indicated that IBD is a significant threat on backyard and commercial poultry productions (Zeleke *et al.,* 2005a and b; Degefu *et al.,* 2010; Kassa and Molla, 2012; Tulu, 2019). Understanding the epidemiology and designing comprehensive mitigation strategies for IBD is essential to safeguard the poultry production which is deeply embedded in the Ethiopian society, kept by all strata of society from the landless rural poor to rich (Wilson, 2010). Various agro-climatic conditions in our country helps most of the rural societies raised indigenous chicken under scavenging management system (Azage *et al*., 2010; Abiyu *et al*., 2019).

Prevention and control of IBD relying on appropriate management and vaccination measures, which are very interrelated practices. Vaccinating breeder flocks is the vital practice that helps the chickens to have efficient immunity to resist IBDV infection and facilitates high level maternal antibody transfer in to young chicks. The presence and persistence of maternally derived antibody interferes with vaccination time. Early vaccination of chicks post hatching may leads to vaccine neutralization by the maternal antibody. The booster vaccine of Gumboro disease had principal effect that strengthens the humoral response by harmonizing the neutralizing effect of maternally derived antibodies. These preventive measures are favored by the use of immune modulators, which are capable of promoting the functioning of the immune system with the aim to improve resistance to infections. Different research reports have shown that dietary manipulation of nutrients leads to immunoregulatory effects with the occurrence of infectious disease (Jankwoski *et al*., 2014; Shanker, 2020).

In Ethiopia, poultry health services especially to the backyard production system are very limited (Asfaw *et al* 2021). Promoting traditional knowledge and alternative disease management practice may help in overcoming the impact of highly infectious diseases, including IBD. Moreover, feed additives have been reported to reduce incidence of diseases through improving cellular and humoral immunity system (Ayalew *et al* 2022). Meanwhile, local poultry producers are seen to use amino acid, mineral and vitamins based growth promoting feed supplement to mitigate the impact of IBD and other diseases in their farm. Vitamins and amino acids are proved immune

enhancers that provide additional disease tolerance capacity to humans and animals (Li *et al* 2007, Montout *et al* 2021, Shakoor *et al* 2021, Camelia and Betty, 2022).

There are various kind of poultry feed supplements commercially available in Ethiopia. However, "NEOBRO" and "AD3 COMBAT" are the most commonly used feed supplements as growth promoter. The core active ingredients of NEOBRO are multivitamins and amino acids and for AD3COMBAT vitamins are the essential ingredients. This combination is very effective to promote growth of broilers. No study has assessed the potential use of these supplements as immune booster to improve chickens resistance to IBDV infection. To this end, the present study evaluated cellular and humoral immune responses variations among experimental chickens using IBD vaccine, and NEOBRO and AD3 COMBAT supplement as treatment variable under experimental setting.

General objective

- To evaluate the effects of vaccine, amino acids and vitamins on herd immunity of broiler chickens

Specific objectives

- To measure maternally derived IBDV antibody transfer to the offspring in broiler chickens
- To evaluate IBD antibody titer levels pre and post vaccination in broiler chickens
- To determine level of humoral and cellular immunity response to vitamin and protein based feed supplements

2. LITRATURE REVIEW

2.1. Etiology of IBD

Infectious bursal disease also known as Gumboro disease is a contagious economically important poultry disease. The etiological factor is infectious bursal disease virus which belongs to the genus avibirnaviruse (Nascimento *et al.*, 2017).

2.1.1. Taxonomy and virus strains

Infectious bursal disease virus is a double strand an RNA virus that belongs to the genus *Avibirna*virus of family *Birnaviridae* (Okwor *et al*., 2011; Teshome *et al.*, 2015). There are two serotypes of the virus, IBD virus serotype 1 and IBD virus serotype 2. Serotype 1 is an important pathogen that causes clinical disease of chickens. All commercial vaccines are prepared against this serotype (Van Den Berge *et al*., 2004; Dey *et al*., 2019). Very virulent strains of classical serotype 1 are now common and are causing serious disease in many poultry producing countries (Cubas-Gaona *et al*., 2018). Serotype 1 viruses replicate in the bursa of fabricus and cause clinical disease in chickens in which it damages the lymphoid organ bursa of fabricius and result in immunosuppression and increase the susceptibility of chicken to opportunistic secondary infections such as Marek's and Newcastle disease (Khan *et al*., 2011; Mahgoub, 2012).

2.1.2. Host preference

Infectious bursal disease virus is highly contagious and of major importance to the poultry industry worldwide. Serotype 1 strains are pathogenic in chickens, while serotype 2 lines are non-pathogenic. Although turkeys, ducks, guinea fowl, pheasants and ostriches can be infected, clinical case happens solely in chickens (Campbell, 2001). Young chickens are typically clinically affected. In fully susceptible flocks, mortality and morbidity associated with classic strain infections may range from 1-60% and up to 100% (Muller *et al*., 2003; Eterradossi and Saif, 2013) while, vvIBDV

strains cause mortality of 50-60% in laying hens, 25-30% in broilers (Van den Berg *et al*., 2000).

Despite the use of vaccine, in Ethiopia the occurrence of IBD in most parts of the country have wiped out large number of chickens in private commercial poultry farms and in government owned poultry farms (Zeleke *et al*., 2005; Woldemariam and Wossene, 2007).

2.2. Epidemiology of IBD

2.2.1. Worldwide Distribution

Infectious bursal disease was first reported by Cosgrove in 1962 as a particular disease affecting the bursa of Fabricius in chickens. The first cases were observed in the area of Gumboro, in Delaware in the Bunting farm of USA, which is the origin of the name, although the terms 'IBD' or 'infectious bursitis' are more accurate descriptions. Most regions of the USA affected by IBD between the years 1960 and 1964 then reached Europe in the years 1962 to 1971 (Dwight *et al*., 2005). It recognized in Middle East, Southern and Western Africa and India in the years 1966 to1974. Currently it become an international problem and occurs worldwide in the major poultry producer areas (Van Den Berge *et al*., 2004).

2.2.2. Status of IBD in Ethiopia

Very virulent IBDV (vvIBDV) strains are circulating in Ethiopia (Shegu *et al*., 2020). In 2002, advent and presence of IBD was first reported in Ethiopia at privately owned commercial poultry farm wherein 45-50% mortality rate was documented (Zeleke *et al.,* 2003) and diagnosed first in commercial poultry farm (Zeleke *et al.,* 2005b) thereafter in a government poultry multiplication center (Woldemariam and Wossene, 2007) and a commercial broiler farm (Chanie *et al.,* 2009) with serological tests. Different recent countrywide studies were reported IBDV seropositivity rates in chickens (Zeleke *et al.,* 2005*;* Chaka *et al*., 2012; Jenbreie *et al*., 2012; Tesfaheywet and Getnet, 2012).

Table 1: Reported prevalence of IBD in different parts of Ethiopia

Study area	Prevalence (%)	Authors
Gondar and West Gojam	73.5	Kassa and Molla, 2014
Bahir Dar	29.4	Mazengia *et al*., 2010
Eastern Ethiopia	83	Tadesse and Jenbere, 2014
Debre Brehan	94.7	AHY, 2011
Selected sites of Ethiopia	83.1	Jenberie et al., 2012
Mekele	45.05	Zegeye *et al*., 2015

Source*: (*Teshager, 2015; Tulu, 2019)

2.2.3. Transmission

Infected birds shade the virus in their dropping which contaminates water, feed and litter, where it persists and from where it commonly spreads (Minalu *et al*., 2015). The most common mode of infection is faeco-oral route. Also it may spread through contaminated equipment and aerosol route (Nafi'u *et al*., 2017; Tsegaye and Mersha, 2014). The disease spread very fast in deep liter management system due to free contact of the infected and non-infected birds and direct access to their droppings since the virus can survive for four months in contaminated bedding and premises (Benton *et al*., 1967; Aliyu *et al*., 2016). Distributing chickens from infected poultry multiplication centers to other farms, sharing farm utensils and uncontrolled human exit and entrance spreads Gumboro disease. It was reported that morbidity attributed to IBDV approaches 100% and mortality may be up to 20%-30%, but may also be 0% and also mortality will peak and recede usually in a period of 5-7 days post infection (OIE, 2012).

