EFFECTS OF PREBIOTIC, PROBIOTIC AND SYNBIOTIC ON CLINICO-PATHOLOGICAL CHANGES, ANTIBODY RESPONSE AND PERFORMANCE IN COMMERCIAL PULLETS EXPERIMENTALLY INFECTED WITH INFECTIOUS BURSAL DISEASE VIRUS

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APRIL, 2021

DECLARATION

I hereby declare that this Thesis work entitled, **"Effects of Prebiotic, Probiotic and Synbiotic on Clinico-Pathological Changes, Antibody Response and Performance in Commercial Pullets Experimentally Infected with Infectious Bursal Disease Virus"** has been performed by me in the Department of Veterinary Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria, under the supervision of Professors P. A. Abdu, S. B. Oladele and T. Aluwong. The information derived from the literature has been duly acknowledged in the text and list of references provided. No part of this Thesis has been previously presented for another degree, diploma or certificate in any institution.

Aliyu Andamin DANLAMI	……..	……
Name of Student	Signature	Date

CERTIFICATION

This Thesis entitled, **"EFFECTS OF PREBIOTIC, PROBIOTIC AND SYNBIOTIC ON CLINICO-PATHOLOGICAL CHANGES, ANTIBODY RESPONSE AND PERFORMANCE IN COMMERCIAL PULLETS EXPERIMENTALLY INFECTED WITH INFECTIOUS BURSAL DISEASE VIRUS"** by Aliyu Andamin DANLAMI meets the regulations governing the award of the Doctor of Philosophy Degree in Avian Medicine of Ahmadu Bello University, Zaria, Nigeria, and is approved for its contribution to scientific knowledge and literary presentation.

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DEDICATION

This work is dedicated first and foremost to the Omnipotent, Omniscient Almighty Allah, the fountain of wisdom and knowledge, secondly to my beloved parents; Alh. Danlami Andamin and Hafsatu Muhammad, and lastly to my wife, Rashida Adamu, and children Amina, Aisha, Aliyu, Atika, Aliya and Asiya Aliyu Andamin.

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ABSTRACT

Infectious bursal disease (IBD) is endemic in Nigeria and outbreaks occur despite vaccination. Prebiotic, probiotic, and synbiotic exert beneficial effects on chickens. This study evaluated the mitigative effects of prebiotic, probiotic and synbiotic on clinico-pathological changes, antibody response and performance in commercial pullets infected with infectious bursal disease virus (IBDV). Two hundred and fifty ISA Brown day-old chicks were divided into five groups of 50 each. Groups A, B and C were supplemented from day-old to 49 days of age with molasses, Antox® and EN-FLORAX®, respectively and inoculated with a very virulent infectious bursal disease virus (vvIBDV) at 28 days of age, while groups D and E served as positive and negative controls, respectively. Haemagglutinating inhibition (HI) Ab titre and feed conversion ratio (FCR), bursal/body weight index (BBI), packed cell volume (PCV), lymphocyte counts, glucose (GLC) and total protein (TP), calcium (Ca) and blood urea nitrogen (BUN) concentrations, activities of aspartate aminotransferase (AST), total cholesterol (TC), activities of superoxide dismutase (SOD) and malondialdehyte (MDA) concentrations were determined. The chicks were observed for clinical signs (CS), mortality (MTR) rate was calculated, from one day postinoculation (dpi). At the end of observation 35 days of age (doa) (7 dpi), the clinical sign scores for groups administered supplements were moderate, but severe in positive control. The overall mortality rates were 25.5%, 61.3%, 46.8% and 95.4% in groups A, B, C and D, respectively. The HI Ab titre between groups A (6.45 ± 0.04 $\log_2$), B (4.64 ± 0.02 $\log_2$), C (5.35 ± 0.03 $\log_2$) and D (3.60 ± 0.01 $\log_2$) at 1-wk PV (42 doa), differed ($P \leq 0.05$). The mean in FCR between groups A (0.80), B (0.68), C (0.74) and D (0.92) at 35 doa (7 dpi), differed ($P \leq 0.05$). There was significant difference ($P \leq 0.05$) in PCV between

groups A (22.29 ± 0.12%), B (19.26 ± 0.08%), C (20.28 ± 0.10%) and D (13.56 ± 0.05%) at 35 doa (7 dpi). The lymphocyte counts between groups A (3.34 ± 0.03 × 10^9/l), B (2.29 ± 0.02 × 10^9/l), C (2.53 ± 0.02 × 10^9/l) and D (1.33 ± 0.01 × 10^9/l) at 35 doa (7 dpi) differed ($P \leq 0.05$). The GLC concentrations in groups A (307.54 ± 3.23 mg/dl), B (200.27 ± 2.24 mg/dl) and C (235.35 ± 2.32 mg/dl) were higher ($P \leq 0.05$), compared to that of group D (105.65 ± 1.22 mg/dl) at 35 doa (7 dpi). There was significant difference ($P \leq 0.05$) in TP concentrations between groups A (4.45 ± 0.06 g/dl), B (3.19 ± 0.04 g/dl), C (3.38 ± 0.05 g/dl) and D (2.25 ± 0.02 g/dl) at 35 doa (7 dpi). There were differences in Ca concentrations in groups A (3.36 ± 0.03 mg/dl), B (2.35 ± 0.02 mg/dl), C (2.56 ± 0.02 mg/dl) and D (1.99 ± 0.01 mg/dl) at 35 doa (7 dpi). The BUN concentrations in groups A (4.95 ± 0.04 mg/dl), B (5.93 ± 0.05 mg/d) and C (6.88 ± 0.05 mg/dl) were lower ($P \leq 0.05$), compared to that of group D (7.99 ± 0.07 mg/dl) at 35 doa (7 dpi). The AST between groups A (200.25 ± 2.15 µg/ml), B (283.21 ± 2.23 µg/ml), C (250.23 ± 2.19 µg/ml) and D (350.19 ± 3.22 µg/ml) at 35 doa (7 dpi) differed ($P \leq 0.05$). The TC concentrations between groups A (125.24 ± 1.28 mg/dl), B (105.21 ± 1.24 mg/dl), C (110.22 ± 1.27 mg/dl) and D (69.20 ± 0.25 mg/dl) at 35 doa (7 dpi), differed ($P \leq 0.05$). The MDA concentrations recorded in groups A (56.23 ± 0.27 nmols/mL) and B (76.36 ± 0.39 nmols/mg) were significantly ($P \leq 0.05$) lower than that of D (91.49 ± 0.50 nmols/mg) at 35 doa (7 dpi). There was a significant difference ($P \leq 0.05$) between groups C (65.51 ± 0.35 nmols/mg) and D at 7 dpi. In conclusion, molasses, Antox® and EN-FLORAX® mitigated the negative effects of vvIBDV on FCR, organs/BD weight indices, PCV, heterophil, lymphocyte, GLC, TP, Ca, BUN, AST, TC, and MDA, and reduced the severity of CS and MTR. It is recommended that the supplements could be used to mitigate the negative effects of IBD and the potentials of molasses be evaluated.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Per cent
(*)	Asteric
±SE	Plus or minus standard error of mean
µl	Microlitres
^{o}C	Degree centigrade
Ab	Antibody titre
ADS	Antioxidant defence system
AGID	Agar gel immunodiffusion
AGPT	Agar gel precipitation test
AI	Avian influenza
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
B	Body
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BF	Bursa of Fabricius
CAM	Chorioallantoic membrane
CAT	Catalase
CD	Cluster of differentiation
CEB	Chicken Embryo Bursa
CEF	Chicken Embryo Fibroblast
CEK	Chicken Embryo Kidneys
CF	Crude fibre
ChIFN-ɣ	Chicken Interferon-gamma
ChIL-2	Chicken Interleukin-2

ChIL-y	Chicken Interleukin-gamma
ChIL-1ß	Chicken Interleukin-1beta
CID	Chicken Infective Dose
CM	Centimetre
CMI	Cell mediated immunity
CP	Crude protein
CK	Creatine kinase
COX	Cyclo-oxygenase
ELISA	Enzymes linked-immunosorbent assay
DIC	Disseminated intravascular coagulopathy
Dl	Decilitre
DM	Dry matter
doa	Days of age
dpi	Day postinoculation
e.g	For example
EDTA	Ethylene diamine tetra acetic acid
EE	Ether extract
EID	Enbryo Infected Dose
EOF	Erythrocyte osmotic fragility
FCR	Feed conversion ratio
FOS	Fructo-oligosaccharide
ft	Feet
g	Grams
GALT	Gut-associated lymphoid tissue
GGT	Gamma glutamyl transerase
GDH	Glutamate dehydrogenase
GIT	Gastro-intestinal tract

GDP	Gross Domestic Products
GPx	Glutathione peroxidase
h	Hours
HA	Haemagglutination
HAU	Haemagglutination Units
H/L	Heterophils/lymphocytes ratio
hpi	Hours post-inoculation
Hb	Haemoglobin
HI	Haemaglutination Inhibition
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IB	Infectious bronchitis
IBD	Infectious busal disease
IBDV	Infectious bursal disease virus
iNOS	inducible-Nitric Oxide Synthatase
IFN-γ	Interferon-gamma
IL	Interleukin
KCN	Potassium cyanide
kg	Kilogram
L	Litre
LDH	Lactate dehydrogenase
M	Metre
MDAs	Maternally-derived antibodies
MAPKs	Mitogen-activated protein kinases
MCV	Mean Corpuscular Volume
MCH	Mean Corpuscular Haemoglobin

MCHC	Mean Corpuscular Haemoglobin Concentration
MDA	Malondialdehyde
MHC	Major histo-compatibility complex
min	Minutes
N	Nitrogen
ND	Newcastle disease
NDV	Newcastle disease Virus
NO	Nitric oxide
NT	Nucleotides
PBS	Phosphate Buffered Saline
PCV	Packed cell volume
PI	Post-immunisation
Pi	Post-inoculation
pH	Hydrogen ion concentration
PV	Post Vaccination
RBC	Red blood cell
RNA	Ribonucleic acid
RT-PCR	ReverseTranscription-Polymerase Chain Reaction
ROS	Reactive oxygen species
SDH	Sorbitol dehydrogenase
SPF	Specific Pathogen Free
SRBC	Sheep red blood cells
SOD	Superoxide dismutase
SPL	Spleen
THY	Thymus
TLR	Toll-like receptor
TMB	Tetramethylbenzidine

TNF	Tumour necrotic factor
TWBC	Total white blood cell
USA	United State of American
vvIBDV	very virulent infectious bursal disease virus
VN	Viral Neutralization
WBC	White blood cell
WFI	Weekly feed intake

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Poultry industry In Nigeria is one of the fastest growing segments of the agricultural sub-sector (Ayoola, 2016). The poultry sub-sector is contributing 25 per cent to the nation's Gross Domestic Product (GDP) annually, amounting to the single largest contributor to agriculture (Ayoola, 2016). Unfortunately, this industry is being threatened by various infectious diseases of which infectious bursal disease (IBD), is second to Newcastle disease in prevalence in Nigeria (Obi *et al.,* 2008). Infectious bursal disease has also been reported as the second most important viral diseases in other countries example, Pakistan (Rai *et al.,* 2017).

Prebiotics are non-digestible carbohydrates that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the gastro-intestinal tract (GIT) (Fuller, 1989; Gibson and Roberfroid, 1995). Prebiotics are intended to promote and increased the number of beneficial bacteria with a concomitant reduction in the number of undersirable bacteria (Park *et al.,* 2013). Most prebiotics are oligosaccharides that resist enzymatic degradation and absorption in the upper GIT until reaching the caecum, where they are hydrolysed and fermented by bacteria, such as *Bifidobacteria* and *Lactobacillus* (Park *et al.,* 2013).

Probiotics are products containing viable, defined microorganisms that are non-pathogenic and non-toxic in nature which when administered orally in sufficient number will alter the GIT microflora of the host, thereby exerting beneficial health effects, favourable to the host's health (Guillot, 1998; Schrezenmeir and de Vrese, 2001).

Probiotics must avoid being flushed from the GIT by attaching to the wall or they must grow at a rate faster than the removal rate by peristalsis (Fuller, 1989). Probiotics may consist of a single strain or multiple bacterial strains that colonize the crop, small intestine, caeca and cloaca (Fuller, 1989; Carvalho *et al*., 2018).

Synbiotics are products that contain prebiotics and probiotics. The prebiotic component of the synbiotic mixture selectively favours the probiotic component, thereby creating synergism (Schrezenmeir and de Vrese, 2001; Jung *et al.*, 2008). In order to preserve gut microbiota and to promote host innate defenses, administration of synbiotic (combinations of prebiotics, probiotics and immunomodulators) as alternative approach for promoting production performance and immune responses in modern poultry husbandry is widely accepted (Awad *et al.,* 2009; Sugiharto, 2014).

Infectious bursal disease is an acute, highly contagious, immunosuppressive infection of young chickens (Abdu, 1986). It is caused by a bisegmented (A and B), double-stranded RNA (dsRNA) virus and is enclosed within a non-enveloped icosahedral capsid belonging to the genus *Avian birnavirus* of the family *Birnaviridae* (Kibenge *et al.*, 1988; Lasher and Shane, 1994; Lukert and Saif, 1997; Lukert and Saif, 2003; Muller *et al.*, 2003). The disease mostly affects chickens 1-6 weeks old (Camilotti *et al.*, 2016) but have also been reported in chickens older than 6 weeks, even in up to 22 week-old layer chickens (Okoye and Uzoukwu, 1981; Owoade and Durojaiye, 1995; Igbokwe *et al.*, 1996; Musa *et al.*, 2010; Shekaro and Josiah, 2015; Aliyu *et al.*, 2016). Infectious bursal disease virus (IBDV) present subclinical and clinical diseases in susceptible chickens, depending on the age of the bird at the time of infection. Subclinical and clinical IBD may cause immunosuppression (Cereno, 2013; Mutinda *et al.*, 2015). Subclinical IBD

occurs in chickens below 3 weeks of age; while the clinical form in 3-8-week old chickens with typical lesions of IBD (Sharma *et al.,* 2000; Ahmed and Akhter, 2003; Cereno, 2013). Irrespective of when the infection occurs, the disease causes immune-suppression making the birds vulnerable to a variety of secondary infections (Mutinda *et al.,* 2015). As a result, infected chickens develop a poor immune response to vaccination against other pathogens (Mutinda *et al.,* 2015). Following infection, IBDV multiplies rapidly in the B-lymphocytes of the bursa of Fabricius (BF), leading to immunosuppression, increased susceptibility to other diseases and reduced growth rate of surviving birds (Kibenge *et al.,* 1988*;* Becht and Muller, 1991). The BF is the principal organ of virus replication and peak virus titres in it can be detected between 3 and 5 days after IBDV infection (Lukert and Saif, 1997). In the BF of chickens infected with IBDV, productive viral replication is often associated with necrosis, apoptosis of lymphoid cells, inflammatory changes, atrophy and haemorrhages (Kim *et al.,* 2000; van den Berg, 2000; Taylor *et al.,* 2008).

Oxidative stress is a detrimental imbalance between oxidative and antioxidative system that occurs in the cells of vertebrates as a result of production of reactive oxygen species (ROS) (Bartsch and Nair, 2006). The activity of superoxide dismutase (SOD) decreases, while those of catalase (CAT) and glutathione peroxidase (GPx) increases during infections (Inal *et al.,* 2001). Antioxidant enzymes, such as SOD, CAT and GPx are involved in scanvenging ROS. The SODs convert superoxide radical into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), while the CAT and GPx convert H_2O_2 into oxygen and water (Weydert and Cullen, 2010). The activities of the enzymes may increase during oxidative stress, resulting in increased lipid peroxidation of cell membranes and organelles (Ji, 1999; Kalpakcioglu and Senel, 2008). Lipid peroxidation

generates a variety of relatively stable decomposition end-products, mainly α, β-unsaturated reactive aldehyde, such as malondialdehyde (MDA), erythrocyte osmotic fragility (EOF) and isoprostances, which can then be measured as an indirect index of oxidative stress biomakers (Jain *et al.*, 1989; Draper *et al.*, 2000; Montuschi *et al.*, 2004; Adenkola *et al.*, 2010).

The diagnostic application of haematology and biochemistry in human and veterinary medicine is a well-established procedure (Ross *et al.*, 1976). The present organization within the poultry industry, where large number of individuals of low genetic variance is maintained in controlled environments, present an ideal situation for the use of clinical chemistry. Clinical examination and clinical chemistry estimates can readily be combined with postmortem examination to complement disease investigation (Ross *et al.*, 1976).

1.2 Statement of Research Problems

Synthetic growth promoters and supplements are expensive, usually unavailable and posses' detrimental effects in birds as well as humans (Portugaliza and Fernandez, 2012). The administration of antibiotics as growth enhancers in poultry at sub-therapeutic levels may result in the development of antibiotic-resistant bacteria, which are hazardious to animal and human health (Portugaliza and Fernandez, 2012). The continuous exposure of poultry to antibiotics have led to serious health problems such as allergies, the spread of drug resistant microorganisms, cancer and a potential harmful effect on human and animal intestinal microflora (Ferrinie *et al.*, 2006; Jafari *et al.*, 2007; Nonga *et al.*, 2010).

In Nigeria, despite routine vaccination, outbreaks of IBD in commercial poultry with varying degree of mortality rates have been reported (Awolaja and Adene, 1995; El-Yuguda and Baba, 2004; Dashe *et al.,* 2009; Musa *et al.,* 2010; Musa *et al.,* 2012; Aliyu *et al.,* 2016). Infectious bursal disease virus has been reported to be one of the very important immunosuppressive agents in modern poultry production, as the virus may induce a temporary or permanent destruction of the BF and other lymphoid tissues leading to immunosuppression (Sharma *et al.,* 2000; Lukert and Saif, 2003; Khatri *et al.,* 2005). The prevention of IBD in Nigeria is largerly dependent on vaccination with single prototype indigeneous and different types of imported IBD vaccines (Okoye and Uzoukwu, 2001). Reversion of attenuated vaccinal strains to more virulent phenotype under field and experimental conditions has been frequently reported (Jackwood *et al.,* 2008). Poor vaccines handling and inappropriate timing of vaccination period in relation to maternal derived antibody decay have posed serious challenge to outcomes of vaccination (Abdu, 1997; Abdu *et al.,* 2001). Studies have shown that, IBD has acquired an endemic status in poultry farms in Nigeria (Nawathe *et al.,* 1978; Durojaiye *et al.,* 1984; Abdu, 1988).

Regrettably, severe outbreaks of IBD still occurred with high mortality rates both in vaccinated and unvaccinated flocks (Okoye, 1983; Abdu, 1986; Sainbury, 2000; Musa *et al.,* 2010), and these makes the control of IBD to be virtually impossible in most farms (Musa, 2009; Musa *et al.,* 2010). Infectious bursal disease virus is resistant to many disinfectants and environmental factors, it remains infectious for at least four months, if the poultry house becomes contaminated; the disease tends to recur in subsequent flocks (Gary and Richard, 2015; Aliyu *et al.,* 2016).

Oxidative stress is the major cause of reduction in poultry growth and increase the incidence of infectious and metabolic diseases in poultry (Sen, 1995). Oxidative stress especially in broilers can results in damage to biomolecules, cells and tissues which decrease immunity and antioxidant status of birds (Peter, 2002; Yun-Zhong *et al.*, 2002; Joachim *et al.,* 2010). Oxidative stress is associated with infectious diseases and is one of the mechanisms responsible for the patho-physiological consequences of several viral infections (Li *et al.,* 2011; De Marco, 2013; Sebastiano *et al.,* 2016). Reactive oxygen species, the major cause of oxidative stress are constantly generated *in vivo* as part of normal metabolism (Jimoh *et al.,* 2017). Oxidative stress results from increased production of free radicals and ROS without the corresponding increase in antioxidant defence (Trevisan *et al.,* 2001; Williams *et al.,* 2002).

Haematology and biochemical values of avian species are significantly influenced by poultry diseases, such as IBD (Panigraphy *et al.,* 1986; Juranova *et al.,* 2001). Zeryehun *et al.* (2012) reported that IBDV causes alterations in different haematological parameters of poultry. In birds, clinical signs of illness are frequently delicate; therefore, clinical chemistry is necessary to evaluate cellular changes (Ritchie *et al.,* 1994). Besides the severe clinical signs and high mortality rate that results from vvIBDV infection in susceptible chickens, it also causes many pathological changes that form part of the pathogenesis of the disease which could basically be explained in terms of biochemical changes that occur in relation to the pathological effect of the virus in several organs such as the BF, spleen, thymus, liver and kidneys (Ley *et al.,* 1983; Nunoya *et al.,* 1992; Lukert and Saif, 1997).

1.3 Justification of the Study

The supplements such as prebiotics, probiotics, synbiotics and other organic compounds are generally believed to be safer, healthier, and less hazardous when compared to other synthetic products (Onyimonyi and Onu, 2009). Improvement in the poultry industry should incorporate emphasis on the prevention and control of diseases that cause economic losses (Okwor *et al.,* 2009). The poultry industry has become economically important in many countries (Griggs and Jacob, 2005). However, the use of antimicrobial agents as a preventive measure has been questioned, given extensive documentation of the evolution of antimicrobial resistance among pathogenic bacteria (Nava *et al.,* 2001). Antibiotics may no longer be used as growth promoters and prophylaxis in some disease conditions of poultry due to concern about residual effect on poultry products and development of bacterial resistance (Nava *et al.,* 2001). Therefore, consumers and manufacturers are looking for alternatives to synthetic products (Fuller, 2001). Prebiotics, probiotics and synbiotics are being considered to fill this gap, and already some farmers are using them in preference to antibiotics (Trafalka and Grazy, 2004).

To develop immunity, the animal needs energy and protein for the manufacture of antibodies and cells as well as minerals (zinc, copper, iron and selenium) and vitamins (A and E) for transmitting signals to parts of the animal's body in addition to other factors, in other to fight infections (Conroy, 2005). Prebiotics, probiotics and synbiotics posses' carbohydrate, protein, minerals vitamins and amino acids (Makkar and Becker, 1999; Kakengi *et al.,* 2003; Oduro *et al.,* 2008). There is little or no information on the effects of prebiotic, probitic and synbiotics on clinico-pathological changes in commercial pullets experimentally infected with vvIBDV in Nigeria.

1.4 Aim of the Study

The aim of the study was to evaluate the mitigative effects of prebiotic, probiotic and synbiotic on clinico-pathological changes, antibody response and performance in commercial pullets experimentally infected with vvIBDV.

1.5 Objectives of the Study

The objectives of the study were to evaluate the mitigative effects of prebiotic, probiotic and synbiotic in commercial pullets experimentally infected with vvIBDV on:

i. Clinical signs, morbidity and mortality rates,
ii. Antibody response to vvIBDV and ND vaccine La Sota,
iii. Production performance indices,
iv. Haematological parameters,
v. Serum biochemical parameters and oxidative stress biomarkers, and
vi. Pathological changes in some immune organs.

1.6 Research Questions

Does administration of prebiotic, probiotic and synbiotic:

i. Mitigate the severity of clinical signs, morbidity and mortality rates,?
ii. Improve antibody immune response to vvIBDV and ND vaccine La Sota,?
iii. Reduce negative effects on production performance indices,?
iv. Lead to changes in haematological parameters,?
v. Lead to changes in serum biochemical parameters and oxidative stress biomarkers,? and
vi. Mitigate the pathological changes in some immune organs of commercial pullets experimentally infected with vvIBDV. ?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Prebiotics

2.1.1 Definition of prebiotics

Prebiotics are non-digestible food ingredients in the GIT, that affect the host by selectively stimulating the growth and/or activity of one or a specified number of bacteria already residing in the colon (Nagpal and Kaur, 2011). They are not hydrolysed or absorbed in the upper GIT (Hajati and Rezaei, 2010) and do serve as sources of carbon and energy for friendly strains of bacteria already inhabiting the colon, where bacterial fermentation of nutrients occur (Kulakowska, 2009). They are dietary fibres not affected by heat, cold, acid or time, provide health benefits, and nourish good bacteria that already exist in the GIT.

2.1.2 Mechanism of action of prebiotics

Prebiotics can either directly bind the pathogens or increase the osmotic value in the intestinal lumen. Mechanism of actions of prebiotics include; lowering gut pH through lactic acid production (Chio *et al.,* 1994; Gibson and Wang, 1994); inhibiting/preventing colonization of pathogens (Morgan *et al.,* 1992; Bengmark, 2001); modifying metabolic activity of normal intestinal flora (Demigne *et al.*, 1986), and stimulation of the immune system (Monsan and Paul, 1995). Prebiotics also increase the bioavailability of minerals, prevent GIT infections, modify inflammatory conditions, regulate metabolic disorders, and reduce the risk of cancer (Roberfroid *et al.,* 2010).

2.1.3 Evaluating the effects of prebiotics on growth performance

Prebiotics act like growth factors in commensal bacteria, directly target the colon, have a selective fermentation and help maintaining a balanced microflora, by being utilized,

promoting species and increased vigor/body weights of birds (Gibson and Roberfroid, 1995). Beside the local effects, other systemic effects may also occur after absorption of fermentation products into the blood stream (Leske *et al.,* 1993). The ultimate goal of prebiotics is to improve body weight and health status of the bird and therefore reduce the risk or burden of disease (Sinovec *et al.,* 2005).

2.1.4 Effects of prebiotics on immune response

Prebiotics can improve gut barrier function through protection of the epithelial tight junction during external stress and activate the immune responses in the gut-associated lymphoid tissue (GALT) (Seth *et al.,* 2008). Most of the gut lymphoid tissue is located in and around the GIT and the bacterial colonization of its mucosa is a key immunological stimulus for the development of the immune system (Smith *et al.*, 2007). Commensals stimulate the GALT with their cell wall components, such as lipopolysaccharides from Gram-negative bacteria (Kelly *et al.,* 2005). Fructo-oligosaccharides contain a pattern recognition receptor, CD14, to which commensals bind. As a result of binding, immune cell differentiates Th1 type immunity as production of regulatory cytokines (i.e, IL-10 and TNF-β) is up- regulated and Th2 type immunity as promoter of allergies is down-regulated (Kelly *et al.,* 2005). Prebiotics, containing fructo-oligosaccharides and manno-oligosaccharides have stimulating effect on the GALT (Janardhana *et al.,* 2010). This is due to lactic acid action which affects the mechanism of non-specific immunity (increase proliferation of macrophages and their phagocytic activity, and natural killer cell production), specific (stimulates macrophages to produce cytokines that activate T-cells) and humoral (stimulates B-lymphocytes to produce antibacterial antibodies, mainly IgA). Immunoglobulin (IgA) is active mainly in the digestive system, and its role is to protect the intestinal epithelium against pathogens (Janardhana *et al.,* 2010).

2.2 Probiotics

2.2.1 Definition of probiotics

Probiotics are products containing viable, defined microorganisms in sufficient number to alter the GIT microflora of the host which when administered orally favour the host thereby exerting beneficial health effects (Guillot, 1998; Schrezenmeir and de Vrese, 2001).

2.2.2 Mechanism of action of probiotics

Probiotic strains have been shown to inhibit pathogenic bacteria both *in vitro* and *in vivo* through several different mechanisms. The mode of action of probiotics in poultry includes (i) maintaining normal intestinal microflora by competitive exclusion and antagonism (Nurmi and Rantala, 1973; Rantala and Nurmi, 1973; Fuller, 1989; Jin *et al.*, 1998; Line *et al.*, 1998; Kizerwetter-Swida and Binek, 2009); (ii) altering metabolism by increasing digestive enzyme activity and decreasing bacterial enzyme activity and production of ammonia (Cole *et al.*, 1987; Yoon *et al.*, 2004); (iii) improving feed intake and digestion (Dierek, 1989; Awad *et al.*, 2006); and (iv) stimulating the immune system (Dalloul *et al.*, 2005; Haghighi *et al.*, 2006; Nayebpor *et al.*, 2007; Mathivanan and Kalairasi, 2007; Apata, 2008; Brisbin *et al.*, 2008).

2.2.3 Evaluating the effects of probiotics on growth performance

It is evident from the result of Kabir *et al.* (2004) that live weight gains were significantly higher in experimental birds as compared to controls at all levels of probiotics administration during the period of 2nd, 4th, 5th and 6th weeks of age, both in IBD vaccinated and non-vaccinated birds. This result is in agreement with that of other investigators (Jin *et al.*, 1998; Zulkifli *et al.*, 2000; Kalavathy *et al.*, 2003; Islam *et al.*,

2004; Apata, 2008; Willis and Reid, 2008) who demonstrated increased live weight gain in probiotic fed birds.

2.2.4 Effects of probiotics on immune response

Kabir *et al.* (2004) evaluated the dynamics of probiotics on immune response of broilers and reported higher antibody production in experimental birds as compared to controls. They also demonstrated that the differences in the weight of the spleen and BF of probiotics and conventional fed broilers could be attributed to different level of antibody production in response to sheep red blood cells (SRBC). Similarly, Khaksefidi and Ghoorchi (2006) reported that the antibody titre in the probiotic supplemented group was higher at 5 and 10 days of post-immunisation (PI) compared to control, when SRBC were injected at 7 and 14 days of age.

2.3 Synbiotics

2.3.1 Definition of synbiotics

Synbiotics are appropriate mixtures of pre- and probiotics that beneficially affect the host by improving the survival and implantation of live microbial dietary supplements in the GIT of the host (de Vrese and Schrezenmeir, 2008; Bandyopadhyay and Narayan, 2014; Mohammed *et al.,* 2018).

2.3.2 Mechanism of action of synbiotics

Synbiotic targets the small and large intestinal tracts. Probiotic bacteria are stimulated by prebiotic oligosaccharides in the colon. Probiotic microorganisms use prebiotic carbohydrates for growth and replication in the gut (Dang *et al.,* 2013). Prebiotics increases the chances of the survival of probiotic organisms. A synbiotic product exerts both a pre- and probiotic effect (Gibson and Roberfroid, 1995). Two types of synergism

between pre- and probiotic have been defined. Both compounds can be synergistic with each other, or alternatively with the host (Schrezenmeir and de Vrese, 2001; Roberfroid, 2007). In the first case, the prebiotic stimulates growth of probiotic bacteria. The second mechanism assumes that the pre- and probiotic act independently in the GIT where they stimulate the development of the host microbiota.

2.3.3 Evaluating the effects of synbiotics on growth performance

Mohnl *et al.* (2007) observed that the synbiotic products (Biomin®, PouItryStar®) had a comparable growth stimulating potential to avilamycin (an antibiotic-based growth stimulant) in broiler chicken. Vicente *et al.* (2007) verified the effect of a synbiotic product containing *Lactobacillus* spp. with the addition of lactose. Some 320 turkeys infected with *Salmonella sp* were bred, and a positive effect of the synbiotic on feed conversion and body weight gain of the study animals was demonstrated. Li *et al.* (2008) assessed the effect of administration of fructo-oligosaccharide (FOS) and *Bacillus subtilis* to broiler chickens on body weight gain were found significantly higher in experimental birds as compared to controls.

2.3.4 Effects of synbiotics on immune response

With regards to immunomodulatory effects of synbiotics, for instance; Biomin®, there are reports that probiotic (*Enterococcus faecium*) of Biomin® enhanced humoral immune responses against SRBC (Mehri *et al.*, 2013). Biomin® also increased most blood parameters including total protein (Akinleye *et al.*, 2008) and higher protein promotes induction of specific antibody titre against avian pathogens. Enhanced effects of Biomin® on antibody titres of chickens against ND, AI, IB and IBD were observed by Alireza *et al.* (2015) who concluded that application of synbiotic (Biomin®) enhanced antibody responses following vaccination against ND, AI, IB and IBD and that it was

more effective in live than killed vaccines and could be used as a feed additive for improving innate and acquired immune responses in chickens.

2.4 Infectious Bursal Disease

2.4.1 Definition of infectious bursal disease

Infectious bursal disease is a highly contagious viral disease of young chickens and causes subclinical or clinical disease. Characterised by trembling, incoordination, inflammation followed by necrosis and atrophy of the BF resulting in immunosuppression following extensive destruction of lymphocytes (Jordan, 1990; Mato *et al*., 2001; Abdu, 2007; Giambrone, 2013).

2.4.2 Infectious bursal disease virus

2.4.2.1 Classification of the infectious bursal disease virus

The virus responsible for IBD is a member of the family *Birnaviridae*, within the *Avibirnavirus* genus (Muller *et al.*, 1979; Murphy *et al.*, 1999).

2.4.2.2 Types and subtypes of infectious bursal disease virus

Two types of IBDV are recognized based on serum neutralization test. Following isolation of variants, McFerran *et al*. (1980) described types 1 and 2. Studies in the US also demonstrated two major serotypes, designated 1 and 2, isolated from turkeys (Jackwood *et al.*, 1982). Serotype 1 viruses can be further categorized into four groups on the basis of their pathogenicity; classical, variants, attenuated and very virulent strains (appendix 1) (Lasher and Davis, 1997).

2.4.2.3 Structure of the infectious bursal disease virus

The structure of the IBDV is based on a T=13 lattices and the capsid sub-units are predominantly trimmer clustered (Bottcher *et al.*, 1997). Meanwhile, the complete

nucleotide (NT) sequences of the two genome segment, A and B have been established (Mundt and Muller, 1995).

2.4.2.4 Physicochemical properties of infectious bursal disease virus

The IBDV is resistant to physical and chemical agents (Benton *et al.,* 1967b). Due to the stability and hardness of the virus, it persists in poultry premises even after thorough cleaning and disinfection (Benton *et al.,* 1967b). The virus is inactivated at pH of 12.0, but not at pH 2.0 (Benton *et al.,* 1967b). Benton *et al.* (1967b) reported that IBDV survived a temperature of 37^{o}C for 90 min and 56^{o}C for h. A marked reduction in infectivity of the virus was observed after treatment with 0.5% formalin for 6 h. The virus remained unaffected by either chloroform, phenol, thiomeasal, staphene and Hyamine 2389 treatments.

2.4.3 Epidemiology of the infectious bursal disease

2.4.3.1 Distribution of infectious bursal disease

Infectious bursal disease is currently an international problem: 95% of the 65 countries that responded to a survey conducted by OIE in 1995 declared cases of infection (Eterradossi *et al.,* 1999), including New Zealand which had been free of the disease until 1993 (Eterradossi *et al.,* 1999). Classical IBDV has traditionally affected poultry worldwide ever since the first outbreak of disease was reported from Delaware, Maryland and Virginia Delmarva region (Cosgrove, 1962). By 1970, the disease had been reported from Canada (Ide and Stevenson, 1973), Mexico (Lucio *et al.,* 1972), Europe (Landgraf *et al.,* 1972; Gukelberger, 1977), Africa (Ojo *et al.,* 1973; Onunkwo, 1975; Onunkwo and Momoh, 1981), the Middle East (el-Zain *et al.,* 1974) and Asia (Mohantey *et al.,* 1971).

2.4.3.2 Hosts of infectious bursal disease virus

The host for IBDV is the young chicken where a clinical disease occurs whilst in older birds the infection is essentially subclinical. Only chickens (*Gallus gallus domesticus*) develop IBD after infection by serotype 1 viruses and inoculation of IBDV in other avian species failed to induce disease. Turkeys (*Meleagris gallopavo*) and Peking ducks (*Anaspeking*) may be asymptomatic carriers of serotype 1 viruses (McFerran *et al.,* 1980). Varying susceptibility of different chicken breeds has been described with higher mortality rates in light than in heavier breeds (Bumstead *et al.,* 1993; Nielsen *et al.,* 1998).

In Nigeria, local chickens are more susceptible to IBD than broilers and slightly more susceptible than pullets, (Salman *et al.,* 1983; Abdu and George, 1986; Okoye, 1987; Okoye and Aba-Adulugba, 1998).). The IBDV has been isolated from Ostriches (Gough *et al.,* 1998), Baltic ducks and herring Gulls (Hitchner, 1970), raptors and passerines in Japan (Nieper and Muller, 1996). The virus has also been documented in the lesser mealworm (*Alphitobius* species) that fed on IBDV contaminated feed. Several studies have suggested that rats, mice, dogs, wild birds, and mosquitoes are reservoirs of IBDV (Howie and Thorsen, 1981; Okoye and Uche, 1986; Park *et al.,* 2009; Pages-Mante *et al.,* 2004; Jeon *et al.,* 2009). Anti-IBDV antibodies have been reported from Antarctic penguins (Gardnar *et al.,* 1997). Neutralizing or precipitating antibodies have been detected in various species of wild duck, goose, tern, puffin, crow and penguin, (Wilcox *et al.,* 1983; Gardner *et al.,* 1997; Ogawa *et al.,* 1998).

