

**EFFECTS OF ANTOX<sup>®</sup> AND BACTOFORT<sup>®</sup> ON CLINICO-PATHOLOGICAL  
CHANGES IN ISA BROWN CHICKS INOCULATED WITH VERY VIRULENT  
INFECTIOUS BURSAL DISEASE VIRUS**

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INFECTIOUS BURSAL DISEASE VIRUS**

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**DEPARTMENT OF VETERINARY MEDICINE,  
AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA**

**MARCH, 2018**

## DECLARATION

I hereby declare that this Dissertation work entitled, “**Effects of Antox<sup>®</sup> and Bactofort<sup>®</sup> on Clinico-Pathological Changes in ISA Brown Chicks Inoculated with Very Virulent 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 Professor P. A. Abdu and Dr. T. Aluwong. The information derived from the literature has been duly acknowledged in the text and list of references provided. No part of this Dissertation has been previously presented for another degree, diploma or certificate in any institution.

Aliyu Andamin DANLAMI  
Name of Student

.....  
Signature

.....  
Date

## CERTIFICATION

This Dissertation entitled, “**EFFECTS OF ANTOX<sup>®</sup> AND BACTOFORT<sup>®</sup> ON CLINICOPATHOLOGICAL CHANGES IN ISA BROWN CHICKS INOCULATED WITH VERY VIRULENT INFECTIOUS BURSAL DISEASE VIRUS**” by Aliyu Andamin DANLAMI meets the regulations governing the award of the Master of Science 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 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 Mohammad, and lastly to my wife, Rashida Adamu and children: Amina, Aisha, Aliyu, Atika and Aliya Aliyu Andamin, respectively.

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## ABSTRACT

Infectious bursal disease (IBD) is endemic in Nigeria and outbreaks occur despite vaccination. Probiotics exert beneficial effects on animals. This study evaluated the ameliorative effects of probiotics, Antox<sup>®</sup> and Bactofort<sup>®</sup> on clinicopathological changes in ISA Brown chicks, inoculated with very virulent infectious bursal disease virus (vvIBDV). Two hundred ISA Brown day-old chicks were divided into four groups of 50 each. Groups A and B were treated from day-old to 42 days of age with Antox<sup>®</sup> and Bactofort<sup>®</sup>, respectively and inoculated with vvIBDV at 28 days of age, groups C and D served as positive and negative controls, respectively. Packed cell volume, haemoglobin concentration (Hb), red blood cell (RBC), total (TWBC) counts and differential leucocyte count, erythrocytic indices, activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and malondialdehyde (MDA) concentration were determined. The birds were observed for mortality from day one postinoculation (pi). Mortality rate in groups A (34.76), B (47.60) and C (72.70) lasted for four, five and eight days. At day, 7 postinoculation (dpi), five chicks from each group were selected and euthanized. Their body (BD), bursae of Fabricius (BF), spleen (SPL) and thymus (THY) were weighed, and there organ-body weight (BD) ratios calculated. There was a significant ( $P < 0.001$ ) difference in RBC count between groups A ( $5.40 \pm 0.29 \times 10^{12}/l$ ), B ( $4.10 \pm 0.43 \times 10^{12}/l$ ) and C ( $3.40 \pm 0.43 \times 10^{12}/l$ ) at 7 dpi differed significantly. The difference in TWBC between group A ( $8.90 \pm 0.29 \times 10^9/l$ ) and C ( $4.30 \pm 0.37 \times 10^9/l$ ) at 7 dpi differed significantly ( $P < 0.001$ ). The TWBC in group B ( $7.00 \pm 0.42 \times 10^9/l$ ) was higher ( $P < 0.01$ ) than that of group C ( $4.30 \pm 0.37 \times 10^9/l$ ) at 7 dpi. There was a significant difference ( $P < 0.05$ ) in heterophil counts in group A ( $2.22 \pm 0.26 \times 10^9/l$ ) and C ( $1.38 \pm 0.29 \times 10^9/l$ ) at 7 dpi, and between group B ( $1.83 \pm 0.05 \times 10^9/l$ ) and C at 7 dpi. The lymphocyte counts between groups A

( $7.42 \pm 00.47 \times 10^9/l$ ), B ( $6.99 \pm 00.42 \times 10^9/l$ ) and C ( $2.79 \pm 00.12 \times 10^9/l$ ) at 7 dpi differed significantly ( $P < 0.001$ ). The GPx activity recorded in group A ( $45.97 \pm 60.90 \mu\text{g/mL}$ ) was significantly ( $P < 0.001$ ) lower than that of C ( $79.80 \pm 40.63 \mu\text{g/mL}$ ) at 7 dpi. There was significant difference ( $P < 0.01$ ) between group B ( $55.59 \pm 40.99 \mu\text{g/mL}$ ) and C ( $79.80 \pm 40.63 \mu\text{g/mL}$ ) at 7 dpi. The MDA concentrations between groups A ( $826.22 \pm 17.24 \text{ nmol/mg}$ ), B ( $873.10 \pm 24.22 \text{ nmol/mg}$ ) and C ( $1406.86 \pm 25.00 \text{ nmol/mg}$ ) at 7 dpi were different ( $P < 0.001$ ). The BD weight in group A ( $233.40 \pm 70.13 \text{ g}$ ) were higher ( $P < 0.001$ ), compared to that of group C ( $140.60 \pm 10.03 \text{ g}$ ) at 7 dpi. The BD between groups B ( $233.40 \pm 90.10 \text{ g}$ ) and C ( $140.60 \pm 10.03 \text{ g}$ ) at 7 dpi differed significantly ( $P < 0.01$ ). The enzyme-linked immune-sorbent antibody titre level in groups A ( $9.12 \pm 00.52$ ) and B ( $8.12 \pm 1.58$ ) was higher compared to that of group C ( $4.42 \pm 1.87$ ) at 7 dpi. In conclusion, Antox<sup>®</sup> and Bactofort<sup>®</sup> ameliorated the negative effects of vvIBDV on RBC, TWBC, heterophil, lymphocyte, BD, GPx, and MDA, and reduced mortality. Antox<sup>®</sup> and Bactofort<sup>®</sup> neutralize most of the vvIBDV, thereby protecting the integrity of BF, SPL and THY. It is recommended that Antox<sup>®</sup> and Bactofort<sup>®</sup> could be used to ameliorate IBD.

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## LIST OF ABBREVIATIONS AND SYMBOLS

|      |                                      |
|------|--------------------------------------|
| %    | Per cent                             |
| (*)  | Asteric                              |
| ±SE  | Plus or minus standard error of mean |
| µl   | Microlitres                          |
| ALP  | Alkaline phosphatase                 |
| ALT  | Alamine aminotransferase             |
| AST  | Aspartate aminotransferase           |
| BD   | Body                                 |
| BF   | Bursa of Fabricius                   |
| CAT  | Catalase                             |
| CD   | Cluster of differentiation           |
| CEF  | Chicken embryo fibroblast            |
| CF   | Crude fibre                          |
| CM   | Centimetre                           |
| CP   | Crude protein                        |
| dl   | Decilitres                           |
| DM   | Dry matter                           |
| dph  | Day-post hatch                       |
| dpi  | Day postinoculation                  |
| e.g. | For example                          |
| EDTA | Ethylene diamine tetra acetic acid   |
| EE   | Ether Extract                        |
| fl   | Femto liters                         |
| ft   | Feet                                 |
| g    | Grams                                |
| GI   | Gastro-intestinal                    |

|               |                                            |
|---------------|--------------------------------------------|
| GPx           | Glutathione peroxidase                     |
| H/L           | Heterophils/lymphocytes ratio              |
| Hb            | Haemoglobin                                |
| IBD           | Infectious busal disease                   |
| IBDV          | Infectious bursal disease virus            |
| IFN- $\gamma$ | Interferon-gama                            |
| IL            | Interleukin                                |
| KCN           | Potassium cyanide                          |
| kg            | Kilograms                                  |
| L             | Litres                                     |
| M             | Metres                                     |
| MCH           | Mean corpuscular haemoglobin               |
| MCHC          | Mean corpuscular haemoglobin concentration |
| MCV           | Mean corpuscular volume                    |
| MDA           | Malondialdehyde                            |
| MHC           | Major histo-compatibility complex          |
| N             | Nitrogen                                   |
| °C            | Degree centigrade                          |
| PCV           | Packed cell volume                         |
| pg            | Picogram                                   |
| pH            | Hydrogen iron concentration                |
| RBC           | Red blood cells                            |
| RNA           | Ribonucleic acid                           |
| SOD           | Superoxidesmutase                          |
| SPL           | Spleen                                     |
| THY           | Thymus                                     |
| TLR           | Toll-like receptor                         |

|        |                                               |
|--------|-----------------------------------------------|
| TNF    | Tumour necrotic factor                        |
| TWBC   | Total white blood cells                       |
| vvIBDV | Very virulent infectious bursal disease virus |
| WBC    | White blood cells                             |

## CHAPTER ONE

### INTRODUCTION

#### 1.1 Background of the Study

Infectious bursal disease (IBD) is an acute, highly contagious, immunosuppressive infection of young chickens (Abdu *et al.*, 1986). It is caused by a member of the genus *Avian birnavirus* in the family *Birnaviridae*, whose genome consists of double-stranded RNA segments, designated A and B, and are enclosed within a non-enveloped icosahedral capsid (Kibenge *et al.*, 1988; Lasher and Shane, 1994; Lukert and Saif, 2003; Muller *et al.*, 2003). Infectious bursal disease virus (IBDV) produces two distinctly different diseases: the sub-clinical and clinical forms in susceptible chickens, depending on the age of the bird at the point of infection. Sub-clinical and clinical infections with IBDV may cause immunosuppression (Cerenó, 2013). The sub-clinical form is observed in chickens below 3 weeks of age; while the clinical form is mostly observed in 3-8-week old chickens with typical lesions of IBD (Sharma *et al.*, 2000; Ahmed and Akhter, 2003; Cerenó, 2013).

The IBD, which emerged in 1957, was documented by Cosgrove (1962) in broiler flocks, located near Gumboro, Delaware, USA; hence, the common eponym “Gumboro disease”. Originally the condition was referred to as “nephritis-nephrosis syndrome of chickens” because of prominent kidney lesions, but lesions in the bursa of Fabricius (BF) were also recognized (Lasher and Shane, 1994). Rapid spread of the infection occurred and most broiler-growing areas in the United States were affected by 1960. The IBD, as it became known, was diagnosed in the UK in 1962 (Etteradossi, 1995). New Zealand was free of IBD

during the mid-1980s (Jones, 1986), but it was diagnosed in late 1993 and became endemic within months of the initial outbreak in Waikato (Lasher and Shane, 1994).

Despite confusion regarding the identity of the causal agent, which was originally isolated by Winterfield *et al.* (1972), Koch's postulates were satisfied by Snedeker *et al.* (1967), who isolated a virus from the BF of affected birds and subsequently produced the disease. This was distinct from the nephron-pathogenic coronavirus (Gray strain of infectious bronchitis virus) isolated from Delmarva broilers in 1960, which was responsible for nephritis-nephrosis syndrome (Winterfield *et al.*, 1972). After initial classification as a picornavirus and then a reovirus, the IBD agent was designated a birnavirus, based on the presence of double-stranded RNA and unique biophysical characteristics (Cho and Edgar, 1969; Dobos, 1979; Dobos *et al.*, 1979; Muller *et al.*, 1979; Lukert and Saif, 1997).

Following infection, IBDV multiplies rapidly in the B-lymphocytes of the 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 the BF 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 (van den Berg, 2000; Kim *et al.*, 2000; Taylor *et al.*, 2008).

Oxidative stress is a harmful imbalance in the oxidative-antioxidative system of cells, in favour of oxidative system (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). SOD, CAT and GPx are antioxidant enzymes in livestock cells involved in scavenging reactive oxygen species. The SODs convert superoxide radical into hydrogen peroxide and molecular oxygen (O<sub>2</sub>), while the GPx and CAT convert hydrogen peroxide into oxygen and water (Weydert and Cullen, 2010). However, the activities of these enzymes may increase during oxidative stress (Kalpakcioglu and Senel, 2008), resulting in increased lipid peroxidation of cell membranes and organelles (Ji, 1999). Lipid peroxidation generates a variety of relatively stable decomposition end-products, mainly  $\alpha$ ,  $\beta$ -unsaturated reactive aldehyde, such as malondialdehyde (MDA) and isoprostanes (Draper *et al.*, 2000; Montuschi *et al.*, 2004), which can then be measured as an indirect index of oxidative stress (Jain *et al.*, 1989). Erythrocyte osmotic fragility (EOF) test has been demonstrated to be an important index of indirect quantification of oxidative stress in livestock (Adenkola *et al.*, 2010).