2.3. Pathogenesis

The virus replicates in gut associated macrophages and lymphoid cells after entering the host through oral ingestion or inhalation and causes primary viremia through portal circulation (Eterradossi and Saif, 2008). After primary viremia, the virus reaches to bursa of fabricius by 11-14hr post infection and after extensive replication in bursal follicles and B cells; the virus enters the bloodstream and cause secondary viremia which leads to disease and death (Van den Berg, 2000; OIE, 2004). Virus spreading to other lymphoid organs takes place mainly during vvIBDV infection of susceptible chickens (Eterradossi and Saif, 2008).

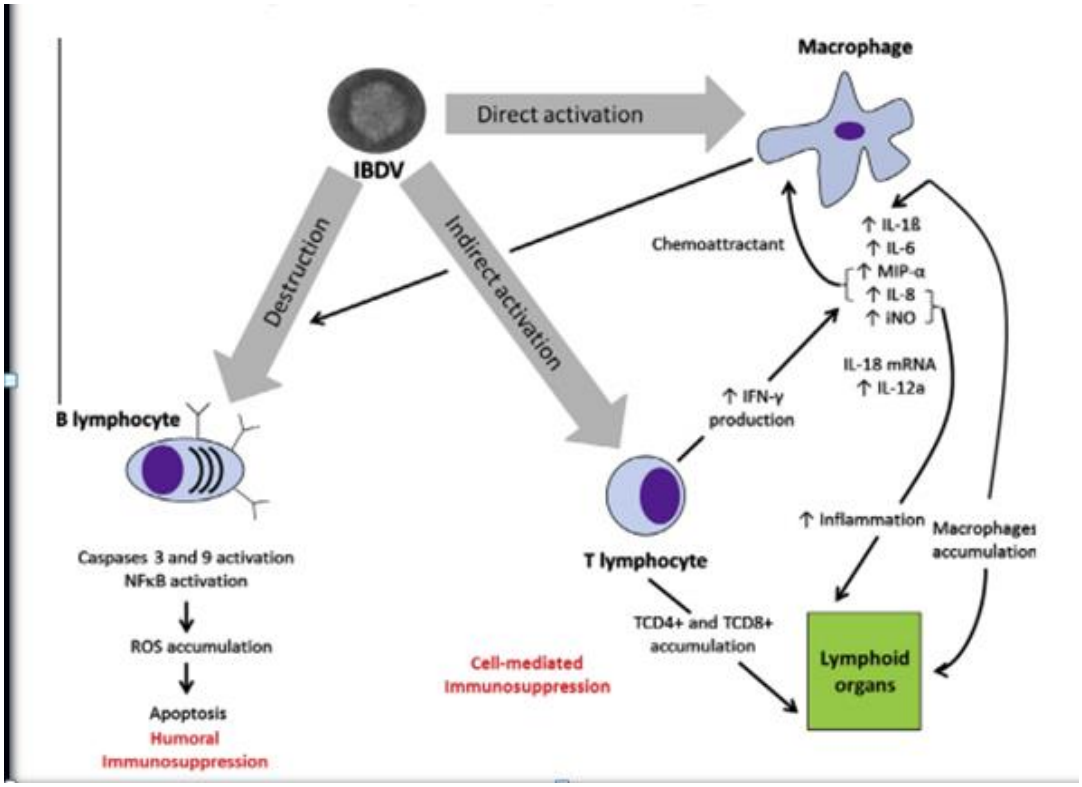

Figure 1: Pathogenesis pathway. ***Source****: (Akinaw, 2021)*

As early as 48 hr post infection, it induces prominent inflammation in the bursa of Fabricius. The primary immune response is persistently suppressed and the number of circulating IgM+ cells dramatically drops when the virus replicates during the acute lytic phase (Sharma *et al*., 2000). The necrotizing action of these viruses on the host tissues causes acute illness and death. Chickens that survived and recovered from this stage of the disease nevertheless have impaired immune systems, making them

vulnerable to opportunistic infections and inhibiting protective responses to vaccines against other pathogens (Van den Berg, 2004; Qin and Zheng, 2017).

2.4. Clinical signs

Severity of clinical signs and immunosuppression depends on the status of immunity of the chicken, age of the chickens, and the virulence of virus strain. Chickens infected at 3 to 6 weeks of age develop the most severe clinical disease within 2-3 days of exposure and lasts about 7 days. The clinical case characterized by acute onset of depression, trembling, white watery diarrhea, ruffled feathers, severe prostration, vent picking, vent feathers soiled with urates, anorexia, dehydration and elevate water consumption then severe atrophy of the organ occurred, and cause severe immunosuppression by destroying B lymphocyte cells, which leads to an increased susceptibility to other pathogens and reduce the growth rate of surviving animals (Zeleke *et al*., 2005; Aliyu *et al*., 2016). Both the clinical and sub-clinical forms of disease result in great economic loss due to its permanent damage on infected birds (Farooq *et al*., 2003).

2.5. Diagnosis

Infectious bursal disease is diagnosed by considering the flock's history, clinical sign and postmortem lesion. Chickens less than 3 weeks and greater than 6 weeks of age didn't express apparent signs (kegne and chanie, 2014). Clinical IBD can be recognized by means of a combination of feature signs and postmortem lesions. However, Differential and confirmatory diagnosis of the disease can be carried out by different laboratory techniques (Banda, 2002; Minalu *et al*., 2015; Sali, 2019). Subclinical IBD can be detected in the laboratory through demonstrating a humoral immune reaction or by identifying viral antigens or viral genome in tissues. In the absence of such exams, histological examination of bursae can be useful (OIE, 2016).

2.5.1. Laboratory diagnosis

The most common laboratory techniques to detect IBDV include cell culture, serology and molecular characterization. Serological tests such as AGID, ELISA, and VNT for detecting antibodies are used for monitoring vaccine responses and might be additional information for diagnosis of infection of unvaccinated flocks (OIE, 2012).

Periodic quantitative serologic test like ELISA is necessary to monitoring the immune status of chickens. The enzyme linked immuno sorbent assay (ELISA) is the most commonly used test for the detection and quantification of IBDV antibodies to check response to vaccination, natural infection and decay of maternal antibody titer. It is low-cost, easy, and quick which tests many samples simultaneously and is adaptive to automation to computer software (Lukert and Saif, 2003). The VN titers accurately correlate with protection of chickens against IBDV (Knoblich *et al*., 2000). However, these methods may not be as rapid and sensitive as molecular methods (Jackwood, 2018).

2.6. Prevention and Control

Management and Hygiene: IBDV is highly contagious and infectious viral disease. It resists to inactivation and survives persistently on poultry farms. Due to the integrated nature of commercial poultry operations, litter reuse and the possibility of interaction with free-living wild birds, the control of IBD in our country faces difficulties (AHA 2009). Therefore, avoiding strictly contact between farm to other farm, contaminated premises and utensils, free people entrance and exit, isolating infected birds in the farm are some of management concerns that help to prevent and control IBD (Van den Berg *et al*., 2000).

The use of therapeutic treatment has been reported to have no effect on the course of infection instead nutritional supplementation had good impact (Cosgrove 1962; Lukert and Saif, 2003).