2.4.4 Transmission of infectious bursal disease virus

The IBDV is spread by direct contact between infected and susceptible flocks. Infected chickens shed IBDV one day after infection and can transmit the disease for at least 14 days (Vindevogel *et al.,* 1976) but not exceeding 16 days (Winterfield *et al.,* 1972).

Indirect transmission of IBDV most probably occurs on fomites (feed, clothing, and litter) or through airborne dissemination of virus-laden feathers, and poultry house dust (Benton *et al.,* 1967a).

2.4.5 Pathogenesis of infectious bursal disease

Under natural conditions, the common mode of infection is via the oral route (Muller *et al.,* 1979). From the gut, the IBDV is transported to other tissues by resident phagocytic macrophages. Although viral antigen has been detected in the liver and kidneys within the first few hours of infection, extensive viral replication takes place primarily in the BF (Muller *et al.,* 1979). Following oral infection, the virus will be present within 4-5 h in the cytoplasm of macrophages and lymphatic cells of the duodenum, jejunum and caecum (de Wit and Baxendale, 2013). Via the portal venous system, the virus reaches the liver within 5 h post infection. Kupfer cells in the liver trap and phagocytose particles (de Wit and Baxendale, 2013). Then the virus travels to the BF via the blood stream, where replications occur. By 13 h post inoculation (pi), most of follicles are positive for virus and by 16 h pi, a second and massive viraemia occurs with secondary replication in lymphatic organs such as THY and SPL including the BF leading to disease and death (Muller *et al,* 1979; de Wit and Baxendale, 2013).

Clinical disease and death occurs within 64-72 h post-infection and IBDV can be found in the BF up to 9 days post infection (Muller *et al.,* 1979; de Wit and Baxendale, 2013). Immature B-lymphocytes in the follicles of the BF are the target cells for viral replication. Actively dividing, surface immunoglobulin M-bearing B cells are lysed by the virus (Hirai and Calnek, 1979; Hirai *et al,* 1981; Rodenberg *et al,* 1994). But cells of the monocyte-macrophage lineage can be infected in a persistent and productive manner, and play a role in dissemination of the virus (Burkhardt and Muller, 1987; Inoue *et al,*

1992; van den Berg *et al*, 1994), and in the onset of the disease (Sharma and Lee, 1983; Kim *et al*, 1998; Lam, 1998).

2.4.6 Immunology of infectious bursal disease

2.4.6.1 Target cells for infectious bursal disease virus

It is the B-cells that are the target cells for the IBDV. During the acute phase of IBD the BF undergoes atrophy as bursal follicles gets depleted of B-cells. Virus replication causes extensive damage to lymphoid cells in medullary and cortical regions of the follicle. Apoptosis of the neighboring B-cells augments the destruction of bursal morphology (Giambrone *et al.*, 1977; Kim *et al.*, 1999). T-cells are resistant to infection by IBDV during the acute phase of IBD as lesions in the thymus quickly disappear within a few days (Hirai *et al.*, 1979; Sharma *et al.*, 2000). A dramatic influx of T-cells is reported in and around the site of virus replication (Poonia and Charan, 2005). The infiltrated T-cells could be detected from 1st and 12th weeks pi although the viral antigen disappears by the 3rd week pi. The IBDV-induced cytotoxic T-cell limits the spread of the virus by destroying the cells expressing the viral antigen and thus can initiate the recovery process (Poonia and Charan, 2005). At the same time IBDV-induced T-cells might enhance the viral lesions by producing inflammatory cytokines. T-helper cells produce inflammatory cytokines like IFN-Υ which activates macrophages to produce nitric oxide (NO) and other cytokines with anti-proliferative activity (Sharma *et al.*, 2000). The NO production after IBDV infection exerts antiviral effect hence immunosuppressed chickens that failed to induce NO had more severe disease and higher degree of virus replication (Poonia and Charan, 2005).

Infectious bursal disease virus does not affect natural killer cells levels in chicken (Sharma *et al.*, 2000). During the course of mitogenic inhibition, T-cells of infected

chicken also failed to secrete IL-2 upon *in vitro* stimulation (Sharma and Fredericksen, 1987; Kim and Sharma, 2000). Intra bursal T-cells and T-cell mediated responses play a significant role in viral clearance and promoting recovering from infection. They defend the host cell by reducing the viral burden but at the same time produce inflammatory cytokines and nitric oxide including factor that enhance tissues destruction and also delay the recovery process (Sharma *et al.,* 2001). Intrabursal T-cells were activated by *in vitro* stimulation with IBDV. The activated cells had increased surface expression of chicken MHC class II molecule, IA and IL-2 receptor CD25. In addition, these cells have an up regulated IFN-Υ gene (Kim *et al.,* 2000). Intrabursal T-cells inhibited the mitogenic response of normal splenocytes by 90%. This bursal T-cell induced mitogen inhibition was found to be dose-dependent and not MHC-restricted (Kim and Sharma, 2000). In contrast to the bursal T-cells, the splenocytes from IBDV exposed chickens did not have suppressive activity (Kim and Sharma, 2000). Mitogenic inhibition by bursal T-cells is mediated by soluble factors (Sharma *et al.,* 2000). Chickens that survive IBD clear the virus and recover from its pathologic effects (Sharman *et al.,* 2000).

2.4.6.2 Target organs for infectious bursal disease virus

The target organ for IBDV is the BF at its maximum development, (Okoye and Uzoukwu, 1984; Saif, 1998; van den Berg *et al.,* 2000; Lukert and Saif, 2003; Mac Lachlan and Dubovi, 2011; Mahgoub, 2012; Ingrao *et al.,* 2013). Bursectomy can prevent illness in chicks infected with virulent virus (Hiraga *et al.,* 1994).

2.4.7 Immunosuppression

2.4.7.1 Mechanism of immunosuppression

Reduction in the number of B-cells in the BF due to viral infection is the major cause of immunosuppression (Abdu, 1985; van den Berg, 2000). Suppression of B-cell function

might be caused by damage to helper T-cells or other cells involved in generating the immune responses (Sharma *et al.,* 1989). Chickens infected with IBDV have suppressor cells in the spleen, which cause *in vitro* mitogenic hypo responsiveness to concavalin A (Sharma *et al.,* 1989). The impairment of T-cells and development of suppressor cells was demonstrated *in vitro* by using proliferation tests (Sharma and Fredericksen, 1987; Confer *et al.,* 1981; Confer and MacWilliam, 1982; Ingrao *et al.*, 2013) or by measuring the cytokine release after mitogen activation of T-cells (Lambrecht *et al.,* 2000). The infiltrated T cells constituting the majority of the bursal population after IBDV infection are unresponsive to mitogen activation at days 4 and 9 dpi (McNeilly *et al.,* 1999; Rauw and Lambrecht, 2007). Moreover, splenocytes from IBDV infected chickens were also shown to be deficient in secretion of ChIL-2 (Sharma and Fredericksen, 1987; Kim *et al.,* 1998 and Ingrao *et al.,* 2013).

2.4.7.2 Effects of immunosuppression

The destruction of the BF by IBDV creates an immunosuppression, which is more serious in younger birds (Allan *et al.,* 1972). The most serious and long-lasting immunosuppression was described when day-old chicks were infected by IBDV (Allan *et al.,* 1972; Faragher *et al.,* 1974) with lasting of up to 6 weeks of age (Giambrone *et al.,* 1977). Recovery from IBD or subclinical infection will be followed by immunosuppression with more serious consequences if the strain is very virulent and infection occurs early in life (Giambrone *et al.,* 1977). It has been shown that the more virulent the IBDV the stronger is the suppression of the humoral and cell mediated immunity. Virulent virus also produced a detectable NO in serum (Shamaila, 2005). In addition to IBD impact on production and role in the development of secondary infections, IBDV may affect the immune response of the chicken to subsequent vaccinations, (Ingrao *et al.,* 2013). Immunosuppression has been most evident using

experiments based on the measurement of humoral responses induced by antigens such as *Brucella abortus*, sheep red blood cells (SRBC), or ND vaccines La Sota (Allan *et al.*, 1972; Giambrone *et al.,* 1977; Giambrone and Clay, 1986).

2.4.7.3 Effect of infectious bursal disease virus on innate immunity

Infectious bursal disease virus modulates the macrophage function by altering phagocytic activity (Lam *et al.,* 1998). Macrophages from infected chicken have up regulated cytokine gene expression and produce increased levels of nitric oxide (Kim *et al.,* 1998). During the acute phase of IBD and 1-day post infection (dpi), there is dramatic infiltration of CD4 cells, $CD8^+$ cells and macrophages at the site of virus replication in the BF (Sharma *et al.,* 2000; Withers *et al.,* 2005). Bursal T cells are activated and exhibit up-regulation of gene transcription of pro-inflammatory cytokines, e.g., ChIL-1β, ChIL-6, COX-2 and ChIFN-ϒ (Eldaghayes *et al.,* 2006). High levels of systemic ChIFN-ϒ and ChIL-ϒ were observed during the acute phase of vvIBDV challenge, leading to a "cytokine storm" (Rauw and Lambrecht, 2007). The ChIFN-ϒ, ChIL-6, and inducible nitric oxide synthetase (iNOS) (Palmquist *et al.,* 2006) that may promote cellular dysregulation and accentuate tissue destruction (Digby and Lowenthal, 1995; Karaca and Kim, 1996; Kim *et al.,* 1998). Macrophages and monocytes infected by IBDV are activated to produce high levels of proinflammatory cyctokines, interleukin-1 (IL-1) and IL-6, chemokines (IL-8 and MIP-α and nitric oxide (NO) (Kim *et al.,* 1998; van den Berg, 2000; Khatri and Palmquist, 2005; Rauf *et al.,* 2011). The role of mitogen-activated protein kinases (MAPKs) and NF-kB was tested by using specific pharmacological inhibitors. These results suggest that IBDV uses cellular signal transduction machinery, in particular the p38 MAPK and NF-kB pathways, to elicit macrophage activation (Khatri and Sharma, 2006). The increased production of NO, IL-8 and COX-2 by macrophages may contribute to bursa inflammatory responses seen

during the acute phase of IBDV infection (Khatri *et al.,* 2005). This was confirmed in a study (Rauf *et al.,* 2011), where the over expression of chemokines genes, IL-8 and MIP-α was also higher in IBDV-infected chickens during the early phase of infection (chicken IL-8 acts as a chemo-attractant for heterophils and monocytes). The IBDV trigger both direct (T cell activation) and indirect (macrophage activation) pathways to induce a "cytokine storm" during the acute phase of IBD and the individual susceptibility must be related to the variable intensity of the innate immune response (van den Berg, 2000; Rauw and Lambrecht, 2007; Rauf *et al.,* 2011). Results of a study revealed that genes of tall-like receptor (TLR) pathway play important role in the pathogenicity of IBDV infection (Guo *et al.,* 2012).

2.4.7.4 Effect of infections bursal disease virus on humoral immunity

Infectious bursal disease virus has a predilection for immature (Sivanandan and Maheswaran, 1980) actively dividing B-lymphocytes and causes lytic infection of IgM bearing B cells resulting in decrease in circulating IgM^+ cells. Infected chicken produces less level of antibodies against antigen (Kim *et al.,* 1999). Only primary antibody responses are affected. Secondary responses remain unaltered (Sharma *et al.,* 1989; Rodenberg *et al.,* 1994). Infectious bursal disease virus induced humoral deficiency is reversible and overlaps with the restoration of bursal morphology (Sharma *et al.,* 2000). Chickens infected with IBDV at 1 day of age were found to be completely deficient in serum immunoglobulin G (IgG) and produced only a monomeric IgM (Ivanyi, 1975; Ivanyi and Morris, 1976). The IgG levels varied depending on the age at the time of infection (Hirai *et al.,* 1979). The adverse effect on antibody responses is due to the damage to the B-cells in the BF and the blood (Sivanandan and Maheshwaran, 1980).

2.4.7.5 Effect of infectious bursal disease on cellular immunity

The effect of IBDV on cellular immune response is transient and less pronounced than the effect on humoral response (Shamaila, 2005). Infected chickens show a poor cellular response to certain antigens and show increased susceptibility of diseases that are under the control of cellular immune defence (Anderson *et al.,* 1977). T-cells from infected chickens during the early stages of IBDV infection fail to respond optimally to mitogens *in vitro* (Confer *et al.,* 1981). Maximum depression in the cellular immunity was shown to occur at 6 weeks pi by using the lymphoblast transformation assay (Sivanandan and Maheshwaran, 1981). *In vitro* mitogen hypo-responsiveness of T-cell is mediated by the suppressor cells in the spleens of the infected chickens (Lam, 1991).

Infectious bursal disease infected chickens were shown to have a normal natural killer cell levels, mononuclear phagocytic activity and delayed–type hyper sensitivity reaction (Hudson *et al.,* 1975; Giambrone, 1977). Neither did virus infection alter the normal proportions of CD4 and CD8 T-cells in the circulation and spleen (Sharma *et al.,* 1993). It was reported that variant A strain of IBDV had a significantly higher effect on CMI as compared to the standard Edgar strain when given to 1-day-old chicken which lingered on until 5 weeks (Sharma *et al.,* 1993). Broilers infected with IBDV at 3 weeks of age had reduced antibody titres to *Brucella abortus* (T-cell independent antigen) and SRBC (a T-cell dependent antigen) in extracts from Harderian gland were evident at a later time as compared to SRBC antibody response (Craft *et al.,* 1980).

2.4.8 Persistence of infectious bursal disease virus in organs

The BF and spleen were reported to have higher concentration of IBDV as compared to other tissues (Winterfield *et al.,* 1972). The BF harvested from chickens at 72 hpi

yielded high virus titres followed by the spleen and kidneys. No virus was detected beyond 10 dpi (Winterfield *et al.*, 1972). The virus was not detected beyond 11 dpi in commercial pullet chickens when inoculated at 1, 7 or 14 days of age (Mackenzie and Spradbrow, 1981). The IBDV was re-isolated most consistently from the BF and less frequently from thymus, liver, kidneys, lungs, and spleen and no virus was isolated from the pancreas (Mackenzie and Spradbrow, 1981). In birds inoculated at 21 days of age, IBDV was re-isolated up to 8 days only (Mackenzie and Spradbrow, 1981). Precipitating antigen was detected only in the BF and only at 3rd, 4th and 5th dpi but was not detected in any organ of chicken infected at 21 days of age (Mackenzie and Spradbrow, 1981). When chickens were inoculated with an attenuated cell culture adapted virus at day of age, the virus could be detected in BF, spleen, thymus, liver, kidneys, and lungs for up to 10 and 14 dpi (Skeeles and Lukert, 1979). When specific pathogen free (SPF) chickens were inoculated with IBDV at 3 weeks of age, viral RNA was detected by RT/PCR up to 21 dpi. Infectious bursal disease virus was detected by embryo inoculation up to 7 dpi in the BF of SPF chickens inoculated at 2 or 3 weeks of age, whereas the viral RNA was detected by RT/PCR for up to 28 dpi (Abdel-Alim and Saif, 2001a). In SPF chickens inoculated at 1 day of age, the bursa-derived virus RNA was detected at 7 and 14 dpi when inoculated as a high dose of 10^4 EID_{50}/bird or at a low dose of $10^{2.5}$ EID_{50}/bird (Abdel-Alim and Saif, 2001a). In commercial 1-day-old broiler chickens, the bursa-derived virus was detected at 7 and 14 dpi when inoculated at a high dose (10^4 EID_{50}/bird), whereas the virus was detected only at 14 dpi when inoculated at a low dose ($10^{2.5}$ EID_{50}/bird). In SPF and commercial chickens, vaccinated with a modified live IBDV vaccine, the virus persisted in the BF of SPF chickens till 3 weeks (Abdel-Alim and Saif, 2001).

2.4.9 Clinical manifestation of infectious bursal disease

2.4.9.1 Subclinical infectious bursal disease

Severity of the signs depends on the virus strain and the age, breed, type and immune status of the infected bird prior to onset of infection (van den Berg *et al*., 1991; Mbuko *et al*., 2010; Cereno, 2013). The subclinical form of IBD occurs when chickens are exposed to IBDV during the first two weeks of life (de Wit and Baxendale, 2013). Chickens present no clinical signs but are characterized by bursal atrophy, severe immunosuppression and resultant increased susceptibility to secondary infections such as *Escherichia coli.* Secondary infections in broilers, mainly *E. coli,* result in continuous daily mortality and poor feed conversion (de Wit and Baxendele, 2013). Due to immunosuppression, there can be poor response to other vaccinations (Hirai *et al*., 1979; Abdu, 2007; Toro *et al*., 2009; de Wit and Baxendele, 2013).

2.4.9.2 Acute clinical infectious bursal disease

The clinical form of IBD affects chicks between 3 and 6 weeks of age. It is characterized by a sudden onset, severe depression, ruffled feathers; vent picking, presence of urate stains on the vent, whitish or watery diarrhoea, anorexia, elevated water consumption, trembling, severe prostration and finally death (Cereno, 2013). Morbidity and mortality begins three days pi, peaks and recedes in a period of 5-7 days (de Wit and Baxendele, 2013). Mortality may be negligible or as high as 90% in case of vvIBDV but most commonly a mortality of 10-20% is usually seen (de Wit and Baxendele, 2013). In field situation, mortality in pullet chicks is generally higher than broiler chicks (de Wit and Baxendele, 2013).

2.4.10 Pathotypic variation of infectious bursal disease virus

Different pathotypes of the virus and cell culture adapted strains differ markedly in virulence. The criteria used for the characterization of IBDV strains include antigenicity,

genetic relatedness and pathogenicity (Kibenge *et al.,* 1988). In addition to antigenic differences in serotypes and subtypes, the viral strains have been classified according to their virulence (van den Berg, 2000). IBDV strains can be defined as apathogenic (serotype 2); mild, intermediate or "hot" (serotype 1 vaccines); classical virulent (IBDV), variant, or very virulent (serotype 1). Serotype 2 strains cause neither mortality nor bursal lesions in specified pathogen free (SPF) birds. Virulent serotype 1 strains induce mortality and bursal lesions (van den Berg, 2000).

Based on pathogenicity, IBDV antigenic serotype I can be classified into 4 different strains; classical, variant, attenuated and very virulent strains (Snyder, 1990; Lim *et al.,* 1999). Birds infected with classical strain of IBDV suffer severe lymphoid necrosis and inflammation of the BF, thereby resulting in immunodeficiency and moderate mortality of 20-30% in SPF chickens (Lim *et al.,* 1999). Attenuated strains of IBDV are apathogenic and used as live vaccines (Sharma, 1991). They are generated by adapting classical and variant strains of IBDV to chicken embryo fibroblasts (CEF) or other cell lines (Lim *et al.,* 1999). Serotype 1 vaccines cause no mortality but possess residual pathogenicity with bursal lesions varying from mild to moderate or severe. Antigenically, variant strain of IBDV are known for their ability to escape cross neutralisation by antiserum against the classical strain and cause a rapid and a severe bursal atrophy with no clinical signs of illness (Vakharia *et al.,* 1994; Lim *et al.,* 1999). The feature of vvIBDV is the ability to induce high mortality. The clinical sign induced by infection with a vvIBDV is similar to that observed when the classical strain of IBDV is involved and the same incubation period of 4 days except that infection with a vvIBDV is more acute and the disease and lesions are more pronounced in individual bird and are generalised in flocks. Haemorrhages are pronounced in the BF and muscles with a rapid bursal and thymic regression. However, the microscopic lesions of vvIBDV

are similar to those observed in the classical strain of IBDV (van der Berg *et al.*, 1991). Very virulent IBD is characterized by a high mortality range of 5-25% in broilers and 60-100% in layers (van der Berg, 2000). Very virulent strains can breakthrough the immunity provided by maternal antibodies (van den Berg, 2000) (Appendix 1).

2.4.11 Pathology due to infectious bursal disease

2.4.11.1 Gross pathological lesions

The tissue distribution and severity of lesions is dependent on the subtype and pathogenicity of the IBDV (Rosenberger and Cloud, 1986; Tanimura *et al.*, 1995). Infected birds are dehydrated and have dark pectoral muscles. Haemorrhages occur in the thigh and pectoral muscles, and on the mucosa at the proventriculo-ventriculus junction (Hanson, 1962). There is increased mucus in the intestine (Lukert and Saif, 2003). Chickens that die or are scarified four days after infection show a doubling in size of the BF due to oedema (Tanimura *et al.*, 1995). A straw-coloured viscous transudate may surround the BF, which is pale yellow and shows striations (Ley *et al.*, 1979). On section, the BF is hyperaemic and intrafollicular haemorrhages may be present (Helmboldt and Garner, 1964). From the fifth day after infection the BF decreases in size and within eight days may be one-third of the weight of the BF of unaffected chicken (Lukert and Saif, 1997; 2003). Using the Edgar strain of IBDV, Cheville (1967) recorded the bursal weights for 12 dpi (Mahgoub, 2012). The BF began to increase in size and weight due to oedema and hyperaemia on 3 dpi and by day 4 it doubled in size (Cheville, 1967). By 5 dpi, the BF returned to its normal weight (Cheville, 1967). By day 2 or 3 dpi, the BF had a gelatinous yellowish transudate covering the serosal surface. Longitudinal striations became prominent and the colour changed from white to creamy. The transudate disappeared as the BF returned to its normal size and the organ turned gray during the period of atrophy (Lukert and Saif, 2003).

The Delaware variant strains of type 1 IBDV do not cause acute bursal enlargement but infection results in rapid and profound atrophy (Rosenberger and Cloud, 1986; Rosenberger *et al.,* 1987). Bursal size is quantified as a ratio of bursal (numerator) to body mass (denominator) (Lucio and Hitchner, 1979). Authors quote ratios based on different formulae; most incorporate a factor (10^2 or 10^3) to compensate for the numerical difference between body and bursal mass expressed in grams (Lasher and Shane, 1994).

Pathologic changes in the spleen and thymus were less prominent than those of the BF (Cosgrove, 1962; Inoue *et al.,* 1994; Lukert and Saif, 2003; Okoye, 2005). The spleen might be enlarged and usually had small gray foci uniformly dispersed on the surface (Lukert and Saif, 2003) but later atrophied (Okoye, 2005). Lesions in the spleen and thymus were noticed at the same time as the changes in the BF and resolved within 1 or 2 days of appearance (Helmboldt and Garner, 1964). Spleenic enlargement was documented by Morales and Boclair (1993) who showed differences in bursal: spleen weight ratio of 2.4 for controls compared with 0.9 in chicks seven days after challenge.

Kidneys show enlargement and pallor with accumulation of crystalline urate in tubules, visible as white flecks beneath the capsule (Cosgrove, 1962; Hitchner, 1971; van den Berg *et al.,* 2000). Renal changes observed in carcasses and chickens sacrificed in extremilly dehydration due to water deprivation associated with recumbency (Lukert and Saif, 1997; Lukert and Saif, 2003).

2.4.11.2 Histopathological lesions

Degeneration and necrosis of individual lymphocytes in the medullary region of the BF occur as early as 1 dpi (Lukert and Saif, 2003). Lymphocyte degeneration is

accompanied by nuclear pyknosis and formation of lipid droplets in the cytoplasm (Cheiville, 1967). Degenerating lymphocytes are surrounded by macrophages. Lymphocytes are soon replaced by heterophils, pyknotic debris and hyperplastic reticuloendothelial cells (Cheiville, 1967). By day 3 or 4 pi, all lymphoid follicles are affected. Severe oedema, hyperaemia, and marked accumulation of heterophils are evident (Lukert and Saif, 2003; Mahgoub, 2012). Cystic cavities develop in the follicular medulla. These cystic cavities are caused by necrosis and phagocytosis by heterophils and macrophages. During the stage of bursal atrophy, fibroplasia of the bursal tissue becomes evident (Mahgoub, 2012). In addition, the bursal epithelium becomes proliferative, forming a glandular-like structure, which consists of the bursal columnar epithelium containing globules of mucin. In the late stages, scattered lymphocyte foci appear (Mahgoub, 2012).

In the spleen, hyperplasia of the reticulo-endothelial cells around the adenoid sheath arteries has been observed in early stages of IBDV infection (Lukert and Saif, 2003). At day 3 pi, focal areas of necrosis of lymphoid nodules and periarteriolar lymphoid sheath were observed (Ley *et al.,* 1983). Okoye (1984) reported more severe tissue destruction in areas near the spleenic capsules.

Evidence of lymphocyte necrosis in the inner cortex of the thymus appears at 2 dpi in 4-day-old and 2-weeks-old chickens infected with IBDV (Inoue *et al.,* 1994). Many altered lymphocytes were detected throughout the entire cortex and lymphocyte depletion was prominent in areas of the cortex at 3 and 4 dpi (Inoue *et al.,* 1994). Lymphocyte depletion was observed at 5 dpi in the whole cortex. At 7 dpi, cortical atrophy was greatest but visible lymphocytes remained focally and diffusely in the outer cortex (Inoue *et al.,* 1994). At 14 dpi, the cortex showed apparent repopulation and recovery. In

the medulla, macrophage and plasma cells infiltration and haemorrhage were prominent from 5-7 dpi (Mahgoub, 2012).

The Harderian gland is reported to be severely affected by the IBDV in 1 day old chickens (Skeeles and Lukert, 1979). Normally, the gland is populated with plasma cells as the chicken ages but the infection prevents this infiltration (Skeeles and Lukert, 1979). Harderian gland of the chickens infected at 1 day of age has 5-10 folds fewer plasma cells than those of uninfected chickens from 1-7 weeks of age (Dohms *et al.,* 1981). However, lymphoid follicles and heterophil population in the Harderian gland are not affected by IBDV infection, nor could necrotic or degenerative changes be found in the acini or excretory ducts (Skeeles and Lukert, 1979).

Histologic lesions appearing in the kidneys were non-specific and resulted from dehydration (Helmboldt, 1964). Interstitial haemorrhages and perivascular accumulations of lymphoid cells, oedema, tubular necrosis and glomerular nephrosis in the kidneys of IBDV-infected birds have been reported (Mandelli *et al.,* 1966; Del Bono *et al.,* 1968; Ley *et al.,* 1983). The liver had some perivascular infiltration of monocytes (Parkhurst, 1964).

2.4.12 Diagnosis of infectious bursal disease

2.4.12.1 Tentative diagnosis

Tentative diagnosis of IBD is based on flock history, clinical signs, course of the disease mortality pattern, and pathological changes observed in the BF, pectoral, and thigh muscles (Abdu, 2007; de Wit and Baxendale, 2013).

2.4.12.2 Clinical diagnosis

The clinical diagnosis of the acute forms of IBD is based on disease evolution (a mortality peak followed by recovery in 5-7 days), and relies on the observation of clinical signs and gross lesions, in particular of the BF (Lukert and Saif, 2003).

2.4.12.3 Histological diagnosis

Histological diagnosis is based on the detection of modifications occurring in the BF and other lymphoid organs (van den Berg, 2000).

2.4.12.4 Viral isolation

Infectious bursal disease virus may be detected in the BF of chicks in the acute phase of infection, within the first three days following the appearance of clinical signs (van den Berg, 2000). Isolation of the virus is achieved when filtered homogenate of the BF is inoculated in nine to eleven day-old embryonated eggs originating from hens free of anti-IBDV antibodies (Lukert and Saif, 1997). The most sensitive route of inoculation is the CAM; yolk sac route is also practicable, and the intra-allantoic route is the least sensitive (van den Berg, 2000). In the absence of lesions, the embryos from the first passage should be homogenized in sterile conditions and clarified, and two additional serial passages should be performed (Hitchner, 1970; Rosenberger, 1989; Lukert and Saif, 1997). Classic viruses usually kill the embryos in 3-5 days and produce lesions of vascular congestion and subcutaneous hemorrhages in the embryos (Hitchner, 1970; Lambrecht *et al.,* 2000). Variant viruses however, do not kill the embryos but cause embryo stunting, discolouration, splenomegaly and hepatic necrosis (Lukert, 1986). Primary cell cultures of chicken embryo fibroblasts (CEF), bursa (CEB) and kidneys (CEK) have been used to propagate the IBDV.

2.4.12.5 Serological detection

Serological tests used for the detection of antibodies to IBDV are ELISA, VN and AGPT. The ELISA is the commonly used test for detection of antibodies to IBDV (Lukert, 1986). It is economical, simple, quick, and tests a large number of samples at the same time and is adaptive to automation of computer software (Lukert, 1986). The ELISA allows the quantification of antibodies to IBDV and is used for monitoring the immune status of the chicken flocks (Lukert and Saif, 2003), to check response to vaccination, natural field exposure and decay of maternal antibody titre (Lasher and Shane, 1994; Lukert and Saif, 2003). The VN titres accurately reflect the relative protection of chickens to IBDV (McFerran *et al.*, 1980; Jackwood *et al.*, 1985; Jackwood and Saif, 1987; Ismail and Saif, 1991). However, VN is laborious and time consuming and therefore its used is limited to research applications. Although *in vitro* VN tests can be used for detection of antigenic differences between the virus strains, *in vivo* cross protection studies are essential for determining immunogenicity of the virus and complete evaluation of host response (Jackwood and Saif, 1987). Another method used for detect antibodies to IBDV is the agar gel immunodiffusion (AGID) test (Cullen and Wyeth, 1975). This test has been adapted to the quantitative format (Cullen and Wyeth, 1975); it is rapid but insensitive. It does not detect serotypic differences and measures primarily group-specific soluble antigens (Lukert, 1986).

2.4.12.6 Molecular detection

Reverse transcription-polymerase chain reaction (RT-PCR) is a molecular tool frequently applied in IBDV diagnosis. RT-PCR in combination with restriction enzyme analysis allows for rapid identification of vvIBDV (Lin *et al.*, 1993; Jackwood and Jackwood, 1994; Zierenberg *et al.*, 2001). Restriction fragment length polymorphism (RFLP) has also been used to form six different molecular groups of IBDV (Ture *et al.*,

1998; Jackwood and Sommer, 1999). Nucleotide sequencing of RT-PCR products is widely used for further characterization of IBDV strains (Sapats and Ignjatovic, 2000; Zierenberg *et al.,* 2000; Islam *et al.,* 2001; Liu *et al.,* 2002; Viswa *et al.,* 2002).

2.4.12.7 Differential diagnoses

The conditions most likely to be mistaken for IBD are avian coccidiosis, ND, stunting syndrome, chicken infectious anaemia, mycotoxicoses and nephropathogenic forms of infectious bronchitis, inclusion body hepatitis, and lymphoid leucosis (Faragher, 1972; Okeke *et al.,* 1982). In all acute cases, the presence of bursal lesions allows for a diagnosis of IBD (Lukert and Saif, 2003). In subclinical cases, an atrophy of the BF may be confused with diseases such as Marek's disease or infectious anaemia. A histological examination of the BF will allow differentiation between these diseases (Lukert and Saif, 1997). Visceral form of ND has been observed to cause severe degeneration of lymphocytes in the medullary region of the BF (Lukert and Saif, 2003; Okoye, 2005), but necrosis and later replacement of lymphocytes by heterophils are additional features of IBD.

2.4.13 Treatment of infectious bursal disease

There is no specific treatment for IBD, but supportive measures such as increasing heat, ventilation and water consumption are beneficial. Vitamins and minerals and/or sugar can be added to drinking water to prevent dehydration, replace lost electrolytes and provide energy boost (Giambrone, 2013).

2.4.14 Prevention and control of infectious bursal disease

2.4.14.1 Vaccination

In practice, control of IBD is greatly dependent upon the use of vaccines. A satisfactory vaccine should protect against IBD, especially the acute form, and the consequences of

the disease, namely immunosuppression (Ingrao *et al.,* 2013). There is indeed a close correlation between titres in neutralizing antibodies and protection against IBD (Ingrao *et al.,* 2013). Passive protection is provided by maternally-derived antibodies (MDAs) against mortality, lesions of the BF and immunosuppression, respectively. The half–life of MDAs, depending on blood volume, varies between 3 (broilers) and 5 days (layers) (Ingrao *et al.,* 2013). However, MDAs can interfere with vaccination and prevent active antibody production (Block *et al.,* 2007; Mahgoub, 2012; Ingrao *et al.,* 2013). The MDA are transmitted from the mother to her offspring through the yolk and protect the chicks until the development of the adaptive immune response (Ingrao *et al.,* 2013). Thus, if one knows the antibody titre of a chick at hatch, one can determine the time of maximum susceptibility to the vaccine. This information is very important when establishing vaccination programmes (Abdu, 1985). Neutralizing antibodies are protective and can be provided by immunizing chickens with live attenuated vaccines given in drinking water (de Herdt *et al.,* 2005). In addition, a live vaccine will induce a strong CMI response (Ingrao, 2013). To obtain high levels of MDAs in the progeny, parent stocks are vaccinated between 4 and 10 weeks of age with live vaccine and again at 16-18 weeks of age with inactivated oil-adjuvanted vaccine. In the progeny, the MDA levels wane with time, but protects against virulent challenge up to between 2 to 7, weeks of age (Abdu, 1985; van den Berg, 2000; Mahgoub, 2012; Ingrao *et al.*, 2013). As MDA levels may vary within a flock and between flocks repeated vaccination is practiced in order to ensure that chicks are actively immunized as soon as the MDAs have waned to a level at which they do not interfere with vaccination (Abdu, 2014). Intermediate and hot vaccine strains can induce bursal lesions and cause immunosuppression (Mazariegos *et al.,* 1990). The intensive use of intermediate/ or hot vaccines increased preoccupations about residual pathogenicity and about the decreasing

effectiveness of vaccines against other diseases (Mazariegos *et al.,* 1990). Most live IBDV vaccines are based on classical virulent strains (Mazariegos *et al.,* 1990). Those classified as "mild" vaccines exhibit only poor efficacy in the presence of certain levels of MDAs and against vvIBDV. 'Intermediate' and 'intermediate plus' or 'hot' vaccines have better efficacy and may breakthrough higher levels of MDAs, but they can induce moderate to severe bursal lesions and, thus, cause corresponding levels of immunosupression (Mazariegos *et al.,* 1990; Tsukamoto *et al.,* 1995; Kumar *et al.,* 2000; Rautenschlein *et al.,* 2005). The "mild" vaccines may not fully protect chickens against infection by the vvIBDV strains (Rautenschlein *et al.,* 2005) or by antigenic variants. Safety and efficacy of "mild" vaccines still remain a major concern (Rautenschlein *et al.,* 2005).

2.4.14.2 Biosecurity

There is evidence, that thorough cleaning and disinfection of houses between flocks and the practice of all-in all-out farming methods, cleaning and disinfection of premises, and observance of a 'down time' (a period of rest between depopulation and restocking) can reduce infection rates (Heine and Boyle, 1993). Given the contagious nature of IBD and resistance of the IBDV, certain essential steps in the cleaning/disinfection process should be adhered to (Benton *et al.,* 1967a). Prior to cleaning, all insects and pests must be eliminated as soon as the farm premises are empty. Old bedding and dung must be eliminated and composted. All farm equipment must be disassembled and stored in cleaning rooms located outside the farm buildings. The buildings, immediate surroundings and farm equipment must be dry-cleaned first, in order to eliminate all dust, and then hosed down using hot water (60 °C) with a detergent, at a pressure of 80 bar to 150 bar (Benton *et al.,* 1967a). A second disinfection of the full premises must be performed before the introduction of new chicks. Feed silos must be emptied completely

and cleaned inside and outside. Under no circumstances may feed remains from previous flocks be reused. Disinfection is to be undertaken only after all the buildings have been cleaned. The quantity of disinfectant solution to be used is 4 liters per $15m^2$ (Higashihara *et al.,* 1991) reduces the challenge virus. It may also delay challenge thus allowing more time for vaccines to induce immunity (Ingrao *et al.,* 2013).