Probiotics are microorganisms that are non-pathogenic and non-toxic in nature, which when administered through the oral route are favourable to the host's health (Guillot, 1998). Commercial bacteria living in harmony with and benefit the host in a variety of ways, including organic acid production and immunomodulation (Tse and Chadee, 1991). Organic acids inhibit pathogenic bacteria growth by disrupting bacteria cell membrane transport, preventing the bacteria from reaching equilibrium with their environment (Cherrington *et al.*, 1991). Probiotics, which are beneficial microbial cultures, may be administered to stimulate the local immune response and enhance epithelial innate immunity-related gene expression through anti-inflammatory cytokine (Fuller, 1989; Gibson and Roberfroid, 1995; Netherwood *et al.*, 1999; Revollo *et al.*, 2006; Amit-Romach *et al.*, 2010; Pagnini *et al.*,

2010). Furthermore, with the presence of microorganisms in the gut, innate immune system associated germ line-encoded pathogen-pattern recognition receptors called toll-like receptors (TLR) may induce expression of various pro-inflammatory cytokines and antimicrobial peptides (such as defensins), which are direct effector molecules of the innate immune response (Birchler *et al.*, 2001; Ganz, 2003; Kaiser, 2010).

The species used in probiotic preparations are varied and many (Fuller, 1989). These include *Lactobacillus bulgaricus*, *L. acidophilus*, *L. casei*, *L. helveticus*, *L. lactis*, *L. salivarius*, *L. plantarum*, *Streptococcus thermophiles*, *S. faecium*, *Enterococcus faecium*, *E. faecalis*, *Bifidobacterium* spp and *Escherichia coli*, species are intestinal strains, except *Lactobacillus bulgaricus* and *Streptococcus thermophiles*, which are yoghurt starter organisms (Fuller, 1989). Some other probiotics are microscopic fungi such as strains of yeast, belonging to *Saccharomyces cerevisiae* species (Guillot, 1998; Thomke and Elwinger, 1998). Addition of probiotics (Anthox<sup>®</sup> and Bactofort<sup>®</sup>) to livestock feed have been shown to improve the nutritive quality of feed and performance of animals (Martin *et al.*, 1989; Glade and Sist, 1998). Non-antibiotic growth promoters such as organic acids and probiotics are increasingly being produced for animal nutrition (Windisch *et al.*, 2008). The probiotics have been used as an alternative to antibiotics in animals and humans and their efficiency in animals has been widely established (O'Sullivan, 2001; Siriken *et al.*, 2003; Park *et al.*, 2005).

## **1.2 Statement of Research Problems**

Studies in Nigeria have shown that IBD has acquired an endemic status (Oluwayelu *et al.*, 2007). Due to its hardy nature, IBDV persists in poultry houses despite thorough cleaning

and disinfection (Alexander and Chettle, 1998; Mandeville *et al.*, 2000). During the 63rd General Session of the Office International des Epizooties (OIE, 1995), it was estimated that IBD has considerable socio-economic importance at the international level as the disease is present in more than 95% of member countries (Etteradossi, 1995).

One of the major challenges facing the poultry industry in the developing world, including Nigeria, is the improvement of production efficiency (Fuller, 2001). In an attempt to address this problem, concerted efforts have been made to incorporate antimicrobials and other natural products such as yeasts in animal feeds (Muihead, 1992). Prevention and control of diseases have led to a substantial increase in the use of veterinary drugs in recent years (Fuller, 2001). Infectious and enteric diseases are of great concern to the poultry industry because of loss of productivity, increased morbidity, mortality and the associated damage due to pathologic lesions on poultry meat for human consumption (Walker and Duffy, 1998).

### **1.3 Justification of the Study**

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 cease to be used as growth promoters and prophylaxis in some diseases condition of poultry due to concern about residual effect on poultry products and development of resistance. Consumer and manufacturer are therefore

looking for alternatives (Fuller, 2001). Probiotics are being considered to fill this gap, and already some farmers are using them in preference to antibiotics (Trafalka, 2004).

With increasing concern about antibiotic resistance, the ban on sub-therapeutic antibiotic usage in Europe and the potential for a ban in the United States of America, there is increasing interest in finding alternative to antibiotics for poultry production (Menten, 2001). The roles of probiotics in bacterial infection in the performance indices of broilers have been investigated in Nigeria (Aluwong *et al.*, 2013). However, there is no information on the effects of probiotics on clinico-pathological changes in commercial chickens inoculated with vvIBDV in Nigeria. In addition, the use of Antox<sup>®</sup> and Bactofort<sup>®</sup> in chickens inoculated with vvIBDV to ameliorate oxidative stress, improve performance indices and immunodulatory effects in ISA Brown chicks has not been investigated.

#### **1.4 Aim of the Study**

The aim of the study was to investigate the ameliorative effects of Antox<sup>®</sup> and Bactofort<sup>®</sup> on clinico-pathological changes, oxidative stress and antibody response in ISA Brown chicks inoculated with vvIBDV.

#### **1.5 Objectives of the Study**

The objectives of the study were to evaluate the effects of two patented probiotics in ISA Brown chicks inoculated with vvIBDV using the following indices:

- i. Clinical signs, morbidity, mortality and pathological changes in some immune organs,

- ii. Some haematological parameters,
- iii. Oxidative stress biomarkers,
- iv. Antibody response,

### **1.6 Research Questions**

- i. Did treatment with Antox<sup>®</sup> and Bactofort<sup>®</sup> reduce the severity of clinical signs, morbidity, mortality and pathological changes on some immune organs in ISA Brown chicks inoculated with vvIBDV?
- ii. Due the effects of Antox<sup>®</sup> and Bactofort<sup>®</sup> on some haematological parameters in ISA Brown chicks inoculated with vvIBDV?
- iii. Due the effects of Antox<sup>®</sup> and Bactofort<sup>®</sup> on oxidative stress biomarkers in ISA Brown chicks inoculated with vvIBDV?
- iv. Due the antibody response of Antox<sup>®</sup> and Bactofort<sup>®</sup> in ISA Brown chicks inoculated with vvIBDV?

## CHAPTER TWO

### LITERATURE REVIEW

#### 2.1 Definition of Infectious Bursal Disease

Infectious bursal disease virus (IBDV) is the causative agent of infectious bursal disease (IBD) that affects young chickens about 3-6 weeks of age (Lukert and Saif, 2003). It is a highly contagious and acute viral disease that is characterised by the destruction of lymphoid cells in the bursa of Fabricius (BF) of chickens and rarely of turkeys (Cheville, 1967; Faragher, 1972; Chettle *et al.*, 1985; Abdu *et al.*, 1986; Lukert and Saif, 2003). The disease has the following synonyms: avian-nephrosis; Gumboro disease; infectious bursitis and avian infectious bursitis (Cosgrove, 1962; Hitchner, 1970; Faragher, 1972).

#### 2.2 History of Infectious Bursal Disease

Infectious bursal disease was first described by Cosgrove in 1962 in the town of Gumboro, a community in Sussex County, Southern Delaware, USA; hence the name 'Gumboro disease' was coined after the geographic location of the first recorded outbreaks (Muller, 2003). Since the first report, IBD has been reported in chickens all over the world (Saif, 1998). Ever since the disease was recognised over 50 years ago, it posed has a threat to the commercial poultry industry (Okoye, 2005).

## **2.3 Aetiology of Infectious Bursal Disease**

### **2.3.1 Description of the 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). It is non-enveloped with a single-shelled icosahedral capsid and has a diameter between 58 nm – 60 nm (Hirai and Shimakura, 1974; Kibenge *et al.*, 1988). The genus name *Birnavirus* was proposed to describe viruses with 2 segments of double stranded RNA (Murphy *et al.*, 1999). Other viruses included in this group are infectious pancreatic necrotic virus (IPNV) of fish, tellina virus, oyster virus, blotched snakehead virus (BSVN) (Dobos *et al.*, 1979) and crab virus of bivalve mollusk belonging to Aquabirnavirus, while Drosophila X virus (*Drosophila melanogaster*) belongs to genus Birnavirus. All of these contain two segments of double stranded RNA surrounded by a single protein capsid of icosahedral symmetry (Kibenge *et al.*, 1988).

### **2.3.2 Types and subtypes of infectious bursal disease virus**

Two serotypes 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). Comparative studies have confirmed that the US types 1 and 2 are equivalent to the European types 1 and 2, respectively (McNulty and Saif, 1988). Although antibodies to both types 1 and 2 occur in both domestic fowl and turkeys, only infection with type 1 in chickens results in clinical signs.

Serotype 1 viruses can be further categorized into 4 groups on the basis of their pathogenicity: classical strains, variants, attenuated strains and very virulent strains (Lasher and Davis, 1997). Different pathotypes of the virus and the cell culture adapted strains differ markedly in virulence. The three criteria currently being used for the characterization of IBDV strains include antigenicity, genetic relatedness and pathogenicity Kibenge *et al.*, 1988). Classical IBDV has traditionally affected poultry worldwide since the first reported incident from Gumboro. Classical strains cause bursal inflammation and severe lymphoid necrosis in infected chicken, resulting in immunodeficiency and moderate mortality from 20 – 30% in specific Pathogen Free (SPF) chicken (Lim *et al.*, 1994). Variant strains appeared in the US in 1983. These strains were antigenically different from classical strains and caused a rapid and severe bursal atrophy (Vakhari, 1994) and in contrast to classical strains produced no clinical signs of illness. Antigenic variants have been recognized by their ability to escape cross-neutralisation by antiserum against the classical strains (Lim *et al.*, 1999).

Attenuated strains have been generated by adapting the classical and variants strains to chicken embryo fibroblast (CEF) or other cell lines (Lim *et al.*, 1999). Since they are not pathogenic they have been used as live vaccines (Sharma, 1991). The emergence of the very virulent strains during the 1980's in Europe, Japan and China resulted in dramatic losses to the poultry industry (van den Berg, 2000). The vvIBDV strains have been characterised by severe clinical signs and high mortality ranging from 60 – 100%. Very virulent strains can break through the immunity provided by the maternal antibodies (van den Berg, 2000). The vvIBDVs produce similar signs as the classical strains and the same incubation period of 4

days but the acute phase is more severe and more generalised in the affected flocks (van den Berg, 2000).

### **2.3.3 Physicochemical properties of infectious bursal disease virus**

The virus is non – enveloped and quite resistant to physical and chemical agents (Benton *et al.*, 1967). Due to the stability and hardness of the virus, it persist in poultry premises even after thorough cleaning and disinfection (Benton *et al.*, 1967). The virus is inactivated at pH of 12.0, but not at pH 2.0 (Benton *et al.*, 1967). Benton *et al.* (1967) reported that IBDV survived a temperature of 37°C for 90 min and 56°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. The virus survived treatments with various concentrations of three disinfectants (an iodine complex, a phenolic derivative and a quarternary ammonium compound) for a period of 2 min at 23°C only the iodine complex had any deleterious effects (Becht *et al.*, 1988).

Cho and Edgar (1969) reported that the IBDV was inactive by exposure for 1 h to 1% formalin, 1% cresol and 1% phenol. It remained stable at 60°C for 90 min and was still infectious at room temperature for approximately 25°C for 21 days. Petek (1973) observed that IBDV was more resistant than Reovirus to heat, ultraviolet irradiation and photodynamic inactivation. The hardness of the virus makes it difficult to be eradicated from poultry houses after an outbreak of IBD (Alexander and Chettle, 1998). Heat resistance of IBDV is an important factor to be considered in trade of poultry due to extensive international trade of processed and partially processed poultry meat (Shamaila,

2005). Alexander and Chettle (1998) constructed the heat inactivation curves of classical IBDV at 70°C, 75°C and 80°C. These biphasic multiple kinetic curves showed an initial rapid drop in infectivity followed by a more gradual decline. In the second phase it took 18.8 min at 70°C, 11.4 min at 75°C and 3.0 min at 80°C for reduction of the infectivity by 1 by 10. Lam (1991) showed that IBDV survived at 60°C but did not survive at 70°C for 30 min and 0.5% chloramines killed the virus after 10 minute. Invert soaps with 0.05% sodium hydroxide either inactivated or had a strong inhibitory effect on the virus (Schnitzler *et al.*, 1993).

The virus is unusually resistant to inactivation by cooking so there is a risk of introduction to the backyard flocks through uncooked chicken meat products since viable virus might be present in meat from apparently healthy chickens (Lam, 1991). Drumsticks and chicken patties experimentally injected with  $10^7 - 10^9$  TCID<sub>50</sub> of virus and cooked to internal temperature of 71°C and 75°C, respectively in hot oil or steam in a flame grill still contained the infectious IBDV (Lukert and Davis, 1974).