Nutrition has impacts on body function in a multiple aspects Dietary constituents and different level of nutrients has an immune modulatory effect on the bird's immune response to different infectious diseases. Nutrition is vital for the improvement and effective functioning of the immune system (Dalgaard *et al*., 2018; Bartlett & Smith *et al.*, 2003). Now a day the consumers preference for antibiotic residue free poultry food sources, searching a potential alternatives are obligatory (Low *et al*., 2021).

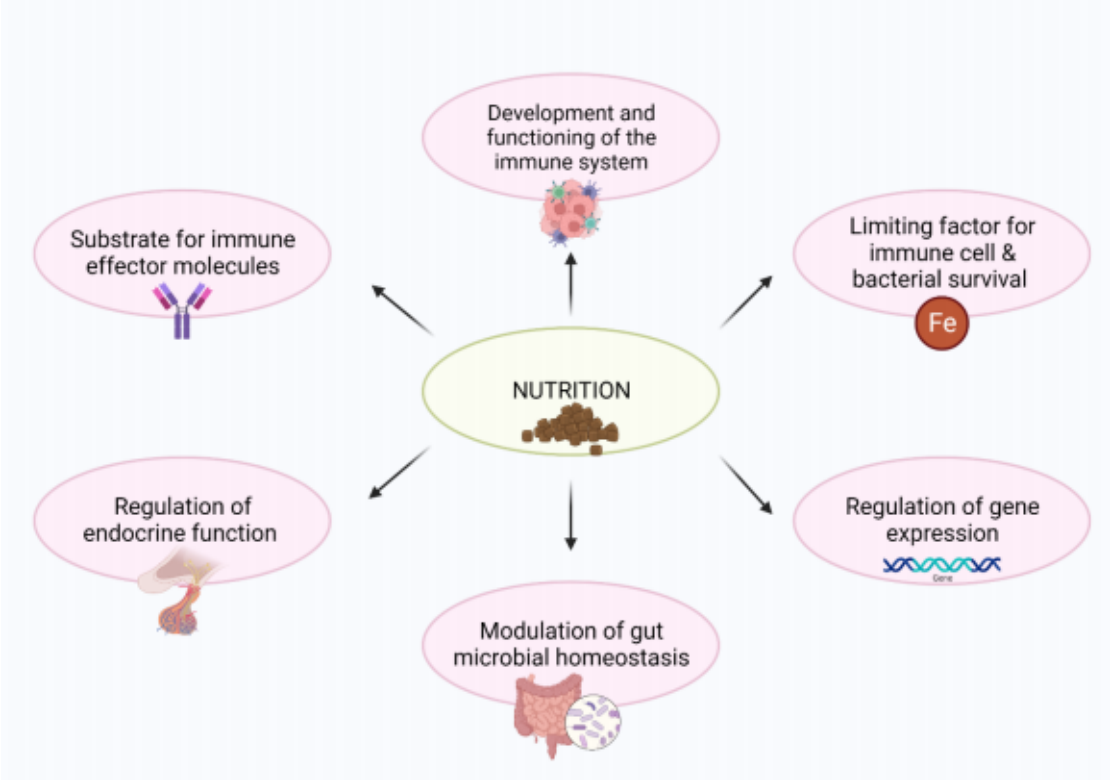

Figure 2: Impact of nutrition on immune system. **Source:** (Li *et al*., 2007)

Even though a strict bio-security program is vital for the prevention of IBDV infection, also vaccination is very important to prevent and reduce the incidence and impact of IBD in the poultry industry (Eterradossi and Saif, 2008). The unique characteristic of IBDV is ability to survive long time in the environment without inactivated by most disinfectants and environmental extremes, thus principally controlled by vaccination on optimal time schedule (Dacic *et al*., 2008; OIE, 2012). Depending on disease pattern, maternally derived antibody status, level of biosecurity, and vaccine availability, vaccines and vaccination programs vary greatly. Research

reports proved maternally derived antibodies against IBDV protected chicks for the early age (Dormitorio *et al*., 2007).

Live attenuated and killed vaccines are widely used vaccine types to control IBD. Classical and variant IBDV strains are live attenuated vaccines strains which differ by their virulence and antigenicity. Depending on virulence /residual virulence and the level of attenuation, vaccine strains are classified as mild, mild intermediate, intermediate, intermediate plus, or "hot (OIE, 2004; Islam, 2005; Camilotti, 2016). Live-attenuated vaccines are administered via drinking water which is favorable for mass vaccination and can induce strong humoral and cellular immunity. The frequent occurrence of outbreaks of IBD in several parts of the country despite vaccination suggests that there might be a mismatch between vaccines and field strains and vaccination schedule. (Rautenschlein *et al*., 2007; Hailemichael, 2016). Killed vaccines are not ideal for stimulating a primary antibody response; therefore, they used to boost chicks that have been immunized before or naturally infected by field strain (etteradossi and Saif, 2008).

2.7. Economic importance of IBD

The presence of disease might also limit opportunities in the market place, either regionally or internationally. IBD outbreak has impacts on different aspects of production and productivity. It might results in disruption of the enterprise and its regular marketing styles. Immunosuppressive viral diseases are a great concern for the poultry industry for several years. Indeed the re-emergence of IBDV in variant or highly virulent forms has been the cause of significant economic losses (Rautenschlein *et al*., 2001). IBDV has an economic impact not only due to specific mortality but also to the indirect losses as it causes severe immunosuppression that exposed chickens for other infection and vaccine failure. This results in retarded growth, poor weight gain low quality products. (Sharma *et al*., 2000; Naqi *et al*., 2001).

Furthermore the increase use of antibiotics and chemicals to fight against opportunistic infections is a major concern of human health, if we consider the risks

linked to the presence or residues in meat products, the release of residues into environment and increased antibiotic resistance (Marian, 2001). It can be stated that there is a significant variation in body weight in Gumboro affected broilers due to the existing and imposed vaccination program under farm condition and thus the imposed vaccination program should be recommended for use in farm condition to attain better body weight (Paul *et al*., 2004). Proper vaccination and maintenance of better hygiene and management should be ensured to increase productivity and profitability of broilers.

3. MATERIALS AND METHODS

3.1. Description of Study Area

An experimental study was conducted from November, 2022 to May 2023 in Bishoftu town Addis Ababa University-College of Veterinary Medicine and Agriculture poultry farm which prepared for experimental purpose with the aim of evaluating the effects of vaccine, amino acids and vitamins on herd immunity of broiler chickens. Bishoftu is a town where several commercial farms of different scale, hatcheries and breeding farms are found. It is located 47 km far from Addis Ababa in East direction at a latitude of 8° 45´08" N and longitude of 38° 58′42"E at altitude of 1,920 m above sea level. Bishoftu has an annual rainfall of 866mm and 84% humidity in the long rainy season (June to September). The mean annual maximum and minimum temperatures are 26°C and 14°C respectively, with mean relative humidity of 61.3% (NMSA, 2003).