2.4.15 Economic significance of infectious bursal disease

The economic impacts of IBD include; losses due to morbidity and mortality, immunosuppression in surviving chickens, reduction in the chicken's ability to respond to vaccination and risk of introduction to exotic places from importing infected poultry products (Eterrodossi, 1995; Van den Berg, 2000; Shamaila, 2005).

2.5 Oxidative Stress

2.5.1 Definition of oxidative stress

Oxidative stress is a consequence of either over production of ROS or a decreased efficiency of the antioxidant defence system (ADS), or both (Rains and Jain, 2011; Yonar *et al.,* 2012; Wei *et al.,* 2013). Excess production of ROS exerts pressure on the ADS of the organism, thereby depleting its components. The term "oxidative damage" refers to cell and tissue damages that result from the excess ROS in oxidative stress.

2.5.2 Effects of free radicals on biological system

Reactive oxygen species are extremely reactive and not depleted in the chemical reactions they induces (Kumar *et al.,* 2012). They instead form more radicals, which in turn form more radicals, resulting in a radical chain reaction (Yin and Porter, 2011). Thus, one ROS molecule is capable of destroying irreversibly several biomolecules and tissues in a chain oxidation reaction (Galli *et al.,* 2005; Gallego *et al.,* 2013). ROS

induce cellular injury by reacting with biomolecules such as nucleic acids, nucleotides, polysaccharides, protein, non–protein, thiols, thereby altering their structure and function. ROS also covalently bind to membrane components (proteins, lipids, enzymes, receptors, and transport systems), thereby altering cellular homeostasis and initiating lipid peroxidation (Comporti, 2012).

Superoxide radical is the first free radical formed from the electron transport chain. It is a precursor of ROS such as singlet oxygen and hydroxyl radicals (Acharya *et al.,* 2011). It is thus not only a physiological oxidant but also a pro–oxidant (Reiter and Rusnak, 2002; Cadenas and Boveris, 2011). The superoxide radical is able to oxidize biomolecules including DNA and enzymes. It is capable of directly inactivating CAT and GPx, thus exhausting the ADS (Shah *et al.,* 2013). Hdyroxyl radical is extremely reactive and can oxidize almost all low-molecular weight organic compounds. It is highly unstable and is thus the most destructive ROS. It is generated through the breakdown of H_2O_2 in the presence of copper or iron (Maezono *et al.,* 2011). Peroxyl radical have been shown to mediate lipid peroxidation and its activity alters cell homeostasis as well its deleterious effects are seen on the cell membrane (Salama *et al.,* 2013). Regulation of ROS is therefore critical for cell viability, proliferation, and organ function (Birben *et al.,* 2012). Although notorious from the harm they cause on biomolecules in the cells, ROS are important physiologically. At low concentrations, they have been shown to act as intracellular second messengers involved in intracellular signaling (Finkel, 2011; Kruk *et al.,* 2013). They activate cascade 3, an important mediator of apoptosis (Ma *et al.,* 2013; Wang *et al.,* 2013). Injury-induced ROS production is an important regulator of tissue regeneration as seen in *Xenopustadpole* tail regeneration (Love *et al.,* 2013). ROS, being products of radical chain reactions

(secondary radicals), they serve several physiological roles in the living cell as signaling molecules and have been shown to be important immunological substances, as they mediate inflammatory responses and possess antimicrobial effects (Koskenkorva-Frank *et al.,* 2013), but, like ROS, in nitrosamine stress, they induce extensive cellular damage when in excess (Tripathi *et al*., 2013).

2.5.3 Oxidative stress biomarkers

Oxidative stress biomarkers are measures of antioxidant enzymes; superoxide dismutase (SOD), catalase (CAT), glutathionine peroxidase (GPx) and non-enzymatics; malondialdehyde (MDA), erythrocyte osmotic fragility (EOF) concentrations in the body as a result of damage caused by oxidative stress status from the oxidation of lipids, proteins and DNA (Kurutas *et al.,* 2015; Sarawoot and Phanit, 2015).

2.5.4 Antioxidants

Antioxidants are compounds which act as inhibitors of oxidation (Naik, 2003). When present at low concentration in relation to oxidizable substrates they inhibit or delay oxidative processes, while being oxidized themselves (Kumar, 2011). Some antioxidants are synthesized endogenously while most are absorbed from food and supplements (Tewari *et al.*, 2014).

2.5.5 Types of antioxidants

Antioxidants are classified based on: their source and mode of action. Based on source, antioxidants are classified into natural and artificial antioxidants (Mishra and Bisht, 2011). Natural antioxidants occur in nature and are derived from plant sources. They are mostly phenolic compounds such as flavonoids and phenolic acid, vitamins and volatile compounds (Ahmad *et al.,* 2013). Natural antioxidants are often preferred to artificial

antioxidants as they are safe and have little or no interference with the body's ability to use free radicals constructively (Wolfe *et al.,* 2003). Artificial antioxidants are (butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) which are the products of chemical synthesis. They are effective and commonly used as preservatives in processed food, but have side effects and may be carcinogenic (Jaafar *et al.,* 2013).

2.5.6 Mode of action of antioxidants

Antioxidants bind to and sequester ROS and transition of metal ions, such as iron and copper, which contain unpaired electrons and accelerate free radical formation (Cui *et al.,* 2004). The mode of action of an antioxidant is via one or more of the following routes:

(1) Chain-breaking reaction: eg α-tocopherol (vitamin E) act in lipid phase by donating electrons to form tocopheroxyl radical, thereby terminating the chain reaction.

(2) By reducing concentration of ROS; eg glutathione (Pang and Panee, 2014).

(3) By scavenging initiating radicals which act in the lipid phase to trap superoxide free radicals.

(4) By chelating transition metal catalyst: a group of antioxidant compounds act by sequestration of transition metals that are well established peroxidases. For example, transferrin, lactoferrin and ferritin function to keep iron induced oxidant stress in check while eruloplasmin and albumin are copper sequestrants (Vaya and Aviram, 2001; Kumar, 2011).

(5) By repairing damages caused by ROS, antioxidants remove damaged biomolecule before they can accumulate and their presence results in altered cell metabolism and viability (Noguchi *et al.,* 2000).

2.6 Haematology of Birds

2.6.1 Packed cell volume

Normal PCV for many bird species ranges between 20% and 40% (Abdu, 2014). Anaemic birds have a PCV of less than 35% (Thrall, 2004). According to Polo *et al.*, (1998), psittacines normal PCV is greater than 45%, therefore references ranges for individual species should be consulted.

2.6.2 Erythrocytes

2.6.2.1 Red blood cell

Total red blood cell, and haemoglobin (Hb) concentrations, and packed cell volume (PCV) are influenced by age, sex, hormones and other factors. Packed cell volume and total erythrocyte count are higher in male birds than in female and increase with age (Herbert *et al.*, 1989). Because erythrocytes turnover is more rapid, birds tend to have higher percentages of polychromatophils in health than mammals. The mean corpuscular values (RBC indices) provide information about the haemoglobin content and size of RBC which include the average red blood cell size (MCV), haemoglobin amount per red blood cell (MCH), and amount of haemoglobin relative to the size of the cell (Hb) per red blood cell (MCHC). These RBC indices are used to diagnose the presence and type of anaemia (Campbell, 2004a). Birds suffering from heavy metal toxicosis, especially lead poisoning, often reveal an inappropriate release of immature erythrocytes in a non-anaemic patient. Animals that are responding to anaemia may exhibit an increase in the number of immature erythrocytes in the peripheral blood film (Campbell, 2004b). The major function of erythrocytes is oxygen transport, but it has been reported that bird erythrocytes, as non-immune cells, are able to participate in some immune responses that contribute to host defence (Passantino *et al.*, 2007).

2.6.3 Thrombocytes

Thrombocytosis an increase in the size of thrombocytes may be seen with chronic inflammation in birds (D'Aloia *et al.,* 1994). Avian thrombocytes produce thromboplastin and aggregate in a site of vascular injury, forming a haemostatic plug (Mitchell and Johns, 2008; Claver and Quaglia, 2009). Avian thrombocytes have phagocytic abilities. They play an important role in removing foreign materials from the blood and have some functions in non-specific immunity (Edmonds, 1968; Grecchi *et al*., 1980; Bounous and Stedman, 2000). Increased destruction or use of thrombocytes in conditions such as septicaemia or disseminated intravascular coagulopathy (DIC), thrombocytopaenia can be seen. Thrombocytopaenia can be seen as a component of pancytopaenia in some viral diseases, such as psittacine circovirus or polyomavirus infections (Fudge, 1997) and in lymphoid leukemia (Latimer, 1994).

2.6.4 Leucocytes

2.6.4.1 Heterophils

Heterophils are the most common granulocytes in circulation in majority of birds (Mitchell and Johns, 2008; Claver and Quaglia, 2009). Owing to their highly phagolytic activities, they are capable of a broad spectrum of antimicrobial activity (Claver and Quaglia, 2009). There are two types of changes observed in heterophils during the course of disease processes in birds. One change is the presence of immature cells in peripheral blood, representing recruitment of cells from the bone marrow in response to cytokines and other inflammatory mediators (Campbell and Ellis, 2007). A degenerative left shift, in which the number of immature heterophils exceeds the number of mature heterophils indicates intense tissue demand for cells and carries a poor prognosis (Mitchell and Johns, 2008). The other change observed in avian heterophils during disease is toxic change. Generally, when toxic heterophils are seen, all the heterophils in

the film appear toxic and usually to the same degree unless the condition is caught in the per acute stage or is resolving (Campbell, 2004c). Conditions that cause an increase in heterophils include; infection (bacterial, fungal, viral and parasitic), inflammatory stress, certain toxicities, trauma, and leukemia (Gildersleeve *et al.,* 1987; Andreasen *et al.,* 1993; Harmon, 1998; Bienzle and Smith, 1999). Infectious agents that commonly lead to heterophilia includes; *Mycobacterium, Chlamydophilia*, *Aspergillus* and *Mycoplasma* species. Heterophilia associated with these organisms is accompanied by monocytosis (Branton *et al.,* 1997). Heterophilia with toxic substances change is indicative of severe systemic illness such as septicaemia, chlamydophilosis, fungal infection, or viremia. The development of toxic change may indicate lack of control of an infectious process and carries a poor prognosis (Campbell and Ellis, 2007). Some toxic substances (organophosphate) can lead to heterophilia (Heatley and Jowett, 2000). In addition, heterophilia has been observed in zinc toxicosis, as a result of GIT inflammation, stress, and decreased resistance to pathogens (Campbell and Ellis, 2007). Macaws may demonstrate marked leukocytosis with heterophilia as a result of transport and handling. Corticosteroids results in an increase in circulating heterophils and lymphopaenia in birds (Harmon, 1998). A decrease in heterophil number can be seen with increased use of cells or decreased production. Overwhelming infection such as septicaemia can lead to a degenerative left shift which indicates bone marrow depletion.

2.6.4.2 Lymphocytes

An increased presence of lymphocytes is observed in birds that have infectious disease (Mitchell and Johns, 2008). Lymphocytosis usually occurs as a result of antigenic stimulation. This can occur in psittacines with viral diseases, such as herpes virus or *Psittacine circovirus* (Fudge and Joseph, 2000). Lymphocytosis also occurs with wound healing, inflammatory diseases, parasitic infections, and viral diseases (Campbell,

2004a) and lymphoid leukemia (Latimer, 1994). Lymphopaenia may be seen as a result of excess endogenous or exogenous corticosteroids (Harmon, 1998) as observed in stressful conditions and malnourished birds (Campbell, 2004b).

2.6.4.3 Monocytes

Monocytosis occurs in infectious and/or inflammatory disease, especially with granulomatous diseases such as aspergillosis or mycobacteriosis. *Chlamydophila psittaci* infections in birds also results in monocytosis usually due to the production of chemotactic agents that attract monoyctes (Campbell, 1994). Although monocytosis is common with chronic inflammation, acute infections, such as *Mycoplasma* species infections, may lead to monocytosis in addition to heterophilia and lymphopaenia (Branton *et al.,* 1997). Monocytosis has also been observed in birds fed a zinc-deficient diet (Wight *et al*., 1980).

2.6.4.4 Basophils

Basophils play important role in the initial phases of acute inflammation and immediate hypersensitivity reactions. This, however, does not always result in peripheral basophilia (Montali, 1988; D'Aloia et al., 1994; Maxwell and Robertson., 1995; Campbell and Ellis, 2007). Severe stress is an underlying cause for increased basophilic response in birds (Maxwell, 1993; Altan *et al.,* 2003; Bedăñovă *et al.,* 2007; Campbell and Ellis, 2007).

2.6.4.5 Eosinophils

Studies have shown eosinophilia with generalized inflammation in birds; thus avian eosinophils play a role in delayed hypersensivity (Montali, 1988). Severe eosinophilias have also been observed in cases of poxvirus infection in red-tailed hawks (Mitchell and Johns, 2008).

2.6.4.6 Heterophil-to-lymphocyte ratio

In birds, the heterophil-to-lymphocyte (H/L) ratio is a useful tool for monitoring stress response (El Lethey *et al.*, 2003; Post *et al.*, 2003; Davis *et al.*, 2008), and infection status for some diseases (Davis *et al.*, 2004; Chakarov et *al.*, 2008; Fokidis *et al.*, 2008; Norte *et al.*, 2009). Acute stress is known to increase H/L ratio (Lazarevic *et al.*, 2000; Ewenson *et al.*, 2001; Ruiz *et al.*, 2002; Scope *et al.*, 2002; El Lethey *et al.*, 2003; Bedăňovă *et al.*, 2007; Davis *et al.*, 2008).

2.7 Serum Biochemistry of Birds

2.7.1 Metabolites and nutrients

2.7.1.1 Glucose

In periods of starvation, glucose is derived from the breakdown of fats and proteins, primarily from muscle tissue (Hochleithner, 1994). The normal plasma glucose concentration of birds ranges between 200 and 500 mg/dl. Plasma glucose concentration remains stable during 1-5 days of fasting in birds (Campbell, 1998). Hypoglycaemia is extremely rare in birds and when present, is almost never associated with starvation (Harris, 2000). Hypoglycaemia, however, occurs with septicaemia, severe liver disease, enterotoxaemia and prolonged starvation (Campbell, 1998; Harris, 2000). Glucose concentration can be artificially decreased during storage if the blood sample is contaminated with bacteria (Hochleithner, 1990). Plasma glucose levels are higher in juvenile than adult budgerigars (Hochleithner, 1989b). Variations also occur due to time of day and amount of environmental stress (Lewandowski *et al.*, 1986). Hyperglycaemia occurs due to catecholamine release (as seen in exertion and excitement) or after meals, glucocorticosteroid excess, and diabetes mellitus (Woerpel and Rosskopf, 1984; Amand, 1985; Glystorff and Grimm, 1987; Lumeij, 1987a' Campbell, 1998; Harris, 2000). Diabetes mellitus in birds is associated with glucose concentration greater than 800

mg/dl (Campbell, 1998). Transient evaluations in glucose have been reported in cockatiels with egg-related peritonitis (Woerpel and Rosskopf, 1984). Decrease in plasma glucose levels can be due to hepatic dysfunction (Pacheco's disease virus), impaired glucose production or its excessive utilization (septicaemia, neoplasia, aspergillosis) (Rosskopf, 1982; Woerpel and Rosskopf, 1984).

2.7.1.2 Total protein

Total protein concentration ranges between 2.5 and 4.5 g/dl in normal birds (Campbell, 1998). Campbell and Dein (1984) reported that the total plasma protein values lower than 3.0 g/dl are hypoproteinaemic and birds with values less than 2.5 g/dl have poor prognosis. Amand (1985) reported that birds with total plasma proteins below 3.5 g/dl appear to have less of a chance to recover from their illness (Lumeij, 1987c). Advancing age has been associated with increases in total protein in several bird species (Glystorff and Grimm, 1987; Clubb *et al.*, 1991a, b). Hypoproteinaemia can reflect reduced synthesis caused by chronic hepatopathies, malasorption caused by chronic enteropathies (enteritis, tumours, and parasitism), increased loss caused by proteinuria due to renal disease, blood loss and malignant tumours or starvation and malnutrition (Hocheleithner, 1994). Hyperproteinaemia may be induced by chronic infectious diseases that stimulate the synthesis of gamma globulin. It also has been seen with chronic lymphoproliferative disease (Lewandowski *et al.*, 1986) and myelosis in budgerigars (Hochleithner, 1991). Dehydration should always be determined and ruled out as a cause of hyperproteinaemia (Hochleithner, 1994). Albumin in birds functions primarily in the maintenance of colloid osmotic pressure, as a rapid substitute for amino acids, assuring glucose through gluconeogenesis in transport of minerals and hormones, in build-up of enzymes and immune system in the organism (Harris, 2000; Filipovic *et*

al., 2007). Decreases in albumin concentration can occur from decreased synthesis due to chronic liver disease or chronic inflammation, increased albumin loss due to renal disease, parasitism or over-hydration (Quesenberry and Moroff, 1991). A decrease in albumin causes oedema because of a decrease in oncotic pressure (Hochleithner, 1994).

2.7.2 Electrolytes

2.7.2.1 Calcium

As a major constituent of bone, calcium plays a role in the structure of the body. It also has important physiologic functions involving the transmission of nerve impulses, the permeability and excitability of all membranes, the activation of enzyme systems (blood clotting), calcification of egg shells and contraction of the uterus during oviposition (Hochleithner, 1994). Two fold or greater elevations of calcium occur with ovulation. Causes of lowered blood calcium levels include; hypocalcaemic syndrome in African grey parrots, glucocorticoid, hypomagnesaemia and insufficient exposure to light (Hochleithner, 1989b, c; Lumeij, 1990; Hochleithner, 1991b; Harris, 2000). Elevated calcium levels have been reported in conjunction with elevated albumin (Ivans *et al.*, 1985; Harris, 2000). Young birds have lower calcium concentrations than adults (Hannon, 1979; Hochleithner, 1989a). Elevated levels of calcium have been associated with vitamin D3 toxicity, osteolytic bone tumours, renal adenocarcinoma and dehydration.

2.7.2.2 Phosphorus

Diets that consist mostly of seeds may lead to increased phosphorus levels. Juvenile budgerigars were found to have higher phosphorus concentrations than adults (Hochleithner, 1989a). Elevated plasma phosphorus is observed in avian renal failure (Woerpel and Rosskopf, 1984; Amand, 1985; Hochleithner, 1991b; Harris, 2000).

Increase in phosphorus has also been reported in hypoparathyroidism (Woerpel and Rosskopf, 1984; Lewandowski *et al.*, 1986) and nutritional secondary hyperparathyroidism (Hochleithner, 1989b, c; Harris, 2000). Haemolysis may result in increase in phosphorus concentration (Hochleithner, 1994). Decreased phosphorus values may be associated with vitamin D deficiency and malabsorption because of phosphate binding agents in the diet and long term glucocorticoid therapy (Hochleithner, 1994; Harris, 2000).

2.7.3 Kidney function biomarkers

2.7.3.1 Blood urea nitrogen

The tubular reabsorption of urea in conditions of renal failure accompanied by a low urine flow (dehydration), causes an increase in plasma urea concentrations which consequently results in elevated urea: uric acid ratio (Lumeij, 1987b). An increase in the BUN concentration suggests prerenal azotaemia in racing pigeons (Lumeij, 1987b; Campbell, 1998). Normal plasma urea nitrogen concentrations of non-carnivorous birds are less than 5 mg/dl, while the carnivores have higher normal values (Balasch *et al.*, 1976; Gee *et al.*, 1981; Ferrer *et al.*, 1987, Campbell, 1998). Urea concentration rises during periods of fast as a consequence of the catabolism of tissue proteins as an energy source of protein (Okumura and Tasaki, 1969; Garcia-Rodriguez *et al.*, 1987; Ferrer and Dobado-Berrios, 1998; Totzke *et al.*, 1999).

2.7.3.2 Creatinine

Creatinine originates from muscle metabolism and is formed from creatine phosphate. In all avian species, the reference intervals for creatinine have been between 0.1 to 0.4 mg/dl (Lumeij, 1987b, c; Hochleithner, 1994). Elevated values of creatinine have been recorded in cases of dehydration in racing pigeons (Lumeij, 1987b) and kidney disease,

but creatinine is not considered to be a reliable indicator of renal function (Harris, 2000). Elevations have been described in connection with egg peritonitis, septicaemia (chlamydiosis) and nephrotoxic drugs (Lewandowski *et al.,* 1986). Prerenal azotaemia can be expected to be a common complication of many disease conditions in birds (heart failure, dehydration) (Lumeij, 1987b).

2.7.4 Liver enzymes

Anoxia causes cell membranes to lose their integrity so that soluble enzymes from cytosol can leak into the serum (Hochleithner, 1994). Anoxic RBCs, leak cytosolic lactate dehydrogenase (LDH) into serum, causing an increase. Some liver enzymes assayed include alanine aminotransferase (ALT or GPT), alkaline phosphatase (ALP), aspertate aminotransferase (AST or GOT), creatine kinase (CK or CPK), creatinase, gamma glutamyl transferase (GGT), glutamate dehydrogenase (GDH), sorbitol dehydrogenase (SDH) and lactate dehydogenase (LDH) (Hochleithner, 1994).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Location and Period

The experiment was carried out in the Department of Veterinary Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria. Zaria is located in the Northern Guinea Savannah zone of Nigeria between longitudes $7^{o}42'$E and latitude $11^{o}3'$N with an altitude of 550-700 metres above sea level and a total land mass of about 300 square kilometres (Clackson, 1957*)*. Zaria is characterised by a tropical climate, a monthly mean ambient temperature, ranging between 13.8 and 36.7^{o}C and an annual rainfall of 1,092.8 mm. The main occupation of the people of Zaria is agriculture; approximately 40-75% of the population's livelihood is from agriculture (ABU, 2000).The experiment was conducted between Septembers to November 2019. During the rainy season to harmattan period (Clackson, 1957)

3.2 Ethical Clearance

Ethical clearance was sought and obtained from the Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC), with Reference Number: ABUCAUC/2019/19 (Appendix 3).

3.3 Materials

3.3.1 Experimental chickens

A total of two hundred and fifty ISA Brown day-old chicks were obtained from a commercial hatchery located in Ibadan, Nigeria. The chicks were transported using vehicle from the hatchery to the Poultry Research Pen of the Ahmadu Bello University Veterinary Teaching Hospital (ABUVTH) Zaria.

3.3.2 Housing and management

The chicks were brooded on deep litter and provided with a floor space of 0.10 square metres per bird. The Poultry Research Pen was thoroughly cleaned, washed with water and detergents, disinfected with Diskol® (5% benzalkonium chloride, 7.5% glutaraldehyde, 7.5% formaldehyde, stabilisers and antioxidants 9.5%) and Virkon-S® (Potassium peroxymonosulfate and sodium chloride) and fumigated with 10% formalin twice at two weeks' intervals before the chicks arrived. Rodents and insects control were achieved using rodenticide and insecticide; push-out® (Zinc phosphide 80%) and insecticide DD Force® (Diclovos-Organophospharous), respectively twice at one-week interval before the arrival of the chicks. Wood shaving served as litter material and feeders and drinkers (5 each) were provided. One hundred-watt bulb (100-watt bulb) was provided in each of the five compartments to supply light and heat during brooding. Upon arrival the birds were quickly removed from the chick boxes and randomly assigned one chick at a time into five different groups.

3.3.3 Feed and feeding

The chicks were fed with chick mash from 0 to 49 days of age. Feed and water were provided *ad libitum*. The chick mash was purchased from a commercial feed distributor in Zaria, Nigeria and proximate analyses carried out at the Department of Animal Science Laboratory, ABU Zaria (Apppendix 2).

3.3.4 Prebiotic, probiotic and synbiotic

Prebiotic (Molasses) in liquid form (Aappendix 4). Produced by Savannah Sugar Company, located in Gyewana, Lamurde Local Government Area of Adamawa State, Nigeria. Probiotic (Antox®) in liquid form (Appendix 5) obtained, from a Company Sole Agent (AHA Pharma Company Limited), GG No.: 16 Benin Street by Kano road,

Kaduna State, Nigeria. Manufactured by Montajat Pharmaceuticals, Bioscience Division, Dammam 31491, Saudi Arabia, and Synbiotic (EN-FLORAX®) in powdered form (Appendix 6) obtained, from a Company Sole Agent (FDH Agro-vet. Nig. Ltd), No.: 77, Iwo Road Ibadan, Oyo State, Nigeria. Manufactured by EKSPOL s.c, ul, Romana Maya 1, 62-030 lubañ, Poland.

3.3.5 Newcastle disease vaccine La Sota

Newcastle disease vaccine La Sota manufactured by the National Veterinary Research Institute, Vom, Plateau State, Nigeria with the following details: Batch No.: 04/2019; Expiry date: March, 2020 and Dose: 200 was purchased from a sole agent in Samaru, Sabon-Gari Local Government Area, Kaduna State, Nigeria.

3.3.6 Infectious bursal disease virus inoculum

A characterised vvIBDV (Adamu *et al.*, 2015) were obtained from the Department of Veterinary Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University Zaria, Nigeria.

3.3.7 Enzyme-linked immunosorbent assay titre kit

Enzyme-linked immunosorbent assay (ELISA) titre kit was obtained from IDEXX Laboratories, Incorporate, Westbrooke, Maine 04092, USA.

3.3.8 Newcastle disease antigen and antiserum for haemagglutination and haemagglutination inhibition tests for the determination of antibody against Newcastle disease vaccine La Sota

Newcastle disease vaccine La Sota strain obtained from the NVRI, Vom, Nigeria was used as antigen and the antiserum were obtained from a confirmed outbreak of Newcastle disease (ND) in a backyard poultry farm in Zaria, Kaduna State, Nigeria which recorded mortality rate of more than 80%, for the HA and HI tests.

3.4 Methods

3.4.1 Experimental design

Two hundred and fifty ISA Brown day-old chicks were individually weighed and assigned in a complete randomised design into five different groups A, B, C, D, and E of 50 chicks each. Chicks group A were administered molasses at 2 mL/litre of drinking water from day-old to 49 days of age and inoculated with vvIBDV at 28 days of age at 0.05 mL/chick orally. Chicks group B were administered Antox® at 1.5 mL/litre of drinking water from day-old to 49 day of age and inoculated with a vvIBDV at 28 days of age at 0.05 mL/chick orally. Chicks in group C were administered EN-FLORA® at 1 g/litre of drinking water from day-old to 49 days of age and inoculated with a vvIBDV at 28 days of age at 0.05 mL/chick orally. Chicks group D were inoculated with a vvIBDV at 28 days of age at 0.05 mL/chick orally and no supplements were administered and it served as positive control. Chicks group E was not administered supplements nor inoculated with vvIBDV, but distilled water was administered at 0.05 mL/chick orally at 28 days of age and served as negative control. On days 1, 7, 14, 21, 28, 35, 42, and 49 of age of supplements administration and post-inoculation, respectively, five chicks from each group were weighed, bled, euthanised and their BD, BF, SPL and THY harvested and weighed. Blood collected was evaluated for the presence and titre of IBD and ND antibodies, haematological parameters, serum biochemicals, activities of liver enzymes and oxidative stress biomarkers. Feed intake, and feed conversion ratio were evaluated. The BD weights, organ (BF, SPL and THY) weights to body index and histopathological changes were determined. At 28 days of age post-inoculation with vvIBDV, the chicks were observed for clinical signs, morbidity and mortality rates and gross lesions of IBD. At 35 days of age (1 week post

inoculation with vvIBDV) the chicks were vaccinated with ND vaccine La Sota to monitor antibody response (Table 3.1)

3.4.2 Inoculation of chicks with infectious bursal disease virus

Each of the chicks in a group was inoculated with 0.05 ml of the vvIBDV suspension containing $10^{8.46}$ CID_{50} via oral route at 28 days of age.

3.4.3 Clinical observation and determination of morbidity and mortality rates

3.4.3.1 Clinical observation

Following inoculation of chicks with vvIBDV, the onset of clinical signs was noted. The number of birds exhibiting each of the clinical sign of IBD was recorded daily. The percentage of birds exhibiting each of the clinical signs was calculated using the total number of bird in each group on a particular day. The percentages of all the clinical signs exhibited were summed and divided by the total number of clinical signs exhibited on a particular day to arrive at the daily % clinical sign for that day. When clinical signs disappeared, daily % clinical signs were summed and divided by the total number of days the clinical signs were observed to obtain the average % clinical signs. The average % clinical signs were grouped into five categories; 1-20%, 21-40%, 41-60%, 61-80% and 81-100%. The overall severity of clinical signs for each group was assigned scores on a scale of five using the average % clinical signs as; 1 = mild (1-20%), 2 = moderate (21-40%), 3 = severe (41-60%), 4 = very severe (61-80%), and 5 = grave (81-100%) (Appendix 7).

Table 3.1: Experimental design for evaluating the effects of molasses, Antox®, and EN-FLORAX® on clinico-pathological changes, antibody response and performance indices in commercial pullets experimentally infected with infectious bursal disease virus

						Age in days								
						Pre inoculation				Post inoculation				
						1	7	14	21	28	35	42	49	
Group	No. of chicks	Treatment	Age (days) of commencement of supplementation with M, A & E-F	Age (days) of inoculation with vvIBDV	Age (days) at vaccination with ND vaccine	Number of chicks bled, euthanised and weighed								Organs weighed and collected for histopathology
A	50	molasses + vvIBDV	1	28	35 doa	5	5	5	5	5	5	5	5	BF, spleen & thymus
B	50	Antox® + vvIBDV	1	28	35 doa	5	5	5	5	5	5	5	5	BF, spleen & thymus
C	50	EN-FLORAX® + vvIBDV	1	28	35 doa	5	5	5	5	5	5	5	5	BF, spleen & thymus
D	50	Z supplements + vvIBDV	Nil	28	35 doa	5	5	5	5	5	5	5	5	BF, spleen & thymus
E	50	Z supplements + Dw	Nil	Nil	35 doa	5	5	5	5	5	5	5	5	BF, spleen & thymus
Total	250					25	25	25	25	25	25	25	25	

KEY: M = Molasses, A = Antox®, E-F = EN-FLORAX®, BF = Bursa of Fabricius, vvIBDV = very virulent infectious bursal disease virus, Z = Zero, Dw = Distilled water.

3.4.3.2 Determination of morbidity and mortality rates

Morbidity and mortality rates in different groups, were monitored, and recorded daily. The morbidity and mortality rates were calculated using the formulae outlined by Babiker *et al.* (2008) as follows; (1-2):

$$\text{Morbidity rate} = \frac{\text{Number of sick chicks}}{\text{Number of chicks inoculated}} \times 100\% \quad (1)$$

$$\text{Mortality rate} = \frac{\text{Number of dead chicks}}{\text{Number of chicks inoculated}} \times 100\% \quad (2)$$

3.4.4 Determination of feed intake, weight gain and feed conversion ratio

Feed consumption were assess based on daily weighed feed given to each group of chicks and the amount of weighed feed left in the feeders the following day. The final weight of feed was subtracted from the initial weight of feed given to obtain the total feed consumed by each group. The average daily weight gains and feed conversion ratios (total feed consumed/total weight gain) for each group were determined as described by Ayssiwede *et al.* (2011) using the formulae below; (3 – 4):

Daily feed intake (g/bird/day) = {(Quantity of feed offered – Quantity of feed left)/day ÷ Number of chicks/group} (3)

Feed conversion ratio = Feed intake during a period (g) ÷ Weight gain of the period (g) (4)

3.4.5 Determination of body weight and organ body weight index

Their body (BD), BF, SPL and THY weights were measured using a digital weighing scale (manufactured by Philip Harris, in Church Bridge House, Henry Street, Accrington, BB5 4EE, with Company Number: 01135827, VAT Number: GB125688644. England).

The organ/body weight ratio and organ/body index was calculated as described by Lucio and Hitchner (1979) and Eterradossi *et al.* (2004) using the formulae; (5):

$$\text{Organ: body index} = \frac{\text{Mean organ/body weight ratio of test group}}{\text{Mean organ/body weight ratio of negative control}} \quad (5)$$

3.4.6 Collection of blood

One to two millilitres of blood was collected through the heart of each chick using a sterile plastic disposable 5 mL syringe and 25 x 7 mm gauge needle. The blood samples were divided into two. One part was emptied into a commercial sample bottles containing ethylene diamine tetra acetic acid (EDTA) for haematology and the other into a commercial plain sample bottles (without anticoagulant) and allowed to clot to produce sera according to the methods described by Okeudo *et al.* (2003). Serum was separated from the clot by centrifugation at 447.2 g for 10 min using a Hermle® centrifuge (Model No.: Z 364 B. HERMLE GmbH & CO., Germany). Each of the sample bottles was labelled using a permanent marker and stored at -20 ^{o}C until examined for IBD and ND antibodies, serum biochemicals and oxidative stress biomarkers.

3.4.7 Serology

3.4.7.1 Evaluation of humoral immune response to infectious bursal disease virus

Enzyme-linked immunosorbent assay (ELISA) was carried out according to the method described by IDEXX Laboratories, Incorporate, Westbrooke, Maine 04092, USA. Briefly, the antigen coated plates and the ELISA titre kit reagents were adjusted to room temperature. The test serum was diluted to five hundred folds (1:500) with sera sample diluent. A 100 µL of diluted serum sample was then poured into each well of the plate. This

was followed by 100 µL of undiluted negative control serum sample into well A1 and A2, and 100 µL of undiluted positive control serum sample into well A3 and A4. The plates were incubated for 30 min at room temperature. Each well was then washed with 350 µL of distilled water three times. Goat anti-chicken conjugate (100 µL) was dispensed into each well. The plate was incubated at room temperature for 30 min, followed by washing each well with 350 µl of distilled water three times. Tetramethylbenzidine (TMB) solution (100 µL) was dispensed into each well. The plate was then incubated at room temperature for 15 min. Finally, 100 µL of stop (stopper) solution was dispensed into each well to stop the reaction. The absorbance values were measured and recorded at 650 nm using an ELISA reader (Blankfard and Silk, 1989). Infectious bursal disease antibody titre level was calculated automatically, using software by Blankfard and Silk (Blankfard and Silk, 1989). Serum samples with the S:P ratio (S = Serum sample, P = Positive control) less than 45 $\log_2$ or equal to 35 $\log_2$ were considered not protective. Ratios above 45 $\log_{10}$ (titres higher than 65 $\log_{10}$) were considered protective as indicated in the IDEXX ELISA Kit manual.

3.4.7.2 Evaluation of humoral immune response to Newcastle disease La Sota vaccine

Preparation of 1% chicken red blood cells

Five millilitres of blood was collected from unvaccinated chickens and pooled into equal volume of Alserver's solution. Red blood cells were washed three times in phosphate buffered saline (PBS) (pH 7.2) and centrifugation at 800 g for five minutes (OIE, 2009). One millilitre of the chicken chicken red blood cells (RBCs) was added to ninety-nine millilitres of PBS making 1% suspension of chicken RBCs.

Determination of the titre of Newcastle disease antigen using haemagglutination test

The titre of Newcastle disease (ND) antigen was determined using haemagglutination (HA) test as described by OIE (2009). Twenty-five microlitres of PBS was dispensed into each well of a plastic V-shape bottomed microtitre plates from well 2 to 12. Twenty-five microlitres of the antigen was then dispensed into the first and second well and a 2-fold serial dilution of 25 µL of the antigen was made across the plates up to well 11 and then followed by 25 µL of 1% chicken RBC across the plate. The solution was mixed by gently tapping the plates and then allowing it to settle for about 40 min at room temperature. Haemagglutination was determined by tilting the plate and observing for the presence or absence of tear shaped streaming of the RBCs. The titration was read to the highest dilution giving complete HA (no streaming) this value represents 1 haemagglutination units (HAU) and the 4 HAU was obtained by dividing the titre value of the 1 HAU by 4.