#### **2.3.4 Pathotypic variation of infectious bursal disease virus**

In addition to antigenic differences in serotypes and subtypes, the viral strains can also be classified according to their virulence (van den Berg, 2000). But there has been a great deal of confusion in these definitions. In particular, the term “very virulent” has been used to describe both European hypervirulent strains and variant American strains that cause less than 5% mortality but are able to multiply to a higher degree in the BF of vaccinated birds (van den Berg, 2000). In the absence of the identification of specific virulence determinants, the only valuable criteria for the classification of IBDV strains as “pathotypes” should refer

to their virulence (mortality or lesions) in 3–6 week–old specific pathogen free birds and not to any antigenic specificity. Classification of IBDV strains as pathotypes. 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 birds. Serotype 1 vaccines cause no mortality but possess residual pathogenicity with bursal lesions varying from mild to moderate or even severe. Virulent serotype 1 strains induce both mortality and bursal lesions (van den Berg, 2000). (Appedix 1).

#### **2.4 Epidemiology of the 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 (Etteradossi *et al.*, 1999), including New Zealand which had been free of disease until 1993 (Jones, 1986). Classical IBDV has traditionally affected poultry worldwide ever since the first outbreak of disease was reported from Delaware, Maryland and Virginia Delmarva) region (Cosgrove, 1973). 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 (Onunkwo, 1975; Onunkwo and Momoh, 1981), the Middle East (el-Zain *et al.*, 1974) and Asia (Mohantey *et al.*, 1971).

#### **2.5 Transmission of Infectious Bursal Disease Virus**

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

*al.*, 1972). Indirect transmission of virus 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). Operation of multi-age broiler and pullet replacement farms, defects in biosecurity, or proximity of farms to roads used to transport poultry will contribute to a high prevalence of infection (Vindevogel, *et al.*, 1976).

## **2.6 Pathogenesis of Infectious Bursal Disease**

### **2.6.1 Role of B-cells in the immunopathogenesis of infectious bursal disease virus**

Under natural conditions, the most common mode of infection appears to be via the oral route (Muller *et al.*, 1979). From the gut, the virus is transported to other tissues by phagocytic cells, most likely resident macrophages. Although viral antigen has been detected in liver and kidneys within the first few hours of infection, extensive viral replication takes place primarily in the bursa of Fabricius (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). By way of the portal venous system, virus reaches the liver within 5 h post infection. Kupfer cells in the liver trap and phagocytose a considerable amount of virus particles (de Wit and Baxendale, 2013). On reaching the main blood stream, IBDV is circulated to other organs including the BF. Immature B-lymphocytes in the follicles of the BF are the target cells for viral replication. By 13 h post infection most follicles in the BF are virus positive, by 16 h post infection, a second massive viraemia occurs (Muller *et al.*, 1979; de Wit and Baxendale, 2013). There is infection and secondary viral replication in other lymphatic organs such as THY and SPL. 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).

### **2.6.2 Role of T-cells in the immunopathogenesis of infectious bursal disease virus**

Appearance of viral antigen in BF is accompanied by an infiltration of T-cells, while IgM + cells undergo a precipitous decrease and the immunoglobulin level remains the same (Lejal *et al.*, 2000). Infiltrating T-cells were detected at 1st day post-inoculation through flow-cytometry and were shown to persist till 12 weeks (Sharma *et al.*, 2000). The ratio of CD4 and CD8 cells were the same during the 1st seven days postinoculation (dpi), but CD8 cells became predominant afterwards (Lajal *et al.*, 2000). Infections bursal disease virus induced bursal T-cells have increased surface expression of MHC-II and IL-2 receptors, elevated expression of cytokine genes like IFN- $\gamma$  and IL-6 like factor (Sharma *et al.*, 2000). T cells from the BF of recovered bird proliferate when exposed *in vitro* to purified IBDV. While the spleen cells from IBDV exposed-chicken produced nitric oxide stimulating factor when stimulated *in vitro* with purified IBDV (Sharma *et al.*, 2000). Bursal T cells also suppressed the mitogenic proliferation of the spleen from normal, virus free chicken (Sharma *et al.*, 2001). T-cell immunodeficiency can modulate pathogenicity of the virus since it has been shown that in the bursal cells the birds have a higher viral burden, lower inflammatory lesions, down regulate IFN- $\gamma$  and IL-2 genes, have a lower incidence of apoptosis bursal cells (Sharma *et al.*, 2000), and they undergo follicular recovery than T-cell (Sharma *et al.*, 2001).

### **2.6.3 Effect of infectious bursal disease virus on innate immunity**

Infectious bursal disease virus has been shown to modulate the macrophage function by altering the *in vitro* 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). There is growing evidence for a role of innate immunity, particularly proinflammatory mediators, in the pathogenesis of IBD (Ingrao *et al.*, 2013). Indeed, during the acute phase of IBD and as early as 1 day post-infection (dpi), there is a dramatic infiltration of CD4<sup>+</sup> cells, CD8<sup>+</sup> cells and macrophages at and near the site of virus replication, mainly 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; for example, ChIL-1 $\beta$ , ChIL-6, CXCL2 and ChIFN- $\gamma$  (Eldaghayes *et al.*, 2006). High levels of systemic ChIFN- $\gamma$  and ChIL- $\gamma$  were also observed during the acute phase following vvIBDV challenge demonstrating the role of an exacerbated innate immune response in the acute phase of the disease, leading to a “cytokine storm” (Rauw *et al.*, 2007). The ChIFN- $\gamma$ , ChIL-6, and inducible nitric oxide synthetase (iNOS) (Palmquist *et al.*, 2006) may promote cellular dysregulation and accentuate tissue destruction (Digby and Lowenthal, 1995; Karaca *et al.*, 1996; Kim *et al.*, 1998). Moreover, macrophages and monocytes infected by IBDV are directly activated to produce high levels of mediators such as pro-inflammatory cytokines, interleukin-1 (IL-1) and IL-6, chemokines (IL-8 and MIP- $\alpha$  and nitric oxide (NO) (Kim *et al.*, 1998; van den Berg, 2000; Khatri *et al.*, 2005; Rauf *et al.*, 2011a).

The signal transduction pathways involved in macrophage activation have also been examined (Khatri and Sharma, 2006). The role of mitogen-activated protein kinases

(MAPKs) and NF- $\kappa$ B was tested by using specific pharmacological inhibitors. The addition of p38 MAPK inhibitor, SB -203580 and NF- $\kappa$ B inhibitor Bay 11-7082, suppressed IBDV-induced NO production and mRNA expression of iNOS, IL-8 and COX-2 (Khatri and Sharma, 2006). These results suggest that IBDV uses cellular signal transduction machinery, in particular the p38 MAPK and NF- $\kappa$ B pathways, to elicit macrophage activation. The increased production of NO, IL-8 and COX-2 by macrophages may contribute to bursa inflammatory responses commonly seen during the acute phase of IBDV infection (Khatri *et al.*, 2005). This was confirmed in a more recent study (Rauf *et al.*, 2011b), where the over expression of chemokines genes, IL-8 and MIP- $\alpha$  was also higher in IBDV-infected chickens during the early phase of infection (chicken IL-8 acts as a chemoattractant for heterophils and monocytes). In summary, IBDV appears to trigger both direct (T-cell activation) and indirect (macrophage activation) pathways to induce a “cytokine storm” during the acute phase of the disease and the individual susceptibility must be related to the variable intensity of the innate immune response (van den Berg, 2000; Rauw *et al.*, 2007; Rauf *et al.*, 2011b).

In one study, an Agilent microarray was used to investigate different transcriptional profiles of the TLR pathway and related genes of chicken bursa at 48 hour after infection with IBDV, compared with simulated infection. Expression of 58 genes changed significantly (Guo *et al.*, 2012). Forty-six genes associated with chicken bursa proinflammatory effects, chemotactic effects, and T-cell stimulation were upregulated, which meant enhancement of these features. Twelve genes that are related to proliferation and differentiation of bursal cells were downregulated, implying suppression of these features. These results revealed

that gens of the TLR pathway play an important role in the pathogenicity of IBDV infection (Guo *et al.*, 2012).

#### **2.6.4 Role of cytokine in the pathogenesis of infectious bursal disease virus**

Infectious bursal disease virus modulates the T cell functions (Sharma *et al.*, 2001). During the acute phase of the disease septic shock-like symptoms have been observed (Hack *et al.*, 1997). In septic shock syndrome, there is an up-regulation of the cytokine gene resulting in an excessive immune response and increased levels of IFN- $\gamma$  and TNF- $\alpha$  (Hack *et al.*, 1997). The TNF- $\alpha$  is a macrophage-produced cytokine involved in inflammation and septic shock. Chicken IFN- $\gamma$  can activate macrophages and enhance their anti-microbial activity (Kim *et al.*, 1998). The ChIFN- $\gamma$  and TNF- $\alpha$  level in serum were measured by capture ELISA and cytotoxic bioassay, respectively. The increase in the levels of cytokines like ChIFN- $\gamma$  and TNF- $\alpha$  correlated with the acute phase of the disease. Levels of circulating ChIFN- $\gamma$  and TNF- $\alpha$  increased as the disease progresses and were highest in the birds that died of infection. The TNF- $\alpha$  level lasted longer than the ChIFN- $\gamma$  levels (Sharma *et al.*, 2001).

### **2.7 Immunology of Infectious Bursal Disease Virus**

#### **2.7.1 Target cells for infectious bursal disease virus**

It is the IgM<sup>+</sup> that are the target cells for the IBDV. During the acute phase of the IBD the bursa undergoes atrophy as the bursal follicles get 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 region of the bursal morphology. By this time an ample amount of viral antigen can be detected in other immune organs (Giambrone, 1977; Kim *et al.*, 1999). T-cells are resistant to infection by IBDV (Hirai *et al.*, 1979). During the acute phase of the IBD lesions appear in the thymus which is quickly overcome within a few days (Sharma *et al.*, 2000). A dramatic influx of T-cells is reported in and around the site of virus replication. The infiltrated T-cells could be detected in the lymphocyte from 1<sup>st</sup> and 12<sup>th</sup> weeks pi although the viral antigen disappears by the 3rd week. 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. 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- $\gamma$  which activates the macrophages to produce nitric oxide (NO) (Sharma *et al.*, 2000). The IBDV causes a transient inhibition of *in vitro* proliferative activity of T-cells to mitogens. The virus stimulates the macrophages to produce T-cell cytokine like IFN- $\gamma$  to produce NO and other cytokines with anti-proliferative activity. Both humoral and cellular arms of the immune system are compromised during IBDV infection due to lysis of the B-cells and altered antigen-presenting cells (Ingrao *et al.*, 2013).

Infectious bursal disease does not affect natural killer cells levels in chicken (Sharma *et al.*, 2000). The NO production after IBDV infection exerts antiviral effect since the immune – suppressed chickens that failed to induce NO had more severe disease and higher degree of virus replication, but does not seem to correlate with the haemorrhagic lesions which result from the reaction of host-factors and the determinants responsible for virus virulence and

virus clearance (Poonia *et al.*, 2005). The IBDV-induced damage to humoral immunity is reversible. Antibody production correlates with the morphologic restoration of the bursal follicles. Mitogenic response of T cells returned to the normal levels. During the course of mitogenic inhibition, T-cells of infected chicken also failed to secrete IL-2 upon *in vitro* stimulation (Sharma *et al.*, 1987; Kim *et al.*, 1996). 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).

Intra bursal 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- $\gamma$  gene (Kim *et al.*, 2000). Intra bursal 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, the nature of which is still unknown (Sharma *et al.*, 2000). Chickens that survive the disease clear the virus and recover from its pathologic effects (Sharman *et al.*, 2000). It has been shown that the more virulent the virus the stronger is the suppression of the humoral and cell mediated immunity. Virulent virus also produced a detectable NO production in serum (Shamaila, 2005).

### **2.7.2 Target organs for infectious bursal disease virus**

The target organ for IBDV is the BF at its maximum development, which is a specific source of mature B-lymphocytes in avian species (Cheville, 1967; Okoye and Uzoukwu, 1984; Saif, 1998; van den Berg *et al.*, 2000; Lukert and Saif, 2003; Mac Lachlan and Dubovi, 2011; Mahgoub, 2012; Ingraio *et al.*, 2013). Bursectomy can prevent illness in chicks infected with virulent virus (Okoye and Uzuokwu, 1990; Hiraga *et al.*, 1994). The highest percentages are age susceptibility between 3 and 6 weeks, when the BF is at its maximum level of development (Muller *et al.*, 2003; Mbuko *et al.*, 2010). This age susceptibility is extended in the case of vvIBDV infection (Nunoya *et al.*, 1992; van den Berg *et al.*, 1991 and van den Berg, 2000). Depletion of lymphoid B-cells in the BF after IBDV infection is due to both necrosis and apoptosis. Actively dividing, surface immunoglobulin M-bearing B-cells are lysed by IBDV infection (Hiria and Calnek, 1979; Hirai *et al.*, 1981; Rodenberg *et al.*, 1994). Chickens infected with IBDV immediately after hatching develop a subclinical infection with atrophy of BF and B-cell depletion (Winterfield *et al.*, 1972; Hudson *et al.*, 1975).