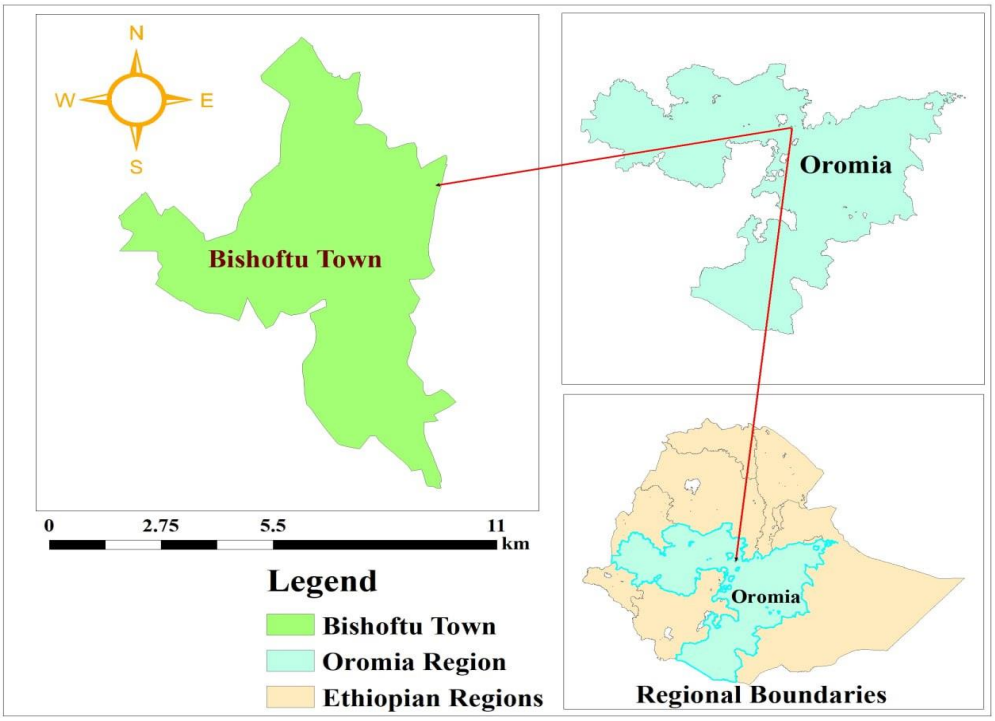

Figure 3: The study area, developed from Ethiopian shape files using QGIS software.

3.2. Preparation of experimental house and other utensils

The poultry shade and experimental pens were constructed by the college in which the roof and half of the wall is made up of iron sheet and the floor is concrete. The area of the experimental house was divided into 12 pens on one side and 10 pens on other side. This poultry house first swept very well then washed by water and allowed it to dry, then after the wall and floor was sprayed by caustic soda, kept for 5 days closed. Then the roof, wire mesh and floor were disinfected by bio-safe. Finally, the litter was distributed on the floor of all experimental pens and sprayed by bio-safe. The external environment around the house also cleaned by sweeping and sprayed by bio-safe and footbath prepared. The feeder and drinking materials was cleaned by clean water and soap daily to the end of the experiment.

3.3. Experimental animals and management

The target study populations were day old Cobb500 broiler chickens which had been bought from ELFORA agroindustry poultry farm. The chicks were randomly assigned into three experimental groups (group1: have chickens that received supplement A; group 2: chickens that received supplement B and Control group) and managed intensively. The chicks were brooded by 250 watt bulb (in each pens) hanging at the optimal height to ensure each chick get adequate heat and light. Heating was reduced gradually every week and at the finisher stage it totally stopped and just only light was supplied.

The chicks were vaccinated for Gumboro disease and Newcastle disease based on the schedule. The commercial feed (starter, grower, and finisher) which purchased from Alema Koudijs poultry feed producer was supplied to the chicks. From 20g/bird/day up to 160g/bird/day feed was offered twice a day and has free access to clean and fresh drinking water. For chickens in group 1 and group2 the supplements were given in 5g/10lit and 2ml/10lit drinking water respectively. Supplement A composed of multivitamins and amino acids (Methionine, Lysine, Sodium Salicylate, Vitamin B1, B2, B6, B12, C, A, D3, E, Nicotinamide, Copper, Manganese) and supplement B composed of vitamins (Vitamin A and D3).

3.4. Sample size determination and Sampling method

The sample size required for this study computed using the following formula obtained from Charan and Biswas (2013).

$$N = \frac{2SD^2\left(Z_{\frac{\alpha}{2}} + Z_{\beta}\right)^2}{d^2}$$

Whereas,

SD – Standard deviation = from previous studies or pilot study

$Z_{\frac{\alpha}{2}}$ =1.96 (From *Z* table) at type 1 error of 5%

Z_{β} = 0.842 (From *Z* table) at 80% power

d = effect size = difference between mean values

According to Legese *et al* 2022, the Live freeze-dried vaccine of IBDV intermediate standard strain produced by the National Veterinary Institute [NVI], Ethiopia was reported to bring mean antibody titer level deference of 1,385 in vaccinated group compared to unvaccinated chicken units with standard deviation of 2368.48. Accordingly, the sample size required for present study was computed as a follow:

$$N = \frac{2(2368.48)^2(1.96+0.842)^2}{(1385)^2}$$

N= 45, therefore for this study randomly selected 45 broiler chicks were used in each group

3.5. Study design

Completely randomized trial was conducted to evaluate the effects of vaccine, amino acids and vitamins on herd immunity of broiler chickens.

3.6. Experimental procedures and vaccination

One hundred thirty five day old Cobb 500 broiler chicks were bought from ELFORA poultry farm and assigned randomly into one of the three experimental groups (Group 1 to 3, Table 1) with 45 chickens per group and each group with three replicates of 15

chickens per replicate. These chickens didn't received IBD vaccinations prior to the age of day eight. Chickens in group 1 had taken supplement A and chicken in group 2 had taken supplement B until 28 days. All chickens, were vaccinated with IBD vaccine via drinking water as outlined by the manufacturer (National Veterinary Institute/NVI, Ethiopia), at the age of Day-9, and received a booster vaccination at Day-19.

In order to monitor vertical antibody transmission and its persistence, a total of 27 blood samples (G1=11; G2=11 and G3=5) 3ml/bird were collected from the wing vein of chickens at day eight immediately before first vaccination. Blood samples were left at room temperature for 24hours then the serum was collected into labeled cryovials and stored in -20℃ deep freezer till ELISA analysis. In order to evaluate antibody response to respective vaccination and supplement effects on humoral response, a total of 38 blood samples (G1=15; G2= 15 and G3=8) at day eighteen and 25 blood sample (G1=10; G2= 10 and G3= 5) at day twenty eight were collected and serum was harvested and stored in -20 deep freezer. To check the supplement effect on cellular responses, a total of 15 whole blood samples, five chickens from each group at day 28 were collected using EDTA vacutainer tube and differential count was done.

Table 2: Completely randomized controlled trial protocol

Experimental Unit	N	IBD vaccination[1]	Supplement[2]
Group 1	36	Yes	Yes (A)
Group 2	36	Yes	Yes (B)
Group 3	18	Yes	No(control)

Notes: Group 1: supplement A; Group 2: supplement B Group3: control

1: Vaccine was given at day 9 and booster dose at day 19

2: Supplement A and B was given daily base at 5g/10lit and 2ml/10lit drinking water, respectively.

3.7. Blood sample and Sera collection, transportation and storage

Blood sample was collected from the wing vein of individual chicken using 3 ml gauge 21 sterile disposable syringe as described by Alcorn (2001) and pouring the blood directly in to plain vacutainer tube. Blood samples were kept for 24 hours a room temperature to facilitate blood clotting for separation of sera and sera samples were collected into cryovials, labeled and transported using Ice box into AAU-CVMA microbiology laboratory and kept in -20℃ deep freezer until the serological assay was done. After the collection of all serum samples, it was taken in to National Animal Biotechnology Research Center (NABRC) animal biotechnology laboratory for serological test. For differential leukocyte count, blood was collected by EDTA vacutainer tube and the count was done in AAU-CVMA physiology laboratory.

3.8. Laboratory tests

3.8.1. Enzyme Linked Immuno Sorbent Assay (ELISA) Test

The collected and stored sera have been tested for IBDV specific antibodies using a commercial IBDV-ELISA kit (ProFLOK^{TM} IBD Ab) zoetis France, following the manufacturer's recommended protocol.