Determination of the Newcastle disease antibody titre in sera using haemagglutination inhibition test

Haemagglutination inhibition (HI) test was used to determine Newcastle disease virus (NDV) antibody (Ab) titre using the method described by OIE (2009). Twenty-five microliters of PBS were dispensed into each well of a plastic V-bottomed microtitre plate followed by addition of 25 µL of the test serum into the first and second well, then 2-fold dilution were make up to the 11 well. The 4 HAU of the antigen in 25 µL was then added to each well from the first to eleven well. Twenty-five microlitre of 1% (v/v) of chicken RBCs was added to well 1 upto well 12 and then mix gently by tapping the plate and this was allowed to stand at room temperature for 40 min. The HI titre was considered to be the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The inhibition

was assessed by tilting the plates so that RBCs in the well streamed at the same rate as the control well (well 12) were considered positive. The titre of that particular test serum is the reciprocal of the dilution that completely inhibited agglutination of RBCs. The titre was then transformed to $\log_2$. The mean titre of the sera collected from a particular group is the sum of all the titre divided by the total number of the positive samples in that group.

3.4.8 Determination of haematological parameters

3.4.8.1 Evaluation of packed cell volume

The packed cell volume (PCV) was determined using the technique described by Rehman *et al.* (2003). Non-heparinised capillary tube was filled up to about ¾ of its length from one end and the second end was heat-sealed using a Bunsen burner. The blood in the sealed capillary tube was then centrifuged for 5 min at 4,383 x *g* using the Saitexiangyi TG12MX® Micro-haematocrit centrifuge. Then the proportion of cells in the total volume of blood was measured and recorded as a percentage using the Hawksley® Micro-haematocrit reader.

3.4.8.2 Evaluation of haemoglobin concentration

Haemoglobin concentration was assayed colorimetrically as cyanomethhaemoglobin as described by Drabkin, (1945) and modified by Waiser (2012) and Jelalu, (2014). The Five millilitres of HICN (Drabkin) solution was measured using a 5 ml syringe into plastic test tubes. Twenty microlitres of blood was measured using a micropipette and added to Drabkin solution in the test tube and properly mixed by gently shaking the test tube (Appendix 8). The blood was centrifuged at 1,509 x *g* for 15 min to separate the empty RBCs from interfering with the reading. The supernatant was decanted into a plastic sample

bottle. The supernatant was absorbed into audiocomb serum auto analyser (Bayer Express Plus, Bayer Germany and Serial Number 15950). After the wiggling pump stops working, the value displayed on the screen was recorded in g/dl as the haemoglobin concentration.

3.4.8.3 Evaluation of red blood cell and leucocyte counts

Red blood cell (RBC) and total white blood cell (TWBC) counts were determined with the Natt-Herrick solution (1:200 dilutions) and improved Neubauer haemocytometer (Campbell and Ellis, 2007). The blood samples were slightly agitated and an RBC diluting pipette was used to pipette the blood to the 0.5 mark. The tip of the pipette was cleaned properly using a tissue paper without touching the distal opening of the pipette tip with tissue. The diluting (Natt-Herrick) solution was also prepared to the 101 mark (1:200) without entirely immersing the pipette tip into the diluting fluid. The mixture was shaken very well for 1 min to obtain equal distribution then emptied into a clean cuvette. The Neubauer haemocytometer and cover slip was cleaned using a dry, lint free cloth. The cover slip was properly placed on the haemocytometer, the mixture was then agitated a little and a capillary tube was used to withdraw a small aliquot. Both sides of the haemocytometer was filled up (charged) by gently touching the intersection between the cover slip and haemocytometer with the loaded capillary tube avoiding air bubbles, then under-filling or over-filling, and allowed to stay for 5 min for cells to settle down. The light microscope (Olymopus-XSZ-107BN), at low power magnification (× 40) was used to view the cells and counting was done using a tally counter (Appendix 9).

For RBC count, the cells contained in the four corners and central squares in the mid-section of the haemocytometer were counted. Following the **"L"** rule; cells that touch the

centre triple lines of the ruling on the left and the bottom sides were counted but cells that touch the centre triple lines of the ruling on the right and the top sides were not counted. The RBC count was calculated using the formula (Campbell and Ellis, 2007) (6):

$$N/100 = RBC \times 10^{12}/L \quad (6)$$

Where N = number of RBCs counted in the five squares in the mid-section of the haemocytometer (or in 160 squares). Note that both charged sides of the haemocytometer were counted for both the RBC and TWBC and average calculated.

For total leucocyte count, the leucocytes in the four outer large squares of the haemocytometer were counted and calculated using the formula (Campbell and Ellis, 2007) (7):

$$N/20 = WBC \times 10^{9}/L \quad (7)$$

Where N = number of cells counted in the four outer large squares (or in 64 small squares).

3.4.8.4 Evaluation of thrombocyte count

An estimated thrombocyte count was obtained from the stained blood film using the same formula for the indirect estimation of total WBC (Campbell and Ellis, 2007). Valid and reliable results were not obtained where there was evidence of thrombocyte clumping. The absolute number of thrombocytes was estimated by using the formula (Campbell and Ellis, 2007) (8):

$$\frac{\text{Number of thrombocytes counted}}{100} \times TWBC = \text{Absolute thrombocytes} \times 10^{9}/l \quad (8)$$

3.4.8.5 Evaluation of differential leucocyte count

For all the chicks that were sampled, a pair of smears for each sample was made. A small drop (about 2 µL) of blood was immediately used for the preparation of blood smears each using the standard slide-to-slide technique as descrbed by Campbell (1988), Campbell and Ellis (2007). The air dried smears were properly labeled using a pencil on the frosted end of the slide and then fixed in 70% methanol inside a fixing jar for 3 min and air-dried. Staining was done by flooding the smears with Wright-Giemsa stain for 3 min. An equal amount of Sørensen's buffer (pH 6.8) was added then mixed gently by blowing using a pipette until green metallic sheen forms on the surface. This was allowed to stand for a further 6 min. The smears were rinsed with the Sørensen's buffer and allowed to stand for a minute for differentiation. The stained slides were then washed copiously with the Sørensen's buffer and the back of the smear was wiped with tissue paper to remove the excess stain and allowed to air dry. The stained slides were neatly packed into a slide box until viewed (Appendix 10).

Examination of the blood smears was done using a light microscope (Olympus-XSZ-107BN) under high-power magnification with oil immersion (× 100). One hundred WBCs were counted and classified based on their morphologic features (Campbell, 1988; Campbell and Ellis, 2007). The counting was done using the Marble® blood cell calculator. The differential WBC count was then expressed as a percentage of the individual cell group. The percentage of each cell was then converted into absolute numbers by reference to the total WBC using the formula (Campbell and Ellis, 2007) (9):

$$\frac{\text{Percentage of WBC counted} \times \text{Total WBC}}{100} = \text{Absolute number of WBC x } 10^9/\text{L} \quad (9)$$

3.4.9 Determination of serum biochemicals and lipid profiles

3.4.9.1 Evaluation of glucose concentration

The sera were then well mixed and left at room temperature for 30 min. Absorbance was then read using spectrophotometer (audiocomb serum auto analyser (Bayer Express Plus, Bayer Germany and Serial Number 15950) at 540 nm. These instruments were zeroed with blank solutions. The control and test blanks were read. The blank reading was then subtracted as described by Cheesbrough (1991).

3.4.9.2 Evaluation of total protein concentration

Biuret method was used to determine total protein, using the method described by Benjamin (1978). A black test tube, standard and the test sample tubes were labeled. 1,000 microlitre of biuret reagent was pipetted into each tube, and 200 microlitres of distilled water, standard and test samples were pipetted into the respective tubes. They were thoroughly mixed and incubated for 30 min at 25 ^{o}C. The spectrophotometer (model 6400/6405 made by Jenway, Barloworld Scientific, England) was set at 546 nm and zero using blank test tube, the absorbance was read, and the total protein calculated using the formula (10)

$$T_P = \frac{A_1 \times C}{A_2} \quad (10)$$

Where A_1 = Absorbance of the test sample, A_2 = Absorbance of the standard, C = Standard concentration.

3.4.9.4 Evaluation of calcium and phosphorus concentrations

Serum calcium and phosphorus concentrations were determined using cresolpthalein complexone and ammonium molybdate methods by Baginski (1973) and Gomorri (1942), respectively. Calcium reacts with o-cresolphthalein complexone in the presence of 8-

hydroxyquinoline-5-sulfonic acid to form a purple complex. The intensity of the final reaction colour is proportional to the amount of calcium in the specimen

3.4.9.5 Evaluation of potassium concentrations

Using compressed air, diluted serum was sprayed as a fine mist of droplets into a non-luminous pass through flame. These then give the colour of emission characteristics of sodium and potassium metallic ions. A light filter selects the light of a wavelength corresponding to the metal being measured. This was then allowed to fall on a photosensitive detector system which then emits light; the amount of light depends on the concentration of metallic ion. The value was then evaluated in mg/dl as described by Cheesbrough (1991).

3.4.9.4 Evaluation of creatinine and urea nitrogen

Once the serums thawed, blood urea nitrogen and creatinine, were assayed using the Audiocomb Serum Auto analyser (Bayer Express Plus, Bayer Germany and Serial Number 15950).

3.4.9.5 Evaluation of liver enzymes activity

Sera were analysed using colourimetric methods to determine the activity of liver enzymes, aspartate aminotransferase (AST), glutamate dehydrogenase and sorbitol dehydrogenase method as described by Reitman and Frankel (1957); Cheesbrough (1991). The sera were mixed in wells and left at room temperature for 5 min. Then absorbance was read using spectrophotometer at 505 nm (the instrument was zeroed with blank solution in the tube) values obtained in μg/mL from calibration graph.

3.4.9.6 Evaluation of total cholesterol concentration

All cholesterol esters present in serum were hydrolyzed quantitatively into free cholesterol and fatty acids by microbial cholesterol esterase. In the presence of oxygen, free cholesterol was oxidized by cholesterol oxidase to cholest-4-en-3-one. The H_2O_2 reacts in the presence of peroxidase (POD) with phenol and 4-aminophenazone to form an o-quinone-imine dye. The intensity of the colour was proportional to the cholesterol concentration and was measured photometrically.

3.4.9.7 Evaluation of triglycerides concentration

This method uses microbial lipase to promote rapid and complete hydrolysis of triglycerides to glycerol with subsequent oxidation to dihydroxyacetone phosphate and hydrogen peroxide. The peroxide reacts with 4-aminophenol in a Trinder reaction to a colorimetric endpoint.

3.4.10 Determination of oxidative stress biomarkers

3.4.10.1 Evaluation of superoxide dismutase activity

Sera were diluted (1:100 v/v) in phosphate buffered saline (PBS) (pH 7.3) before determination of the activities of superoxide dismutase (SOD). The level of antioxidant enzymes (SOD) in serum was evaluated using commercial test kit for SOD. Standard protocol of the North West Life Science Specialist, Vancouver, Canada was used to measure the SOD concentrations (Martin *et al*., 1987). The method for determination of SOD was based on monitoring the autoxidation rate of haematoxylin as originally described by Martin *et al.* (1987), with modification to increase robustness and reliability.

3.4.10.2 Evaluation of malondialdehyde concentration

Malondialdehyde (MDA) concentration was determined using thiobarbituric acid (TBA) reactive substance. The level of thiobarbituric acid reactive substance was evaluated in the serum using the double heating method of Draper and Hadley, (1990) as modified by Yavuz *et al.* (2004). The concentration of MDA in the sera was calculated by the absorbance coefficient of MDA-TBA complex 1.6 × 105/cm/M and expressed as nmol/mL of protein. The MDA was assayed after mixing in a microcentrifuge vial, containing butylated hydroxytoluene reagent, serum sample, phosphoric acid and 2-thiobarbituric acid. The mixture was then incubated for 60 min at 60^{o}C using a GallenKamp® incubator (Model: IH-100; Made in England), and centrifuged at 10,000 x *g* for 2-3 min using a Hermle® centrifuge (Model No.: Z 364, B. HERMLE GmbH & CO., Germany). The reacted mixture was transferred into a cuvette and the absorbance of the test sample read at 548 nm using a spectrophotometer (Spectrumlab® 23A; Q/SEEK2: Model No.: 23A07025 Huangwei, China).

3.4.11 Pathology

3.4.11.1 Gross-pathological lesions score

Following inoculation of chicks with vvIBDV, the onset of mortality was noted and postmortem examination conducted on dead birds daily. The severity of gross lesions observed per group was estimated based on organs with lesions, types and extent of the lesions. The organs of each of the bird examined were the BF, SPL, THY, caecal tonsils, kidneys, pancreas, liver, trachea, lungs, heart, proventriculus, ventriculus, breast, leg and thigh muscles. The types and extent (assigned scores) of lesions observed were; enlargement (assigned a score of 1), congestion, (2), haemorrhage (3), necrosis (4) and

atrophy (5). For example, a bird with congested lungs, liver and spleen, and an enlarged, and haemorrhahgic BF would have an assigned lesion score of 2 + 2 + 2 + 1 + 3/4 = 2.5. The assigned lesion score for each bird examined daily were summed and divided by the number of birds examined per group. The final assigned lesion score per group were graded and categorized into five groups: 1.0-1.9 = mild, 2.0-2.9 = moderate, 3.0 – 3.9 = severe, 4.0-4.9 = very severe, > 5.0 extremely severe (Appendix 11).

3.4.11.2 Histopathological lesions score

Sections of the BF, SPL, and THY, were obtained and fixed in 10% phosphate-buffered neutral formalin solution. The tissues were cut into blocks, identified and dehydrated through a series of graded alcohol (70%, 80%, 90%, and 100%). Thereafter, the blocks were cleared in xylene and then infiltrated with molten paraffin wax. Sections of 5 microns (µm) were cut from embedded tissue. The tissues were mounted on grease free clean glass slides and kept at room temperature and then stained alternatively with Haematoxylin and Eosin (H and E). The processed and stained slides was examined using a light microscope at × 40, × 100, × 200, and × 400 power objectives lens for histopathological changes (Hair-Bejo *et al.,* 2000; Babiker *et al.,* 2008). The lesions were scores based the grade assigned to the lesions (Appendix 12)

3.4.12 Data analyses

GraphPad Prism 4.0 for windows (GraphPad Software, San Diego, California USA) was used for statistical analysis. Descriptive statistics was used to express clinical signs, morbidity and mortality rates, feed intake and feed conversion ratio. Data of BD weight, antibody titre level, BF/BD, SPL/BD and THY/BD weight index, haematological

parameters, serum biochemicals, liver enzymes, and oxidative stress biomarkers, H/L ratio obtained were expressed as mean ± standard error of the mean values (Mean ± SEM). Means were subjected to one-way analysis of variance (ANOVA), to compare the differences between the means, obtained from the control and treated birds. Tukey's multiple comparison *post-hoc* tests; using Values of $P \leq 0.05$ were considered significant. Results were presented in tables.

CHAPTER FOUR

4.0 RESULTS

4.1 Clinical Signs Manifested by ISA Brown Chicks Administered Molasses, Antox® and EN-FLORAX® and Infected with Infectious Bursal Disease Virus

Chicks in groups A, B, C, and D inoculated with vvIBV at 28 days of age showed somnolence, prostration, ruffled feathers, depression, weakness, recumbence, huddling, anorexia, dispnoea and diarrhoea. The severity of clinical signs in chicks in groups A, B, and C were mild at 6 dpi compared to severe in group D (Table 4.1). By 35 doa chicks in groups A, B, and C had fully recovered while chicks in D still had moderate clinical signs (Appendix 13).

4.2 Morbidity and Mortality Rates

Onset of morbidity was observed 3 dpi in all the groups inoculated with vvIBDV and lasted for 6 days in groups A, B and C and 7 in group D. The highest morbidity rates, were recorded 4 dpi in groups A (42.6%); B (55.2%); C (50.5%) and D (75.6%). Mortality was observed 3 dpi in all the groups inoculated with vvIBDV with mortality lasting 3 days in group A, 4 in groups B and C and 5 in group D. The overall mortality rates were 25.5%, 61.3%, 46.8% and 95.4% in groups A, B, C, and D, respectively (Table 4.2).

4.3 Enzyme-linked Immuno-sorbent Antibodies Before and After Infection with Infectious Bursal Disease Virus

Before inoculation with vvIBDV, Ab titres decreased as chicks grew older with Ab values from 7 to 28 days of age being highest in group C. The Ab titres were highest at 1 day of age in all the groups. The Ab titres were $50.72 \pm 0.26 \log_{10}$, $35.82 \pm 0.19 \log_{10}$, $40.89 \pm 0.22 \log_{10}$ and $25.65 \pm 0.14 \log_{10}$ in groups A, B, C, and D, respectively at 35 doa. The Ab titres in A, B, C, and D were significantly different ($P \leq 0.05$) at 42 and 49 doa (Table 4.3, Appendix 15).

Table 4.1: Mean clinical signs (%) of ISA Brown chicks administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation							
		2	3	4	5	6	7	Overall average % of chicks with signs	Severity of clinical signs
Group	Treatment	Daily average (%) of chicks with clinical signs							
A	Molasses	0.00	28.91	31.26	23.32	0.00	0.00	27.83	2 (moderate)
B	Antox®	0.00	39.03	46.25	34.59	25.00	0.00	36.22	2 (moderate)
C	EN-FLORAX®	0.00	35.38	40.32	28.21	17.05	0.00	30.24	2 (moderate)
D	Positive control	0.00	46.52	54.83	44.35	39.44	34.25	44.08	3 (severe)
E	Negative control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0 (none)

Table 4.2: Mean morbidity and mortality rates (%) of ISA Brown chicks administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Days post-inoculation							
		1	2	3	4	5	6	7	
Group	Treatment	Daily morbidity rate (%)							Highest morbidity rate
A	Molasses	00.0	00.0	18.9	42.6	19.2	00.0	00.0	4 (42.6)
B	Antox®	00.0	00.0	29.1	55.2	29.2	10.1	00.0	4 (55.2)
C	EN-FLORAX®	00.0	00.0	25.2	50.5	23.1	09.2	00.0	4 (50.5)
D	Positive control	00.0	00.0	38.1	75.6	45.1	24.3	13.2	4 (75.6)
E	Negative control	00.0	00.0	00.0	00.0	00.0	00.0	00.0	0 (00.0)
		Days post-inoculation							
		1	2	3	4	5	6	7	
Group	Treatment	Daily mortality rate (%)							Overall mortality rate
A	Molasses	00.0	00.0	08.3	13.8	03.4	00.0	00.0	25.5
B	Antox®	00.0	00.0	16.7	19.1	14.7	10.8	00.0	61.3
C	EN-FLORAX®	00.0	00.0	13.2	16.7	10.3	06.6	00.0	46.9
D	Positive control	00.0	00.0	20.1	27.9	22.3	16.7	08.4	95.4
E	Negative control	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0

Table 4.3: Mean (± SE) enzyme linked immunosorbent assay antibody titre (log_{10}) level of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean IDEXX enzyme linked immunosorbent assay antibody titre level (log_{10}) before and after inoculation with vvIBDV							
A	Molasses	65.54 ± 0.27	56.67 ± 0.32	45.37 ± 0.25	33.89 ± 0.19	27.99 ± 0.16	50.72 ± 0.26*****	45.99 ± 0.19*****	35.89 ± 0.15*****
B	Antox®	66.55 ± 0.28	57.77 ± 0.33	49.38 ± 0.24	35.26 ± 0.20	29.22 ± 0.17	35.82 ± 0.19***	25.65 ± 0.11***	20.34 ± 0.08***
C	EN-FLORAX®	67.56 ± 0.28	59.85 ± 0.32	54.59 ± 0.29	39.42 ± 0.21	32.23 ± 0.19	40.89 ± 0.22****	35.85 ± 0.14****	25.45 ± 0.10****
D	Positive control	65.54 ± 0.27	49.37 ± 0.33	38.84 ± 0.26	25.78 ± 0.17	18.95 ± 0.09	25.65 ± 0.14**	15.36 ± 0.06**	05.15 ± 0.01**
E	Negative control	66.55 ± 0.28	48.45 ± 0.34	39.86 ± 0.27	24.76 ± 0.18	19.94 ± 0.10	05.55 ± 0.02*	00.25 ± 0.01*	00.05 ± 0.00*

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$)in the same column differed significantly

4.4 Presence of Antibodies Before and After Vaccination with Newcastle Disease Vaccine La Sota

The Ab titre was highest at 1 doa in all groups. Before vaccination ND Ab titre decreased as chicks grow older with Ab values from 7 to 28 doa being highest in group C. The ND Ab titres were 6.45 ± 0.04 $\log_2$, 4.64 ± 0.02 $\log_2$, 5.35 ± 0.03 $\log_2$ and 3.60 ± 0.01 $\log_2$ in groups A, B, C, and D, respectively one-week post vaccination (42 doa). By two weeks post vaccination (49 doa) the mean ND Ab titres of groups A, B, C, and D decreased to 5.72 ± 0.03 $\log_2$, 3.55 ± 0.01 $\log_2$, 4.63 ± 0.02 $\log_2$ and 2.81 ± 0.00 $\log_2$, respectively. The ND Ab titre in group A, were significantly ($P \leq 0.05$) higher than in B, C and D at one and two weeks post vaccination (Table 4.4, Appendix 16).

4.5 Performance Indices of ISA Brown Chicks Administered molasses, Antox® and EN-FLORAX® and Infected with Infectious Bursal Disease Virus

4.5.1 Changes in feed intake

Before inoculation with vvIBDV, weekly feed intake (WFI) increased as chicks grow older with values from 7 to 28 days of age being highest in group C. The WFI in groups A, B, and C decreased from 224.45 g, 227.54 g and 229.58 g, at 28 doa to 200.25 g, 155.15 g and 174.20 g at 35 doa, but the decrease in WFI were significantly ($P \leq 0.05$) lower when compared to that of positive control group D (125.35 g) at 35 doa. The WFI in A, B, C, and D were significantly different ($P \leq 0.05$) at 42 and 49 doa (Table 4.5, Appendix 17).

4.5.2 Changes in feed conversion ratios

Before inoculation with vvIBDV, feed conversion ratios (WFCR) increased as chicks grow older with values from 7 to 28 doa being highest in group C. The WFCR in groups A, B, and C increased from 0.67, 0.62 and 0.62 at 28 doa to 0.80, 0.68 and 0.74 at 35 doa

Table 4.4: Mean (± SE) haemagglutinating antibody titre (Log_2) before and after vaccination at 35 days of age with Newcastle disease vaccine La Sota of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean (Log_2) haemagglutinating antibody titre before and after vaccination with Newcastle disease vaccine La Sota							
A	Molasses	8.42 ± 0.04	6.48 ± 0.03	4.86 ± 0.02	3.84 ± 0.01	3.05 ± 0.00	2.05 ± 0.00****	6.45 ± 0.04****	5.72 ± 0.03****
B	Antox®	7.92 ± 0.05	6.89 ± 0.03	5.25 ± 0.03	4.25 ± 0.02	3.42 ± 0.01	2.43 ± 0.00**	4.64 ± 0.02**	3.55 ± 0.01**
C	EN-FLORAX®	8.45 ± 0.04	7.25 ± 0.04	6.05 ± 0.03	4.92 ± 0.02	3.83 ± 0.01	2.84 ± 0.00***	5.35 ± 0.03***	4.63 ± 0.02***
D	Positive control	7.89 ± 0.04	5.55 ± 0.03	3.55 ± 0.02	2.11 ± 0.01	1.65 ± 0.00	0.00 ± 0.00*	3.60 ± 0.01*	2.81 ± 0.00*
E	Negative control	7.90 ± 0.04	5.50 ± 0.03	3.60 ± 0.01	2.12 ± 0.00	1.64 ± 0.00	0.00 ± 0.00*	8.25 ± 0.05*****	7.45 ± 0.04*****

Key**:** n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly

Table 4.5: Weekly average feed intake (g per bird) of ISA Brown chicks administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age in days						
		7	14	21	28	35	42	49
Group	Treatment	Average feed intake (g per bird)						
A	Molasses	77.35	119.20	175.34	224.45	200.25****	245.40****	289.45****
B	Antox®	79.40	120.23	177.36	227.54	155.15**	193.25**	230.35 **
C	EN-FLORAX®	80.45	121.25	179.39	229.58	174.20***	208.35***	256.40***
D	Positive control	64.55	110.32	158.50	215.45	125.35*	145.45*	189.47*
E	Negative control	65.50	109.35	157.55	214.40	249.45*****	285.50*****	325.55*****

Key: Average values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly

but the increase in WFCR were significantly ($P \leq 0.05$) lower when compared to that of positive control group D (0.92) at 35 doa. The WFCR in A, B, C, and D were significantly different ($P \leq 0.05$) at 42 and 49 doa (Table 4.6, Appendix 18).

4.5.3 Changes in live body weights

Before inoculation with vvIBDV, live body weights (LBW) increased as chicks grow older with values from 7 to 28 doa being highest in group C. The LBW in groups A, B, and C decreased from 336.61 ± 3.12 g, 369.72 ± 3.15 g and 372.64 ± 3.16 g, at 28 doa to 248.91 ± 2.15 g, 227.60 ± 2.13 g and 235.77 ± 2.35 g at 35 doa, but the decrease were significantly lower when compared to that of positive control group D (136.64 ± 1.17 g), at 35 doa. The LBW in A, B, C and D were significantly different ($P \leq 0.05$) at 42 and 49 doa (Table 4.7, Appendix 19).

4.5.4 Changes in bursa, spleen and thymus to body weight index

Before inoculation with vvIBDV, bursa to body weight index (BBI) increased as chicks grow older with values from 7 to 28 doa being highest in group C. The BBI in groups A, B, and C increased from 1.20 ± 0.02 g, 1.23 ± 0.02 g and 1.24 ± 0.02 g at 28 doa to 1.27 ± 0.02 g, 1.78 ± 0.02 g and 1.51 ± 0.02 g at 35 doa, but the increase were significantly lower when compared to that of positive control group D (2.34 ± 1.03 g), at 35 doa. The BBI in A, B, C, and D were significantly different ($P \leq 0.05$) at 42 and 49 doa (Table 4.8, Appendix 21).

Before inoculation with vvIBDV, spleen to body weight index (SBI) increased as chicks grow older with values from 7 to 28 doa being highest in group C. The SBI in groups A, B, and C increased from 1.22 ± 0.01 g, 1.23 ± 0.01 g and 1.25 ± 0.02 g at 28 doa to 1.38 ± 0.02 g, 1.87 ± 0.02 g and 1.77 ± 0.02 g at 35 doa,

Table 4.6: Feed conversion ratios (per bird) of ISA Brown chicks administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age in days in						
		7	14	21	28	35	42	49
Group	Treatment	Feed conversion ratios (per bird)						
A	Molasses	0.52	0.49	0.59	0.67	0.80**	0.64***	0.58***
B	Antox®	0.53	0.49	0.59	0.62	0.68*****	0.56*****	0.53*****
C	EN-FLORAX®	0.53	0.49	0.59	0.62	0.74****	0.58****	0.57****
D	Positive control	0.99	1.06	0.89	0.97	0.92*	0.89*	0.73*
E	Negative control	0.97	1.07	0.89	0.98	0.78***	0.62**	0.61**

Key**:** Ratios with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly

Table 4.7: Mean (± SE) live body weight (g) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean (± SE) live body weight (g)							
A	Molasses	32.98 ± 0.16	147.44 ± 1.20	240.82 ± 2.12	287.96 ± 2.24	336.61 ± 3.12	248.91 ± 2.15****	387.24 ± 3.24****	485.50 ± 4.32****
B	Antox®	31.27 ± 0.15	149.87 ± 1.22	243.36 ± 2.15	299.71 ± 2.30	369.72 ± 3.15	227.60 ± 2.13**	343.34 ± 3.22**	437.29 ± 4.28**
C	EN-FLORAX®	29.75 ± 0.12	152.77 ± 1.24	247.26 ± 2.17	305.33 ± 3.34	372.64 ± 3.16	235.77 ± 2.19***	358.69 ± 3.25***	469.97 ± 4.30***
D	Positive control	30.53 ± 0.14	65.47 ± 0.24	103.64 ± 1.15	175.44 ± 1.35	221.79 ± 2.13	136.64 ± 1.17*	193.22 ± 1.28*	259.32 ± 2.35*
E	Negative control	31.38 ± 0.16	67.48 ± 0.22	102.62 ± 1.14	177.48 ± 1.37	219.78 ± 2.12	320.99 ± 3.28*****	457.43 ± 4.34*****	531.40 ± 5.44*****

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.01$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly

Table 4.8: Mean (± SE) bursa of Fabricius/body weight index of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean bursa of Fabricius/body weight index							
A	Molasses	0.97 ± 0.00	1.08 ± 0.00	1.13 ± 0.01	1.16 ± 0.01	1.20 ± 0.02	1.27 ± 0.02****	0.51 ± 0.00****	0.77 ± 0.00****
B	Antox®	0.97 ± 0.00	1.10 ± 0.00	1.15 ± 0.01	1.18 ± 0.01	1.23 ± 0.02	1.78 ± 0.02**	0.38 ± 0.00**	0.54 ± 0.00**
C	EN-FLORAX®	0.98 ± 0.00	1.11 ± 0.00	1.18 ± 0.01	1.20 ± 0.01	1.24 ± 0.02	1.51 ± 0.02***	0.46 ± 0.00***	0.66 ± 0.00***
D	Positive control	0.97 ± 0.00	0.98 ± 0.00	1.01 ± 0.00	1.02 ± 0.00	1.09 ± 0.00	2.34 ± 0.03*	0.26 ± 0.00*	0.40 ± 0.00*
E	Negative control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00*****	1.00 ± 0.00*****	1.00 ± 0.00*****

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly

but the increase were significantly lower when compared to that of positive control group D (2.61 ± 0.03 g), at 35 doa. The SBI in A, B, C, and D were significantly different ($P \leq 0.05$) at 42 and 49 doa (Table 4.9, Appendix 22).

Before inoculation with vvIBDV, thymus to body weight index (TBI) increased as chicks grow older with values from 7 to 28 doa being highest in group C. The TBI in groups A, B, and C increased from 1.18 ± 0.00 g, 1.21 ± 0.01 g and 1.23 ± 0.01 g at 28 doa to 1.44 ± 0.02 g, 1.78 ± 0.02 g and 1.68 ± 0.02 g at 35 doa, but the increase were significantly lower when compared to that of positive control group D (2.33 ± 0.03 g), at 35 doa. The TBI in A, B, C, and D were significantly different ($P \leq 0.05$) at 42 and 49 doa (Table 4.10, Appendix 23).

4.6 Haematological Parameters of ISA Brown Chicks Administered Molasses, Antox® and EN-FLORAX® and Infected with Infectious Bursal Disease Virus

4.6.1 Packed cell volume

Before inoculation with vvIBDV, packed cell volume (PCV) increased as chicks grow older with values from 7 to 28 doa being highest in group C. The PCV in groups A, B and C decreased from 27.46 ± 0.16%, 28.47 ± 0.17% and 29.48 ± 0.18% at 28 doa to 22.29 ± 0.12%, 19.26 ± 0.08% and 20.28 ± 0.10% at 35 doa, but the decrease were significantly lower when compared to that of positive control group D (13.56 ± 0.05%), at 35 doa. The PCV in A, B, C and D were significantly different ($P \leq 0.05$) at 42 and 49 doa (Table 4.11, Appendix 24).

4.6.2 Haemoglobin concentration

Before inoculation with vvIBDV, haemoglobin (Hb) concentration increased as chicks grow older with values from 7 to 28 doa being highest in group C.

Table 4.9: Mean (± SE) spleen/body weight index of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean spleen/body weight index							
A	Molasses	1.02 ± 0.00	1.08 ± 0.00	1.20 ± 0.01	1.21 ± 0.01	1.22 ± 0.01	1.38 ± 0.02****	0.53 ± 0.00****	0.88 ± 0.00****
B	Antox®	1.03 ± 0.00	1.10 ± 0.00	1.21 ± 0.01	1.22 ± 0.01	1.23 ± 0.01	1.87 ± 0.02**	0.37 ± 0.00**	0.58 ± 0.00**
C	EN-FLORAX®	1.02 ± 0.00	1.12 ± 0.00	1.22 ± 0.01	1.23 ± 0.01	1.25 ± 0.02	1.67 ± 0.02***	0.47 ± 0.00***	0.65 ± 0.00***
D	Positive control	1.02 ± 0.00	1.03 ± 0.00	1.05 ± 0.00	1.07 ± 0.00	1.09 ± 0.00	2.61 ± 0.03*	0.24 ± 0.00*	0.34 ± 0.00*
E	Negative control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00*****	1.00 ± 0.00*****	1.00 ± 0.00*****

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.01$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly

Table 4.10: Mean (± SE) thymus/body weight index of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean thymus/body weight index							
A	Molasses	1.01 ± 0.00	1.05 ± 0.00	1.10 ± 0.00	1.11 ± 0.00	1.18 ± 0.00	1.44 ± 0.02****	0.52 ± 0.00****	0.89 ± 0.00****
B	Antox®	1.00 ± 0.00	1.07 ± 0.00	1.18 ± 0.00	1.19 ± 0.00	1.21 ± 0.01	1.78 ± 0.02**	0.36 ± 0.00**	0.52 ± 0.00**
C	EN-FLORAX®	1.01 ± 0.00	1.09 ± 0.00	1.20 ± 0.01	1.21 ± 0.01	1.23 ± 0.01	1.68 ± 0.02***	0.43 ± 0.00***	0.66 ± 0.00***
D	Positive control	1.00 ± 0.00	1.01 ± 0.00	1.03 ± 0.00	1.05 ± 0.00	0.07 ± 0.00	2.33 ± 0.03*	0.20 ± 0.00*	0.43 ± 0.00*
E	Negative control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00*****	1.00 ± 0.00*****	1.00 ± 0.00*****

Key**:** n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly

Table 4.11: Mean (± SE) packed cell volume (%)of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean (± SE) packed cell volume (%)							
A	Molasses	15.25 ± 0.07	19.30 ± 0.09	21.32 ± 0.11	23.35 ± 0.13	27.46 ± 0.16	22.29 ± 0.12****	26.32 ± 0.16****	31.46 ± 0.19****
B	Antox®	16.26 ± 0.07	20.31 ± 0.10	22.33 ± 0.12	24.36 ± 0.14	28.47 ± 0.17	19.26 ± 0.08**	23.28 ± 0.13**	26.30 ± 0.15**
C	EN-FLORAX®	15.26 ± 0.06	21.32 ± 0.11	23.35 ± 0.13	25.37 ± 0.15	29.48 ± 0.18	20.28 ± 0.10***	24.30 ± 0.14***	29.34 ± 0.17***
D	Positive control	16.27 ± 0.07	17.28 ± 0.08	19.30 ± 0.09	21.32 ± 0.11	24.37 ± 0.14	13.56 ± 0.05*	19.20 ± 0.09*	23.25 ± 0.13*
E	Negative control	15.26 ± 0.07	18.29 ± 0.09	20.32 ± 0.10	22.33 ± 0.12	25.37 ± 0.15	29.48 ± 0.18*****	35.55 ± 0.20*****	39.63 ± 0.22*****

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly.

The mean Hb concentration in groups A, B, and C decreased from 10.78 ± 0.09 g/dl, 10.99 ± 0.08 g/dl and 11.61 ± 0.10 g/dl at 28 doa to 7.44 ± 0.05 g/dl, 5.89 ± 0.03 g/dl and 6.98 ± 0.04 g/dl at 35 doa, but the decrease were significantly lower when compared to that of positive control group D (3.59 ± 0.01 g/dl), at 35 doa. The Hb concentration in A, B, C, and D were significantly different ($P \leq 0.05$) at 42 and 49 doa (Table 4.12, Appendix 25).

4.6.3 Red blood cell count

Before inoculation with vvIBDV, red blood cell (RBC) count increased as chicks grow older with values from 7 to 28 doa being highest in group C. The RBC count in groups A, B, and C decreased from 2.74 ± 0.02 $\times 10^{12}$/L, 2.76 ± 0.03 $\times 10^{12}$/L and 2.79 ± 0.03 $\times 10^{12}$/L at 28 doa to 1.95 ± 0.01 $\times 10^{12}$/L, 1.46 ± 0.00 $\times 10^{12}$/L and 1.59 ± 0.02 $\times 10^{12}$/L at 35 doa, but the decrease were significantly lower when compared to that of positive control group D (0.92 ± 0.00 $\times 10^{12}$/L), at 35 doa. The RBC count in A, B, C, and D were significantly different ($P \leq 0.05$) at 42 and 49 doa (Table 4.13, Appendix 26).