### **2.7.3 Immunosuppression**

Immunosuppression has been defined as a ‘state of temporary or permanent dysfunction of the immune response resulting from damage to the immune system and leading to increased susceptibility to disease’ (Dohms and Saif, 1984) and often a suboptimal antibody response (Lutticken, 1997). However, this definition considers more the consequences (increased disease incidence) than the causes, of which mechanisms, beyond the destruction of a specific cell type, are still not fully understood (Ingraio *et al.*, 2013). The destruction of the BF by IBDV creates an immunosuppression, which will be more serious in the younger and

infected bird. In addition to its zootechnical impact and its role in the development of secondary infections, it may affect immune response of the chicken to subsequent vaccinations, essential in all types of intensive farming (Ingrao *et al.*, 2013). The immunosuppression has been most often evident using experimental models based on the measurement of humoral responses induced by different antigens such as *Brucella abortus*, sheep red blood cells, or ND vaccines (Allan *et al.*, 1972; Giambrone, 1976; Giambrone *et al.*, 1976;). The best assessment is clearly the measurement of vaccinal protection against a challenge infection by the Newcastle virus as described by OIE (2012). However, OIE only gave a partial picture of the immunosuppression as, according to the clonal nature of immunity, it will depend on the number of NDV specific clones that will be destroyed (OIE, 2012). Using OIE diagnostic tests, 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 duration up to the age of 6 weeks (Giambrone *et al.*, 1976). In field conditions, chickens tend to become infected toward the age of 2 – 3 weeks, when maternal antibodies decline (Guittet *et al.*, 1992).

In any case, 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. Although the immunosuppression caused by IBDV is principally directed towards B-lymphocytes, an indirect effect on cell-mediated immunity (CMI) has also been demonstrated (Sharma and Fredericksen, 1987; Sharma *et al.*, 1989; Cloud *et al.*, 1992; Rauw *et al.*, 2007) therefore increasing the impact of IBDV on the immunocompetence of the chicken. 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 *et al.*, 2007). Moreover, splenocytes from IBDV infected chickens were also shown to be deficient in secretion of ChIL-2 (Fredericksen and Sharma, 1987; Kim *et al.*, 1998 and Ingraio *et al.*, 2013). It is now well accepted that macrophages are the central effector cells of the innate immune system and influence the nature of the adaptive immune response.

#### **2.7.4 Effect of infections bursal disease virus on humoral immunity**

Infectious bursal disease virus has a predilection for the immature (Sivanandan and Maheswaran, 1980) actively dividing B-lymphocytes and causes lytic infection of IgM bearing B cells resulting in decrease in circulating IgM<sup>+</sup> cells. Infected chicken produce less level of antibodies against the 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 IgG and produced only a monomeric IgM (Ivanyi, 1975; Invanyi and Morris, 1976). The IgG levels varied depending on the age at the time of infection (Hirai and Shimakura, 1979). The adverse effect on antibody responses is due to the damage to the B-cells in the BF and the blood (Sivanadan and Maheswaran, 1980).

#### **2.7.5 Effect of infectious bursal disease on cellular immunity**

The effect of IBDV on CMI 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, 1977). The thymic lesions were transient and appeared within the first week of infection peaked at 3-4 days pi and then subsided (Okoye and Uzoukwu, 1990). The presence of thymic lesions were not associated with active viral replication of the virus in the thymic cells as shown by the immunofluorescence (IF) and antigen capture ELISA (Confer *et al.*, 1981). In addition, T-cells from infected chickens during the early stages of virus 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. The reason for the delay in this response is not clear considering that the virus persists in the host for approximately 3 weeks (Confer *et al.*, 1981). It was speculated that this depression is the overall depression of T-cell function during the virus infection (Sivanandan and Maheshwaran, 1981). It was reported that IBDV had an inconsistent blastogenic response of spleen cells to phytohaemagglutinin (Sharma and Lee, 1983). *In vitro* mitogen hypo-responsiveness of T-cell is mediated by the suppressor cells in the spleens of the infected chickens; the mechanism of reduced *in vivo* cellular immunocompetence is not known (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.*, 1973). 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.*, 1973). Broilers infected with IBDV at 3 weeks of age had reduced antibody titres to *Brucella abortus* (T-cell independent antigen) and sheep red

blood cells (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).

### **2.7.6 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 and Metz, 1989). Chickens infected with IBDV have suppressor cells in the spleen, which cause *in vitro* mitogenic hypo responsiveness to concavalin A (Sharma and Metz, 1989). The impairment of T-cells and development of suppressor cells (Sharma and Fredericksen, 1987) was demonstrated *in vitro* by using proliferation tests (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).

### **2.7.7 Apoptosis**

Apoptosis, or programmed cell death, is a process where, in response to specific stimuli, cells die in a controlled, programmed manner (Shamaila, 2005). Besides lymphocyte lysis, apoptosis also plays a role in immunosuppression (Vasconcelos and Lam, 1994; Ojeda *et al.*, 1997; Tanimura and Sharma, 1998). Apoptosis could occur in a variety of organs (Allan *et al.*, 1997) like thymus (Inoue, 1994) BF and spleen (Vasconcelos and Lam, 1995; Lam, 1997). The CEF and Vero cells infected with IBDV show the biochemical features of apoptosis (Tham and Moon, 1996). In addition to causing necrosis, IBDV can also induce apoptosis in avian lymphocytes *in vitro* (Vasconcelos and Lam, 1994). Viral proteins like VP2 and VP5 have been implicated in the induction of apoptosis (Fernandez-Arias *et al.*,

1997; Yao *et al.*, 1998). Expression of VP243 polyprotein in transiently transferred DT40 B lymphocyte culture suppresses cell growth and proliferative responses to mitogen stimulation indicating that IBDV polyprotein is a mediator of immunosuppression (Peter and Wu, 2004). Apoptotic cells have also been observed in antigen negative bursal cells indicating that immunological mediators like cytokines might be involved in the process (Tanimura and Sharma, 1998).

Many different cells species can undergo apoptosis but immature B and T cells are particularly susceptible to apoptotic cell death. Only 20% of the lymphoid cells in the BF contain replicating IBDV (Shamaila, 2005). The severe damage to the bursa can be ascribed to apoptosis (Muller, 1986; Burkhardt and Muller, 1987). In addition to necrosis, marked atrophy of the BF occurs without eliciting an inflammatory response that is a characteristic sign of the apoptotic process (Burkhardt and Muller, 1987). Replication of the virus in BF results in secondary viraemia thus spread the virus to other tissues. The IBDV infection of a susceptible chicken has been shown to induce apoptosis in the bursa as well as thymus (Vasconcelos and Lam, 1995; Ojeda *et al.*, 1997; Tanimura and Sharma, 1997; Tanimura and Sharma, 1998).

Morphological and biochemical features of apoptosis were also observed after *in vitro* infection of IBDV in chicken peripheral blood lymphocytes (Vaconcelos and Lam, 1994) and chicken embryo fibroblasts (Than and Moon, 1996). Apoptosis occurs in lymphocytes of various organs like thymus (Inoue, 1994) bursa and spleen (Lam, 1997). Some researchers believed that apoptosis induced by IBDV in cell cultures following *in vitro* infection was an early genetic response of the host cells and was independent of virus

replication while others showed that appearance of CPE coincided with virus replication. (Than and Moon, 1996). Jungmann *et al.* (2001) however, showed that proportion of apoptotic cells increased from 5.8% at 4 h post-infection (pi) to 64.5% at 48 h p.i. in CE cells after infection with IBDV strain CU-1 (Jungmann *et al.*, 2001). However, treatment of CE cell cultures with UV inactivated IBDV did not induce apoptosis.

Double labeling studies for apoptotic or antigen positive cells revealed that apoptosis in the BF occurs both in IBDV positive and IBDV negative cells (Tanimura and Sharma, 1998) whereas apoptosis in the thymus occurs in the antigen negative cells only (Tanimura and Sharma, 1998). It was concluded that IBDV induced apoptosis indirectly in non-bursal organs (Tanimura and Sharma, 1998). It has been postulated that IBDV impairs the withdrawal of apoptotic cells and therefore results in the increased number of the apoptotic cells (Ojeda *et al.*, 1997). Apoptotic cells were located mostly in an area between the cortex and medulla whereas majority of cells positive for viral antigens were found in the medulla (Tanimura and Sharma, 1998). Indirect mechanisms might also be involved in the induction of apoptosis and could have induced apoptosis *in vivo*, resulting in rapid depletion of cells in BF (Jungmann *et al.*, 2001). In the infected follicles large numbers of cells were apoptotic but very few contained the viral antigen (Tanimura and Sharma, 1998; Jungmann *et al.*, 2001). Interferon production occurs after IBDV infection and is thought to be the major apoptosis-inducing factor in the neighbouring cells along with TNF- $\alpha$  (Jungmann *et al.*, 2001).

## **2.8 Clinical Manifestations of Infectious Bursal Disease**

### **2.8.1 Subclinical infectious bursal disease**

The subclinical form of the disease occurs when chickens are exposed to IBDV during the first two weeks post hatch (de Wit and Baxendale, 2013). Chickens present no clinical signs but grossly 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 a continuous daily mortality and poorer feed conversions (de Wit and Baxendale, 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 Baxendale, 2013).

### **2.8.2 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 diarrhea, 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 Baxendale, 2013). Mortality may be negligible or as high as 90 % in case of very virulent IBDV but most commonly a mortality of 10-20 % is usually seen (de Wit and Baxendale, 2013). In field situation, mortality in layer type birds is general higher than meat type birds (de Wit and Baxendale, 2013).

## 2.9 Pathology of Infectious Bursal Disease

### 2.9.1 Gross pathological lesions

The tissue distribution and severity of lesions is dependent on the subtype and pathogenicity of the virus (Rosenberger and Cloud, 1986; Tanimura *et al.*, 1995). Infected birds are dehydrated and have darkened discoloration of pectoral muscles. Individuals that die or are scarified approximately 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 organ, which is pale yellow in colour and shows striations (Ley *et al.*, 1979). On section, the bursa is hyperaemic and intrafollicular haemorrhages may be present (Helmboldt and Garner, 1964). From the fifth day after infection the organ decreases in size and within eight days may be only one-third of the weight of the bursae of unaffected chicken (Lukert and Saif, 1991; 2003). Haemorrhages occur in the thigh and pectoral muscles and are also reported from the mucosa at the proventriculus-ventriculus junction and on the serosal surface and plica of the BF (Hanson, 1962). There is increased mucus in the intestine and renal changes are observed in diseased birds that are due to dehydration (Lukert and Saif, 2003).

In a detailed study using the Edgar strain of the virus, Cheville recorded the bursal weights for 12 days post-inoculation (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; Lukert and Saif, 2003; Mahgoub, 2012). By day 5, the bursa returned to its normal weight (Cheville, 1967; Lukert and Saif, 2003; Mahgoub, 2012). 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 organs 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 of BF (Rosenberger and Cloud, 1986; Rosenberger *et al.*, 1987). Bursal size is usually quantified as a ratio of bursal (numerator) to body mass (denominator) (Lucio and Hitcher, 1979). Various authors quote ratios based on different formulae. Most papers 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). General comparisons cannot be made among publications unless the same factor is applied, but bursal atrophy can be characterized in a specific trial using a common formula to calculate bursal to body ratio (Lasher and Shane, 1994). In a trial reported by van den Berg *et al.* (1991) a bursal: body weight ratio of 1.3 was determined in specific pathogen free Leghorn pullets 10 days after challenge with vvIBDV, strain 849VB. In contrast, non-infected, unvaccinated male and female controls yielded a bursal: body ratio of 5.9. Similar results were shown by Mazariegos *et al.* (1990), who infected 1 day-old specific pathogen free chicks with Edgar strain IBDV, which resulted in a bursal:body weight ratio of 2.3 at 14 days compared to controls with a value of 10.0 ratios.

Pathologic changes in the spleen and thymus were less prominent than those of the BF (Cosgrove, 1964; Inoue *et al.*, 1994; Lukert and Saif, 2003; Okoye, 2005). The spleen might be slightly enlarged and usually had small gray foci uniformly dispersed on the surface (Lukert and Saif, 2003). Lesions in these organs were noticed at the same time as the changes occurred in the BF and resolved within 1 or 2 days of appearance (Helmboldt and Garner, 1964). Kidneys show enlargement and pallor with accumulation of crystalline urate

in tubules, visible as white flecks beneath the capsule (Cosgrove, 1962; Hitchner, 1978; van den Berg *et al.*, 2000). The renal lesions were prominent in early outbreaks which were described in the USA (van den Berg *et al.*, 2000). Renal changes which are observed in carcasses and in chickens sacrificed *in extremis* represent water deprivation associated with recumbency (Lukert and Saif, 1991). Splenic enlargement was documented by Morales and Boclair (1993) who showed highly significant differences in bursal: spleen weight ratio of 2.4 for controls compared with 0.9 in chicks seven days after challenge. The spleen may be slightly enlarged and very often has small grey foci uniformly dispersed on the surface (Lukert and Saif, 2003) but later atrophic (Okoye, 2005).