The procedures were as follows: The test sera was thawed and thoroughly mixed then diluted in sample dilution micro plate wells using dilution buffer according to the established kit instructions. After incubation under the appropriate conditions, the test serum was discarded from the dilution micro plates and the wells afterwards washed three times thoroughly. About 100 µl conjugate was dispensed into the wells, and put the plates incubated for 30 min. About 100 µl substrate solutions was dispensed into each test well and again incubated for 15 min at room temperature. After a final incubation, the substrate/ chromogen reaction will be stopped by adding about 100 µl stop solution and the color reactions was quantified by reading the micro plate by using an ELISA micro plate reader set at optical density of each well at 405–410 nm. Interpretation of serum sample positive (SP) control ratio necessarily required for the test interpretation. Accordingly, the following equation will be applied:

$$SP = \frac{(sample\ OD)x\ (average\ normal\ control\ OD)}{corrected\ positive\ control}$$

A test serum with SP value ≤ 0.180 was considered to be negative for IBD antibody and when SP value >0.180, accounted as positive.

ELISA titer was calculated by the following formula:

$$Log_{10}\ Titer = (1.172 * Log_{10}^{SP}) + 3.614$$

3.8.2. Differential count

Wright's staining is a commonly used staining technique in laboratory settings to visualize blood cells. Wright's stain is a polychromatic stain thus the different blood cells were appear differently stained under the microscope, allowing for identification and counting. Differential leukocyte count was done to evaluate the effects of the supplements on cell immunity. This was done by preparing thin smear from the previously collected whole blood and allowed to air dry, after putting the slides on staining racks flooded with Wright's stain and was kept for 3minutes. Then the slides were washed off by distilled water and blot the slides gently to remove water drops. Finally the slides were observed via microscope with 100x objective.

3.9. Data Management and analysis

Data generated from laboratory investigations was recorded and coded using Windows 10 Microsoft Excel spread sheet then it was analyzed by using statistical software R version 4.12. Specifically, One-way ANOVA and Tukey's multiple comparison tests were computed to compare the mean antibody titer and mean count leucocytes of experimental groups. The differences were considered as statistically significant at the level of $p \leq 0.05$.

3.10. Ethical clearance

The experiment was conducted following welfare and ethical research protocols prescribed and approved by the College of Veterinary Medicine and Agriculture Animal Research Ethics committee with reference No.VM/ERC/30/03/15/2023 (Annex 3) (Annex IV).

4. RESULTS

4.1. Serological test result

A total of 90 sera samples were examined using proFLOK IBD ELISA kit. Among the samples, 35 (38.8%) reacted positive to antibody against infectious bursal disease virus (IBDV). There was difference in detection of antibody level across the sampling dates. Higher number of reactors were noted from day eight samples (77.78%), followed by samples from day 28th (36%) and the least reactors were recorded during the second bleeding on 18th day (13.16%). Interestingly, the current result revealed loss of maternal immunity in more than 20% of the chicken within a week and further decreased to second bleeding weeks. First bleeding time there was a higher antibody production against IBDV as compared to day 18 and 28 bleeding times (Table 3). This indicated possible maternal antibody transfer from theirs parent.

Table 3: Summary of ELISA test results

	Bleeding time					
		Day eight	Day eighteen		Day twenty eight	
	N	Positive (%)	N	Positive (%)	N	Positive (%)
Group 1	11	8 (72.73)	15	2 (13.33)	10	3 (30)
Group 2	11	10 (90.91)	15	2 (13.33)	10	4 (40)
Group 3	5	3 (60)	8	1 (12.50)	5	2 (40)
Total	27	21 (77.78)	38	5 (13.16)	25	9 (36)

The mean antibody titer was 1747±244.99 at day eight sampling date; this result was higher as compared to the mean titer level at day eighteen and twenty eight bleeding times. While the mean titer at day twenty eight (856.56±237.65) was higher as compared to the mean titer at day eighteen (figure 4). This result indicated that the booster vaccine might be had enhancing effect on humoral response Table 4.

Table 4: Mean titer among different bleeding times

Bleeding time	Mean titer	Min	Max	SD	Se	N
Day 8	1747	61	4073	1273.005	244.9899	27
Day 18	325.6842	12	3019	620.1932	100.6086	38
Day 28	856.56	20	3890	1188.267	237.6533	25
Total	899.5444	12	4073	1171.786	123.5171	90

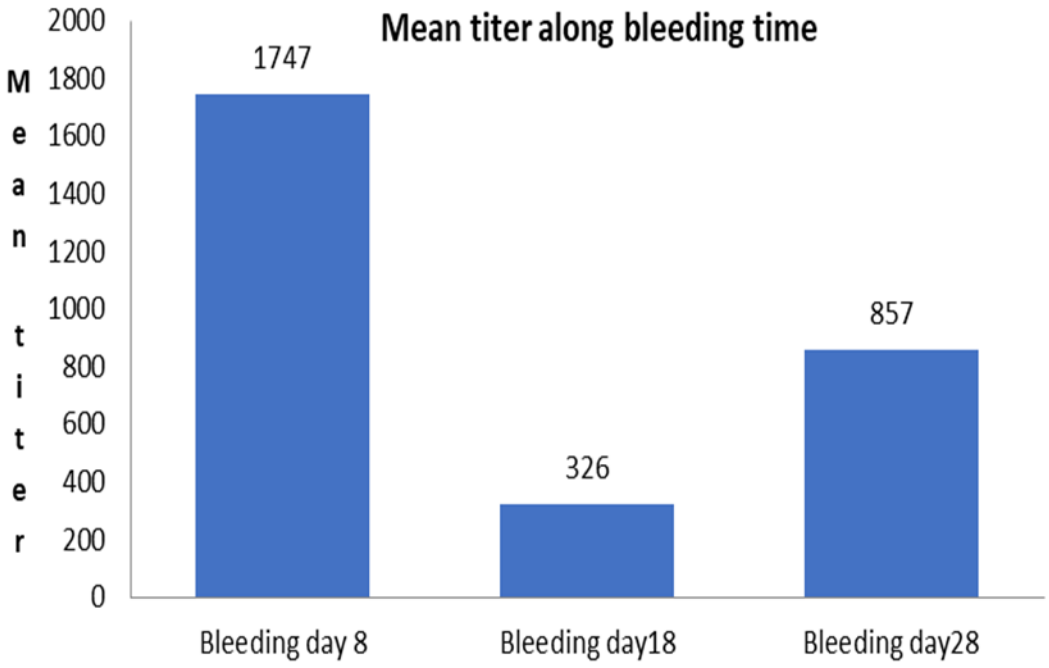

Figure 4: Mean titer with different sampling times

The analysis of variance showed that the mean antibody titer among the three bleeding times had a significant difference (P= 0.000) as shown in table 5.

Table 5: *Comparison of mean titer among bleeding times*

Source	SS	Df	MS	F	Prob>F
Between groups	31951068	2	15975534	15.40	0.0000
Within groups	90253190.4	87	1037392.99		
Total	122204258	89	1373081.55		

4.2. Effects of supplements on humoral response

As depicted in Figure 4, the mean antibody titers from the serum samples of group1, group2 and control group at day eight sampling date were 1613±417, 2246±359 and 943±362, respectively. This result indicated that the mean titer of group 2 was higher as compared to group1 and control group. While at day eighteen were 430±210, 277±134 and 218±120, which was lower as compared to day eight. The mean antibody titers at day twenty eight were 1227±719, 512±191 and 1014±448, respectively. The higher mean antibody titers were recorded in treatment groups compared to control ones (Figure 5).