4.6.4 Thrombocyte count

Before inoculation with vvIBDV, thrombocyte count increased as chicks grow older with values from 7 to 28 doa being highest in group C. Thrombocytes count in groups A, B, and C decreased from 9.77 ± 0.07 $\times 10^{9}$/L, 9.85 ± 0.08 $\times 10^{9}$/L and 9.97 ± 0.08 $\times 10^{9}$/L at 28 doa to 7.84 ± 0.05 $\times 10^{9}$/L, 6.78 ± 0.04 $\times 10^{9}$/L and 6.88 ± 0.04 $\times 10^{9}$/L at 35 doa, but the decrease were significantly lower when compared to that of positive control group D (4.96 ± 0.02 $\times 10^{9}$/L), at 35 doa. The thrombocyte count in A, B, C, and D were significantly different ($P \leq 0.05$) at 42 and 49 doa (Table 4.14, Appendix 27).

Table 4.12: Mean (± SE) haemoglobin concentration (g/dl) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean (± SE) haemoglobin concentration (g/dl)							
A	Molasses	3.59 ± 0.01	5.57 ± 0.02	7.79 ± 0.05	9.66 ± 0.08	10.78 ± 0.09	7.44 ± 0.05****	8.67 ± 0.06****	9.59 ± 0.08****
B	Antox®	3.58 ± 0.01	5.89 ± 0.03	7.99 ± 0.05	9.89 ± 0.07	10.99 ± 0.08	5.89 ± 0.03**	6.58 ± 0.04**	7.68 ± 0.06**
C	EN-FLORAX®	3.68 ± 0.02	6.31 ± 0.04	8.52 ± 0.07	10.45 ± 0.09	11.61 ± 0.10	6.98 ± 0.04***	7.91 ± 0.05***	8.89 ± 0.07***
D	Positive control	3.71 ± 0.02	4.65 ± 0.02	5.31 ± 0.03	6.55 ± 0.04	7.65 ± 0.05	3.59 ± 0.01*	4.48 ± 0.02*	5.37 ± 0.03*
E	Negative control	3.68 ± 0.02	4.67 ± 0.02	5.33 ± 0.03	6.56 ± 0.04	7.64 ± 0.05	8.77 ± 0.07*****	9.86 ± 0.08*****	10.99 ± 0.10*****

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly.

Table 4.13: Mean (± SE) red blood cell count (x 10^{12}/l) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean (± SE) red blood cell count (x 10^{12}/l)							
A	Molasses	0.95 ± 0.00	1.48 ± 0.01	1.79 ± 0.01	2.19 ± 0.02	2.74 ± 0.02	1.95 ± 0.01****	2.34 ± 0.02****	2.99 ± 0.03****
B	Antox®	0.96 ± 0.01	1.53 ± 0.02	1.83 ± 0.02	2.21 ± 0.03	2.76 ± 0.03	1.46 ± 0.00**	1.87 ± 0.01**	2.25 ± 0.02**
C	EN-FLORAX®	0.95 ± 0.00	1.55 ± 0.02	1.85 ± 0.02	2. 24 ± 0.03	2.79 ± 0.03	1.59 ± 0.02***	1.92 ± 0.02***	2.35 ± 0.02***
D	Positive control	0.96 ± 0.01	1.19 ± 0.00	1.46 ± 0.01	1.89 ± 0.01	2.22 ± 0.02	0.92 ± 0.00*	1.27 ± 0.01*	1.66 ± 0.01*
E	Negative control	0.95 ± 0.00	1.18 ± 0.00	1.47 ± 0.01	1. 88 ± 0.01	2.23 ± 0.02	2.77 ± 0.02*****	3.33 ± 0.03*****	3.98 ± 0.03*****

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$) or (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly.

Table 4.14: Mean (± SE) thrombocyte count (x 10^9/l) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean (± SE) thrombocyte count (x 10^9/l)							
A	Molasses	4.66 ± 0.02	6.55 ± 0.03	7.62 ± 0.04	8.53 ± 0.05	9.77 ± 0.07	7.84 ± 0.05****	8.87 ± 0.07****	9.69 ± 0.08****
B	Antox®	4.69 ± 0.03	6.85 ± 0.04	7.87 ± 0.05	8.88 ± 0.06	9.85 ± 0.08	6.78 ± 0.04**	7.66 ± 0.06**	8.68 ± 0.07**
C	EN-FLORAX®	4.68 ± 0.02	6.99 ± 0.04	7.95 ± 0.05	8.98 ± 0.06	9.97 ± 0.08	6.88 ± 0.04***	7.95 ± 0.05***	8.97 ± 0.07***
D	Positive control	4.69 ± 0.03	5.59 ± 0.03	6.64 ± 0.04	7.79 ± 0.05	8.83 ± 0.06	4.96 ± 0.02*	5.87 ± 0.03*	6.95 ± 0.04*
E	Negative control	4.65 ± 0.02	5.57 ± 0.03	6.65 ± 0.04	7 78 ± 0.05	8.82 ± 0.06	9.96 ± 0.08*****	10.94 ± 0.04*****	11.89 ± 0.09*****

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly.

4.6.5 Total white blood cell count

Before inoculation with vvIBDV, total white blood cell (TWBC) count increased as chicks grow older with values from 7 to 28 doa being highest in group C. The TWBC count in groups A, B, and C decreased from 4.97 ± 0.04 $\times 10^9$/L, 5.35 ± 0.05 $\times 10^9$/L and 5.45 ± 0.05 $\times 10^9$/L at 28 doa to 3.95 ± 0.03 $\times 10^9$/L, 3.19 ± 0.03 $\times 10^9$/L and 3.33 ± 0.03 $\times 10^9$/L at 35 doa, but the decrease were significantly lower when compared to that of positive control group D (1.95 ± 0.01 $\times 10^9$/L), at 35 doa. The TWBC count in A, B, C, and D were significantly different ($P \leq 0.05$) at 42 and 49 doa (Table 4.15, Appendix 28).

4.6.6 Heterophil and lymphocyte count

Before inoculation with vvIBDV, heterophils count increased as chicks grow older with values from 7 to 28 doa being highest in group C. The heterophils count in groups A, B, and C decreased from 2.85 ± 0.02 $\times 10^9$/L, 2.97 ± 0.02 $\times 10^9$/L and 3.24 ± 0.03 $\times 10^9$/L at 28 doa to 2.00 ± 0.02 $\times 10^9$/L, 1.33 ± 0.01 $\times 10^9$/L and 1.52 ± 0.01 $\times 10^9$/L at 35 doa, but the decrease were significantly lower when compared to that of positive control group D (0.29 ± 0.00 $\times 10^9$/L), at 35 doa. The heterophils counts in A, B, C, and D were significantly different ($P \leq 0.05$) at 42 and 49 doa (Table 4.16, Appendix 29).

Before inoculation with vvIBDV, lymphocytes count increased as chicks grow older with values from 7 to 28 doa being highest in group C. The lymphocytes count in groups A, B, and C decreased from values of 4.85 ± 0.04 $\times 10^9$/L, 4.99 ± 0.04 $\times 10^9$/L and 5.23 ± 0.05 $\times 10^9$/L at 28 doa to 3.34 ± 0.03 $\times 10^9$/L, 2.29 ± 0.02 $\times 10^9$/L and 2.53 ± 0.02 $\times 10^9$/L at 35 doa, but the decrease were significantly lower when compared to that of positive control group D (1.33 ± 0.01 $\times 10^9$/L), at 35 doa. The lymphocyte counts in A, B, C, and D were significantly different ($P \leq 0.05$) at 42 and 49 doa (Table 4.17, Appendix 30).

Table 4.15: Mean (± SE) total white blood cell count (x 10^9/l) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean (± SE) total white blood cell count (x 10^9/l)							
A	Molasses	1.83 ± 0.01	2.93 ± 0.02	3.75 ± 0.03	4.36 ± 0.04	4.97 ± 0.04	3.95 ± 0.03****	4.87 ± 0.04****	5.57 ± 0.05****
B	Antox®	1.84 ± 0.01	2.96 ± 0.02	3.85 ± 0.03	4.88 ± 0.04	5.35 ± 0.05	3.19 ± 0.03**	3.55 ± 0.02**	3.99 ± 0.03**
C	EN-FLORAX®	1.83 ± 0.01	2.99 ± 0.02	3.95 ± 0.03	4.97 ± 0.04	5.45 ± 0.05	3.33 ± 0.03***	3.89 ± 0.03***	4.36 ± 0.04***
D	Positive control	1.84 ± 0.01	2.33 ± 0.02	2.89 ± 0.02	3.39 ± 0.03	3.93 ± 0.03	1.95 ± 0.01*	2.37 ± 0.02*	2.98 ± 0.02*
E	Negative control	1.83 ± 0.01	2.34 ± 0.02	2.88 ± 0.02	3. 38 ± 0.03	3.94 ± 0.03	4.59 ± 0.04*****	5.68 ± 0.05*****	6.71 ± 0.06*****

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly

Table 4.16: Mean (± SE) heterophils count (x 10^9/l) of ISA Brown chicks (n = 5) administered Molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean (± SE) heterophils count (x 10^9/l)							
A	Molasses	0.53 ± 0.00	1.45 ± 0.01	1.86 ± 0.01	2.34 ± 0.02	2.85 ± 0.02	2.00 ± 0.02****	2.55 ± 0.02****	3.17 ± 0.03****
B	Antox®	0.52 ± 0.00	1.65 ± 0.01	1.99 ± 0.01	2.52 ± 0.02	2.97 ± 0.02	1.33 ± 0.01**	1.87 ± 0.01**	2.19 ± 0.02**
C	EN-FLORAX®	0.53 ± 0.00	1.85 ± 0.01	2.26 ± 0.02	2. 87 ± 0.02	3.24 ± 0.03	1.52 ± 0.01***	2.33 ± 0.02***	2.89 ± 0.02***
D	Positive control	0.52 ± 0.00	0.98 ± 0.00	1.59 ± 0.01	1.93 ± 0.01	2.38 ± 0.02	0.29 ± 0.00*	0.89 ± 0.00*	1.15 ± 0.01*
E	Negative control	0.53 ± 0.00	0.99 ± 0.00	1.58 ± 0.01	1.92 ± 0.01	2.39 ± 0.02	2.98 ± 0.02*****	3.88 ± 0.03*****	4.47 ± 0.04*****

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly

Table 4.17: Mean (± SE) lymphocytes count (x 10^9/l) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean (± SE) lymphocytes count (x 10^9/l)							
A	Molasses	1.42 ± 0.01	2.55 ± 0.02	3.17 ± 0.03	4.37 ± 0.04	4.85 ± 0.04	3.34 ± 0.03****	3.98 ± 0.03****	4.58 ± 0.04****
B	Antox®	1.43 ± 0.01	2.62 ± 0.02	3.38 ± 0.03	4.55 ± 0.04	4.99 ± 0.04	2.29 ± 0.02**	2.84 ± 0.02**	3.08 ± 0.03**
C	EN-FLORAX®	1.42 ± 0.01	2.78 ± 0.02	3.63 ± 0.03	4.96 ± 0.04	5.23 ± 0.05	2.53 ± 0.02***	2.98 ± 0.02***	3.57 ± 0.03***
D	Positive control	1.43 ± 0.01	2.25 ± 0.02	2.64 ± 0.02	3.29 ± 0.03	3.79 ± 0.03	1.33 ± 0.01*	1.95 ± 0.01*	2.38 ± 0.02*
E	Negative control	1.42 ± 0.01	2.26 ± 0.02	2.65 ± 0.02	3.28 ± 0.03	3.80 ± 0.03	4.32 ± 0.04*****	4.84 ± 0.04*****	5.43 ± 0.05*****

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly

4.6.7 Heterophil/lymphocyte ratios

Before inoculation with vvIBDV, heterophil/lymphocyte (H/L) ratio increased as chicks grow older with values from 7 to 28 doa being highest in group C. There was a decrease in H/L ratio in groups A, B, and C from values of 0.59 ± 0.05 $\times 10^9$/L, 0.60 ± 0.05 $\times 10^9$/L and 0.62 ± 0.06 $\times 10^9$/L at 28 doa to 0.60 ± 0.06 $\times 10^9$/L, 0.58 ± 0.05 $\times 10^9$/L and 0.60 ± 0.05 $\times 10^9$/L at 35 doa, but the increases were significantly lower compared to that of positive control group D (0.22 ± 0.00 $\times 10^9$/L.), at 35 doa. The H/L ratio in A, B, C, and D were significantly different ($P \leq 0.05$) at 42 and 49 doa (Table 4.18, Appendix 31).

4.8 Changes in Serum Biochemical Parameters of ISA Brown Chicks Administered Molasses, Antox® and EN-FLORAX® and Infected with Infectious Bursal Disease Virus

4.7.1 Changes in metabolites

Before inoculation with vvIBDV, glucose concentration increased as chicks grOw older with values from 7 to 28 doa being highest in group A. The glucose concentration in groups A, B, and C decreased from 384.75 ± 3.33 mg/dl, 317.56 ± 3.31 mg/dl and 337.62 ± 3.32 mg/dl at 28 doa to 307.54 ± 3.23 mg/dl, 200.27 ± 2.24 mg/dl and 235.35 ± 2.25 mg/dl at 35 doa, but the decrease was significantly ($P \leq 0.05$) lower when compared to positive control group D (105.65 ± 1.22 mg/dl) at 35 doa (Table 4.19, Appendix 32).

Before inoculation with vvIBDV, total protein (TP) concentration increased as chicks grow older with values from 7 to 28 doa being highest in group A. The TP concentration in groups A, B, and C decreased from 6.99 ± 0.17 g/dl, 6.61 ± 0.16 g/dl and 6.74 ± 0.17 g/dl at 28 doa to 4.45 ± 0.06 g/dl, 3.19 ± 0.04 g/dl and 3.38 ± 0.05 g/dl at 7 dpi, but the decrease was significantly ($P \leq 0.05$) lower when compared to positive control group D (2.25 ± 0.02 g/dl) at 35 doa (Table 4.20, Appendix 33).

Table 4.18: Mean (± SE) heterophil/lymphocyte ratios of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean (± SE) heterophil/ lymphocyte ratio							
A	Molasses	0.37 ± 0.00	0.57 ± 0.05	0.59 ± 0.03	0.54 ± 0.05	0.59 ± 0.05	0.60 ± 0.06****	0.64 ± 0.06**	0.69 ± 0.08**
B	Antox®	0.36 ± 0.00	0.63 ± 0.05	0.59 ± 0.03	0.55 ± 0.05	0.60 ± 0.05	0.58 ± 0.05**	0.82 ± 0.05****	0.71 ± 0.06***
C	EN-FLORAX®	0.37 ± 0.00	0.67 ± 0.05	0.62 ± 0.03	0.58 ± 0.05	0.62 ± 0.06	0.60 ± 0.05***	0.78 ± 0.01***	0.81 ± 0.06****
D	Positive control	0.36 ± 0.00	0.44 ± 0.00	0.60 ± 0.05	0.59 ± 0.03	0.63 ± 0.03	0.22 ± 0.00*	0.46 ± 0.00*	0.48 ± 0.00*
E	Negative control	0.37 ± 0.00	0.44 ± 0.00	0.60 ± 0.05	0.59 ± 0.03	0.63 ± 0.06	0.69 ± 0.00*****	0.80 ± 0.08*****	0.82 ± 0.08*****

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly

Table 4.19: Mean (± SE) glucose concentration (mg/dl) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean (± SE) glucose concentration (mg/dl)							
A	Molasses	102.21 ± 1.23	226.48 ± 2.28	289.55 ± 2.33	328.68 ± 3.28	384.75 ± 3.33	307.54 ± 3.23****	338.39 ± 3.28****	385.25 ± 3.33****
B	Antox®	103.22 ± 1.24	165.33 ± 1.29	215.42 ± 2.27	252.48 ± 2.32	317.56 ± 3.31	200.27 ± 2.24**	224.20 ± 2.27**	255.24 ± 2.30**
C	EN-FLORAX®	104.21 ± 1.23	185.36 ± 1.28	235.47 ± 2.30	285.55 ± 2.35	337.62 ± 3.32	235.35 ± 2.25***	265.25 ± 2.29***	299.30 ± 2.32***
D	Positive control	102.22 ± 1.24	135.25 ± 1.29	184.33 ± 1.34	225.38 ± 2.27	273.45 ± 2.32	105.65 ± 1.22*	132.32 ± 1.27*	196.48 ± 1.29*
E	Negative control	104.21 ± 1.22	134.26 ± 1.27	183.34 ± 1.32	224.39 ± 2.26	275.44 ± 2.31	335.71 ± 3.27*****	399.75 ± 3.32*****	425.83 ± 4.27*****

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly

Table 4.20: Mean (± SE) total protein concentration (g/dl) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean (± SE) total protein concentration (g/dl)							
A	Molasses	4.33 ± 0.05	5.55 ± 0.08	5.94 ± 0.11	6.65 ± 0.14	6.99 ± 0.17	4.45 ± 0.06****	4.88 ± 0.08****	5.51 ± 0.10****
B	Antox®	4.32 ± 0.04	5.35 ± 0.07	5.74 ± 0.10	6.45 ± 0.13	6.61 ± 0.16	3.19 ± 0.04**	3.46 ± 0.06**	3.99 ± 0.08**
C	EN-FLORAX®	4.33 ± 0.05	5.40 ± 0.08	5.78 ± 0.11	6.55 ± 0.14	6.74 ± 0.17	3.38 ± 0.05***	3.84 ± 0.07***	4.25 ± 0.09***
D	Positive control	4.32 ± 0.04	4.83 ± 0.06	5.25 ± 0.08	5.87 ± 0.10	6.23 ± 0.12	2.25 ± 0.02*	2.44 ± 0.03*	2.98 ± 0.05*
E	Negative control	4.33 ± 0.05	4.84 ± 0.07	5.24 ± 0.09	5.88 ± 0.11	6.24 ± 0.13	6.89 ± 0.15*****	7.44 ± 0.17*****	7.98 ± 0.19*****

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly

4.7.2 Changes in minerals/electrolytes

Before inoculation with vvIBDV, calcium (Ca) concentration increased as chicks grow older with values from 7 to 28 doa being highest in group A. The Ca concentration in groups A, B, and C decreased from 4.95 ± 0.06 mg/dl, 4.87 ± 0.06 mg/dl and 4.91 ± 0.06 mg/dl at 28 doa to 3.36 ± 0.03 mg/dl, 2.35 ± 0.02 mg/dl and 2.56 ± 0.02 mg/dl at 35 doa, but the decrease was significantly ($P \leq 0.05$) lower when compared to positive control group D (1.99 ± 0.01 mg/dl) at 35 doa (Table 4.21, Appendix 34).

Before inoculation with vvIBDV, phosphorus (P) concentration increased as chicks grow older with values from 7 to 28 doa being highest in group A. The P concentration in groups A, B and C decreased from 3.45 ± 0.05 mg/dl, 3.31 ± 0.05 mg/dl and 3.38 ± 0.05 mg/dl at 28 doa to 2.52 ± 0.02 mg/dl, 2.15 ± 0.01 mg/dl and 2.25 ± 0.01 mg/dl at 35 doa, but the decrease was significantly ($P \leq 0.05$) lower when compared to positive control group D (1.15 ± 0.00 mg/dl) at 35 doa (Table 4.22, Appendix 35).

Before inoculation with vvIBDV, potasium (K) concentration increased as chicks grew older with values from 7 to 28 doa being highest in group A. The K concentration in groups A, B, and C decreased from 5.35 ± 0.05 mg/dl, 5.21 ± 0.05 mg/dl and 5.28 ± 0.05 mg/dl at 28 doa to 4.55 ± 0.03 mg/dl, 3.23 ± 0.02 mg/dl and 3.34 ± 0.02 mg/dl at 35 doa, but the decrease was significantly ($P \leq 0.05$) lower when compared to positive control group D (2.09 ± 0.01 mg/dl) at 35 doa (Table 4.23, Appendix 36).

4.7.3 Changes in kidneys function

Before inoculation with vvIBDV, creatinine level increased as chicks grew older with values from 7 to 28 doa being highest in group B.

Table 4.21: Mean (± SE) calcium concentration (mg/dl) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age (days)							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean (± SE) calcium concentration (mg/dl)							
A	Molasses	2.44 ± 0.02	3.55 ± 0.03	3.97 ± 0.04	4.45 ± 0.05	4.95 ± 0.06	3.36 ± 0.03****	3.94 ± 0.04****	4.45 ± 0.05****
B	Antox®	2.43 ± 0.02	3.34 ± 0.03	3.85 ± 0.04	4.35 ± 0.05	4.87 ± 0.06	2.35 ± 0.02**	2.84 ± 0.03**	3.46 ± 0.04**
C	EN-FLORAX®	2.43 ± 0.02	3.45 ± 0.03	3.92 ± 0.04	4.40 ± 0.05	4.91 ± 0.06	2.56 ± 0.02***	2.95 ± 0.03***	3.57 ± 0.04***
D	Positive control	2.44 ± 0.02	2.83 ± 0.02	3.52 ± 0.03	3.98 ± 0.04	4.49 ± 0.05	1.99 ± 0.01*	2.45 ± 0.02*	2.95 ± 0.03*
E	Negative control	2.43 ± 0.02	2.84 ± 0.02	3.54 ± 0.03	3.96 ± 0.04	4.48 ± 0.05	4.88 ± 0.06*****	5.54 ± 0.07*****	5.95 ± 0.08*****

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly

Table 4.22: Mean (± SE) phosphorus concentration (mg/dl) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean (± SE) phosphorus concentration (mg/dl)							
A	Molasses	1.03 ± 0.00	2.26 ± 0.02	2.85 ± 0.03	3.15 ± 0.04	3.45 ± 0.05	2.52 ± 0.02****	2.94 ± 0.03****	3.14 ± 0.04****
B	Antox®	1.02 ± 0.00	2.24 ± 0.02	2.65 ± 0.03	3.10 ± 0.04	3.31 ± 0.05	2.15 ± 0.01**	2.49 ± 0.02**	2.89 ± 0.03**
C	EN-FLORAX®	1.03 ± 0.00	2.25 ± 0.02	2.74 ± 0.03	3.12 ± 0.04	3.38 ± 0.05	2.25 ± 0.01***	2.54 ± 0.02***	2.95 ± 0.03***
D	Positive control	1.02 ± 0.00	1.63 ± 0.01	2.15 ± 0.02	2.48 ± 0.03	2.83 ± 0.04	1.15 ± 0.00*	1.43 ± 0.01*	1.92 ± 0.02*
E	Negative control	1.02 ± 0.00	1.64 ± 0.01	2.14 ± 0.02	2.49 ± 0.03	2.82 ± 0.04	3.18 ± 0.05*****	3.43 ± 0.06*****	3.89 ± 0.07*****

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly

Table 4.23: Mean (± SE) potassium concentration (mg/dl) of ISA Brown chicks (n = 5) administered Molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean (± SE) potassium concentration (mg/dl)							
A	Molasses	3.10 ± 0.02	3.95 ± 0.03	4.48 ± 0.04	4.93 ± 0.04	5.35 ± 0.05	4.55 ± 0.03****	4.94 ± 0.04****	5.29 ± 0.04****
B	Antox®	3.10 ± 0.02	3.78 ± 0.03	4.35 ± 0.04	4.75 ± 0.04	5.21 ± 0.05	3.23 ± 0.02**	3.55 ± 0.03**	3.82 ± 0.03**
C	EN-FLORAX®	3.11 ± 0.02	3.84 ± 0.03	4.42 ± 0.04	4.85 ± 0.04	5.28 ± 0.05	3.34 ± 0.02***	3.64 ± 0.03***	3.95 ± 0.03***
D	Positive control	3.10 ± 0.02	3.45 ± 0.02	3.85 ± 0.03	4.27 ± 0.03	4.59 ± 0.04	2.09 ± 0.01*	2.43 ± 0.02*	2.85 ± 0.02*
E	Negative control	3.11 ± 0.02	3.46 ± 0.02	3.84 ± 0.03	4.28 ± 0.03	4.61 ± 0.04	4.98 ± 0.04*****	5.24 ± 0.05*****	5.55 ± 0.05*****

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly

The creatinine level in groups A, B, and C increased from 2.16 ± 0.02 mg/dl, 2.18 ± 0.02 and 2.14 ± 0.02 at 28 doa to 4.05 ± 0.03 mg/dl, 4.89 ± 0.04 mg/dl and 4.57 ± 0.04 mg/dl at 35 doa, but the increase was significantly ($P \leq 0.05$) lower when compared to positive control group D (7.28 ± 0.06 mg/dl) at 35 doa (Table 4.24, Appendix 37).

Before inoculation with vvIBDV, blood urea nitrogen (BUN) level increased as chicks grew older with values from 7 to 28 doa being highest in group B. The BUN level in groups A, B, and C increased from 2.25 ± 0.02 mg/dl, 2.45 ± 0.02 mg/dl and 2.38 ± 0.02 mg/dl at 28 doa to 4.95 ± 0.04 mg/dl, 5.93 ± 0.05 mg/dl and 5.88 ± 0.05 mg/dl at 35 doa, but the increase was significantly ($P \leq 0.05$) lower when compared to positive control group D (7.99 ± 0.07 mg/dl) at 35 doa (Table 4.25, Appendix 38).

4.7.4 Changes in liver enzymes activity

Before inoculation with vvIBDV, aspartate aminotransferase (AST) activity increased as chicks grew older with values from 7 to 28 doa being highest in group B. The AST activity in groups A, B, and C increased from mean value of 120.54 ± 1.30 U/L, 130.42 ± 1.40 U/L and 125.46 ± 1.35 U/L at 28 doa to 200.25 ± 2.15 µg/l, 283.21 ± 2.23 U/L and 250.23 ± 2.19 U/L at 35 doa, but the increase was significantly ($P \leq 0.05$) lower when compared to positive control group D (350.19 ± 3.22 U/L) at 35 doa (Table 4.26, Appendix 39).

Before inoculation with vvIBDV, glutamate dehydrogenase (GDH) activity increased as chicks grew older with values from 7 to 28 doa being highest in group B. The mean GDH activity in groups A, B, and C increased from mean value of 110.44 ± 1.22 U/L, 120.43 ± 1.28 U/L and 115.46 ± 1.26 U/L at 28 doa to 200.85 ± 1.19 U/L, 260.88 ± 2.24 U/L and 235.87 ± 2.27 U/L at 35 doa,

Table 4.24: Mean (± SE) creatinine concentration (mg/dl) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean (± SE) creatinine concentration (mg/dl)							
A	Molasses	0.33 ± 0.00	1.15 ± 0.01	1.45 ± 0.01	1.75 ± 0.01	2.16 ± 0.02	4.05 ± 0.03****	3.65 ± 0.02****	3.35 ± 0.02*****
B	Antox®	0.32 ± 0.00	1.35 ± 0.01	1.64 ± 0.01	1.95 ± 0.01	2.18 ± 0.02	4.89 ± 0.04**	4.05 ± 0.03**	3.95 ± 0.03**
C	EN-FLORAX®	0.33 ± 0.00	1.34 ± 0.01	1.74 ± 0.01	1.88 ± 0.01	2.14 ± 0.02	4.57 ± 0.04***	3.99 ± 0.03***	3.79 ± 0.03***
D	Positive control	0.33 ± 0.00	1.85 ± 0.01	2.15 ± 0.02	2.48 ± 0.02	2.99 ± 0.02	7.28 ± 0.06*	6.45 ± 0.05*	5.62 ± 0.04*
E	Negative control	0.32 ± 0.00	1.83 ± 0.01	2.17 ± 0.02	2.47 ± 0.02	2.98 ± 0.02	3.19 ± 0.03*****	3.44 ± 0.03*****	3.85 ± 0.03****

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly

Table 4.25: Mean (± SE) blood urea nitrogen concentration (mg/dl) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean (± SE) blood urea nitrogen (mg/dl)							
A	Molasses	1.13 ± 0.00	1.44 ± 0.01	1.73 ± 0.01	2.05 ± 0.02	2.25 ± 0.02	4.95 ± 0.04****	4.64 ± 0.04****	4.25 ± 0.04****
B	Antox®	1.14 ± 0.00	1.63 ± 0.01	1.95 ± 0.01	2.15 ± 0.02	2.45 ± 0.02	5.93 ± 0.05**	5.64 ± 0.05**	5.45 ± 0.05**
C	EN-FLORAX®	1.14 ± 0.00	1.58 ± 0.01	1.84 ± 0.01	2.10 ± 0.02	2.38 ± 0.02	5.88 ± 0.05***	5.52 ± 0.05***	5.28 ± 0.05***
D	Positive control	1.13 ± 0.00	1.62 ± 0.01	2.15 ± 0.02	2.47 ± 0.02	2.89 ± 0.02	7.99 ± 0.07*	7.75 ± 0.07*	7.32 ± 0.07*
E	Negative control	1.13 ± 0.00	1.60 ± 0.01	2.14 ± 0.02	2.45 ± 0.02	2.88 ± 0.02	3.28 ± 0.03*****	3.54 ± 0.03*****	3.89 ± 0.03*****

Key**:** n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly

Table 4.26: Mean (± SE) aspartate aminotransferase activity (U/L) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28 days-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean (± SE) aspertate aminotransferase activity (U/L)							
A	Molasses	100.14 ± 1.22	105.19 ± 1.15	110.34 ± 1.20	115.45 ± 1.25	120.54 ± 1.30	200.25 ± 2.15****	185.34 ± 1.10****	158.39 ± 1.08*****
B	Antox®	100.12 ± 1.22	115.15 ± 1.25	120.25 ± 1.30	125.35 ± 1.35	130.42 ± 1.40	283.21 ± 2.23**	255.28 ± 2.15**	225.32 ± 2.10**
C	EN-FLORAX®	100.13 ± 1.23	110.17 ± 1.20	115.31 ± 1.25	120. 38 ± 1.30	125.46 ± 1.35	250.23 ± 2.19***	225.31 ± 2.13***	200.37 ± 2.09***
D	Positive control	100.15 ± 1.23	120.17 ± 1.28	132.25 ± 1.33	144.32 ± 1.38	153.44 ± 1.43	350.19 ± 3.22*	300.23 ± 3.19*	275.28 ± 2.15*
E	Negative control	100.13 ± 1.22	121.16 ± 1.27	133.24 ± 1.32	143.35 ± 1.37	154.43 ± 1.42	166.42 ± 1.47*****	174.49 ± 1.52*****	189.53 ± 1.57****

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly

but the increase was significantly ($P \leq 0.05$) lower when compared to positive control group D (300.80 ± 3.35 U/L) at 35 doa (Table 4.27, Appendix 40).

4.7.5 Changes in lipids profiles

Before inoculation with vvIBDV, total cholesterol (TC) concentration increased as chicks grew older with values from 7 to 28 doa being highest in group C. The TC concentration in groups A, B, and C decreased from mean value of 153.42 ± 1.40 mg/dl, 159.43 ± 1.45 mg/dl and 172.45 ± 1.48 mg/dl at 28 doa to 125.24 ± 1.28 mg/dl, 105.21 ± 1.24 mg/dl and 110.22 ± 1.27 mg/dl at 35 doa, but the decrease was significantly ($P \leq 0.05$) lower when compared to positive control group D (69.20 ± 0.25 mg/dl) at 35 doa (Table 4.28, Appendix 41).

Before inoculation with vvIBDV, triglyceride (TG) concentration increased as chicks grow older with values from 7 to 28 doa being highest in group C. The TG concentration in groups A, B, and C decreased from mean value of 120.38 ± 1.37 mg/dl, 126.39 ± 1.39 and 137.41 ± 1.40 at 28 doa to 100.25 ± 1.23 mg/dl, 85.16 ± 0.20 mg/dl and 95.20 ± 0.22 mg/dl at 35 doa, but the decrease was significantly ($P \leq 0.05$) lower when compared to positive control group D (56.12 ± 0.16 mg/dl) at 35 doa (Table 4.29, Appendix 42).

4.8 Oxidative Stress Biomarkers of ISA Brown Chicks Administered Molasses, Antox® and EN-FLORAX® and Infected with Infectious Bursal Disease Virus

4.8.1 Superoxide dismutase activity

Before inoculation with vvIBDV, superoxide dismutase activity increased as chicks grew older with values from 7 to 28 doa being highest in group C.

Table 4.27: Mean (± SE) glutamate dehydrogenase activity (U/L) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28 days-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean (± SE) glutamate dehydrogenase activity (U/L)							
A	Molasses	90.22 ± 0.10	95.27 ± 1.13	100.32 ± 1.16	105.39 ± 1.19	110.44 ± 1.22	200.85 ± 1.19****	198.43 ± 1.13****	185.20 ± 1.09*****
B	Antox®	90.23 ± 0.11	105.28 ± 0.15	110.33 ± 1.20	115.37 ± 1.24	120.43 ± 1.28	260.88 ± 2.24**	225.46 ± 2.19**	200.24 ± 2.14**
C	EN-FLORAX®	90.24 ± 0.10	100.29 ± 1.14	105.35 ± 1.18	110.41 ± 1.22	115.46 ± 1.26	235.87 ± 2.27***	205.44 ± 2.22***	195.25 ± 1.17***
D	Positive control	90.22 ± 0.11	115.26 ± 0.18	130.30 ± 1.23	145.35 ± 1.26	161.40 ± 1.30	300.80 ± 3.35*	285.40 ± 2.23*	255.29 ± 2.19*
E	Negative control	90.23 ± 0.10	116.27 ± 0.19	132.31 ± 1.22	144.36 ± 1.27	162.41 ± 1.31	173.48 ± 1.34*****	183.54 ± 1.37*****	192.58 ± 1.40****

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly

Table 4.28: Mean (± SE) total cholesterol concentration (mg/dl) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28 days-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean (± SE) total cholesterol concentration (mg/dl)							
A	Molasses	100.23 ± 1.24	110.28 ± 1.27	130.32 ± 1.34	155.40 ± 1.45	153.42 ± 1.40	125.24 ± 1.28****	135.29 ± 1.33****	147.34 ± 1.39****
B	Antox®	100.22 ± 1.26	115.27 ± 1.29	137.33 ± 1.35	147.38 ± 1.40	159.43 ± 1.45	105.21 ± 1.24**	115.26 ± 1.27**	125.31 ± 1.30**
C	EN-FLORAX®	100.22 ± 1.24	120.28 ± 1.30	145.34 ± 1.36	155.40 ± 1.45	172.45 ± 1.48	110.22 ± 1.27***	120.27 ± 1.30***	130.32 ± 1.35***
D	Positive control	100.23 ± 1.25	105.26 ± 1.27	114.30 ± 1.30	128.35 ± 1.34	138.40 ± 1.36	69.20 ± 0.25*	80.25 ± 0.30*	92.30 ± 0.35*
E	Negative control	100.22 ± 1.24	106.25 ± 1.26	112.29 ± 1.29	129.34 ± 1.32	136.39 ± 1.35	148.44 ± 1.38*****	155.49 ± 1.41*****	167.54 ± 1.45*****

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly

Table 4.29: Mean (± SE) triglyceride concentration (mg/dl) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean (± SE) triglyceride concentration (mg/dl)							
A	Molasses	82.14 ± 0.22	89.23 ± 0.24	99.28 ± 0.27	109.33 ± 1.32	120.38 ± 1.39	100.25 ± 1.23****	110.30 ± 1.28****	120.35 ± 1.33****
B	Antox®	83.13 ± 0.23	91.24 ± 0.25	105.29 ± 1.29	115.34 ± 1.34	126.39 ± 1.39	85.16 ± 0.20**	95.21 ± 0.25**	105.26 ± 1.30**
C	EN-FLORAX®	83.14 ± 0.23	94.26 ± 0.26	115.31 ± 1.31	125.36 ± 1.36	137.41 ± 1.40	95.20 ± 0.22***	103.25 ± 1.26***	113.30 ± 1.31*
D	Positive control	82.13 ± 0.22	86.19 ± 0.23	93.24 ± 0.26	100.29 ± 1.29	112.34 ± 1.32	56.12 ± 0.16*	66.17 ± 0.20*	76.23 ± 0.26*
E	Negative control	82.15 ± 0.22	85.21 ± 0.24	92.26 ± 0.27	101.31 ± 1.30	113.36 ± 1.33	125.48 ± 1.36*****	137.50 ± 1.39*****	145.55 ± 1.43*****

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly

The superoxide dismutase activity in groups A, B, and C decreased from mean value of 55.32 ± 0.21 U/mL, 53.44 ± 0.19 U/mL and 58.47 ± 0.22 U/mL at 28 doa to 34.49 ± 0.14 U/mL, 25.52 ± 0.09 U/mL and 28.58 ± 0.08 U/mL at 35 doa, but the decrease was significantly ($P \leq 0.05$) lower when compared to positive control group D (14.57 ± 0.04 U/mL) at 35 doa (Table 4.30, Appendix 43).