### **2.9.2 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 (Cheville, 1967). Degenerating lymphocytes are surrounded by macrophages. Lymphocytes are soon replaced by heterophils, pyknotic debris and hyperplastic reticuloendothelial cells (Cheville, 1967). By day 3 or 4 pi, all of the lymphoid follicles are affected. Severe oedema, hyperaemia, and marked accumulation of heterophils are evident, which cause the increased bursal weight (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, with consists of the bursal columnar epithelium containing

globules of mucin. In the late stages, scattered lymphocyte foci appear without the ability to form functional follicles (Mahgoub, 2012).

The thymus exhibited some cellular reaction in the lymphoid tissues. Evidence of lymphocyte necrosis in the inner cortex appears at 2 dpi in 4-day-old and 2-weeks-old chickens infected with IBD (Inoue, 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, 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, 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).

In the spleen, hyperplasia of the reticulo-endothelial cells around the adenoid sheath arteries has been observed in the early stages of 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 splenic capsules. Complete lymphocytes regeneration was observed in the spleen and thymus unlike in the BF (Ley *et al.*, 1983; Okoye, 1984; Lukert and Saif, 2003).

The Harderian gland is reported to be severely affected by the virus 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).

The kidneys in contract, the broilers infected at 3 weeks of age have a 51% reduction in plasma cells content at 5-14 dpi, (Helmboldt, 1964; Dohms *et al.*, 1988). Plasma cell numbers reduction was temporary and levels became normal after 14 days. 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 kidney of IBDV-infected birds have been reported (Mandelli *et al.*, 1966; Del Bono *et al.*, 1968; Ley *et al.*, 1983).

The liver had some slight perivascular infiltration of monocytes (Parkhurst, 1964). Indeed, using various immunostaining methods, a higher frequency of antigen-presenting cells could be demonstrated after infection of birds with vvIBDV than with other strains (Nunoya *et al.*, 1992; Sharma *et al.*, 1993; Inoue *et al.*, 1994).

## **2.10 Diagnosis of Infectious Bursal Disease**

### **2.10.1 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).

### **2.10.2 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 post-mortem examination of the lesions, in particular of the BF (Lukert and Saif, 2003).

### **2.10.3 Histological diagnosis**

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

### **2.10.4 Virus detection**

The virus can be found in other organs such as the thymus, liver and bone marrow but in significantly low quantities than in the bursa (Cheville, 1967; Solano *et al.*, 1985). The inoculum for virus isolation is prepared by homogenising the tissue sample in antibiotic containing buffer that is centrifuged to remove larger tissue particles and is used for inoculating embryonated eggs and tissue culture (Lukert, 1986).

The chorioallantoic route of inoculation in 9-11 days old embryos is the most sensitive route for the isolation of the virus (Hitchner, 1970; Lambrecht, 2000). 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, 2000). Variant viruses however, do not kill the embryos but cause embryo stunting, discoloration, 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 virus. Macreadie *et al.* (1990) reported that 3 out of 7 chicken isolates failed to grow in chicken embryo fibroblast (CEF)

cells but propagated well in embryonating eggs. Some strains grow well in embryos but are not readily adapted to grow in CEF or CEK (Lasher, 1997).

### **2.10.5 Molecular detection**

Nowadays, 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.*, 2001a; Liu *et al.*, 2002; Viswa *et al.*, 2002).

### **2.10.6 Serological tests**

Serological tests generally used for the detection of IBDV are ELISA, VN and AGP. The ELISA is the most commonly used test for the detection of antibodies of 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 therefore 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; Ismial and Saif, 1990). It is essential to use appropriate indicator viruses in VN tests to avoid artificially

lower titres due to the existence of several antigenic variants (Ismail and Saif, 1990). Most chicken sera have high levels of neutralizing antibodies to a broad spectrum of antigenically diverse viruses due to vaccine and field exposure (Jackwood and Saif, 1987). However, VN is laborious and time consuming and therefore its use 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 detection of 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).

#### **2.10.7 Virological isolation**

Infectious bursal disease virus may be detected in the BF of chicks in the acute phase of infection, ideally 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 bursa of Fabricius 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; the yolk sac route is also practicable, and the intra-allantoic route is the least sensitive (van den Berg, 2000). The specificity of the lesions observed must be demonstrated by neutralising the effect of the virus with a monospecific anti-IBDV serum. Isolation in embryonated eggs does not require adaptation of the virus by serial passages, and is suitable for vvIBDVs. 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).

### **2.10.8 Differential diagnoses**

The conditions most likely to be mistaken for IBD are avian coccidiosis, Newcastle disease, stunting syndrome, chicken infectious anaemia, mycotoxicoses and nephropathogenic forms of infectious bronchitis, inclusion body hepatitis and lymphoid leukosis (Barron *et al.*, 1966; Faragher, 1972; Okeke, 1984). 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 Newcastle disease has been observed to cause severe degeneration of lymphocytes in the medullary region of the BF (Okoye, 2000; Lukert and Saif, 2003), but necrosis and later replacement of lymphocytes by heterophils are additional features of IBD.

### **2.11 Treatment of Infectious Bursal Disease**

There is no specific treatment, 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 an energy boost (Giambrone, 2013).

## **2.12 Prevention and Control of Infectious Bursal Disease**

### **2.12.1 Vaccination**

In practice, control of IBD is greatly dependent upon the use of vaccines. Taking all the previous considerations into account, a satisfactory vaccine should protect against the disease, especially the acute phase, and the consequences of the disease, namely immunosuppression (Ingrao *et al.*, 2013).

### **2.12.2 Biosecurity and eradication**

There is evidence, however, 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) (Heine and Boyle, 1993) can reduce infection rates. Given the very contagious nature of the disease and the resistance of the virus, certain essential steps in the cleaning/disinfection process should be adhered to (Benton *et al.*, 1967). Prior to cleaning, all insects and pests (for example, rats and mice) 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 hose down using hot water (60 °C) with a detergent, at a pressure of 80 bar to 150 bar (Benton *et al.*, 1967). A second disinfection of the full premises must be performed before the introduction of the 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. All disinfectants are more active at a temperature above 20°C; however, chlorinated and iodinated disinfectant cannot be heated above 43°C (Higashihara, 1991). The quantity of disinfectant solution to be used is approximately 4 liters per 15m<sup>2</sup> (Higashihara, 1991) reduces the challenge virus. It may also delay challenge thus allowing more time for vaccines to induce immunity (Ingrao *et al.*, 2013). Rigorous biosecurity measures have to be implemented in order to stop the spread of virus from one flock to the next (Higashihara, 1991). Integrated nature of commercial poultry operations and vectors like lesser mealworm, mosquitoes and rats pose extra problems for the control of this infection (Howie and Thorsen, 1981; Okoye and Uche, 1986; McAllister *et al.*, 1995).

### **2.13 Economic Significance of Infectious Bursal Disease Virus**

Uncomplicated IBDV with original type 1 isolates results in up to 90% flock morbidity and 20% mortality in susceptible 3-8 week-old hybrid Leghorn replacement pullets (van den Berg *et al.*, 1991; Lasher and Shane, 1994; Okoye, 2005). In broilers aged 3 – 6 weeks lower flock morbidity (50%) occurs following infection and primary mortality from IBD seldom exceeds 3% (van den Berg *et al.*, 1991; Nounaoya *et al.*, 1992; van den Berg *et al.*, 2000; Okoye, 2005). In broilers, a high prevalence of viral respiratory infections and elevated mortality due to airsacculitis and colisepticaemia during the terminal third of the 6-8 week growing cycle are donated by immunosuppression (Lasher and Shane, 1994). In addition, the plant condemnation rate due to septicaemia – toxaemia syndrome may be increased ten fold from 0.5% to over 5% in affected flocks (Lasher and Shane, 1994).

A 14% depression in financial return from broiler flocks with subclinical IBD compared with unaffected flocks surveyed in Ireland was documented by McIlloy *et al.* (1989). Lasher

and Shane (1994) documented an 11% depression in net income in flocks which showed serological evidence of IBD during a mean 42-day growing period, in comparison to non – exposed broilers. The 10% reduction in profit for the 991 flocks in the study affected with IBDV was attributed to relative depression in body mass and feed conversion efficiency, but not in liveability, compared with non-exposed flocks. The appearance of an IBDV strain of moderate pathogenicity on the Island of Mauritius in 1992 resulted in a 4% depression in broiler live mass. A 2-5% elevation in mortality was recorded, divided evenly between acute 18-25 day death from IBD and subsequent losses due to septicaemia during weeks 5-6 of the growing cycle (Lasher and Shane, 1994).

Increased susceptibility to respiratory viruses, including Newcastle disease (Faragher *et al.*, 1974) and avian infectious bronchitis (Pejkovski *et al.*, 1979), leads to depression in egg production and deterioration in egg shell and internal quality in commercial laying flocks. Chick yield per breeder is diminished by reduced egg numbers and hatchability (Lucio and Hitchner, 1979). The advent of IBDV infection has imposed additional costs on the poultry industry through enhanced decontamination and preventive procedures (Lasher and Shane, 1994). Immunization programmes dictate the purchase, storage, and administration of both live and inactivated vaccines. Monitoring vaccination efficiency requires collection of blood samples and laboratory determination of antibody titre (Lasher and Shane, 1994). The capital investment in analytical equipment and computer software and reagents, commercial kits, and laboratory personnel has become a significant but necessary component of veterinary costs (Lasher and Shane, 1994).

Outbreaks of vvIBD resulted in up to 30% mortality in infected broiler flocks, especially in areas with a high density of poultry. In studies on vvIBD van den Berg and Meulemans

(1991) showed that infection of 38 day-old hybrid Leghorn pullets with 100 chickens LD<sub>50</sub> of the 849 VB isolate result in 60% losses. In contrast, broiler chicks infected at the same age showed 17% mortality (Lasher and Shane, 1994). In Nigeria, outbreaks of IBD have rendered investment in poultry farming fearful and unrealistic to individuals and organizations (Okoye, 1983; Abdu, 1986; Lukert and Saif, 1997; Musa *et al.*, 2012). An estimated economic loss of over three billion naira was reported during a three year study (2009-2011) by Musa *et al.* (2012).

## **2.14 Probiotics**

### **2.14.1 Definition of probiotic**

Probiotics are live microorganisms of nonpathogenic and nontoxic in nature, which when administered through the oral route favour the host's health (Guillot, 1998).

### **2.14.2 History of probiotics**

Over the years the word probiotic has been used in several different ways. It was originally used to describe substances produced by one protozoan or bacteria that stimulated by another organisms (Lilly and Stillwell, 1965), but it was later used to describe animal feed supplements, which had a beneficial effect on the host by affecting its gut flora (Parker, 1974).

#### **2.14.2.1 Antox<sup>®</sup> Probiotic**

Antox<sup>®</sup> (*Saccharomyces cerevisiae* 4.125×10<sup>6</sup>cfu/mL, Citric acid 6 g, Lactic acid 2 g, Vitamin B<sub>1</sub> 100 mg, Vitamin B<sub>2</sub> 7.5 mg, Vitamin B<sub>6</sub> 80 mg, Vitamin B<sub>12</sub> 0.6 mg, Biotin 1.5 mg, Nicotinamide 1 g, Calcium chlorine (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) is rich in biologically important proteins, B-complex vitamins, trace minerals and several unique 'Plus Factors'. Other identified beneficial factors include the enhancement of phosphorus availability (Glade and Biesik, 1986; Brake, 1991; Moore *et al.*, 1994) and nutrient utilization by animals (Thayer *et al.*, 1978; Erdman, 1989; Pagan, 1990), reduction in cases of disease infection (Line *et al.*, 1997) and improvement of feed efficiency (Onifade and Babatunde, 1996).

#### 2.14.2.2 Bactofort<sup>®</sup> Probiotic

Bactofort<sup>®</sup> are used for preservation of food by fermentation for thousands years. It can serve a dual function by acting as agents for food fermentation and in addition, potentially imparting health benefits (Guillot, 1998). In broilers nutrition, Bactofort<sup>®</sup> have a beneficial effect on their performance (Tortuero, 1973; Ashayerizadeh *et al.*, 2009), modulation of intestinal microflora and pathogen inhibition (Jin *et al.*, 1998; Mountzouris *et al.*, 2007), intestinal histological changes (Kabir *et al.*, 2005; Chichlowski *et al.*, 2007), immunomodulation and certain haemato-biochemical parameters (Mountzouris *et al.*, 2007), improving sensory characteristics of dressed broiler meat and promoting microbiological meat quality of broiler (Kabir *et al.*, 2005).