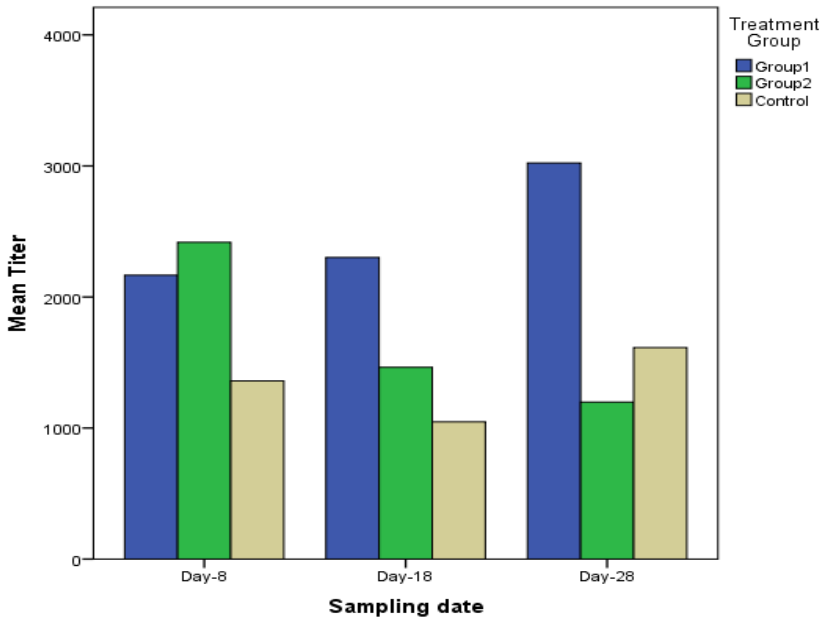

Figure 5: Mean titer level of treatment groups across the sampling dates

From the one-way ANOVA test result shown in Table 6, there was a statistically significant variation (P<0.004) among treatment groups.

Table 6: ANOVA mean effect of treatment on Ab title level

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	6353265.581	2	3176632.790	9.538	.004
Within Groups	3663464.133	11	333042.194		
Total	10016729.714	13			

Tukey's multiple comparisons

As shown in Table 7, the mean titer of group1 had a statistically significant difference with the mean titer of group2 and control group. This indicated that supplement A had higher effect on antibody production against IBD than treatment B. The mean titer level in group2 had a significant difference from the mean titer level in group1 while there was no significant difference with the mean titer level in control group which indicates supplement B had no immune boosting effect.

Table 7: Multiple Comparisons of treatments on Ab titer level using Tukey HSD

(I) Treatment Group	(J) Treatment Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Group1	Group2	1447.867*	349.450	.004	504.05	2391.68*
	Control	1309.200*	421.453	.025	170.92	2447.48*
Group2	Group1	-1447.867*	349.450	.004	-2391.68	-504.05*
	Control	-138.667	408.070	.939	-1240.81	963.47
Control	Group1	-1309.200*	421.453	.025	-2447.48	-170.92*
	Group2	138.667	408.070	.939	-963.47	1240.81

*. The mean difference is significant at the 0.05 level.

Univarate logistic analysis

Logistic regression analysis was conducted to determine the main factor contributing for the variation of antibody titer in the experimental unit. Accordingly, sampling date was determined to be statistically significant predictive variable (Table 8).

Table 8: Univariate logistic regression analysis of predictor variables

Coefficients[a]							
Model	Unstandardized Coefficients		Standardized Coefficients			95% Confidence interval for B	
	B	Std. Error	Beta	T	Sig.	Lower bound	Upper bound
1 (Constant)	2154.4	412.89		5.22	.00	1333.77	2975.1
Sampling date	-503.45	148.37	-340	-3.39	.001	-798.35	-208.55
Treatment groups	-157.84	150.64	-105	-1.05	.298	-457.26	141.57

a. *Dependent variable: Titer*

The model that contains the bleeding time variable explains more than 88% of change in humoral immunity variation among the study unit (Table 9) and remaining variation could be attributed to the feed additives

Table 9: Model strength of the univariate logistic regression analysis

Model summary[b]									
Modal	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change statistics R Square Change	F Change	df 1	df2	Sig.F Change
1	.341[a]	.116	.106	1069.98	.116	11.586	1	88	.001

a. Predictors: (Constant), Sampling date b. Dependent variable: Titer

4.3 Effects of supplement on cellular immunity

The total mean count of hetrophil was 45.87±.872; lymphocyte was 30.8±1.087; eosinophil was 3.93±.739; basophil was 1.4±.254 and monocyte was 18±.632. The mean count of hetrophils (46.6±2.014) and eosinophil was higher in control group as compared to supplemented groups (Table 10). The mean count of lymphocyte was higher in treatment 1 (32.8±.316) and lower in control group (27.4±2.6). The mean count of basophil was relatively higher (1.8±.374) in group 2 and lower in group1 (1±.375) Table 10.

Table 10: Differential White blood cell count across experimental units

	Statics	Hetrophill	Lymphocyte	Eosinophil	Basophil	Monocyte
Group1	Mean	45.6±.509	32.8±.316	3.8±1.113	1±.375	16.8±.663
	Max	47	34	8	2	19
	Min	44	32	2	0	15
	SD	1.14	0.84	2.49	0	1.48
Group 2	Mean	45.4±1.860	32.2±1.068	3.6±1.720	1.8±.374	17±1.140
	Max	50	35	10	3	20
	Min	41	29	0	1	14
	SD	4.16	2.39	3.85	0.84	2.55
Control	Mean	46.6±2.014	27.4±2.6	4.4± 1.208	1.43±.6	20.2±.8
	Max	53	37	8	3	22
	Min	41	22	1	0	18
	SD	4.5	5.81	2.7	1.34	1.79
Total	Mean	45.87±.872	30.8±1.087	3.93±.739	1.4±.254	18±.632
	Max	53	37	10	3	22
	Min	41	22	0	0	14
	SD	3.38	4,2	2.86	0.98	2.45

The analysis of variance indicated that there was no statistically significant difference in the mean count of hetrophil (P= 0.854), eosinophil (P= 0.912) and basophil (P=0.471) among the groups. But, there was a relatively higher mean count of

hetrophils (46.6±2.014) in control group as compared to supplemented groups (table 10). The mean count of eosinophil was relatively higher in control group (4.4±1.208) and lower in treatment 2 (3.6±1.72), whereas the mean count of basophil was relatively higher (1.8±.374) in treatment 2 and lower in treatment 1 (1±.375) (table 10). The mean count of lymphocyte (P=0.047) and monocyte (P= 0.0331) had a significant difference among the three groups (Table 11).

Table 11: Comparison of mean count of leukocytes

Count	Source	SS	Df	MS	F	P-value
Hetrophil	Between groups	4.13	2	2.07	0.16	0.854
	Within groups	155.6	12	12.96		
	Total	159.73	14	11.41		
Lymphocyte	Between groups	87.6	2	43.8	3.47	0.047
	Within groups	160.8	12	13.4		
	Total	248.4	14	17.74		
Eosinophil	Between groups	1.73	2	.87	0.09	0.912
	Within groups	113.2	12	9.43		
	Total	114.93	14	8.21		
Basophil	Between groups	1.6	2	.8	0.80	0.471
	Within groups	12	12	1		
	Total	13.6	14	.97		
Monocyte	Between groups	36.4	2	18.2	4.59	0.033
	Within groups	47.6	12	3.97		
	Total	84	14	6		

5. DISCUSSION

The study demonstrated herd immunity against IBDV in chicken both before and after vaccination. The level of herd immunity was significantly higher in flocks that were not exposed to vaccine ($P<0.05$). The current result indicated that the presence of strong transfer of maternally derived antibody against IBDV from parent herds to their offspring. The mean titer at day eighteen (325.68 ±100.61) was lower than the mean titer of day eight and day twenty eight. This indicated that the maternally derived antibody might be interferes with the action of first vaccination.