4.8.4 Malondialdehyde concentration

Before inoculation with vvIBDV, malondialdehyde concentration decreased as chicks grew older with values from 7 to 28 doa being highest in group D. The malondialdehyde concentration in groups A, B, and C increased from mean value of 31.18 ± 0.20 nmol/mL, 42.14 ± 0.18 nmol/mL and 24.12 ± 0.15 nmol/mL at 28 doa to 56.23 ± 0.27 nmol/mL, 76.36 ± 0.39 µ/mL and 65.33 ± 0.35 nmol/mL at 35 doa, but the increase were significantly ($P \leq 0.05$) lower when compared to positive control group D (91.49 ± 0.50 nmol/mL) at 35 doa (Table 4.31, Appendix 44).

4.9 Gross Lesions in ISA Brown Chicks Administered Molasses, Antox® and EN-FLORAX® and Infected with Infectious Bursal Disease Virus

4.9.1. Gross lesions in the bursa of Fabricius

The BF was severely oedematous, hyperaemic, with a gelatinous yellowish exudate covering the serosal surface in chicks inoculated with vvIBDV. There were fewer gross lesions in the groups A, B and C when compared to positive control group (D) at 35 doa. On the mucosal surface, the BF showed petechial haemorrhages in groups A, B, C, and D. The BF haemorrhages were extensive in positive control group D when compared to groups A, B, and C (Plate I).

Table 4.30: Mean (± SE) superoxide dismutase activity (U/mL) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean (± SE) superoxide dismutase activity (U/mL)							
A	Molasses	10.53 ± 0.05	23.92 ± 0.09	35.49 ± 0.13	46.44 ± 0.19	55.32 ± 0.21	34.49 ± 0.14****	41.24 ± 0.18****	49.42 ± 0.20****
B	Antox®	10.48 ± 0.04	21.41 ± 0.08	32.67 ± 0.12	43.55 ± 0.16	53.44 ± 0.19	25.52 ± 0.09**	32.39 ± 0.13**	41.57 ± 0.15**
C	EN-FLORAX®	10.44 ± 0.04	25.46 ± 0.07	38.58 ± 0.16	49.67 ± 0.18	58.47 ± 0.22	28.58 ± 0.08***	39.34 ± 0.15***	45.29 ± 0.17***
D	Positive control	10.54 ± 0.02	14.05 ± 0.04	19.99 ± 0.07	26.47 ± 0.13	30.49 ± 0.16	14.57 ± 0.04*	21.54 ± 0.09*	32.40 ± 0.12*
E	Negative control	10.50 ± 0.03	15.04 ± 0.05	20.98 ± 0.08	25.95 ± 0.12	31.39 ± 0.17	46.51 ± 0.19*****	52.20 ± 0.22*****	59.73 ± 0.25*****

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$) or (****), ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly

Table 4.31: Mean (± SE) malondialdehyde concentration (nmols/mL) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Age (days)							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean (± SE) malondialdehyde concentration (nmol/mL)							
A	Molasses	93.55 ± 0.53	74.32 ± 0.38	58.25 ± 0.29	42.20 ± 0.25	31.18 ± 0.20	56.23 ± 0.27****	43.20 ± 0.22****	35.15 ± 0.17****
B	Antox®	94.54 ± 0.50	76.35 ± 0.37	62.23 ± 0.27	51.19 ± 0.22	35.14 ± 0.18	75.36 ± 0.39**	65.25 ± 0.27**	50.20 ± 0.22**
C	EN-FLORAX®	95.53 ± 0.54	69.34 ± 0.34	49.21 ± 0.25	33.17 ± 0.20	24.12 ± 0.15	65.33 ± 0.35***	51.23 ± 0.25***	43.18 ± 0.20***
D	Positive control	95.55 ± 0.52	89.44 ± 0.47	77.38 ± 0.35	65.32 ± 0.30	54.27 ± 0.25	91.49 ± 0.50*	87.44 ± 0.46*	72.35 ± 0.40*
E	Negative control	94.54 ± 0.51	88.43 ± 0.48	75.36 ± 0.39	66.30 ± 0.34	53.25 ± 0.29	42.20 ± 0.25*****	35.15 ± 0.20*****	25.10 ± 0.15*****

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means values with asterics (*) ($P \leq 0.05$), (**) ($P \leq 0.01$), (***) ($P \leq 0.001$), (****) ($P \leq 0.0001$) or (*****) ($P \leq 0.00001$) in the same column differed significantly.

4.9.2 Gross lesions in the spleen

Spleen (SPL) was congested, enlarged, oedematous and atrophied in chicks inoculated with vvIBDV groups (A, B, C, and D) but the lesions were less severe in groups A, B, and C when compared to D at 35 doa (Plate I).

4.9.3 Gross lesions in the thymus

The observable gross lesions in the thymus (THY) of chicks in groups A, B, C, and D inoculated with vvIBDV were congestion, enlargement and atrophy, but the lesions were less severe in groups A, B and C when compared to D at 35 doa (Plate I).

4.9.4 Gross lesions in other organs

There were petechial and ecchymotic haemorrhages in the proventriculus and ventriculus, breast, thigh and legs muscles in chicks in groups A, B, C, and D but the haemorrhages were less severe in groups A, B, and C when compared to group D at 35 doa (Plate II, III & IV). There was enlargement, congestion and oedema in liver (Plate V), lungs, kidneys and pancreas, respectively at 35 doa in chicks in groups A, B, C, and D but the lesions were less severe in groups A, B, and C when compared to group D at 35 doa.

4.9.5 Gross pathological lesion scores

After seven days of observations the average gross pathological lesion scores on a scale of 5 for chicks in groups A, B, C, D, and E were 2.44 (moderate), 2.71 (moderate), 2.60 (moderate), 3.54 (severe), and 0.00 (normal), respectively (Table 4.32, Appendix 10).

Table 4.32: Percentage (%) gross pathological lesions score of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

		Days post-inoculation					
		3	4	5	6	7	
Group	Treatment	Daily gross lesions score					Average gross lesions score
A	Molasses	2.47	2.53	2.33	0.00	0.00	2.44 (moderate)
B	Antox®	2.60	3.20	2.55	2.48	0.00	2.71 (moderate)
C	EN-FLORAX®	2.57	3.17	2.38	2.26	0.00	2.60 (moderate)
D	Positive control	3.54	4.32	3.84	3.55	2.47	3.54 (severe)
E	Negative control	0.00	0.00	0.00	0.00	0.00	0.00 (normal)

Key: n=totalnumberofbirdssample

4.10 Microscopic Lesions in ISA Brown Chicks Administered Molasses, Antox® and EN-FLORAX® and Infected with Infectious Bursal Disease Virus

4.10.1 Microscopic lesions in the bursa of Fabricius

Plate VI shows bursal of Fabricius with intact follicles from chick in the negative control group, while Plate XII shows depletion of follicle and oedemal fluid in the bursa of Fabricius of chick from the positive control group. Microscopic lesions in bursae of Fabricius of chicks in groups A, B, and C were follicular vacuolation and oedemal fluid (Plates IX, X and XI).

4.10.2 Microscopic lesions in the spleen

Photomicrograph of spleen section of chick from the negative control group revealed intact white and red pulps (Plate VII). On the other hand, the spleen section from chick in the positive control group revealed haemorrhage and mononuclear cells infiltrations (Plate XVI). The spleen sections from chicks in groups A and C were normal with intact white and red pulps (Plates VIII and XV), while spleen section from chick in group B was haemorrhagic (Plate XIV).

4.10.3 Microscopic lesions in the thymus

Intact thymic cortex and medulla was observed microscopically in the thymus of chick from negative control group (Plate VIII), while haemorrhage and depleted thymus was observed from the positive control group (Plate XX). Haemorrhagic lesions were observed in thymuses of chicks in groups A, B, and C (Plates XVII, XVIII and XIX).

4.10.4 Histopathological lesions score

After seven days of observations the average histopathological lesion scores on a scale of 5 for chicks in groups A, B, C, D and E were 1.00 (mild), 2.17 (moderate), 2.00 (moderate), 3.33 (severe), and 0.00 (normal), respectively at 35 doa (7dpi) (Table 4.33, Appendix 11).

Table 4.33: Histopathological lesions score of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

Group	Treatment	Histopathological score	Grade
A	Molasses	1.00	Mild
B	Antox®	2.17	Moderate
C	EN-FLORAX®	2.00	Moderate
D	Positive control	3.33	Severe
E	Negative control	0.00	Normal

Key: n = total number of birds sampled, seven days post inoculation

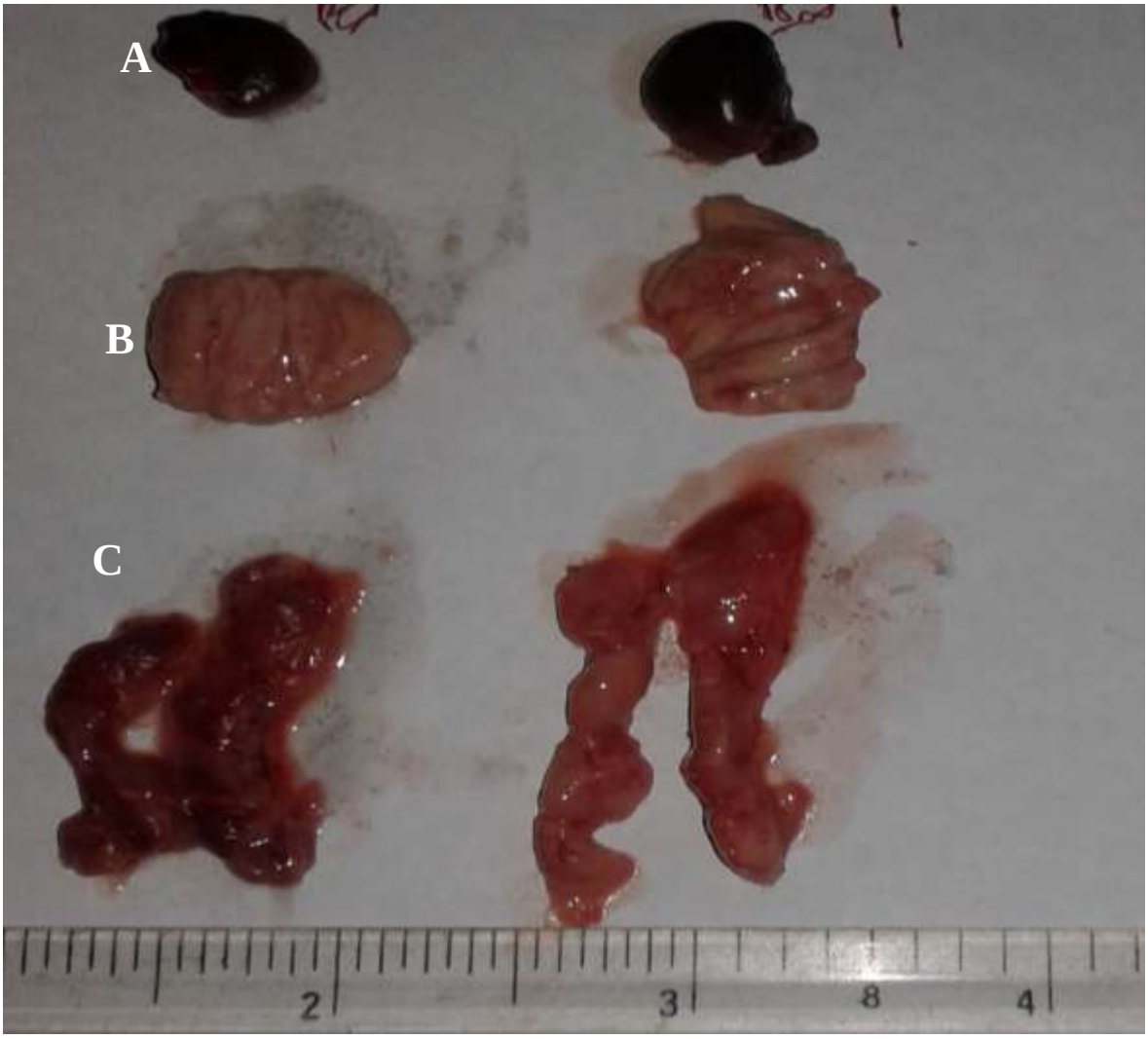


Plate I: Grossly congested spleen (A), enlarged bursa of Fabricius (B) and haemorrhagic and congested thymus (C) in chick from positive control group (D), infected with a very virulent infectious bursal diseases virus at 35 das of age

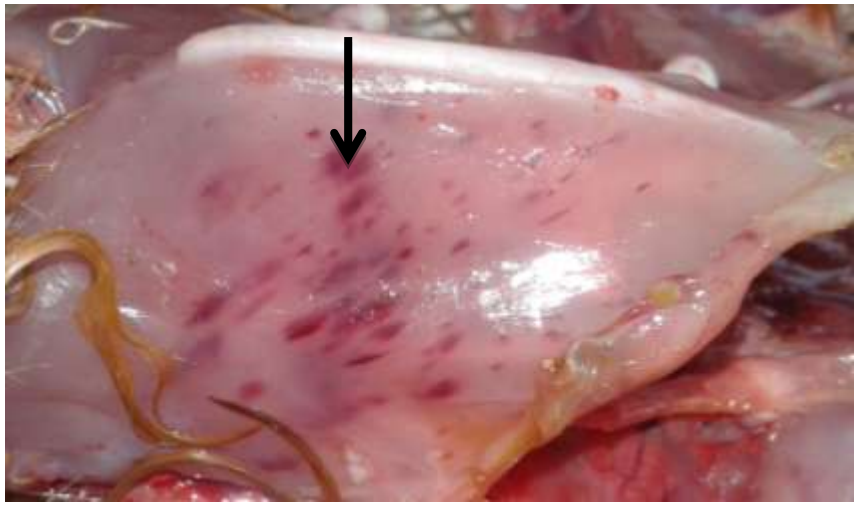


Plate II: Haemorrhagic breast muscles (arrow) in chick from positive control group (D), infected with a very virulent infectious bursal disease virus 35 days of age

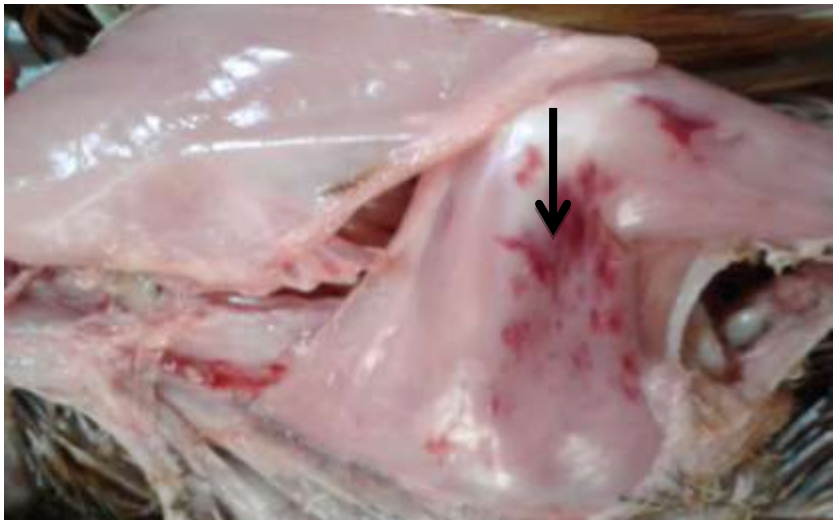


Plate III Showed; Haemorrhagic thigh and leg muscles (arrow) in chick from positive control group (D), infected with a very virulent infectious bursal diseases virus at 35 days of age

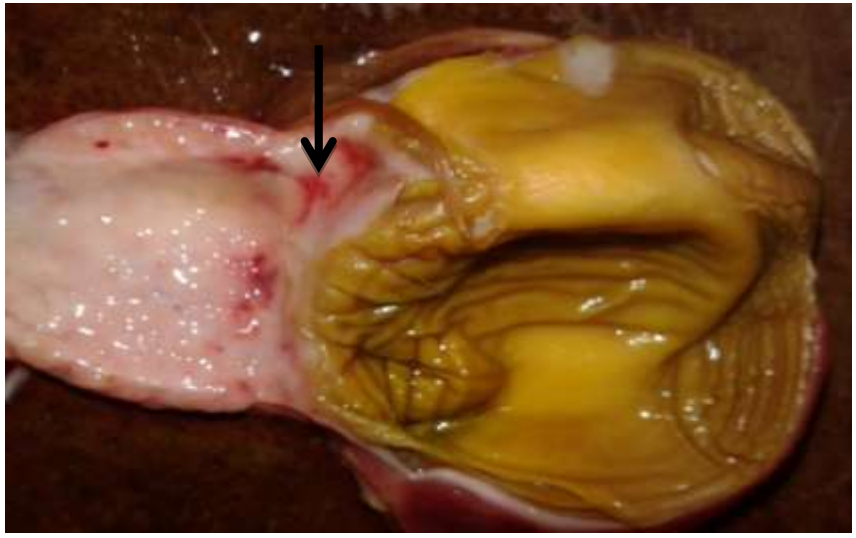


Plate IV: Haemorrhage (arrow) at the proventiculo-ventricular junction of chick in positive control group (D), infected with a very virulent infectious bursal disease virus at 35 days of age

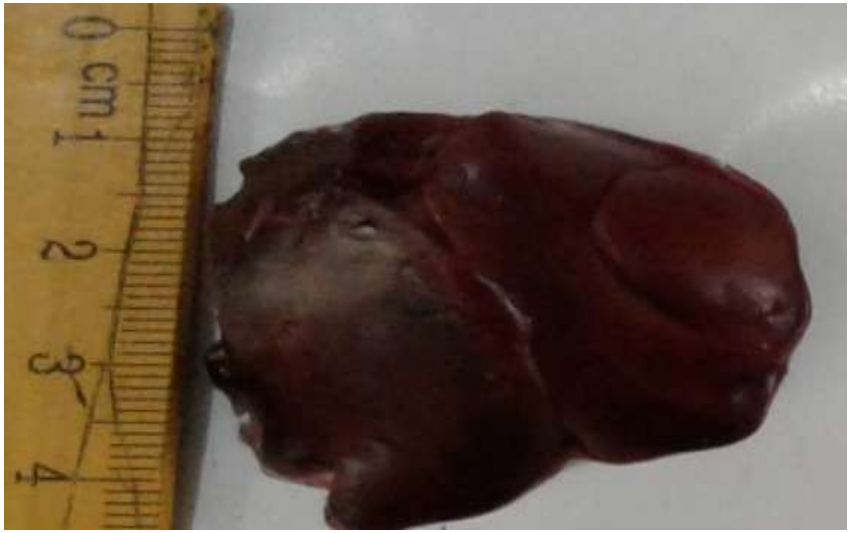


Plate V: Showed enlargement, congestion and oedematous liver of chick in positive control group (D), infected with a very virulent infectious bursal disease virus at 35 days of age

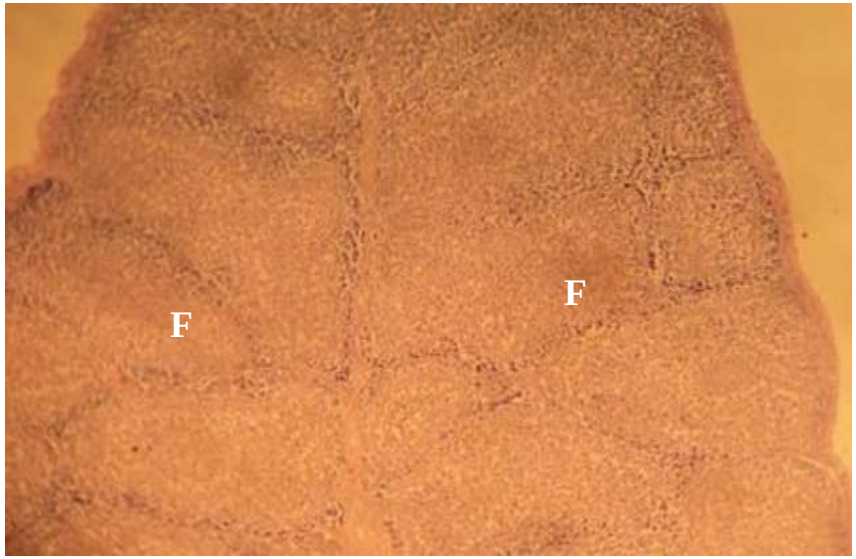
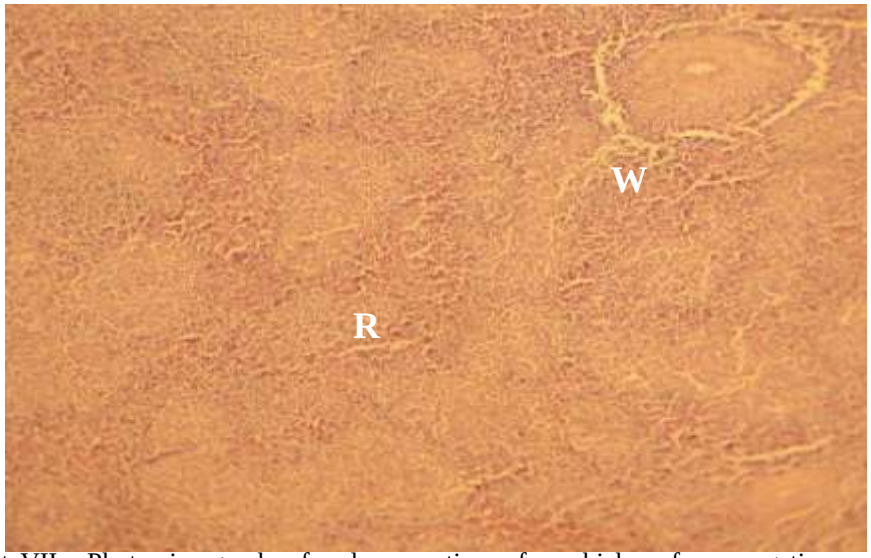


Plate VI: Photomicrograph of bursa of Fabricius section of a chicken from negative control group E (not pretreated with molasses, Antox®, EN-FLORAX® and not inoculated vvIBDV) showing intact follicles (F). H & E x 200.



PlateVII: Photomicrograph of spleen section of a chicken from negative control group E (not pretreated with molasses, Antox®, EN-FLORAX® and not inoculated vvIBDV) showing intact white (W) and red (R) pulps. H & E x 200.

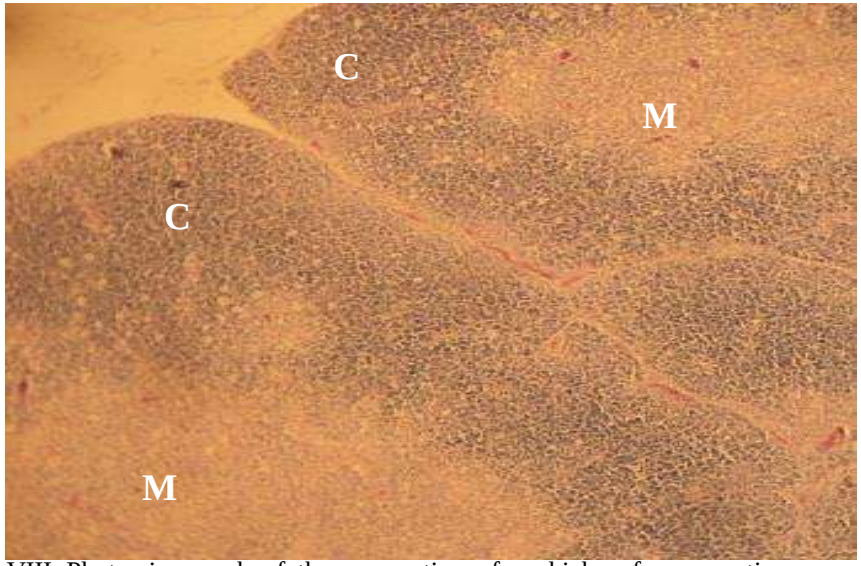


Plate VIII: Photomicrograph of thymus section of a chicken from negative control group E (not pretreated with molasses, Antox®, EN-FLORAX® and not inoculated vvIBDV) showing intact cortex (C) and medulla (M). H & E x 200.

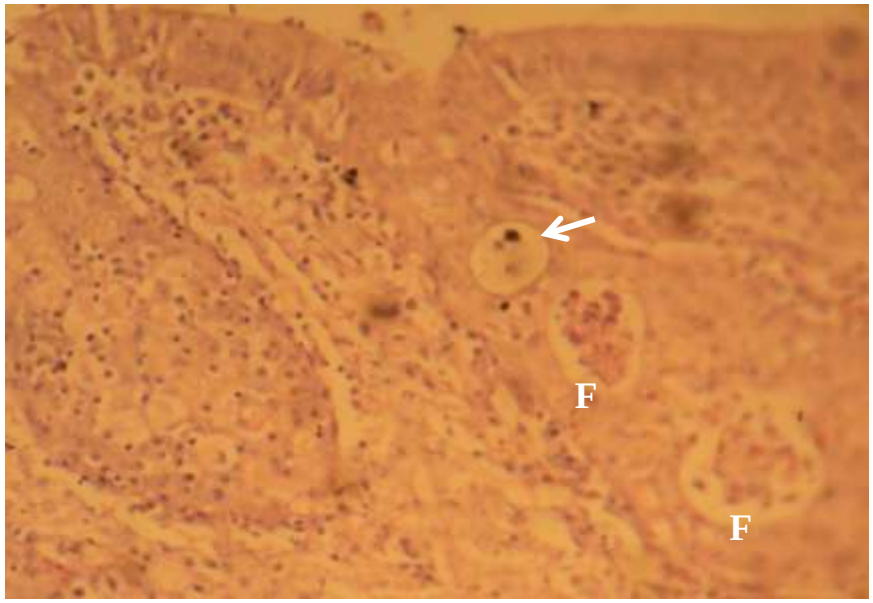


Plate IX: Photomicrograph of bursa of Fabricius section of group A chicken administere molasses an infected with a vvIBDV showing vacuolation (arrow) and oedema fluid (F). H & E x 200.

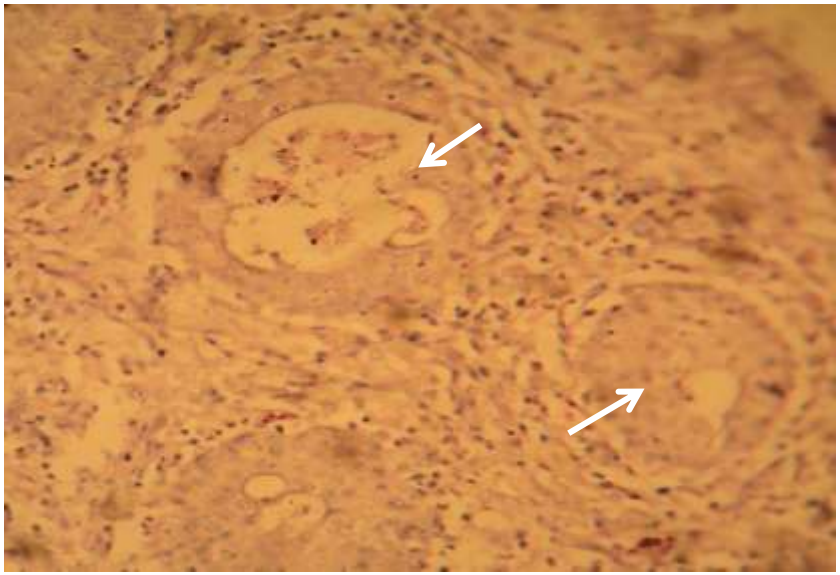


Plate X: Photomicrograph of bursa of Fabricius section of group B chicken administered Antox® and infected with a vvIBDV showing vacuolation (arrow) and oedema fluid (F). H & E x 200.

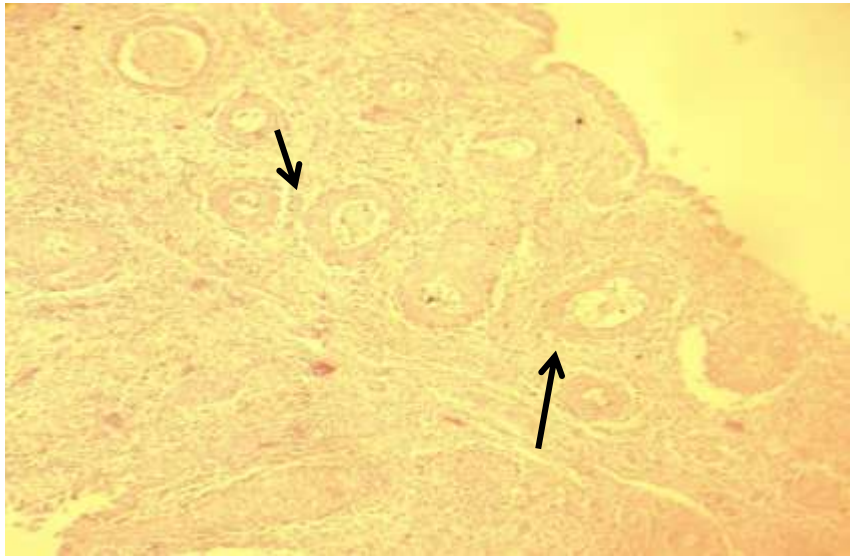


Plate XI: Photomicrograph of bursa of Fabricius section of group C chicken administered EN-FLORAX® and infected with a vvIBDV showing depilated follicles with vacuolation (arrows). H & E x 200.

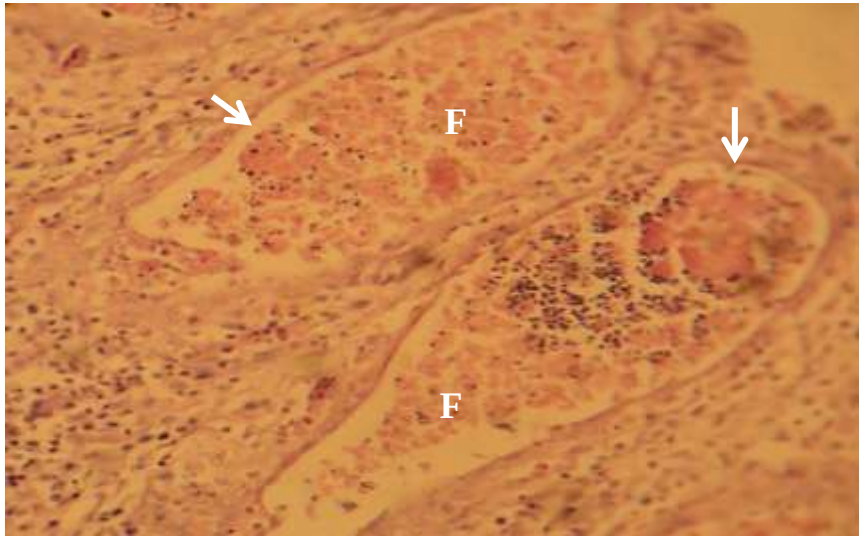


Plate XII: Photomicrograph of bursa of Fabricius section of group D (positive control) chicken infected with a vvIBDV and no supplements were administered showing depleted follicles (arrow) and oedema fluid (F). H & E x 200.

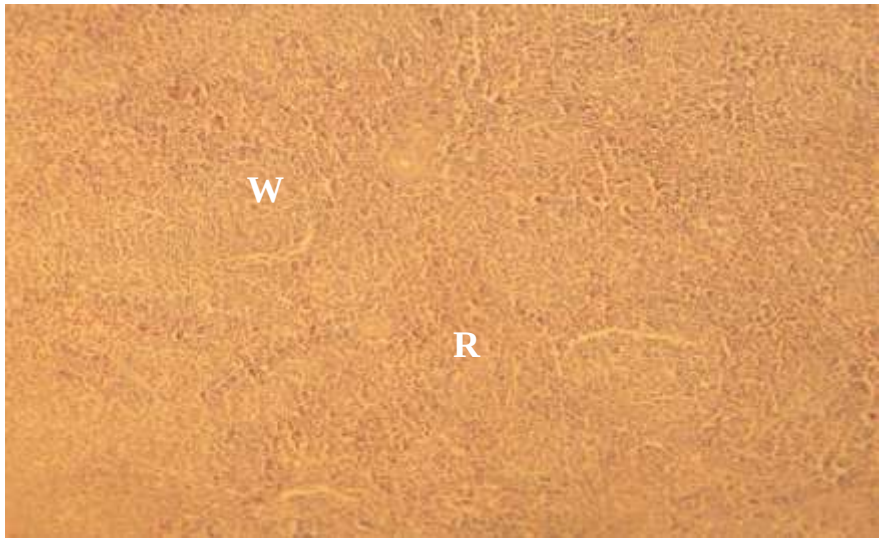


Plate XIII: Photomicrograph of spleen section of group A chicken administered molasses an inoculated with a vvIBDV showing intact white (W) and red (R) pulps. H & E x 200.

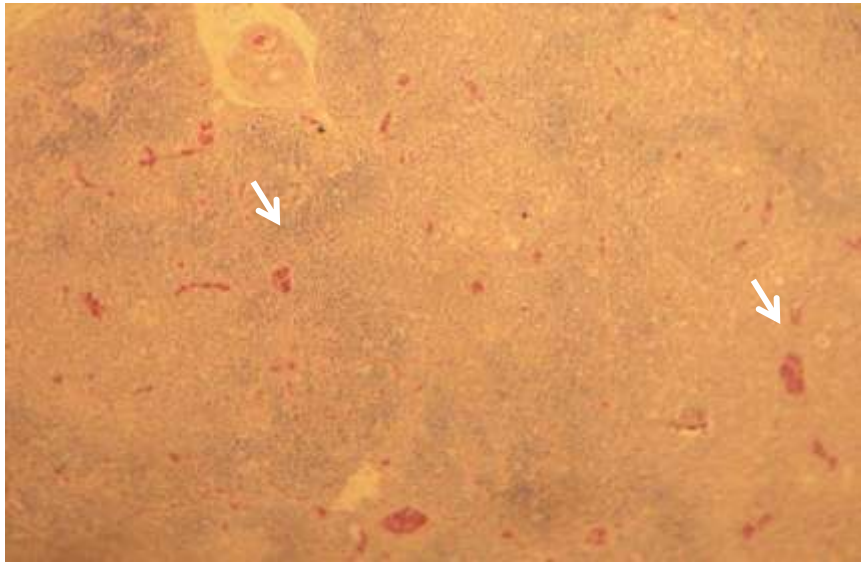


Plate XIV: Photomicrograph of spleen section of group B chicken administered Antox® and inoculated with a vvIBDV showing haemorrhages (arrows) in white and red pulps. H & E x 200.