#### 2.14.3 Mechanism of action of probiotics

Enhancement of colonisation resistance and/or direct inhibitory effects against pathogens is important factors where probiotics have reduced the incidence and duration of diseases. 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 (Direk, 1989; Awad *et al.*, 2006); and (iv) stimulating the immune system (McCracken, and Gaskins, 1999; Huang *et al.*, 2004; Kabir *et al.*, 2004; Koenen *et al.*, 2004; Dalloul *et al.*, 2005; Haghghi *et al.*, 2006; Nayebpor *et al.*, 2007; Mathivanan *et al.*, 2007; Apata, 2008; Brisbin *et al.*, 2008).

Upon consumption, probiotics deliver many lactic acid bacteria into the gastro-intestinal tract. These microorganisms have been reputed to modify the intestinal milieu and to deliver enzymes and other beneficial substances into the intestines (Marteau and Rambaud, 1993). Supplementation of *L. acidophilus* or a mixture of *Lactobacillus* cultures to chickens significantly increased the levels of amylase after 40 days of feeding (Jin *et al.*, 2000). The lactobacilli colonising the intestine may secrete the enzyme, thus increasing the intestinal amylase activity (Duke, 1977; Sissons, 1989). It is well established that probiotics alter gastro-intestinal pH and flora to favor an increased activity of intestinal enzymes and digestibility of nutrients (Direk, 1989). Duke (1977), they postulated that active amylolytic and proteolytic enzymes residing in *Aspergillus oryzae* may influence the digested nutrients. Similarly, it was reported that an increase in the digestibility of dry matter was closely related to the enzymes released by yeast (Han *et al.*, 1999). In addition, probiotics may

contribute to the improvement of health status of birds by reducing ammonia production in the intestines (Chiang and Hsieh, 1995).

Mechanisms by which probiotics improve feed conversion efficiency include alteration in intestinal flora, enhancement of growth of non-pathogenic facultative anaerobic and gram positive bacteria forming lactic acid and hydrogen peroxide, suppression of growth of intestinal pathogens, and enhancement of digestion and utilisation of nutrients (Yeo and Kim, 1997). However, it has been shown that probiotics stimulate different subsets of immune system cells to produce cytokines, which in turn play a role in the induction and regulation of the immune response (Maassen *et al.*, 2000; Christensen *et al.*, 2002; Lammers *et al.*, 2003). Stimulation of human peripheral blood mononuclear cells with *Lactobacillus rhamnosus* strain GG *in vitro* resulted in the production of interleukin 4 (IL-4), IL-6, IL-10, tumor necrosis factor alpha- and gamma-interferon (Schultz *et al.*, 2003). Other studies have provided confirmatory evidence that Th2 cytokines, such as IL-4 and IL-10, are induced by lactobacilli (Christensen *et al.*, 2002; Lammers *et al.*, 2003; Rakoff-Nahoum *et al.*, 2004). The outcome of the production of Th2 cytokines is the development of B cells and the immunoglobulin isotype switching required for the production of antibodies. The production of the mucosal IgA response is dependent on other cytokines, such as transforming growth factor (Lebman and Edmiston, 1999). Importantly, various species and strains of lactobacilli are able to induce the production of transforming growth factor (Blum *et al.*, 2002).

#### **2.14.4 Criteria for selection of probiotics in the poultry industry**

The probiotic must fulfill the following conditions: it must be a normal inhabitant of the gut, and it must be able to adhere to the intestinal epithelium to overcome potential hurdles, such as the low pH of the stomach, the presence of bile acids in the intestines, and the competition against other micro-organisms in the gastro-intestinal tract (Nurmi *et al.*, 1983; Chateau *et al.*, 1993). Many *in vitro* assays have been developed for the pre-selection of probiotic strains (Morelli, 2000; Ehrmann *et al.*, 2002; Koenen *et al.*, 2004). The competitiveness of the most promising strains selected by *in vitro* assays was evaluated *in vivo* for monitoring of their persistence in chickens (Garriga *et al.*, 1998). In addition, potential probiotics must exert its beneficial effects (enhanced nutrition and increased immune response) in the host. Finally, the probiotic must be viable under normal storage conditions and technologically suitable for industrial processes (lyophilized).

#### **2.14.5 Evaluating probiotic effects on growth performance**

It is clearly evident from the result of Kabir *et al.* (2004) that the live weight gains were significantly higher in experimental birds as compared to control ones at all levels during the period of 2<sup>nd</sup>, 4<sup>th</sup>, 5<sup>th</sup> and 6<sup>th</sup> weeks of age, both in vaccinated and non-vaccinated birds. This result is in agreement with 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. Lan *et al.* (2003) found higher weight gains in broilers subjected to two probiotic species. Huang *et al.* (2004) demonstrated that inactivated probiotics, disrupted by a high-pressure homogenizer, have positive effects on the production performance of broiler chickens when used at certain concentrations. In addition, Torres-Rodriguez *et al.* (2007) reported that administration of

the selected probiotic (FM-B 11) to turkeys increased the average daily gain and market body weight. However, Karaoglu and Durdag (2005) used *Saccharomyces cerevisiae* as a dietary probiotic to assess performance and found no overall weight gain difference in poultry.

Kabir *et al.*, (2004) reported the occurrence of a significantly higher carcass yield in broiler chicks fed with the probiotics on the 2nd, 4th and 6th week of age both in vaccinated and non-vaccinated birds. Mahajan *et al.* (1999) recorded that mean values of goblets, hot dress weight, cold dress weight and dressing percentage were significantly higher for probiotic (Lacto-Sacc) fed broilers. Mutus *et al.* (2006) found that tibiotarsi weight, length, and weight/length index, robusticity index, diaphysis diameter, modulus of elasticity, yield stress parameters, and percentage calcium contents were not affected by the dietary supplementation with probiotic when given to the birds at appropriate dose, whereas thickness of the medial and lateral wall of the tibia, tibiotarsal index, percentage of ash, and phosphorus contents were significantly improved by the probiotic.

#### **2.15.6 Evaluating probiotic effects on immune response**

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

injected at 7 and 14 days of age. In addition, Haghghi *et al.* (2005) demonstrated that administration of probiotics enhances serum and intestinal natural antibodies to several foreign antigens in chickens. On the other hand, Dalloul *et al.* (2005) examined the effects of feeding a *Lactobacillus*-based probiotic on the intestinal immune responses of broiler chickens over the course of an *E. acervulina* infection and they demonstrated that the probiotic continued to afford some measure of protection through immune modulation despite a fairly overwhelming dose of *E. acervulina*. Brisbin *et al.* (2008) investigated spatial and temporal expression of immune system genes in chicken caecal tonsils and spleen mononuclear cells in response to structural constituents of *L. acidophilus* and found that caecal tonsils cells responded more rapidly than spleen cells to the bacterial stimuli. They also discovered that in both splenocytes and caecal tonsils cells, STAT2 and STAT4 genes were highly induced and the expression of STAT2, STAT4, IL-18, MyD88, IFN-alpha, and IFN-gamma genes were up-regulated in cecal tonsil cells after treatment with *L. acidophilus* DNA. On the other hand, Midiilli *et al.* (2008) showed the ineffectiveness of additive supplementation of probiotics on systemic IgG.

## **2.16 Haematology of Birds**

In evaluating the health of a bird, the physiological and biological parameters commonly used include haematological examinations that check the haemoglobin and haematocrit (for the evaluation of anaemia), the rate of leucocytes or white blood cells (infection indicators) and the fraction of heterophils, lymphocytes (stress indicator), as well as weight and morphometric measures (Děbora *et al.*, 2011). In avian species, the normal values of cellular proportions may differ among species and may also vary between free-living and captive birds (Ewenson *et al.*, 2001; Campbell and Ellis, 2007; Davis, 2009). Blood

components may also be influenced by physiological factors, such as age and species, and by pathological factors (Szabo *et al.*, 2005; Lloyd and Gbson, 2006).

### **2.16.1 Packed cell volume**

In situations where anaemia is suspected, a blood sample should be collected and the PCV and RBC morphology should be evaluated. In general, anaemic birds are defined as having a PCV of less than 35% (Thrall, 2004). According to the work done by Polo *et al.*, (1998), many psittacines however normal PCV is greater than 45%, therefore references ranges for individual species should be consulted.

### **2.16.2 Erythrocytes**

#### *2.16.2.1 Erythrocytes*

Total erythrocyte concentration, packed cell volume (PCV), and haemoglobin concentration (Hb) may be influenced by age, gender, hormones and other factors. Packed cell volume and total erythrocyte count tend to be higher in male birds than in female birds and also tend to increase with age (Herbert *et al.*, 1989). The normal PCV for many bird species ranges approximately between 20% and 40% (Abdu, 2014). The mean corpuscular values (RBC indices) are blood that 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 the amount of haemoglobin relative to the size of the cell (Hb) per red blood cell (MCHC) can be calculated once the red blood cell count, PCV, and Hb have been obtained using the standard formulae. These RBC measures are used to diagnose the presence of anaemia and the types 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. This is reflected in the peripheral blood film by two distinct populations of RBCs, immature cells (that is metarubricytes and polychromatic erythrocytes) and old mature cells with pyknotic nuclei in the blood films from lower vertebrate patients, it is relevant to take note of the number of immature erythrocytes. Animals that are responding to anaemia may exhibit an increase in the number of immature erythrocytes in the peripheral blood film (Campbell, 2004b).

#### *2.16.2.2 Polychromasia*

Polychromatic erythrocytes are often seen in the peripheral blood films of normal birds and these cells usually represent five per cent or less of the erythrocyte population. The degree of polychromasia is a good indicator of the erythrocytic regenerative response. In birds, the degree of polychromasia can be evaluated based upon the average number of polychromatic erythrocytes per 1,000 x monolayer field (Campbell, 2004a; Thrall, 2004). A slight degree of polychromasia (1+) is represented by 2 – 10 polychromatic cells per 1,000 x monolayer field which is a common finding in health birds; one reference interval for health psittacines reports that polychromatophils comprised 0.41% to 6.78% of all erythrocytes (Johns *et al.*, 2008). A mild polychromasia (2+) is indicated by 11 – 14 polychromatic cells per 1,000 x monolayer field. A moderate (3+) and marked (4+) polychromasia are represented by 15 – 30 and greater than 30 polychromatic erythrocytes per 1,000 x monolayer field, respectively. For example, anaemic birds showing ten per cent or greater polychromasia (3+ or 4+ polychromasia) are demonstrating a good regenerative response to their anaemia. Increases in more immature RBCs have been seen with marked regenerative responses in

birds. Lead poisoning of birds can cause an increase in more immature erythrocytes without evidence of anaemia (Campbell, 2004a; Campbell and Ellis, 2007).

#### *2.16.2.3 Hypochromasia*

Hypochromasia, or decreased amount of staining haemoglobin in an erythrocyte, is associated with several disease conditions in birds, including acute blood loss and inflammation (Christopher *et al.*, 2004). Hypochromasia in mammalian erythrocytes is a finding commonly linked to iron deficiency and poorly regenerative anaemia, and similar findings have been reported in birds (Campbell and Ellis, 2007). Inflammation causes redistribution of body iron stores, reducing iron available for erythropoiesis and resulting in functional iron deficiency. Acute blood loss and nutritional iron deficiency can cause hypochromic, poorly regenerative, or non-regenerative anaemia in birds because of absolute iron deficiency. Hypochromasia is also reported with lead and zinc toxicosis in birds. Experimentally, induced haemolytic anaemia attributable to zinc toxicosis in mallards resulted in poorly regenerative anaemia with high percentages of hypochromic cells in birds that died or were euthanised as a result of severe clinical disease (Christopher *et al.*, 2004). The degree of hypochromasia can be rated based upon the average number of hypochromatic erythrocytes per 1,000 X monolayer field where a 1+, 2+, 3+ and 4+ hypochromasia is represented by 1-2, 3-5, 6-10, and greater than 10 hypochromatic cells, respectively (Campbell, 2004b).

#### *2.16.2.4 Poikilocytosis*

Poikilocytosis, particularly fusiform erythrocytes, and erythrocyte nuclear abnormalities have been reported in mallards that experimentally suffered lead and zinc toxicosis.

However, surviving birds from the experiment had an increased percentage of polychromatophils, indicating a regenerative anaemia, and significantly lower levels of poikilocytosis. The impaired regenerative response in the more severely affected birds suggests functional iron deficiency as a cause of decreased erythropoiesis and is compatible with evidence in birds and mammals that excess ingested zinc impairs iron absorption and use (Storey and Greger, 1987; Pimental *et al.*, 1992). Zinc and lead toxicosis can cause regenerative haemolytic anaemia, impaired haeme synthesis, hypochromasia, and a shortened erythrocyte life span in birds, although one clinical report suggests that haemolysis does not occur in zinc-intoxicated birds (Romagnano *et al.*, 1995; Christopher *et al.*, 2004). In early lead toxicosis, hypochromic erythrocytes are described as having cytoplasmic ballooning, sometimes described as “D cells” when eccentric (Fudge, 2000; Christopher *et al.*, 2004). The degree of poikilocytosis is also based upon the average number of abnormal cells in a 1,000X monolayer field. A 1+, 2+, 3+, and 4+ poikilocytosis is indicated by 5-10, 11-20, 21-50, and greater than 50 abnormal erythrocytes, respectively per 1,000X monolayer field (Campbell, 2004a).