At day-8 about 77.8% were positive for the IBDV antibody and this was statically significantly higher than 13.16% and 38.89% of seropositive chickens at day-18 and day-28 respectively. The finding confirmed vertical transmission and persistence of maternal antibody for IBDV. This result agrees with the findings of Van den Berg, (2000); and Zaheer and Saeed, (2003) who reported persistent presence of IBDV antibody in young chicken after hatching and pre-vaccination. In present study, vaccination was conducted at day-9 as recommended and second blood collection for ELISA test was done after ten days with consideration for humoral immunity development. Nevertheless, antibody for the virus were only detected in 13.16% of the chicken unlike Zaheer and Saeed (2003) flock that reacted positive until the 21st-day. The evidence implies that protection of young chicken from IBD is achievable both through active and passive immunization. Therefore, purchasing day old chicks from vaccinated parent is as important as vaccination of young population to prevent IBD outbreak in broiler farms.

Regarding the antibody titer at different bleeding time, this study revealed that the higher titer was present at day eight (prior to first vaccination) (1747±244.98), and the lower antibody titer (325.68±100.60) was detected at day-18. The low antibody titer post vaccination might be due to the neutralizing effect of maternally derived antibodies. Similarly, the studies (Alam *et al*., 2002; Hair-Bejo *et al*., 2004; Moraes *et al*., 2005; Ahmed *et al*., 2003) reported that immune response against different IBD vaccines varied in accordance with the vaccination schedule, and the levels of maternal antibody against IBDV in the chicks. Zaheer and Saeed, 2003 also reported that maternal antibodies against IBDV may intervene with earlier vaccine-mediated

protection. Sometimes IBD vaccine virus may even be completely neutralized by maternally derived antibodies.

The statistical comparison of the mean titer with ANOVA indicated that the mean antibody titer among the three bleeding times had a significant difference (P= 0.000). This result was in line with findings by (Moraes *et al*., 2005; Hamal *et al*., 2006; Suzuki *et al*., 2009) who revealed the probable variation in the mean titer at different ages. Second vaccination was provided at day 19 and as a result higher level of antibody titer was recorded at day-28 compared to the mean titer level at day eighteen. This finding indicated that the booster vaccine of IBD had a vital effect to strengthen the humoral response to promote antibody production by alleviate the neutralizing effect of maternally derived antibodies and optimizing the first dose vaccine. This agrees with the reports by (Farooq *et al*., 2003; Fantay *et al.,* 2015), who describes the crucial effect of booster vaccine to control IBD by optimizing the immune response. This effect improved with the use of booster vaccine since maternal antibody becomes declined gradually and vaccine induced immunity produced uniformly. The current result supposed due to the high level of maternal antibody at day eight and interference with first vaccination then gradual initiation of vaccine induced antibody production.

Supplementation of poultry feed with amino acids and vitamins based elements has been hypothesized to boost the immunity of chicks (Montout *et al* 2021; Camelia and Betty, 2022). To this end, the effects of two commonly used growth promoters were evaluated for their effect on immune level. Accordingly, the mean antibody titer among chickens received supplement A and supplement B were higher as compared to the mean titer of the control group. The current result is in line with suggestions of previous researches such as Muir *et al*., (2002); Singh *et al*., (2006); Lagana *et al*., (2007), Trevisi *et al*., (2015), Akbarian *et al*., (2016); Dey *et al*., (2019), who stated that amino acid and vitamin supplementation improve immune responses by maintaining the functional and structural integrity of immune cells.

The mean titer level recoded in group that was treated with supplement A was significantly higher compared to group 2 and the control. The robust effect of

Supplement A may be related to its diverse active compounds composed of multivitamins and amino acids (Methionine, Lysine, Sodium Salicylate, Vitamin B1, B2, B6, B12, C, A, D3, E, Nicotinamide, Copper, Manganese). Studies have implied synergism effects of multivitamins and amino acids immunomodulatory effect on chicken's defense mechanism (Leshchinsky and Klasing 2001; Farooq *et al*., 2003). Supplement B which is vitamins (Vitamin A and D3) showed modest effect compared to supplement A. However, chicks supplied with the vitamins based supplement did not produce significant immune response compared to the control group. This may imply supplements with amino acids might have a better immune boosting effect. Literatures also suggest amino acids to have specific and multiple biological functions on defense mechanisms in the immune regulatory pathways (Trevisi *et al*., 2015).

According to Dey *et al*., 2019, during disease occurrence, lymphoid cells number and antibodies increase through the use of amino acids. The present study also conducted differential count of immune cells after treatment of chicks with amino acids and vitamin based supplements. Accordingly, the mean count of lymphocyte was higher in treatment group1 (32.8±.316) and lower in the control group (27.4±2.6). This increasing in the mean counts of lymphocytes in response to supplement A was a good evidence for improvement in the immune response of chicken as a result of supplementation. Lymphocytes are known to play a central role in regulating immune response, antibody production, and in effecting cell-mediated immune response. This result agrees with the findings of (Roth *et al*., 2002; Hiscock *et al*., 2003; Curi, 2005; Calton *et al*., 2016;) who stated that amino acid and vitamin supplements support the expression of key lymphocyte cell surface markers and cytokines production.

Also Pompeu *et al*., (2018) reported that supplementation of broilers with some type of vitamins maintains the heterophil/lymphocyte ratio in inflammatory conditions and increases the antigen-specific antibody response following immunization and increase antibody production on B cells. Whereas, the mean count of monocyte was higher in control group (20.2±.8) and lower in treatment group1 (16.8±.663), monocytes were known for their role in antigen presenting and in clearing intracellular infections. The current result might be due to the little effect of some vitamins in monocyte proliferation; the current result was in line with the finding of (Shan, 2020).

In contrary to the present finding, Munteanu and Schwartz (2022) revealed that vitamin supplementation increases the production of monocytes and macrophages, and improves the maturation and proliferation of T- and B-lymphocytes. Even though the mean count of hetrophils (P= 0.854), eosinophils (P= 0.912) and basophils (P=0.471) was not statistically different, the mean count of hetrophil and eosinophil was higher in control group while the mean count of basophil was higher in group 2. This result supposed to the vaccine interaction, the common effects of core composition of the experimental supplements and management factors. From this study the given amino acid and vitamin supplements had promising effect on improving immune responses.

Though the overall effect of supplements on humoral and cellular response noted in present study was positive it is paramount to note that the study could not establish the sole effect of these supplements. The effects seen in the study were confounded by the age of the chicken as it was confirmed in Logistic regression analysis. Therefore, result of the study on treatment effect should be interpreted with caution. Future studies that evaluate similar products must standardize for age of study units and vaccination history of parent stock.

6. CONCLUSIONS AND RECOMMENDATIONS

The study detected 78% herd immunity against IBDV among young chicks and high antibody titer was noted even at 8^{th} day post hatching. Statistically significant difference ($P<0.05$) in antibody titer level was recorded between bleeding times Antibody level decreases and increases immediately after first vaccination at day-9 and after booster vaccination at day-19, respectively. Feed supplements constituted with multi amino-acid and multi vitamins have a boosting effect on immune responses in poultry. Increasing herd immunity potential of young poultry population through effective immunization program and proper nutrition could prevent and mitigate the impact of IBD in poultry farm.

Based on the above conclusion the following recommendations are forwarded:

- ✓ Poultry producers are advised to purchase day-old chicks from vaccinated layers' stock
- ✓ Maternal antibody transfer should be determined before the vaccination of chicks
- ✓ Booster vaccine should be provided to chickens at proper age
- ✓ Provision of multi vitamin and multi amino acid based supplement should be help to reduce impact of IBDV outbreak
- ✓ Stakeholders should have awareness about the health benefits of supplements, however, supplements with antimicrobial effect should be handled with caution
- ✓ Further studies are required to identify other potentially immune booster feed additives.

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8. APPENDIX

Annex I: Supplementation

The supplements NEOBRO (5gm/10lit) and AD3 COMBAT (2ml/10lit) were supplied with drinking water for respected group of chicks.