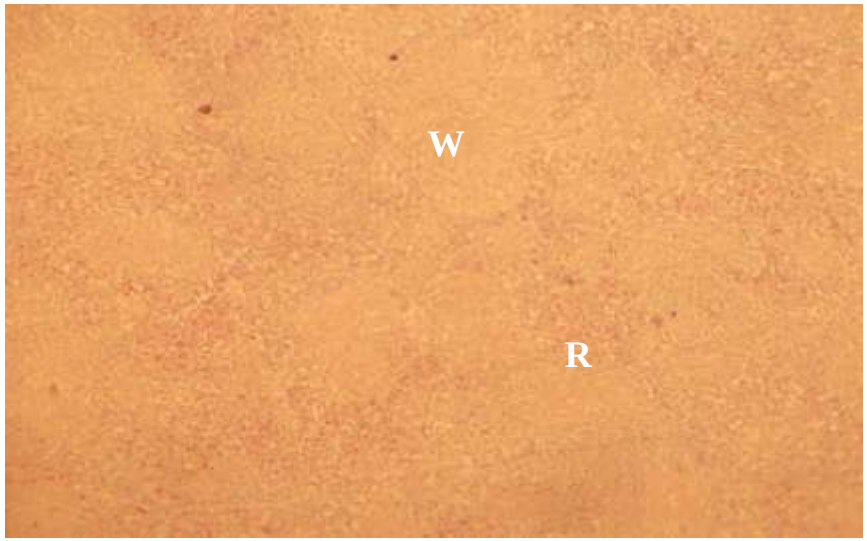


Plate XV: Photomicrograph section of spleen of group C chicken administered EN-FLORAX® and inoculated with a vvIBDV showing intact white (W) and red (R) pulps. H & E x 200.

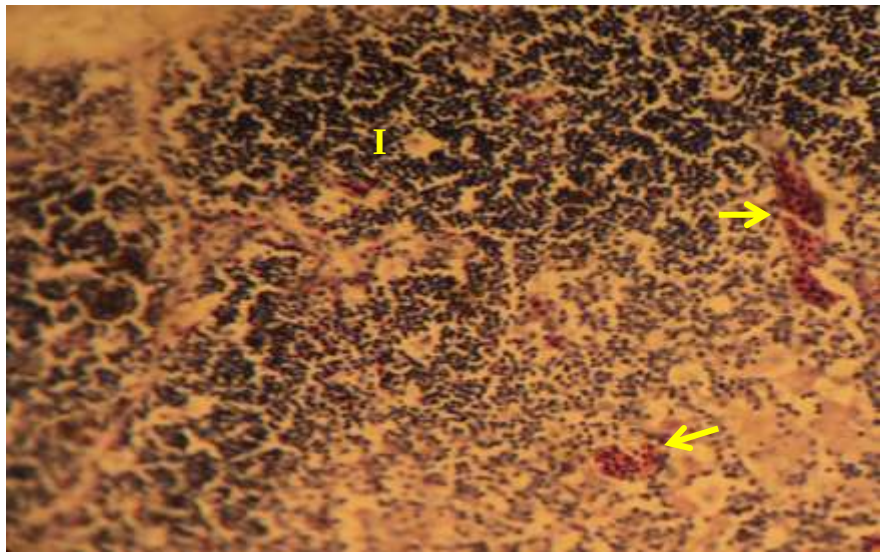


Plate XVI: Photomicrograph section of spleen of group D (positive control) chicken inoculated with a vvIBDV and no supplements were administered showing haemorrhages (arrows) and mononuclear cellular infiltrations (I) in white and red pulps.H & E x 200.

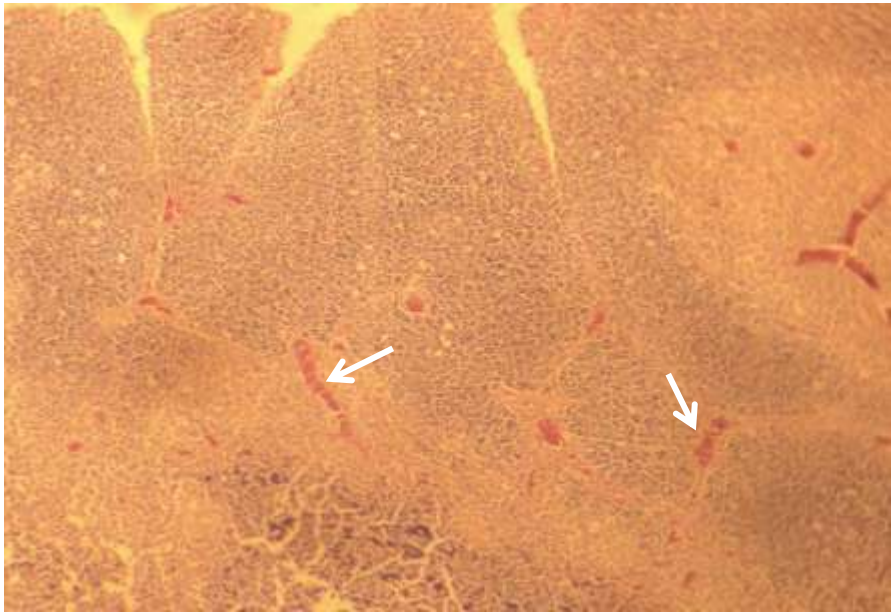


Plate XVII: Photomicrograph section of thymus of group A chicken administered molasses an infected with a vvIBDV showing haemorrhages (arrows).H & E x 200.

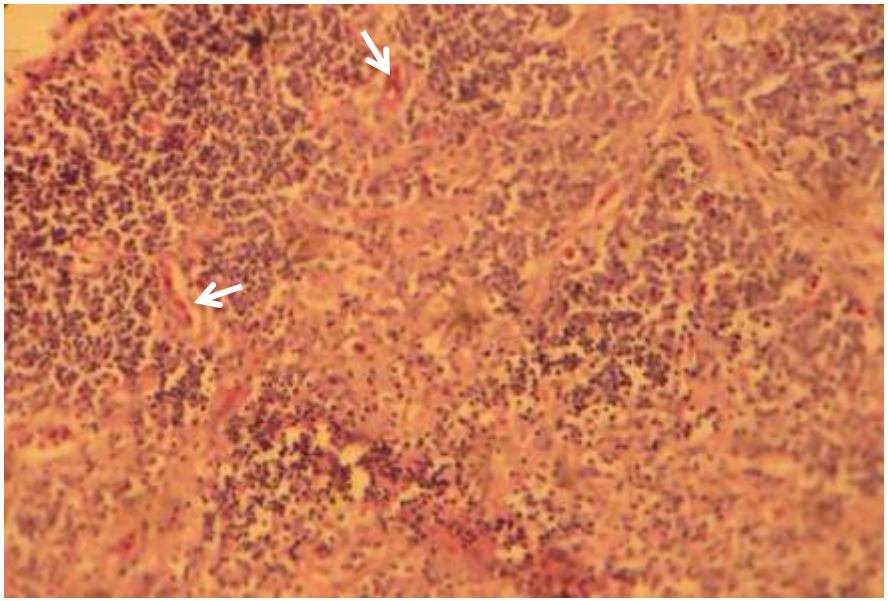


Plate XVIII: Photomicrograph section of thymus of group B chicken administered Antox® and infected with a vvIBDV showing haemorrhages (arrows). H & E x 200.

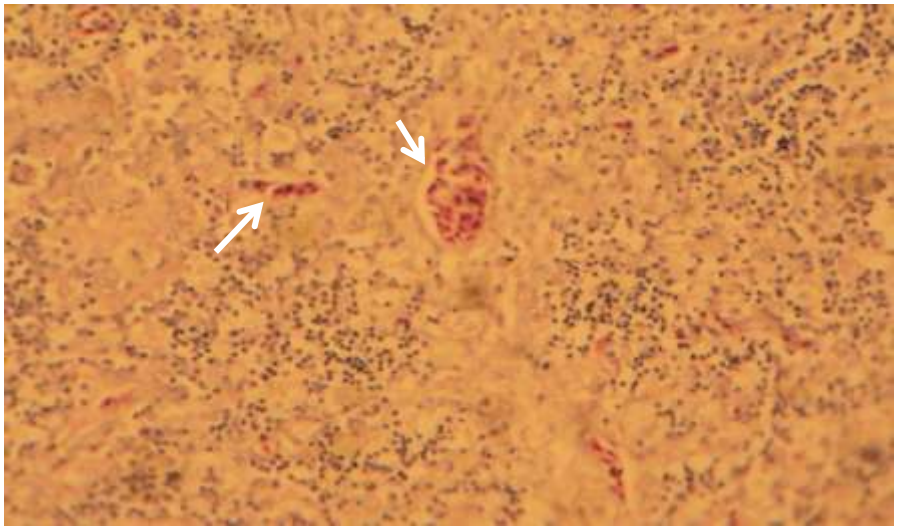


Plate XIX: Photomicrograph of thymus section of group C chicken administered FLORAX® and infected with a vvIBDV showing haemorrhages (arrows). H & E x 200.

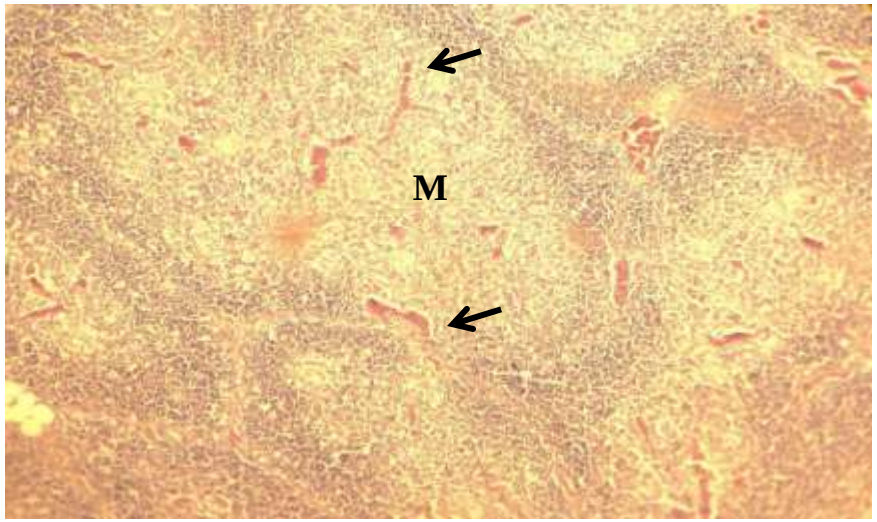


Plate XX: Photomicrograph of thymus section of group D (positive control) chicken infected with a vvIBDV and no supplements were administered showing haemorrhages (arrows) and depleted thymic medulla (M). H & E x 200.

CHAPTER FIVE

5.0 DISCUSSION

The clinical signs observed in chicks inoculated with the vvIBDV in this study have been reported earlier in previous vvIBDV outbreaks (Abdu, 1997; Abdu, 2007; Cereno, 2013; de Wit and Baxendale, 2013). The commonest signs were ruffled feathers, depression, and somnolence in descending order. At the end of seven days of observation the severity of the clinical sign for groups administered supplements were moderate but severe in positive control, because the birds in the supplemented groups takes more water thereby prevented dehydration and allowed the kidneys to excrete appreciable amount of urea which are the main cause of death due to IBD infection. The clinical signs persisted for four days in chicks administered molasses and five days in groups administered Antox® and EN-FLORAX® and seven days in positive control. Therefore, molasses mitigated better the severity of clinical signs due to vvIBDV. This finding agrees with that of Światkiewicz and Koreleski (2007), who reported a decrease in severity of clinical signs following administration of a patented prebiotic which was observed to have stimulated the production of IgM, proliferation of B-lymphocytes and increase antibody titre to IBDV. Kabir *et al.* (2004) evaluated the dynamics of probiotics on immune response of chicks post inoculations with IBDV and reported significantly high antibody production. Alireza *et al.* (2015) concluded that application of synbiotic (Biomin® imbol) enhanced antibody response post inoculation with IBDV and could be used as a feed supplements for improving innate and acquired immune responses in chickens. In the present study, the course of clinical disease after inoculation of chicks with vvIBDV followed the normal pattern; a peak of clinical signs, between 2-3 dpi, and reduction, in 5-7 dpi (Lukert and Saif, 1997; Abdu,

2007; de Wit and Baxendale, 2013). This study further confirms that the chicks died as a result of IBDV infection and there was possibly no other complicating pathogen.

The morbidity and mortality rates in positive control are similar to those of very virulent form of IBD, the pathotype used in this study (Abdu, 2014). This indicates that the chicks were highly susceptible to the vvIBDV at the time of inoculation. Morbidity rate were lower in groups supplemented with molasses, Antox® and EN-FLORAX® when compared to positive control at 4 dpi and mortality rate was lower in groups administered molasses, Antox® and EN-FLORAX® 4 dpi when compared to positive control. The supplements stimulated antibody production that either reduced the destruction of body tissues, multiplication of the vvIBDV or reduced morbidity and mortality rates. Monsan and Paul (1995) have reported that prebiotics increase the bioavailability of minerals for homeostasis and modulation of the immune system to prevent infectious pathogen and invariably reduce morbidity and mortality rates (Lipiñski *et al.,* 2009). The presence of immune-modulatory substances in the supplements could also have helped reduced the high morbidity and mortality rates associated with vvIBDV.

Chicks screened for antibodies (MDAs) to IBDV at day-old showed that they had protective ELISA antibody titre level to IBD which was not protective at 21 days of age. This finding agrees with those of Abdu (1986) who reported a drop in MDAs after 17 days of age and Hair-Bejo *et al.* (2004) who reported a drop in MDAs level at 14 days of age. Moreover, the mean ELISA antibody titre for the groups supplemented with the products was significantly higher than that of positive control, from seven to 28 days of age. The presence of IBD antibody could be attributed to transfer of antibodies from parent stock to chicks and as

supplementation of the products that stimulated the production of antibodies in the supplemented groups (Kabir *et al.,* 2004; Janardhana *et al.,* 2010 and Alireza *et al.,* 2015). There was a significantly higher ELISA antibodies titre in groups supplemented with molasses, Antox® and EN-FLORAX® when compared to positive control at 35 days of age (doa). The supplements were therefore able to induced stronger antibody response. The pre-, pro-, and synbiotics contain substances such as amino acids, vitamins and minerals that enhanced the immune system to produce more immunoglobulins (Kabir *et al.,* 2004; Świątkiewicz and Korelski, 2007; Janardhana *et al.,* 2010; Alireza *et al.,* 2015). This result is in agreement with the findings of Khaksefidi and Ghoorchi (2006), who reported that, the antibody titre level in a probiotic supplemented group was significantly higher at 5 and 10 dpi with vvIBDV when compared to positive control.

The mean serum HI antibodies titre for ND at day-old for all groups indicates the probability of protection of all the chickens against ND (Allan *et al.,* 1978; Verma *et al.,* 1985; OIE, 1996). The higher mean HI titre obtained in all the experimental groups could be attributed to the MDAs inherited from their parent stock at day-old. Moreover, the mean antibody titre for supplemented groups was significantly higher than that of positive control at 35 doa. This could be due the supplementation with molasses, Antox® and EN-FLORAX® to the groups from day-old. This is in agreement with the findings of Świątkiewicz and Korelski, (2007), Kabir *et al.* (2004) and Alireza *et al.* (2015) who concluded that administration of pre, pro and synbiotics to chicks will enhanced the production of antibodies. The groups inoculated with vvIBDV and vaccinated with ND vaccine La Sota at 42 doa was the groups supplemented had protective antibody titres against Newcastle disease when compared to that of positive control at 1 week post

vaccination (42 doa). This indicates that the positive control group was immunosuppressed following exposure to the vvIBDV inoculated.

Reduced feed intake observed in chicks could be as a result of the challenge with vvIBDV, as IBD has been known to cause anorexia (Tsukamoto *et al.,* 1995; Islam *et al.,* 2001a). Moreover, the decrease in weekly feed intake (WFI) was significantly lower in the groups supplemented with molasses, Antox® and EN-FLORAX® compared to positive control. The increase in WFI pre and post inoculation in the chicks supplemented with molasses, Antox® and EN-FLORAX® indicate that the supplements stimulated the chicks appetite and conversely increased their basic metabolic rate when compared to positive control (Karaoglu and Durdag, 2005; Sinopec *et al.,* 2005; Vicente *et al.,* 2007).

It was observed that, feed conversion ratio (FCR) of chicks in supplements groups was lower than that of positive control. It indicates that chicks supplemented with molasses, Antox® and EN-FLORAX® utilized more efficiently the nutrients in the feed they consumed. This could probably be the reason for the increase in the live body weight (LBW) of chicks in supplemented groups compared to positive control. This agrees with the findings of Gibson and Roberfroid (1995), Kabir *et al.* (2004) and Mohnl *et al.* (2007) who recorded lower FCR in chicks fed with pre-, pro-, and synbiotics, respectively and challenged with bacteria (*Salmonella*) compared to positive control.

There was a significant decrease in LBW in vvIBDV inoculated groups. However, the decrease was significantly lower in groups supplemented with molasses, Antox® and EN-FLORAX® compared to that of positive control. The significant increase in the LBW of

chicks in supplemented groups compared to positive control despite challenge with vvIBDV at 35 doa could be attributed to the presence of amino-acids, vitamins and minerals in the supplements which could have aided the increase in the LBW (Gibson and Roberfroid, 1995, Kabir *et al.* 2004; Mohnl *et al,.* 2007). The study showed a steady increase in LBW post-inoculation and slight decrease at 35 doa in chicks in groups supplemented with molasses, Antox® and EN-FLORAX®. This was in contrast to the chicks in positive control that experienced a severe depression of LBW after inoculation with vvIBDV which consequently negatively affected their final LBW.

The lower FCR and increase in LBW of chicks observed in the supplemented groups may be attributed to the rich nutrients in the supplements. The final LBW were found to be higher in the negative control group followed by supplemented groups. This difference observed in the LBW of the chicks in the supplemented groups compared to that of negative control group was due to the challenge with vvIBDV. Although, to the best of the author's knowledge, no literature had reported final LBW of chicks fed with molasses and EN-FLORAX® and inoculated with vvIBDV. Kabir *et al.* (2004) had earlier recorded a decrease in the LBW of broiler chick's supplementation with a probiotic and inoculated with *Salmonella sp.*. The increase observed in the LBW of chicks in the supplemented groups compared to positive control could also be that, chicks in the supplemented groups could have recovered faster from IBD than those in positive control due to the immunostimulant effect of the supplements (Gibson and Roberfroid 1995; Kabir *et al.,* 2004; Mohnl *et al.,* 2007) and they resumed eating faster as the supplements have been reported to aid digestibility, absorption and performance (Sinovec *et al.,* 2005; Karaoglu and Durdag, 2005; Vicente *et al.,* 2007). The improved weight gain of chicks supplemented with molasses,

Antox® and EN-FLORAX® could be attributed to the high protein contents of the products which were efficiently metabolized for growth (Karaoglu and Durdag, 2005; Sinovec *et al.*, 2005; Vicente *et al.,* 2007).

The higher bursal, spleen and thymus to body weight indices observed pre-inoculation with vvIBDV could be an affirmation of the immunoenhancer properties of pre-, pro-, and synbiotics, respectively that have been reported (Kabir *et al.,* 2004; Świątkiewicz and Korelski, 2007; Alireza *et al.,* 2015). Increase in the bursal, spleen and thymus to body weight indices observed in the supplemented groups at 28 doa also indicates that, the supplements could have stimulated the production of more lymphocytes in the BF, spleen and thymus and signify the importance of the supplements with respect to enhanced of the immune response. There was a significant increase in bursal, spleen and thymus to body weight indices in chicks inoculated with vvIBDV at 35 doa. This could be due to inflammatory process that might have mobilized the inflammatory cells to produce endo and exogenous cytokines which could lead to oedema, hyperemia, congestion and enlargement of the BF, spleen and thymus. But the increase was significantly lower in groups supplemented with molasses, Antox® and EN-FLORAX® compared to positive control. This may be attributed to the anti-inflammatory properties of these products (Kabir *et al.,* 2004; Świątkiewicz and Korelski, 2007; Alireza *et al.,* 2015). The decrease in the bursal, spleen and thymus to body weight index observed at 42 doa indicates that, administration of the supplements could not completely prevent the atrophy caused by vvIBDV.

The decrease in PCV, Hb concentration, RBC and thrombocyte counts observed in the groups inoculated with vvIBDV at 7 dpi could be due to anaemia as a result of haemorrhages, destruction of haemopoietic organs and the viraemia usually associated with IBD infection (Skeeles *et al.,* 1980; Moss, 1999). Panigraphy *et al.* (1986) and Kassim, (2014) also reported a significant reduction in the values of haematological parameters at 5 dpi with vvIBDV, when 4 weeks old broilers and cockerels were challenged, respectively. Oladele *et al.* (2005) reported an increase rather than a decrease following sequential haematological parameters changes in broilers chicks inoculated with vvIBDV at 12, 24 and 48 hours interval. The decrease recorded in PCV, Hb concentration, RBC and thrombocyte counts of chicks in the supplemented groups was less severe compared to that of positive control at 35 doa. The substances in the supplements could have enhanced the production of erythropoietin, prevented the destruction of precursor cells in the bone marrow or the haemorrhages usually seen in IBD. The significant increase in haematological parameters at 42 doa observed in the supplements groups suggests that, the minerals, electrolytes and irons found in high amounts in the supplements used may have helped increased the haematological parameters.

The decrease observed in the values of TWBC, heterophils and lymphocytes at 35 doa in vvIBDV inoculated groups indicates a marked leucopaenia due to heteropaenia and lymphopaenia. It has been established that viral infections in chicks are usually associated with heteropaenia and lymphopaenia (Jain, 1986). This is because IBDV destroys B-lymphocytes within the BF before they migrate into the blood stream, causing reduction in the number of circulating TWBC, heterophils and lymphocytes (Weiss and Kaufer-Weiss, 1994). The severe heteropaenia and lymphopaenia observed in positive control at 35 doa

was probably due to multiplication of vvIBDV and possibly subsequent necrosis of B-lymphocytes as observed by Ley *et al.* (2007). At 35 doa after inoculation with vvIBDV, the decrease in TWBC, heterophils and lymphocyte was significantly less severe in the groups administered the supplements compared to positive control. It was possible that the supplements enhanced the production of significant amount of immunoglobulins that neutralized vvIBDV thereby reduced the destruction of leucocytes as observed by Midilli *et al.* (2008).

Increase in H/L ratio has been used as an important indicator of stress in birds (Gross and Siegel, 1983). Stress in birds may vary from water or feeds deprivation, extreme humidity and temperature, constant light or desease usually elevates the number of heterophils and depresses the number of lymphocytes (Gross, 1989; McFarlanje and Curtis, 1989). Heterophil/lymphocyte ratio was significantly higher in positive control compared to the supplements groups. This could be as a result of antioxidant properties that the supplements had which reduced the severity of the stress associated with vvIBDV inoculation in these groups. Scope *et al.* (2002) observed a considerable increase in H/L ratio following stress associated with transporting, handling and viral diseases in birds. Acute stress is known to increase H/L ratio (El Lethey *et al.,* 2003). Therefore, the higher H/L ratio observed in this study could be as a result of the inoculation of the chicks with vvIBDV.

Based on all the haematological parameters studied, molasses mitigated the negative effects of vvIBDV better compared to Antox® and EN-FLORAX®. It is important to note that, molasses contained glucose, fructose and sucrose, B-vitamins, biotin, minerals, essential elements for the formation of RBCs. Also, molasses cannot just be considered as a prebiotic

but rather can serve as a source of nutritional supplement to take care of any deficiency in commercial feed.

The decrease observed in the values of glucose concentration in groups (A, B, C and D) following inoculation with vvIBDV. The decrease was however significantly lower in the supplemented groups when compared to positive control. This may be associated with the high energy content in the supplements. This agrees with the findings of Sinovec *et al.* (2005), Karaoglu and Durdag (2005) and Vicente *et al.* (2007) that pre-, pro-, and synbiotics could have probably stimulated appetite, increase basic metabolic rate, protect tissues of birds from damage and sustained the production of glucose.

The decrease observed in the values of total protein concentration in the supplements groups at 35 doa showed that vvIBDV had affected their concentrations less severely compared to positive control. This finding is similar to those of Afaleq (1998) and Panigraphy *et al*. (1986) following infection with vvIBDV. The findings of this study imply that the supplements can significantly increase serum total protein and mitigate the negative effects of vvIBDV.

The decrease in calcium and phosphorus concentrations observed in the groups inoculated with vvIBDV is associated with IBD infection. But the decrease was significantly lower in the supplemented groups compared to that of positive control at 35 doa. The reduction in the concentrations of calcium and phosphorus observed in this study agrees with the findings of Harris (2000) who reported decrease in calcium and phosphorus during acute IBDV infection. This finding of the study suggests that the amount of calcium and

phosphorus contained in the supplements ameolorate the calcium and phosphorus concentrations in the supplemented groups following challenge with a vvIBDV.

The decreased in potassium concentration observed in this study was higher in positive control compared to supplemented groups A, B and C at 35 doa. This finding could be as a result of high percentage of potassium in the supplements which helped reduced the negative effects of vvIBDV.

The increase in creatinine and BUN levels observed was higher in positive control than in the supplemented groups. This could be as a result of an impaired kidney function due to an immune-mediated glomerulonephritis compatible with immune-complexemia as seen in IBD infection (Ley *et al.,* 1983). The supplements used in the present study may have been responsible for the reduction of the negative effects on the kidneys caused by vvIBDV at 35 doa.

The significant increase in the activities of AST and GDH at 35 doa in the groups inoculated with vvIBDV is suggestive of pathology involving the liver which is a common sequela in IBDV infection, especially following secondary viraemia (Hair-Bejo *et al.,* 2004; Roosevien *et al.,* 2006). Liver injuries are postulated to result from hypoxic state caused by aplastic bone marrow following IBDV infection (Nunoya *et al.,* 1992). The finding of this study is in agreement with that of Tesfaheywet *et al.* (2012) who reported an increase in AST and GDH at 7, 14 and 21 dpi with a vvIBDV. The finding of this study therefore implied that the supplements reduced liver damage and increased liver healing caused by vvIBDV.

The significant decrease observed in the values of TC and TG in the groups inoculated with vvIBDV at 35 doa could be associated with the anorexia and diarrhoea that usually accompany IBDV infection (Dhawale, 2007). This condition (IBD) causes reduce availability and absorption of fatty acid (Dhawale, 2007). The finding of the study therefore implied that the supplements protected the liver and reduced the severity of the pathological damage caused by vvIBDV and allowed proper utilization of TC and TG by the liver.

The supplements improved the activity of SOD during oxidative stress, which agrees with the findings of Bai *et al.* (2017) who reported that Antox®, as an antioxidant, restored the activity of SOD in broilers reared under heat stress. The lower SOD activity in the supplemented groups compared to positive control could be as a result of their antioxidant properties (Birben *et al.,* 2012). Antioxidants prevent lipid peroxidation of cells caused by vvIBDV infection (Birben *et al.,* 2012). Evidence has shown that activity of SOD decrease during viral infections (Inal *et al.,* 2001).

The concentration of MDA increase during viral infections and it is an indirect measurement of oxidative stress (Inal *et al.,* 2001). The groups administered supplements had lower MDA concentration compared to positive control. The lower concentration of MDA in the supplemented groups could be as a result of their antioxidant properties (Birben *et al.,* 2012). Antioxidants prevent lipid peroxidation of cells caused by vvIBDV infection (Birben *et al.,* 2012). MDA is an indicator of lipid peroxidation which occurs in chicks as a result of high oxidative stress. The significant decrease in MDA among the chicks in the supplemented groups at 7, 14, 21 and 28 doa indicates that the supplements were able to prevent lipid peroxidation due to oxidative stress (Birben *et al.,* 2012). In a study, inclusion

of antioxidants such as zinc, potassium, magnesium, cobalt, and manganese decreased the level of MDA (Tawfeek *et al.,* 2014). This is because the afore mentioned minerals are effective in scavenging hydroxyl radical (Sahin *et al.,* (2009).

The molasses, Antox® and EN-FLORAX® have been shown to reduce the negative effects of oxidative stress (Kim *et al.,* 2017) and promote the activities of antioxidant enzymes; thus, they scavenge excess ROS that may cause cell damage, and, consequently improves the health status of the host (Mishra *et al.,* 2015). Based on the activity of antioxidant enzymes and oxidative stress biomarkers studied, administration of molasses mitigated the negative effects of vvIBDV better compared to Antox® and EN-FLORAX® in spite of the fact that it contains only few vitamins and minerals such as vitamins C, E, and zinc, magnesium, potassium and no organism.

The gross pathological lesions that were observed in this study in the BF, SPL and THY were as reported in several IBD outbreaks (Abdu, 1997; Cereno, 2008; de Wit and Baendale, 2013). The lesions were more severe in chicks in positive control compared to groups administered the supplements. The supplements therefore lowered the effect of vvIBDV on the integrity of the BF, SPL and THY. The administration of the supplements may also help in mitigating the pathology caused by other diseases such as Newcastle disease, Marek's disease, avian pox, colibacillosis, fowl cholera, salmonellosis, and coccidiosis prevalent in Nigeria.

The histopatholgical changes in the BF, SPL and THY observed in the groups inoculated with vvIBDV was as a result of inflammatory process that have been reported to stimulate

macrophages leading to production of nitric oxide (NO) and other cytokines with anti-proliferative activity (Sharma *et al.*, 2000). This re-affirms the findings by Cheville, (1967); Ley *et al.* (1983); Chineme and Cho, (1984) that once the chicks are challenged with an IBDV, the inflammatory process will set-in on the immune organs within 48 to 72 h post inoculation particularly in the BF. But this study found that the histopathological lesions were more severe in positive control than in the groups supplemented with molasses, Antox® and EN-FLORAX®. Therefore, the supplements mitigated the effect of vvIBDV on the integrity of the immune organs and reduced the severity of the histopathological lesions.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Based on the findings of the present study, it can be concluded that molasses, Antox® and EN-FLORAX® mitigated the deleterious effects of vvIBDV infection on the:

1. Severity of clinical signs (moderate, moderate, and moderate), morbidity (42.6%, 55.2%, and 50.5%) and mortality rates (25.5%, 61.3%, and 46.8%), and course of disease (4, 4, and 4 days), when compared to positive control (severe, 75.6%, 95.4%, and 5 days).
2. Antibody response to vvIBDV (50.72 ± 0.26 $\log_{10}$, 35.82 ± 0.19 $\log_{10,}$ and 40.89 ± 0.09 $\log_{10}$) when compared to positive control (25.65 ± 0.14 $\log_{10}$) at 35 doa and ND vaccine La Sota (6.40 ± 0.04 $\log_2$, 4.60 ± 0.02 $\log_{2,}$ and 5.30 ± 0.03 $\log_2$) when compared to positive control (3.60 ± 0.01 $\log_2$) 1-week post vaccination,
3. Weekly feed intake (200.25 g, 155.15 g and 174.20 g), and feed conversion ratios (0.80, 0.68, and 0.74), and body to BF (1.27 ± 0.18, 1.78 ± 0.37, and 1.51 ± 0.25), SPL (1.38 ± 0.21, 1.87 ± 0.40, and 1.77 ± 0.28), and THY (1.44 ± 0.19, 1.78 ± 0.38, and 1.68 ± 0.25) weight indices when compared to positive control (125.35 g, 0.92, 2.34 ± 0.50, 2.61 ± 0.68, and 2.33 ± 0.55) at 35 doa.
4. PCV (22.29 ± 0.12%, 19.26 ± 0.08%, and 20.28 ± 0.10%), Hb (7.44 ± 0.05 g/dl, 5.99 ± 0.03 g/dl and 6.98 ± 0.04 g/dl), RBC (1.95 ± 0.01 × 10^{12}/l, 1.46 ± 0.00 × 10^{12}/l and 1.59 ± 0.02 × 10^{12}/l), and thrombocytes counts (7.84 ± 0.05 × 10^9/l, 6.78 ± 0.04 × 10^9/l and 6.88 ± 0.04 × 10^9/l) when compared to positive control (13.56 ± 0.05 %, 3.59 ± 0.01 g/dl, 0.92 ± 0.00 × 10^{12}/l, and 4.96 ± 0.02 × 10^9/l) at 35 doa.

5. TWBC (3.95 ± 0.03 × 10^9/l, 3.19 ± 0.03 × 10^9/l and 3.33 ± 0.03 × 10^9/l), heterophils (2.00 ± 0.02 × 10^9/l, 1.33 ± 0.01 × 10^9/l and 1.52 ± 0.01 × 10^9/l), lymphocytes (3.34 ± 0.03 × 10^9/l, 2.29 ± 0.02 × 10^9/l, and 2.52 ± 0.02 × 10^9/l) and H/L ratio (0.60 ± 0.06, 0.58 ± 0.05 and 0.60 ± 0.05) when compared to positive control (1.95 ± 0.01 × 10^9/l, 0.29 ± 0.00 × 10^9/l, 1.33 ± 0.01 × 10^9/l, and 0.22 ± 0.00) at 35 doa.
6. GLC (307.54 ± 3.23 mg/dl, 200.27 ± 2.24 mg/dl, and 235.35 ± 2.25 mg/dl) and TP (4.45 ± 0.06 mg/dl, 3.19 ± 0.04 mg/dl, and 3.38 ± 0.05 mg/dl) when compared to positive control (105.65 ± 1.22 mg/dl and 2.25 ± 0.02 mg/dl) at 35 doa.
7. Ca, P, and K concentrations (3.36 ± 0.03 mg/dl, 2.35 ± 0.02 and 2.56 ± 0.02 mg/dl), (2.52 ± 0.02 mg/dl, 2.15 ± 0.01 and 2.25 ± 0.01 mg/dl) and (4.55 ± 0.03 mg/dl, 3.23 ± 0.02 and 3.34 ± 0.02 mg/dl) at 7 dpi when compared to positive control (1.99 ± 0.01 mg/dl, 1.15 ± 0.00 mg/dl and 2.09 ± 0.01 mg/dl) at 35 doa.
8. Creatinine and BUN concentrations (3.05 ± 0.03 mg/dl and 4.95 ± 0.04 mg/dl), (4.89 ± 0.04 mg/dl and 5.93 ± 0.05 mg/dl) and (4.57 ± 0.04 mg/dl and 5.88 ± 0.05 mg/dl) when compared to positive control (7.28 ± 0.06 mg/dl and 7.99 ± 0.07 mg/dl) at 35 doa.
9. AST and GDH activities (200.25 ± 2.15 U/mL and 200.85 ± 1.19U/mL), (283.21 ± 2.23 U/mL and 260.88 ± 2.24 U/mL) and (250.23 ± 2.19 U/mL and 235.87 ± 2.27 U/mL) when compared to positive control (350.19 ± 3.22 U/mL and 300.80 ± 3.35 U/mL) at 35 doa.
10. TC and TGC (125.24 ± 1.28 mg/dl and 100.25 ± 1.23 mg/dl), (105.21 ± 1.24 mg/dl and 85.16 ± 0.20 mg/dl), (110.22 ± 1.27 mg/dl and 95.20 ± 0.22 mg/dl) when compared to positive control (69.20 ± 0.25 mg/dl and 56.12 ± 0.16 mg/dl) at 35 doa.

11. SOD (34.49 ± 0.14 U/mL, 25.52 ± 0.09 U/mL, and 28.58 ± 0.08 U/mL) and MDA (56.23 ± 0.27 nmol/mL, 76.36 ± 0.39 nmol/mL, and 65.33 ± 0.35 nmol/mL) when compared to positive control (14.57 ± 0.04 U/mL and 91.49 ± 0.50 nmol/mL at 35 doa.
12. Gross lesions and microscopic changes in the BF, SPL and THY (mild, moderate, and moderate, respectively) when compared to positive control (severe and severe, respectively).

6.2 Recommendations

Based on the findings of the study:

1 Molasses, Antox® and EN-FLORAX® can be used by farmers and veterinarians for the management of IBD in Nigeria.

2 Further studies should be conducted to evaluate the mitigative effects of graded doses and combinations of the supplements on vvIBDV infection.

3 Studies should also be conducted to determine if administration of the supplements for more than seven weeks of age would be safe.

4 The effects of the supplements, on tissue oxidative stress biomarkers, vitamins, amino acids and other minerals following vvIBDV infection should be investigated.

6.3 Limitations of the Study

The values of the measurements of the various data obtained in this study were limited by the sensitivity and accuracy of the analytical tests used because of the tools/equipments variation.