#### 2.16.2.5 Anisocytosis

Prominent anisocytosis may be seen with regenerative anaemia or with dyserythropoiesis, however, and was seen in blood smears from marine birds exposed to crude oil (Leighton, 1985). The degree of anisocytosis in birds can also be rated on a similar scale. A 1+ and 2+ anisocytosis are indicated by an average of 5-10 and 11-20 erythrocytes that vary in size per 1,000X monolayer field, respectively. A 3+ and 4+ anisocytosis is based upon an average of 21-30 and greater than 30 erythrocytes that vary in size per 1,000X monolayer field (Campbell, 2004a).

#### 2.16.2.6 Avian anaemia

Some classical clinical signs of anaemia obvious in birds include weakness, lethargy, collapse and respiratory signs. In addition, on physical examination findings that lead to a suspicion of anaemia include pale oral or cloacal mucous membranes, decreased cutaneous ulnar vein size, poor peripheral arterial pulses, tachycardia and a physiologic heart murmur. There may be obvious signs of blood loss, such as trauma, broken blood feathers, bruising, melena or hematochezia. There may not be an obvious cause for the anaemia, however, an additional diagnostics must be performed (Mitchell and Johns, 2008).

It is important to determine whether the anemia is regenerative or non-regenerative. As mentioned previously, a reticulocyte count is the best method for determining regeneration (Johns *et al.*, 2008). In the absence of this technique regeneration can be estimated using the degree of polychromasia because a small amount of polychromasia is normal in the absence of anemia, moderate or higher polychromasia would be expected with a regenerative anemia. Differentials for regenerative anemia include acute blood loss or haemolysis. Acute blood loss such as is caused by trauma, gastro-intestinal (GI) bleeding (for example, parasitism, GI ulceration, GI neoplasia) or coagulopathy (for example, rodenticides, erythremic myelosis syndrome, secondary to aflatoxicosis), is the most common cause of regenerative anemia in birds (Campbell, 1994; Goodman, 1996).

In birds, haemolytic anaemia has also been reported. Some common causes include haemoparasites, septicaemia (that is salmonellosis), toxins (for example, lead, zinc, petroleum products), and immune mediated haemolytic anaemia (Leighton, 1985; Ochiai *et al.*, 1993; Campbell, 1994; Jones *et al.*, 2002; Johnson *et al.*, 2007). The differential

diagnoses for non-regenerative anaemia include anaemia of chronic disease (especially chlamydophilosis, mycobacteriosis, aspergillosis, West Nile virus, or neoplasia), toxicity (e.g lead toxicosis, aflatoxicosis), iron deficiency, hypothyroidism, and leukaemia (Newell *et al.*, 1991; Campbell, 1994; Tell *et al.*, 2001; Joyner *et al.*, 2006; Campbell and Ellis, 2007).

#### *2.16.2.7 Avian polycythaemia*

Polycythemia refers to an increase in the PCV and RBC count. It is an uncommon finding in birds. Generally, polycythaemia in birds is defined as a PCV greater than 70% (Campbell and Ellis, 2007). Polycythemia is divided into two categories: absolute and relative. Relative polycythaemia results from dehydration and loss of plasma volume. Relative polycythaemia can be corrected by rehydrating the bird and treating the cause of the dehydration. Absolute polycythaemia on the other hand can be further divided into two distinct categories: primary polycythaemia and secondary polycythemia (Campbell and Ellis, 2007). Primary polycythaemia, or polycythaemia vera, is a rare finding in birds but can occur (Campbell and Ellis, 2007). This condition is caused by a myeloproliferative disorder that results in an increase in erythrocytosis. Secondary polycythaemia occurs as a result of an increased need for tissue oxygenation or because of an increase in the production of erythropoietin. Some common diseases that lead to secondary polycythaemia include chronic pulmonary disease, adaptation to high altitude, cardiac disease, iron storage disease, rickets, renal disease or neoplasia leading to increased production of erythropoietin (Samour, 2006; Campbell and Ellis, 2007). 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).

### **2.16.3 Leucocytes**

#### *2.16.3.1 Lymphocytes*

Reactive lymphocytes are small to medium sized with densely clumped chromatin and intensely basophilic cytoplasm. Nucleoli are usually absent, and a pale Golgi zone and vacuolation may be present. Reactive lymphocytes are usually observed in small numbers in peripheral blood smears of healthy birds (Mitchell and Johns, 2008). However, an increased presence of reactive lymphocytes is commonly observed in birds that have infectious disease (Mitchell and Johns, 2008). Blast transformed lymphocytes which are large lymphocytes with smooth dispersed chromatin and may occasionally be observed in avian blood smears. There is abundant blue cytoplasm, and there is often a prominent Golgi zone, the cells are neoplastic, indicating lymphoid leukemia or a leukemic phase of lymphoma, but can also be seen as a result of immunologic stimulation (Mitchell and Johns, 2008). Lymphocytosis usually occurs as result of antigenic stimulation. This can occur in Psittacine birds with viral disease, such as herpes virus or psittacine circovirus (Fudge and Joseph, 2000). This result is in consistent as the same viral diseases may instead result in heterophilia or leukemia (Fudge and Joseph, 2000; Schoemaker *et al.*, 2000). Lymphocytosis also occurs with wound healing, inflammatory diseases, parasitic infections, and viral diseases (Campbell, 2004a) and with lymphoid leukemia (Latimer, 1994). Anaemia and thrombocytopenia may also be present in birds with lymphocytic leukemia (Latimer, 1994). Lymphocytic leukemia's are rare in birds in comparison to lymphosarcoma (Newell *et al.*, 1991). Lymphopenia may be seen as a result of excess endogenous or exogenous corticosteroids (Harmon, 1998) as observed in stressful conditions and malnourished birds (Campbell, 2004b).

### 2.16.3.2 Monocytes

Increased monocyte in peripheral circulation (monocytosis) often occurs in infectious and/or inflammatory disease, especially with granulomatous diseases such as aspergillosis or mycobacteriosis. *Chlamydophila psittaci* infections in birds also often results in monocytosis usually due to the production of chemotactic agents that attract monocytes (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 lymphopenia (Branton *et al.*, 1997). Monocytosis has also been observed in birds fed a zinc-deficient diet (Wight *et al.*, 1980).

### 2.16.3.3 Heterophils

Heterophils are the most common granulocytes in circulation in the majority of birds (Mitchell and Johns, 2008; Claver and Quaglia, 2009). The response of heterophils to infections is similar to that of the mammalian neutrophil, migrating to the sites of inflammation caused by any offending pathogen, hence, killing it (Vegad *et al.*, 1995). Owing to their highly phagolytic activities, they are capable of a broad spectrum antimicrobial activity (Claver and Quaglia, 2009). The avian heterophil contains many lysosomal and non-lysosomal enzymes (Maxwell *et al.*, 1998) that function in phagocytosis and destruction of bacterial organisms (unlike the mammalian neutrophils) which function in phagolysosomal killing and depend primarily on non-oxidative mechanisms, lysosome, and acid phosphatase for antimicrobial activity (Andreasen *et al.*, 1990). 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 the peripheral blood, representing recruitment of

cells from the bone marrow in response to cytokines and other inflammatory mediators (Campbell and Ellis, 2007).

Band heterophils are identified in peripheral blood smears in the first 12-24 h after the initial insult with persistence of leukocytosis for seven days (Latimer *et al.*, 1988). However, the presence of immature heterophils in avian blood smears indicates acute inflammation. 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). Typically, when immature cells are found in the peripheral blood, normal appearing mature heterophils can be found (Campbell, 2004). The other change observed in avian heterophils during disease is toxic change which is similar to those observed in mammalian neutrophils. 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 par acute stage or is resolving (Campbell, 2004). Toxic heterophils are classified on a scale +1, +2, +3 and +4 depending upon the degree of toxicity. A +1 toxic heterophil shows increased cytoplasmic basophilia. A +2 toxic heterophil shows increased cytoplasmic basophilia, slightly abnormal granulation (i.e partial degranulation, coalescing granules, or abnormal appearing granules or vacuolation. A +3 toxic heterophil will show changes that are more severe than +2 toxicity and the nucleus may show slight karyorrhexis or karyolysis. A +4 toxic heterophil will show marked changes in the cytoplasm and nucleus. Toxic heterophils are uncommon and usually seen in birds that are critically ill (Campbell, 2004).

Avian heterophils are involved in controlling bacterial, viral and parasitic infections. Conditions that cause an increase in the heterophils include infection (e.g. bacterial, fungal, viral and parasitic), inflammatory stress, certain toxicities, trauma, and leukemia (Gildersleeve *et al.*, 1987; Andreassen *et al.*, 1993; Harmon, 1998; Bienzle *et al.*, 1999).

Infectious agents that commonly lead to heterophilia include *Mycobacterium* species, *Chlamydophila psittaci*, *Aspergillus* species and birds acutely or chronically infected with *Mycoplasma* species. Heterophilia associated with these organisms is commonly 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 often carries a poor prognosis (Campbell *et al.*, 2007). Some toxic substances (for example, an organophosphate) can lead to heterophilia (Heatley *et al.*, 2000).

In addition, heterophilia has been observed in cases of zinc toxicosis, presumably as a result of GI inflammation, stress, and decreased resistance to pathogens (Campbell *et al.*, 2007). Macaws may demonstrate marked leukocytosis with heterophilia as a result of transport and handling. Corticosteroid administration results in an increase in circulating heterophils and lymphopenia in birds (Harmon, 1998). A decrease in heterophil number can be seen with increased use of cells or decreased production. Utilisation of acute inflammation (Latimer *et al.*, 1988). Overwhelming infection such as septicaemia can lead to a degenerative left shift which indicates bone marrow depletion. Psittacine circovirus can lead to leukopenia with pancytopenia in African Gray parrot (Schoemaker *et al.*, 2000).

#### 2.16.3.4 Basophils

Basophils appear to play important role in the initial phases of acute inflammation and immediate hypersensitivity reactions, but differ from those in mammals by not contributing to delayed hypersensitivity. This, however, does not always result in peripheral basophilia (Montali, 1988; D'Aloia et al., 1994; Maxwell et al., 1995; Campbell and Ellis, 2007). The granules of avian basophils contain histamine, as in mammals (Campbell and Ellis, 2007). Therefore, it is suggested that they function in acute inflammatory and type 1V hypersensitivity reactions, similar to mammalian basophils and mast cells (Mitchell and Johns, 2008). Severe stress has also been proposed as an underlying cause for an increased basophilic response in birds (Maxwell, 1993; Altan *et al.*, 2003; Bedáňová *et al.*, 2007; Campbell and Ellis, 2007).

Nevertheless, there is some clear evidence suggesting that the proportion of basophils among blood-borne leukocytes is much higher in some avian species than the normal physiological values of most mammals (Maxwell *et al.*, 1995). A good example of natural variability in basophil counts was given by Friedl and Edler (2005), who found that the percentage of basophils ranges from 0 to 24% in the Red Bishop (*Euplectes orix*). High levels of basophils in peripheral blood were also reported in some other passerine species (e.g *Pine siskin*, *Carduelis pinus*, or *Pied flycatcher*, *Ficedula hypoleuca*) (Davis 2009). Even in non-passerine such as *Puna ibis* (*Plegadis ridgewayi*) (Coke *et al.*, 2004), the common pheasant (*Phasianus colchicus*) (Lucas and Jamroz, 1961), and some strains of the domestic chicken (Maxwell *et al.*, 1995), normal basophil counts in adults may exceed 10%. Much lower basophil counts (0-5%) were detected in most finches, other than Scarlet Rose finches (Campbell and Ellis, 2007; Davis, 2009). Nevertheless, in the house finch

(*Carpodacus mexicanus*), a species that is closely related to the Scarlet Rose finch, the basophil granulocytes are more frequent than heterophils in the peripheral blood of free-living individuals (Davis *et al.*, 2004; Davis, 2005).

#### 2.16.3.5 Eosinophils

Cytochemically, the avian eosinophil granules, similar to mammalian counterparts contain peroxidase and high concentration of arginine (Montali, 1988; Andreason *et al.*, 1990; Campbell *et al.*, 2007). Additionally, certain hydrolytic (enzymes) including acid phosphatase and aryl sulphatase have been detected in avian eosinophilic granules, supporting the theory that the structures are lysosomal in nature (Andreason *et al.*, 1990; Montali, 1998; Latimer *et al.*, 2000; Campbell *et al.*, 2007).