Annex II: ELISA test procedures

Reagents

Test Sera

Positive control solution

Normal control solution

Conjugate solution

Dilution buffer

Wash solution

Substrate

Stop solution

Materials:

IBD antigen coated micro plate

Micro Pipettes and pipette tips

Graduated cylinders

Sample dilution micro plate

Deionized water

Micro plate reader

Procedures

Sample dilution procedures:

Sera and antigen for use were left at room temperature for half an hour before testing; 300µl dilution buffer was added into all wells of sample dilution micro plate, 6 µl test serum was added from A4 up to H9 and 6 µl Normal control was added into wells A2, H10 and H12 then wells A1, A3 and H11 was aspirated. Before transferring to ELISA micro plate, the diluted serum was put at stable condition for 5 minutes to equilibrate.

ELISA test procedure:

50 µl dilution buffer was added to all test micro plate wells and 50 µl positive control solution was added in to A1, A3 and H11 and pipette tips after each use was removed. 50 µl diluted sample sera was transferred into matching test micro plate wells, after each sample sera transfer pipette tips were changed. It was incubated for 30 minutes. After 30 minutes, solution from all wells was discarded and 300 µl wash solution was added into all test wells and soak for 3minutes. After 3 minutes the test micro plate inverted to remove the residual liquid then this procedure was repeated for 2 more times. 100 µl conjugate solution was added into each test wells and incubated for 30 minutes, then washed by wash solution three times within 3 minute interval. 100µl substrate solution was added into each test wells and incubated for 15 minutes. Finally

stop solution was added to each test wells and the test micro plate was read by ELISA micro plate reader set at optical density of 405 to 410nm.

Test validity check: The ELISA test result was valid since Normal control average optical density is ≤ 0.250 and the corrected positive control 0.250 and 0.900. Therefore, the average OD of this test was 0.21126 and CPC was 0.47017.

Negative: serum samples with IBD Sp ratio value ≤ 0.180, receive a zero titer and presumed negative for IBD antibody. A 0 IBD ELISA titer represents a chicken serum sample that contains an extremely low to insignificant IBD antibody level compared to the IBD ELISA kit positive and normal control sera.

Positives: an ELISA titer value above" 0" indicates only that a chicken serum sample contains a significant and ELISA detectable IBD antibody level compared to the IBD ELISA kit positive and normal control sera.

Absorbance 1												
Wavelength: 405 nm												
Plate 1												
Abs	1	2	3	4	5	6	7	8	9	10	11	12
A	0.6654	0.2265	0.6876	0.1995	0.2004	0.1651	0.1820	0.1956	0.1803	0.2079	0.1980	0.1996
B	0.2100	0.2394	0.1794	0.2020	0.2003	0.1822	0.1926	0.4609	0.1833	0.1866	0.2044	0.1772
C	0.1901	0.2257	0.2733	0.2028	0.4131	0.3594	0.1704	0.1857	0.3596	0.2988	0.2115	0.2244
D	0.1876	0.1744	0.1875	0.1700	0.1932	0.2095	0.2335	0.3517	0.4355	0.6611	0.5483	0.6783
E	0.5950	0.6618	0.4623	0.4031	0.2835	0.4636	0.5477	0.2946	0.3218	0.3124	0.3956	0.5379
F	0.4818	0.6593	0.2933	0.2521	0.4846	0.3685	0.4216	0.5731	0.1787	0.1764	0.1886	0.3972
G	0.2856	0.6200	0.1977	0.2049	0.1399	0.1943	0.3453	0.1920	0.2126	0.6074	0.4236	0.2060
H	0.2278	0.6634	0.2168	0.2000	0.1722	0.2293	0.5014	0.2328	0.3836	0.1877	0.6913	0.2196
Sample	1	2	3	4	5	6	7	8	9	10	11	12
A	PC	NC	PC1	Un0001	Un0002	Un0003	Un0004	Un0005	Un0006	Un0007	Un0008	Un0009
B	Un0010	Un0011	Un0012	Un0013	Un0014	Un0015	Un0016	Un0017	Un0018	Un0019	Un0020	Un0021
C	Un0022	Un0023	Un0024	Un0025	Un0026	Un0027	Un0028	Un0029	Un0030	Un0031	Un0032	Un0033
D	Un0034	Un0035	Un0036	Un0037	Un0038	Un0039	Un0040	Un0041	Un0042	Un0043	Un0044	Un0045
E	Un0046	Un0047	Un0048	Un0049	Un0050	Un0051	Un0052	Un0053	Un0054	Un0055	Un0056	Un0057
F	Un0058	Un0059	Un0060	Un0061	Un0062	Un0063	Un0064	Un0065	Un0066	Un0067	Un0068	Un0069
G	Un0070	Un0071	Un0072	Un0073	Un0074	Un0075	Un0076	Un0077	Un0078	Un0079	Un0080	Un0081
H	Un0082	Un0083	Un0084	Un0085	Un0086	Un0087	Un0088	Un0089	Un0090	NC1	PC2	NC2

Annex III: Differential Leukocytes count (DLC)

Reagents:

Whole Blood

Wright's stain reagent

Distilled water

Materials:

Glass slide

Blood capillary tubes

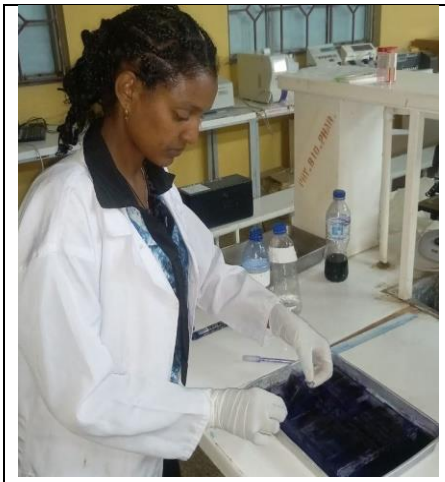
Staining rack

Light microscope and immersion oil

Pipets

Procedures

- A drop of blood was placed near to the one end of a clean and labeled glass slide
- A second slid was hold at 45º attached to the drop of blood to allow the drop to spread along the contact line of the second slide.
- The upper slide was quickly pushed forward along the surface of the first slide to form a thin smear then the smear dried by air
- The slides were put on staining rack and flooded with wright's stain and kept for 3minutes
- After 3minutes the slide was rinsed with distilled water and dried in air.
- The stained smear was put on the microscope and one drop of oil is added and observed using oil-immersion objective (100 xs), 100 leucocytes were counted by Battleship method. Then the result was takes as a relative value.



Annex IV: Ethical Clearance Certificate

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Bishoftu

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/30/03/15/2023

Name and affiliation of applicant: **Helene Aklilu Redie (DVM, MSc student)**
Department of Clinical Studies, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Assessing herd immunity potentials against infectious bursal disease virus before and after vaccination, vitamin and protein treatments in broiler chicken*

Date of application:	**December, 2022**
Nature of the project:	**Experimental investigation**
Target animal species:	**Chicken**
Number of animals involved:	**135**
Study area:	**College of Vet. Medicine and Agriculture, Bishoftu, Ethiopia**

Minutes No. and date of review: **VM/ERC/03/15/022, 25/01/2023**

The Animal Research Ethical Review Committee of the College of Veterinary Medicine and Agriculture of Addis Ababa University has reviewed the above research project and unanimously approved the application of Helen Aklilu.

The Federal Democratic Republic of Ethiopia
Addis Ababa University College of Veterinary Medicine and Agriculture

Professor Getachew Terefe (DVM, PhD)
Chairman

Signature

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ASSESSING HERD IMMUNITY POTENTIALS AGAINST INFECTIOUS BURSAL DISEASE VIRUS BEFORE AND AFTER VACCINATION, VITAMIN AND PROTEIN TREATMENTS IN BROILER CHICKEN

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