6.4 Contributions to Knowledge

Molasses, Antox ® and EN-FLORAX®, reduced the severity of clinical signs, morbidity, and mortality rates, increased antibody response, increased body and immune organs weight and improved performance indices despite infection with vvIBDV. They also reduced the level of anaemia, leucopaenia, loss of metabolites and electrolytes and oxidative stress due to vvIBD infection. Molasses significantly mitigated the deleterious effects of vvIBDV infection and showed a strong potential of a prebiotic at the dose of 2 ml/litre of drinking water.

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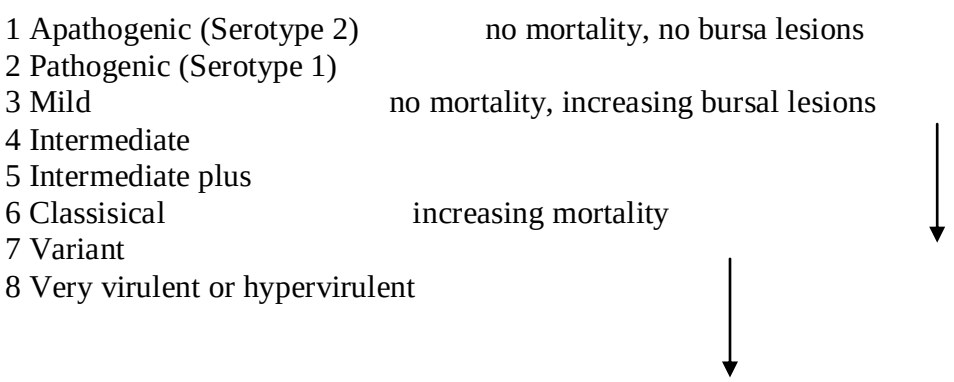
APPENDICES

Appendix 1: Pathotypic variation of infectious bursal disease virus



Appendix 2: Approximate composition of chick mash (Hybrid® feed)

Main constituents	**Components**
Dry matter	97.20%
Ash	13.96%
Ether extra	7.41%
Crude fibre	6.49%
Nitrogen	3.60%
Crude proteins	22.50%

Source: Department of Animal Science Laboratory ABU Zaria

Appendix 3: Ethical approval

DIRECTORATE OF ACADEMIC PLANNING & MONITORING
AHMADU BELLO UNIVERSITY, ZARIA
Vice Chancellor: *Prof. Ibrahim Garba, B.Sc. (Hons) Geology, M.Sc. (Mineral Exploration) A.B.U., Ph.D Geology (London), D.I.C., FNMGS*
Director: Prof. A.A. Tijjani, B.Sc. (Hons) UNIMAID, M.Sc. (UI), Ph.D, D.I.C. (London), FNMS
Tel. Zaria + 234-069-551911 Fax. 234 (069) 550822 Telegram: UNIBELLO Zaria e-mail: dapm@abu.edu.ng

Appl No.: ABUCAUC/2019/Veterinary Medicine/19 31st October, 2019
Approval No: ABUCAUC/2019/19

Prof. P.A. Abdu,
Department of Veterinary Medicine,
Faculty of Veterinary Medicine,
Ahmadu Bello University,
Zaria.

Dear Sir,

APPROVAL OF RESEARCH STUDY 'EVALUATION OF THE EFFECTS OF PRE-, PRO- AND SYNBIOTICS ON CLINICO-PATHOLOGICAL CHANGES IN CHICKS INOCULATED WITH AN INFECTIUOS BURSAL DISEASE VIRUS '

This is to convey the approval of the ABUCAUC to you for the aforestated study domiciled in the Department of Veterinary Medicine. The approval is predicated on the assumption that you shall maintain and care for the Experimental Animals as approved after the visitation of the Committee.

Monitoring of the Research by spot checks, invitations or any other means the Committee deems fit shall be undertaken at the convenience of the Committee.

This approval can and shall be revoked should a significant breach in the terms and condition of the approval occur. It is hence your responsibility to ensure that the agreed terms are maintained to the end of the Study.

The said approval shall be posted on the ABUCAUC Page on the University's website.
Note upon completion of the research, ethical clearance certificate will be issued.

A.B. Abdulkadir
For: Chairman, ABUCAUC.

Cc. Director, DAPM
" Director, IAII&CT
" Head, Department of Veterinary Medicine
" Prof. C.A. Kudi, Chairman, ABUCAUC

Appendix 4: Approximate composition of cane molasses (prebiotic)

Main constituents	Components	Normal range
Water	17–25%	
Sugars	Sucrose	30–40%
Glucose (dextrose)	4–9%	
Fructose (levulose)	5–12%	
Other reducing substances (as invert)	1–4%	
Total reducing substances (as invert)	10–25%	
Other carbohydrates	Gums, starch, pentosans, also traces of hexitols; myoinositol, d-mannitol, and uronic acids	2–5%
Ash	As carbonates	7–15%
Bases (minerals):		
Potassium oxide	(30–50%)	
Calcium oxide	(7–15%)	
Magnesium oxide	(2–14%)	
Sodium oxide	(0.3–9%)	
Metal oxides (as ferric)	(0.4–2.7%)	
Acids:		
Sulfur trioxide	(7–27%)	
Chloride	(12–20%)	
Phosphorus pentoxide	(0.5–2.5%)	
Silicates and insolubles	(1–7%)	
Nitrogenous compounds	Crude protein (as N × 6.25)	2.5–4.5%
True protein	0.5–1.5%	
Amino acids, principally aspartic and glutamic acids, including some pyrrolidine carboxylic acids	0.3–0.5%	
Unidentified nitrogenous compounds	1.5–3.0%	
Nonnitrogenous	Aconitic acid (1–5%), citric, malic, oxalic, glycolic	1.5–6.0%
Mesaconic, succinic, fumaric, tartaric	0.5–1.5%	
Wax, sterols, and phosphatides	0.1–1.0%	
Vitamins		.
Thiamin (B)	2–10 *p.p.m*	
Riboflavin (B)	1–6 *p.p.m.*	
Pyridoxine (B)	1–10 *p.p.m.*	
Nicotinamide	1–25 *p.p.m.*	
Pantothenic acid	2–25 *p.p.m.*	
Folic acid	10–50 *p.p.m.*	
Biotin	0.1–2 *p.p.m.*	

Source: United Molasses Company, London, UK. By courtesy of the Technology Division of Crompton and Knowles Corporation, Mahwah, NJ, USA.

Appendix 5: Approximate composition of Antox® (probiotic)

Main constituents	Components
Micro-organism	
Saccharomyces cerevisiae	4.125×10^{6}cfu/mL
Energy	
Citric acid	6 g
Lactic acid	2 g
Vitamins	
Vitamin B_1	100 mg
Vitamin B_2	7.5 mg
Vitamin B_6	80 mg
Vitamin B_{12}	0.6 mg
Biotin	1.5 mg
Nicotinamide	1g
Minerals	
Calcium chloride	300 mg
Potassium iodide	4.6 mg
Sodium selenite	78.8 mg
Zinc chloride	320 mg
Iron chloride	300 mg
Magnesium chloride hexahydrate	250 mg
Manganese chloride	631 mg
Copper sulphate	32 mg
Cobalt chloride	3.08 mg

Source: Montajat Pharmaceuticals, Bioscience Division, Dammam 31491, Saudi Arabia,

Appendix 6: Approximate composition of EN-FLORAX® (synbiotic)

Main constituents	Components
Carbohydrates	
Inulin	45%
Malto-dextrin	55%
Dextrose	60%
Fructo-oligosaccharide	45%
Oligo-fructose	35%
Micro-orgnisms	
Enterococcus faecium	1.5×10^{-11} cfu/kg
Lactobacillus casei	1.5×10^{-11} cfu/kg
Lactobacillus plantarum	1.5×10^{-11} cfu/kg
Paiococcus acidilactici	1.5×10^{-11} cfu/kg
Protein	
Crude protein	0.04 mg
Crude fibre	0.02 mg
Crude fat	0.01 mg
Crude ash	0.5 mg
Colloidal silico	4,600 mg
Amino-acids	
Lysine	0.01 mg
Methionine	0.01 mg
Vitaamins	
Vitamin B_1	350 mg
Vitamin B_2	250 mg
Nicotinamide	2,000 mg
Vitamin B_6	320 mg
Vitamin B_{12}	1,000 mg
Minerals	
Calcium pantothenate	1,200 mg
Calcium	30,000 mg
Potassium	3,000 mg
Sodium chloride	3.9 mg
Phosphorus	0.01 mg
Magnesium	0.01 mg
Others	
Kwas foliowy	3,000 mg

Source: EKSPOL s.c, ul, Romana Maya 1, 62-030 lubañ, Poland

Appendix 7: Grading of Clinical signs in chickens infected with a very virulent infectious bursal disease virus

Percentage (%) of birds with clical signs	Scale	Grade
01 – 20	1	Mild
21 – 40	2	Moderate
41 – 60	3	Severe
61 – 80	4	Very severe
81 – 100	5	Grave

Source: P. A. Abdu, 2019

Appendix 8: Preparation of Drabkin's solution

Potassium cyanide (KCN) - 0.05 g
Potassium ferricyanide [$K_3Fe(CN)_6$] - 0.2 g
Sodium bicarbonate [$Na_2(CO_3)_2$] - 1g
Dissolve in 1litre of distilled water and store in amber bottle at room temperature

Procedure

1 Measure 5 ml of HICN(Drabkin) reagent into test tube
2 Measure 20 µL of blood using a micro pipette
3 Properly mix with Drabkin reagent
4 Centrifuged at 3000 rpm for 15 minutes to separate the empty RBC from interfering with the reading.
5 Decant supernatant into sample bottle
6 Absorb supernatant into the haemoglobin meter
7 Take reading after wiggling pump stops working

Appendix 9: Preparation of Natt-herrick solution

Sodium chloride (Nacl) - 3.88 g
Sodium sulphate (Na_2SO_4) – 2.50 g
Sodium Phosphate (Na_2HPO_4) - 2.91 g
Potassium phosphate (KH_2PO_4) -0.25 g
Formaline (37%) -7.50 ml
Methyl violet – 0.10 g
+1000 ml with distilled water + filter and store in amber bottle at room temperature

Procedure

1 Use a standard RBC diluting pipette
2 Dilute whole anticoagulated blood with Natt- Herrick's solution at the rate of 1:200
3 Allow diluted blood to mix for a minute or two

4 Discharge into the haemocytometer counting chamber (using a capillary tube). Afetr charging haemocytometer, allow contents to settle for approximately 3 minutes.

Performing the Total Erythrocyte Count

1 Use high dry (40X) objective of the microscope
2 Count total number of RBC (easily recognisaeble by their nuclei) in the four chambers.
3 Count all cells that overlap the top and left border.

NB: Do not count any cells that overlap the bottom or right borders.

The computation for RBC count is as follows:

$$N/100 = RBC \times 10^{12} cells/l$$

Where N= Number of cells counted in the 5 squares in the mid section of the haemocytometer (or in 160 squares)

Performing the total leucocyte Count

1 (as previously noted, the same 1:200 dilution is used for counting white cells) WBC tends to stain dark blue to purple and may exhibit some granulity
2 The total leukocyte count is obtained by counting all leukocytes present in the nine large ruled squares of the haemocytometer.
3 Count all cells that overlap the top and left border

NB: Do not count any cells that overlap the bottom or right borders.

The computation for total leukocyte count is as follows:

$$N/20 = WBC \times 10^{9} cells/l$$

Where N= Number of cells counted in the four outer large squares (or in 64 small)

Appendix 10: Procedure for modified wright- giemsa stain

Working Stain

1 3 g Wright stain powder
2 0.3 g Giemsa stain powder
3 5ml glycerol
4 To 1000 ml absolute methanol (acetone free)
5 Filter and store at room temperature in amber bottle

Preparation of Sorensen's Buffer

Stock Solution A: 0.1 M potassium dihydrogen phosphate. Dissolve 13.61 g KH_2PO_4 in 1000 ml distilled water.

Stock Solution B: 0.1 M disodium hydrogen phosphate. Dissolve 17.8g $Na_2HPO_4.2H_2O$ in 1000ml distilled water.

50 ml stock Solution A + 50ml Stock Solution B = 100ml Sorensen's Buffer of pH 6.81 Refrigerated until required.

Procedure

1 Prepare thin blood smears
2 Place on staining rack
3 Flood smear with Wright- Giemsa stain, allow to stand for 3 minutes
4 Add equal amount of Sorensen's pH 6.5- 6.8 buffers, depending on batch stain.

5 Mix gently by blowing using a pipette until metallic green sheen forms on the surface, allow to satnd for 6 minutes
6 Rinse with buffer, allowing to stand for 1 minute for differentiation
7 Wash copiously with buffer
8 Wipe the back of smear with tissue to remove excess stain
9 Prop in rack until dry.

Appendix 11: Grading of gross pathological lesions in chickens infected with a very virulent infectious bursal disease virus

Observable gross lesions	Lesions score	Organs	Scale	Grade
No lesion	0	Bursal of Fabricius	0	Normal
Congestion	1	Spleen	1	Mild
Oedema	2	Thymus	2	Moderate
Exudate	3	Proventriculus	3	Severe
Enlargement	4	Brest muslces	4	Very severe
Turgid	5	Thigh muscles	5	Grave
Haemorrhages	6	Legs muscles		
Pale	7	Liver		
Necrosis	8	Lunges		
Kidneys urate	9	Kidneys		
Bursal core	10	Pancreas		
Atrophy	11			
Lost of coronary fat	12			

*__Formular__: To get a grade add all the observable gross lesion scores together and divided by the number of observable organs

**__Source__: P.A. Abdu, 2021

Appendix 12: Grading of histopathological lesions in chickens infected with a very virulent infectious bursal disease virus

Observable lesions	Lesion score	Organs	Scale	Grade
No lesion	0	Bursal of Fabricius	0	Normal
Congestion	1	Spleen	1	Mild
Oedema	2	Thymus	2	Moderate
Haemorrhage	3		3	Severe
Necrossis	4		4	Very severe
Cyst formation	5		5	Grave
Thickened interfollicular space	6			

*__Formular__: To get a grade add all the observable histopathological lesion scores together and divided by the number of observable organs

**__Source__: P.A. Abdu, 2021

Appendix 13: Mean (%) clinical signs of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old

			Observable clinical signs										
			S	P	Rf	Dp	W	Re	H	A	Dy		
Group	Treatment	Days post inoculation	Daily mean (%) clinical signs per group									% average of clinical signs	Clinical sign score (Severity of clinical signs)
A	Molasses	3	38.88	27.78	100.00	38.88	33.33	00.00	00.00	27.78	22.22	41.26	3 (severe)
B	Antox®	3	55.00	40.00	100.00	55.00	40.00	00.00	20.00	30.00	30.00	46.25	3 (severe)
C	EN-FLORAX®	3	52.63	36.84	100.00	52.63	36.84	00.00	00.00	26.31	26.31	47.37	3 (severe)
D	Positive control	3	59.10	45.45	100.00	52.55	45.45	04.54	54.55	86.36	45.45	54.83	3 (severe)
E	Negative control	3	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	Normal
A	Molasses	4	06.66	06.66	100.00	26.66	06.66	00.00	26.66	06.66	06.66	23.32	2 (moderate)
B	Antox®	4	39.15	28.46	100.00	35.46	18.07	00.00	33.84	36.15	21.07	39.03	2 (moderate)
C	EN-FLORAX®	4	37.50	18.75	100.00	31.25	12.50	00.00	31.25	31.25	12.50	34.38	2 (moderate)
D	Positive control	4	57.14	42.86	100.00	42.86	28.57	14.28	57.14	50.00	28.57	46.82	3 (severe)
E	Negative control	4	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	Normal
A	Molasses	5	20.00	18.18	100.00	26.66	06.66	00.00	26.66	18.18	00.00	30.91	2 (moderate)
B	Antox®	5	18.18	22.22	100.00	18.18	22.22	00.00	18.18	44.44	33.33	34.59	2 (moderate)
C	EN-FLORAX®	5	27.27	18.18	100.00	33.33	18.18	00.00	27.27	27.27	18.18	33.71	2 (moderate)
D	Positive control	5	38.46	30.76	100.00	38.46	30.76	15.38	46.15	38.46	30.76	41.02	3 (severe)
E	Negative control	5	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	Normal
A	Molasses	6	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	Normal
B	Antox®	6	00.00	00.00	30.00	00.00	20.00	00.00	00.00	00.00	00.00	25.00	2 (moderate)
C	EN-FLORAX®	6	00.00	00.00	20.00	00.00	10.00	00.00	00.00	00.00	00.00	15.00	1 (mild)
D	Positive control	6	30.00	20.00	100.00	40.00	30.00	10.00	30.00	30.00	20.00	34.44	3 (severe)
E	Negative control	6	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	Normal
A	Molasses	7	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	Normal
B	Antox®	7	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	Normal
C	EN-FLORAX®	7	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	Normal
D	Positive control	7	00.00	00.00	100.00	20.00	00.00	10.00	10.00	00.00	00.00	35.44	2 (moderate)
E	Negative control	7	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	Normal

Key: S = Somnelence, P = Prostration, Rf = Ruffled feathers, Dp = Depression, W = Weakness, Re = Recumbent, H = Huddling, A = Anorexia, Dy = Dispnoea

Appendix 14: Mean (± SE) weekly dead body weight (g) of ISA Brown chicks (n = 5) administered Molasses, Antox® and EN-FLORAX® from day-old and inoculated with a very virulent infectious bursal disease virus at 28-day-old as well as positive and negative controls

		Age in days							
		1	7	14	21	28	35	42	49
Group	Treatment	Mean (± SE) weekly average of dead body weight (g)							
A	Molasses	29.85 ± 1.37	140.10 ± 2.86	235.99 ± 3.68	292.94 ± 3.72	361.54 ± 4.99	238.14 ± 7.59***	370.59 ± 14.65****	493.27 ± 10.85****
B	Antox®	28.86 ± 1.36	139.79 ± 3.48	237.87 ± 3.39	294.74 ± 2.73	363.44 ± 3.68	230.73 ± 4.08	366.41 ± 10.17**	482.39 ± 14.61*
C	EN-FLORAX®	27.05 ± 1.37	148.03 ± 5.64	242.42 ± 1.55	300.03 ± 4.10	367.93 ± 2.47	240.01 ± 10.85	371.65 ± 2.61**	494.09 ± 3.71*
D	Positive control	29.31 ± 1.31	63.31 ± 3.54	99.11 ± 6.55	175.69 ± 7.63	216.11 ± 4.91	146.93 ± 3.59	223.34 ± 16.05	294.14 ± 3.22
E	Negative control	29.38 ± 0.84	62.42 ± 2.67	98.19 ± 2.66	171.20 ± 6.78	214.61 ± 9.69	315.62 ± 9.68`	452.41 ± 5.32**	526.42 ± 2.71*

Key: n = total number of birds sampled, Mean ± SE = standard error of the means, Means, with asterics (*) ($P < 0.05$), (**) ($P < 0.01$) or (***) ($P < 0.01$) in the same row differed significantly

Appendix 15: Percentage changes in mean (log_{10}) enzyme linked immunosorbent assay antibody titre level of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	ıtage decrease (-) or increase (+) in mean (log_{10}) enzyme linked immunosorbent assay antibody titre		
A	Molasses	+ 81.21	- 61.45	- 28.22
B	Antox®	+ 22.59	- 12.22	- 30.59
C	EN-FLORAX®	+ 26.87	- 11.23	- 21.04
D	Positive control	+ 35.36	- 18.33	- 72.83
E	Negative control	- 72.17	- 98.75	- 99.75

Appendix 16: Percentage changes in mean (Log_2) haemagglutinating antibody titre before and after vaccination with Newcastle disease vaccine La Sota of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28 days-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in mean (Log_2) haemagglutinating antibody titre before and after vaccination with Newcastle disease La Sota vaccine		
A	Molasses	- 32.79	+ 111.48	- 87.54
B	Antox®	- 28.95	+ 35.67	- 3.80
C	EN-FLORAX®	- 25.85	+ 39.69	- 20.89
D	Positive control	- 100.00	+ 118.18	- 70.30
E	Negative control	- 100.00	+ 403.05	- 354.27

Appendix 17: Percentage changes in average weekly feed intake (per bird) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in average weekly feed intake per bird		
A	Molasses	-10.71	+ 9.38	+ 29.02
B	Antox®	- 31.72	+ 14.98	+ 1.32
C	EN-FLORAX®	- 24.02	+ 9.17	+ 11.79
D	Positive control	- 41.86	+ 32.56	+ 12.09
E	Negative control	+ 16.36	+ 33.18	+ 51.89

Appendix 18: Feed conversion ratio (per bird) of ISA Brown chicks administered Molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in average weekly feed intake per bird		
A	Molasses	+19.40	- 4.48	- 13.42
B	Antox®	- 9.68	- 9.68	- 14.52
C	EN-FLORAX®	+19.35	- 6.45	- 8.06
D	Positive control	- 5.15	- 8.24	- 24.74
E	Negative control	- 20.41	- 36.73	- 37.76

Appendix 19: Percentage changes in mean (± SE) live body weight (g) per bird of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in mean live body weight (g) per bird		
A	Molasses	- 26.19	+ 15.18	+ 44.35
B	Antox®	- 38.48	+ 7.05	+ 18.43
C	EN-FLORAX®	- 36.83	+ 3.76	+ 26.08
D	Positive control	- 38.46	+ 12.67	+ 17.19
E	Negative control	+ 46.12	+ 108.68	+ 142.47

Appendix 20: Percentage changes in mean (± SE) death body weight (g) per bird of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in mean death body weight (g) per bird		
A	Molasses	- 34.13	+ 2.50	+ 36.44
B	Antox®	- 36.51	+ 0.82	+ 32.73
C	EN-FLORAX®	- 34.77	+ 1.01	+ 34.29
D	Positive control	- 32.01	+ 3.35	+ 36.11
E	Negative control	+ 47.07	+ 110.81	+ 145.28

Appendix 21: Percentage changes in mean bursa of Fabricius/body weight index of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in mean bursa of Fabricius/body weight index		
A	Molasses	+ 5.83	- 57.50	+ 35.83
B	Antox®	+ 44.72	- 69.11	+ 56.10
C	EN-FLORAX®	+ 21.77	- 62.90	+ 46.77
D	Positive control	+ 114.68	- 76.15	+ 63.30
E	Negative control	+ 00.00	+ 00.00	+ 00.00

Appendix 22: Percentage changes in mean (± SE) spleen/body weight index of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in mean spleen/body weight index		
A	Molasses	+ 13.11	- 56.56	+ 27.87
B	Antox®	+ 52.03	- 61.79	+ 52.85
C	EN-FLORAX®	+ 33.60	- 62.40	+ 48.00
D	Positive control	+ 139.45	- 77.98	+ 68.81
E	Negative control	+ 00.00	+ 00.00	+ 00.00

Appendix 23: Percentage changes in mean (± SE) thymus/body weight index of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in mean thymus/body weight index		
A	Molasses	+ 22.03	- 55.93	+ 24.58
B	Antox®	+ 47.11	- 70.25	+ 57.02
C	EN-FLORAX®	+ 36.59	- 65.04	+ 46.34
D	Positive control	+ 323.57	- 185.71	+ 514.29
E	Negative control	+ 00.00	+ 00.00	+ 00.00

Appendix 24: Percentage changes in mean (± SE) packed cell volume (%) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in mean packed cell volume (%)		
A	Molasses	- 18.83	+ 4.15	+ 14.57
B	Antox®	- 32.35	+ 18.23	+ 7.62
C	EN-FLORAX®	- 31.21	+ 17.57	+ 0.47
D	Positive control	- 44.36	+ 21.21	+ 4.60
E	Negative control	+ 16.20	+ 40.13	+ 56.21

Appendix 25: Percentage changes in mean (± SE) haemoglobin concentration (g/dl) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in mean haemoglobin concentration (g/dl)		
A	Molasses	- 30.98	+ 19.57	+ 11.04
B	Antox®	- 46.41	+ 40.13	+ 30.12
C	EN-FLORAX®	- 39.88	+ 31.87	+ 23.43
D	Positive control	- 53.07	+ 41.44	+ 29.80
E	Negative control	+ 14.79	+ 29.06	+ 43.85

Appendix 26: Percentage changes in mean (± SE) red blood cell count (x 10^{12}/l) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in mean red blood cell count (x 10^{12}/l)		
A	Molasses	- 19.75	+ 9.21	+ 0.82
B	Antox®	- 31.17	+ 22.23	+ 11.88
C	EN-FLORAX®	- 30.99	+ 20.26	+ 10.03
D	Positive control	- 43.82	+ 33.52	+ 21.29
E	Negative control	+ 12.93	+ 24.04	+ 34.81

Appendix 27: Percentage changes in mean (± SE) thrombocyte count (x 10^9/l) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in mean thrombocyte count (x 10^9/l)		
A	Molasses	- 31.04	+ 24.49	+ 22.23
B	Antox®	- 40.04	+ 33.21	+ 26.12
C	EN-FLORAX®	- 38.06	+ 31.35	+ 24.95
D	Positive control	- 45.75	+ 38.66	+ 29.93
E	Negative control	+ 6.55	+ 15.76	+ 21.61

Appendix 28: Percentage changes in mean (± SE) total white blood cell count (x 10^9/l) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a veryvirulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in mean total white blood cell count (x 10^9/l)		
A	Molasses	- 20.52	+ 2.01	+ 12.07
B	Antox®	- 40.37	+ 33.64	+ 25.42
C	EN-FLORAX®	- 38.90	+ 28.62	+ 20.00
D	Positive control	- 50.38	+ 39.69	+ 24.17
E	Negative control	+ 16.50	+ 44.16	+ 70.30

Appendix 29: Percentage changes in mean (± SE) heterophils count (x 10^9/l) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in mean heterophils count (x 10^9/l)		
A	Molasses	- 29.82	+ 10.53	+ 11.23
B	Antox®	- 55.22	+ 37.04	+ 26.26
C	EN-FLORAX®	- 53.09	+ 28.09	+ 10.80
D	Positive control	- 87.82	+ 62.61	+ 51.68
E	Negative control	+ 24.68	+ 62.34	+ 87.03

Appendix 30: Percentage changes in mean (± SE) lymphocytes count (x 10^9/l) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) tin mean lymphocytes count (x 10^9/l)		
A	Molasses	- 31.13	+ 17.94	+ 5.57
B	Antox®	- 54.11	+ 43.09	+ 38.28
C	EN-FLORAX®	- 51.63	+ 43.02	+ 31.74
D	Positive control	- 64.91	+ 48.55	+ 37.20
E	Negative control	+ 13.68	+ 27.37	+ 42.89

Appendix 31: Percentage changes in mean (± SE) heterophil/lymphocyte ratios of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in mean heterophil/lymphocyte ratios		
A	Molasses	+ 1.69	+ 5.00	+ 16.95
B	Antox®	- 3.33	+ 26.83	- 18.33
C	EN-FLORAX®	- 3.23	+ 25.80	+ 30.65
D	Positive control	- 65.10	+ 26.98	- 23.81
E	Negative control	+ 9.52	+ 26.98	+ 30.16

Appendix 32: Percentage changes in mean (± SE) glucose concentration (mg/dl) in ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in mean glucose concentration (mg/dl)		
A	Molasses	- 20.07	+ 12.05	+ 0.13
B	Antox®	- 36.93	+ 9.77	+ 19.62
C	EN-FLORAX®	- 30.29	+ 21.44	+ 11.35
D	Positive control	- 61.36	+ 51.61	+ 39.17
E	Negative control	+ 21.88	+ 45.13	+ 54.60

Appendix 33: Percentage changes in mean (± SE) total protein concentration (g/dl) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in mean total protein concentration (g/dl)		
A	Molasses	- 36.34	+ 30.19	+ 21.17
B	Antox®	- 51.74	+ 47.66	+ 39.64
C	EN-FLORAX®	- 49.85	+ 43.03	+ 36.94
D	Positive control	- 63.88	+ 60.83	+ 52.17
E	Negative control	+ 10.42	+ 19.23	+ 27.88

Appendix 34: Percentage changes in mean (± SE) calcium concentration (mg/dl) in ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in mean calcium concentration (mg/dl)		
A	Molasses	- 32.12	+ 20.40	+ 10.10
B	Antox®	- 51.75	+ 41.68	+ 28.95
C	EN-FLORAX®	- 47.86	+ 39.91	+ 27.29
D	Positive control	- 55.68	+ 45.43	+ 34.30
E	Negative control	+ 8.93	+ 23.66	+ 32.81

Appendix 35: Percentage changes in mean (± SE) phosphorus concentration (mg/dl) in ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in mean phosphorus concentration (mg/dl)		
A	Molasses	- 26.96	+ 14.78	+ 8.99
B	Antox®	- 35.05	+ 24.77	+ 12.69
C	EN-FLORAX®	- 33.43	+ 24.85	+ 12.72
D	Positive control	- 59.36	+ 49.46	+ 32.16
E	Negative control	+ 12.77	+ 21.63	+ 37.94

Appendix 36: Percentage changes in mean (± SE) potassium concentration (mg/dl) in ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in mean potassium concentration (mg/dl)		
A	Molasses	- 14.95	+ 7.66	+ 1.12
B	Antox®	- 38.00	+ 31.86	+ 26.68
C	EN-FLORAX®	- 36.74	+ 31.06	+ 25.19
D	Positive control	- 54.47	+ 47.06	+ 37.91
E	Negative control	+ 8.03	+ 13.65	+ 20.39

Appendix 37: Percentage changes in mean (± SE) creatinine concentration (mg/dl) in ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in mean creatinine concentration (mg/dl)		
A	Molasses	+ 87.50	- 68.98	- 55.09
B	Antox®	+ 124.31	- 85.78	- 81.19
C	EN-FLORAX®	+ 113.55	-86.45	- 77.10
D	Positive control	+ 143.48	- 115.72	- 87.96
E	Negative control	+ 7.05	+ 15.44	+ 29.19

Appendix 38: Percentage changes in mean (± SE) blood urea nitrogen (mg/dl) of ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) mean blood urea nitrogen (mg/dl)		
A	Molasses	+ 120.00	- 106.22	- 88.89
B	Antox®	+ 142.04	- 130.20	- 122.45
C	EN-FLORAX®	+ 147.06	- 131.93	- 121.85
D	Positive control	+ 176.47	- 168.17	- 153.29
E	Negative control	+ 22.92	+ 22.92	+ 35.07

Appendix 39: Percentage changes in mean aspartate aminotransferase activity (µg/l) in ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in aspartate aminotransferase activity (µg/l)		
A	Molasses	+ 66.13	- 53.76	- 31.40
B	Antox®	+ 117.15	- 95.74	- 72.76
C	EN-FLORAX®	+ 99.45	- 79.59	- 59.71
D	Positive control	+ 128.23	- 95.66	- 79.41
E	Negative control	+ 7.76	+ 12.99	+ 22.73

Appendix 40: Percentage changes in mean glutamate dehydrogenase activity (µg/l) in ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) mean glumate dehydrogenase activity (µg/l)		
A	Molasses	+ 81.86	- 79.67	- 67.69
B	Antox®	+ 116.62	- 87.21	- 66.27
C	EN-FLORAX®	+ 104.29	- 77.93	- 69.11
D	Positive control	+ 86.37	- 127.59	- 76.83
E	Negative control	+ 6.82	+ 13.01	+ 18.58

Appendix 41: Percentage changes in mean total cholesterol concentration (mg/dl) in ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in mean total cholesterol concentration (mg/dl)		
A	Molasses	- 19.42	+ 12.95	+ 5.20
B	Antox®	- 34.01	+ 27.70	+ 21.40
C	EN-FLORAX®	- 36.09	+ 30.26	+ 24.43
D	Positive control	- 50.00	+ 42.02	+ 33.31
E	Negative control	+ 8.83	+ 14.00	+ 22.84

Appendix 42: Percentage changes in mean triglyceride concentration (mg/dl) in ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in mean triglyceride concentration (mg/dl)		
A	Molasses	- 16.72	+ 8.37	+ 0.02
B	Antox®	- 53.98	+ 24.67	+ 16.72
C	EN-FLORAX®	- 30.72	+ 24.86	+ 17.55
D	Positive control	- 50.04	+ 41.10	+ 32.14
E	Negative control	+ 10.69	+ 21.21	+ 28.13

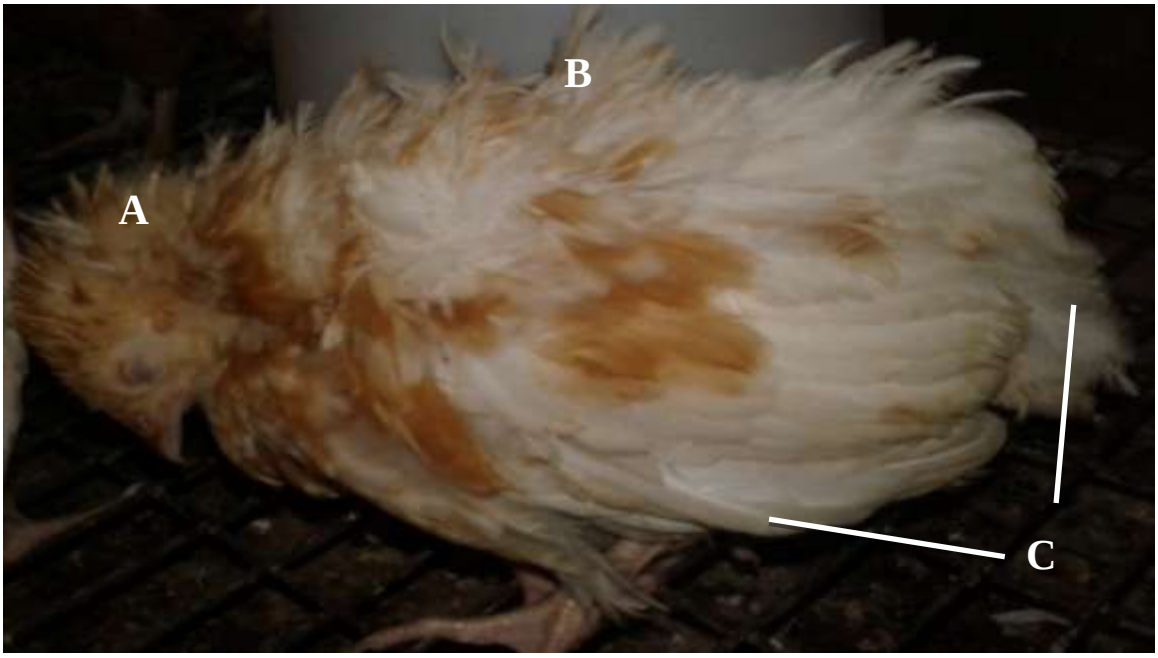
Appendix 43: Percentage changes in mean superoxide dismutase activity (U/mL) in ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in mean superoxide dismutase activity (U/mL)		
A	Molasses	- 37.65	+ 25.45	+ 10.67
B	Antox®	- 52.25	+ 39.39	+ 22.21
C	EN-FLORAX®	- 51.12	+ 32.72	+ 22.54
D	Positive control	- 52.21	+ 29.35	+ 6.26
E	Negative control	+ 48.17	+ 66.29	+ 90.28

Appendix 44: Percentage changes in mean malondialdehyde concentration (nmols/mL) in ISA Brown chicks (n = 5) administered molasses, Antox® and EN-FLORAX® days post inoculation with a very virulent infectious bursal disease virus at 28-day-old

		Days post inoculation		
		7	14	21
Group	Treatment	Percentage decrease (-) or increase (+) in mean malondialdehyde concentration (nmols/mL)		
A	Molasses	+ 80.34	- 38.55	- 12.73
B	Antox®	+ 114.46	- 85.69	- 42.86
C	EN-FLORAX®	+ 170.85	- 112.40	- 79.02
D	Positive control	+ 68.58	- 61.12	- 33.31
E	Negative control	- 20.75	- 33.99	- 52.86

Appendix 45: Photograph of chicks in positive control group (D) inoculated with a very virulent infectious bursal diseases virus at 35 doa exihibitin clinical sign.



Key: Somnolence (A), rouffled feathers (B) and droping of wing/tail feathers (C)