In mammals, eosinophils are modulators of immediate hypersensitivity and suppress parasitic infection. Some have shown a limited association between eosinophils and nematode infection in grouse and water fowls (Maxwell *et al.*, 1985). Eosinophilia has been observed after foreign antigen administration and possibly in association with alimentary tract parasitism (Montali, 1988; Latimer *et al.*, 2000), though parasite antigens do not generally induce eosinophilia in birds (Montali, 1988). Other studies have shown eosinophilia with generalized inflammation in birds. It is possible that avian eosinophils play a role in delayed hypersensitivity, but they have been shown to be related to anaphylaxis or other acute hypersensitivity reactions (Montali, 1988). Severe eosinophilias have also been observed in cases of poxvirus infection in red-tailed hawks, although the mechanism of this response is unknown (Mitchell and Johns, 2008).

#### 2.16.3.6 Heterophil-to-lymphocyte (H/L) 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), as well as infection status for some disease (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; Bedáňová *et al.*, 2007; Davis *et al.*, 2008).

#### 2.16.4 Thrombocytes

. An increased number of immature thrombocytes are seen in birds responding to thrombocytopenia (Campbell, 2004a). Thrombocytosis and an increased in the size of thrombocytes may be seen with chronic inflammation in birds (D'Aloia *et al.*, 1994). Similar to the mammalian platelets, avian thrombocytes produce thromboplastin and they 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 probable have some functions in non-specific immunity (Edmonds, 1968; Gracchi *et al.*, 1980; Bounous and Stedman, 2000). Increased destruction or use of thrombocytes in conditions such as septicaemia or possibly disseminated intravascular coagulopathy (DIC), thrombocytopenia can be seen. Thrombocytopenia can be seen as a component of pancytopenia in some viral diseases, such as psittacine circovirus or polyomavirus infections (Fudge, 1997) and also in cases of lymphoid leukemia (Latimer, 1994).

## **2.17 Antioxidant Enzymes**

### **2.17.1 Oxidative stress**

In normal metazoan cells, there exist a delicate balance between the amount of free radicals and antioxidant defence molecules present in the cell for optimum functioning (Ostun *et al.*, 2014). Oxidative stress is a condition in which reactive oxygen species (ROS) are generated extra-ordinary intracellular and exert toxic effects on cells (Misra *et al.*, 2009). It can be defined as an imbalance between ROS production and/or impaired ROS metabolism that favours them being present in excess of physiological levels (Chrissobolis *et al.*, 2010), or an imbalance between oxidants/antioxidants because of excess oxidant and/or reduced antioxidant capacities, resulting in tissue or organ injuries (Ni *et al.*, 2013; Zeng *et al.*, 2013). It is simply a derangement in the balance between cellular oxidant species production and antioxidant capability, in favour of the oxidants (Elias *et al.*, 2013; Liu *et al.*, 2014), resulting in damage. Assay of antioxidant enzymes activity have, therefore, been employed by many investigators as indices of oxidative stress (Ambali *et al.*, 2011b, c; 2012a, b, c; Ambali and Ayo, 2012; Singh *et al.*, 2013b; Akande *et al.*, 2014; Idris, *et al.*, 2014).

### **2.17.2 Antioxidants**

Antioxidants are compounds which act as inhibitors of the process of oxidation (Naik, 2003). They are substances which, when present at low concentration in relation to oxidizable substrates, significantly inhibit or delay oxidative processes, while often being oxidized themselves (Kumar, 2011). Several types of antioxidants are known, some are synthesized endogenously while most are absorbed from food and supplements (Tewari *et al.*, 2014).

### **2.17.3 Types of antioxidants**

Antioxidants are classified based on two main criteria: their source and their mode of action (that is, mechanism of antioxidant defence). Based on source, antioxidants are classified into natural antioxidants and artificial antioxidants (Mishra and Bisht, 2001). Natural antioxidants occur in nature and in many cases are derived from plant sources. They are mostly phenolic compounds such as flavonoids and phenolic acid, vitamins and volatile compounds found in different fruits, plants, herbs and species (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).

### **2.17.4 Antioxidant defence system**

Physiologically, several cells contain certain endogenous compounds and enzymes (antioxidants) that act as scavengers of ROS “mopping” them “up” before they cause extensive damage to biomolecules and tissues and terminating radical chain reactions. These endogenous antioxidants make up the antioxidant defence system of the organism and are crucial for the survival of all aerobic organisms (Birben *et al.*, 2012; Sachdeva *et al.*, 2012). The antioxidant defence system is a complex mechanism involving many enzymes and substances which plays an important role in the maintenance of cellular homeostasis. The system consists two groups: the antioxidant enzymes such as SOD, CAT and GPx; and the antioxidant molecules such as glutathione, ascorbic acid,  $\alpha$ -tocopherol and melatonin, which are small molecular weight molecules that directly scavenge free radicals and their reactants (Rodríguez *et al.*, 2004; Ognjanovic *et al.*, 2008; Liu *et al.*, 2012; Shafaq, 2012, Reiter *et al.*, 2013). Antioxidant enzymes are the first line of defence against free radicals. They possess a transition metal at their core which takes different valences as they

transfer electrons during ROS detoxification (Burton and Jauniaux, 2011). Superoxide dismutase catalyses the conversion of superoxide radical into  $H_2O_2$  and  $O_2$  (Verma, 2014). Catalase (CAT) on the other hand catalyzes the reduction of  $H_2O_2$  to water and the reduction of other organic hydroperoxides (Naziroglu, 2012). Glutathione peroxidase catalyses reduction of  $H_2O_2$  and a variety of hydroperoxides using reduced glutathione, forming oxidized glutathione (Ferreira *et al.*, 2013).

Antioxidant defences refer to various mechanisms by which living cells are protected from the several deleterious effects of ROS. In principle, protection against such effects can be by prevention, interception and repair (Sies, 1993). Based on their principal mechanism, antioxidant defences are classified into:

- 1 Primary or chain-breaking antioxidants: also called scavenger antioxidants, they neutralize free radicals by donating one of their own electrons, ending the electron “stealing” reaction, and terminating radical chain reactions (Sies, 1993; Bhalerao *et al.*, 2011).
- 2 Secondary or preventive antioxidants: These act through various means to prevent the formation of free radicals. They act through mechanisms like sequestration of transition metal ions to prevent metal catalyzed reactions; removal of peroxides (that can react with transition of metal ions to produce ROS) by catalase peroxides and GPx, as well as removal of ROS (Celikyurt, 2011).
- 3 Tertiary antioxidants defences: The repair processes, which remove damaged biomolecules before they can accumulate and before their presence, result in altered

cell metabolism and viability. Examples include lipases, proteases, transferases, and DNA repair enzymes (Townsend *et al.*, 2010; Celikyurt, 2011).

### **2.17.5 Mode of action of antioxidants**

Antioxidants bind to and sequester reactive oxygen species (ROS) and transition of metal ions, such as iron and copper, which contain unpaired electrons and strongly 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: for example,  $\alpha$ -tocopherol act in lipid phase by donating electrons to form tocopheroxyl radical (which is relatively safe), thereby terminating the chain reaction.
- 2 By reducing concentration of ROS; for example, 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).

## **CHAPTER THREE**

### **MATERIALS AND METHODS**

#### **3.1 Study Location**

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°42'E and latitude 11°3'N with an altitude of 550-700 metres above the sea level and a total land mass of about 300 square kilometers (Clackson, 1957). Zaria is characterised by a tropical climate, a monthly mean ambient temperature, ranging between 13.8 and 36.7°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).

#### **3.2 Materials**

##### **3.2.1 Ethical clearance**

Ethical clearance was sought from the ABU Committee on Animal Use and Care.

##### **3.2.2 Experimental chickens**

Two hundred ISA Brown day-old pullet chicks were purchased from a commercial hatchery in Ibadan, Nigeria.

##### **3.2.3 Housing and feeding**

The chicks were managed intensively in deep litter system with a floor space between 0.024 to 0.10 square metres per bird after thorough washing of the pen with detergent and disinfecting with Diskol<sup>®</sup> (5% benzalkonium chloride, 7.5% glutaraldehyde, 7.5%

formaldehyde, stabilisers and antioxidants 9.5%). Rodent and insect control was achieved using rodenticide push-out<sup>®</sup> (Zinc phosphide 80%) and insecticide DD Force<sup>®</sup> (Diclofos-Organophosphorous), respectively twice one week apart before stocking the pens in the Animal Research Pen of the Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. The chickens were given access to feed and water *ad libitum* for a period of 6 weeks.

### **3.2.4 Feed**

Chick mash was purchased from a commercial feed distributor (Hybrid<sup>®</sup>) in Zaria, Nigeria and proximate analyses were carried out at the Department of Animal Science Laboratory, Ahmadu Bello University Zaria. The feed had the following nutrients: % DM 97.20, % ASH 13.96, % EE 7.41, % CF 6.49, % N 3.60, % CP 22.50 which fit for the requirement.

### **3.2.5 Sources of Probiotics**

Bactofort<sup>®</sup>, containing *Lactobacillus acidophilus* ( $77 \times 10^9$  cfu/kg), *Enterococcus faecium* ( $44 \times 10^9$  cfu/kg), *Saccharomyces cerevisiae* ( $5000 \times 10^9$  cells/kg), and *Bacillus subtilis* ( $2.2 \times 10^9$  cfu/kg) in powder, manufactured by Biofeed Technology Inc., Brossard, QC, Canada, and obtained in Nigeria from Adamore PLC, Kaduna, Nigeria and Antox<sup>®</sup>, containing *Saccharomyces cerevisiae* ( $4.125 \times 10^6$  cfu/mL), Citric acid (6 g), Lactic acid (2 g), Vitamin B<sub>1</sub> (100 mg), Vitamin B<sub>2</sub> (7.5 mg), Vitamin B<sub>6</sub> (80 mg), Vitamin B<sub>12</sub> (0.6 mg), Biotin (1.5 mg), Nicotinamide (1 g), Calcium chlorine (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), in liquid, manufactured by Montajat Pharmaceuticals, Bioscience Division, Dammam 31491, Saudi Arabia were used.

### **3.2.6 Challenge virus**

A characterised vIBDV was obtained from the Department of Veterinary Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria.

## **3.3 Methods**

### **3.3.1 Experimental design for comparing the efficacy of Antox<sup>®</sup> and Bactofort<sup>®</sup> on clinico-pathological changes in ISA Brown chicks inoculated with virulent infectious bursal disease virus**

Two hundred, day old chicks were randomly divided in to four groups A, B, C and D consisting of 50 chicks each. Group A was administered Anthox<sup>®</sup> at the dose rate of 1.5 ml/litre of drinking water from day old to 6 weeks of age and inoculated at 4 weeks of age with vvIBDV at the rate of 0.05 mL/bird intra-ocular, while group B were administered Bactofort<sup>®</sup> at the dose rate of 12.5 g/25 kg of feed from day old to 6 weeks of age and inoculated at 4 weeks of age with vvIBDV. Birds in group C were inoculated with vIBDV at 4 weeks of age and no probiotics was administered. Group D were not treated with probiotics and inoculated with vIBDV. On days 0, 1, 2, 3, 4, 5, and 6 weeks of age of pre-treatment, treatment with probiotics and post-inoculation, respectively, five chicks from each group were bled, sacrificed and their BD, BF, SPL and THY harvested and weighed to evaluate for IBD antibodies, haematological parameters, activities of antioxidant enzymes and histopathological changes. Post-inoculation at 4 weeks with vvIBDV, the birds were

observed for clinical signs, morbidity, mortality and gross pathological lesions of IBD (Table 3.1).

**Table 3.1: Experimental design for comparing the efficacy of Antox<sup>®</sup> and Bactofort<sup>®</sup> on clinico-pathological changes in ISA Brown chicks inoculated with virulent infectious bursal disease virus.**

| Group | No. of birds | Treatment with probiotics + inoculation with vvIBDV           | Age of commencement of treatment with probiotics | Age of inoculation with vvIBDV | Treatment/Inoculation Weeks |    |    |    |    |    |    | Organs weighed and collected for histopathology |
|-------|--------------|---------------------------------------------------------------|--------------------------------------------------|--------------------------------|-----------------------------|----|----|----|----|----|----|-------------------------------------------------|
|       |              |                                                               |                                                  |                                | 0                           | 1  | 2  | 3  | 4  | 5  | 6  |                                                 |
| A     | 50           | a (1.5 mL/litre of water) + vvIBDV (0.05 mL/bird intraocular) | 1-day-old                                        | 28 dph                         | 5                           | 5  | 5  | 5  | 5  | 5  | 3  | BF, spleen & thymus                             |
| B     | 50           | b (12.5 g/25 kg of feed) + vvIBDV (0.05 mL/bird intraocular)  | 1-day-old                                        | 28 dph                         | 5                           | 5  | 5  | 5  | 5  | 5  | 1  | BF, spleen & thymus                             |
| C     | 50           | zp + vvIBDV (0.05 mL/bird intraocular)                        | Nil                                              | 28 dph                         | 5                           | 5  | 5  | 5  | 5  | 5  | 2  | BF, spleen & thymus                             |
| D     | 50           | zp + ow                                                       | Nil                                              | Nil                            | 5                           | 5  | 5  | 5  | 5  | 5  | 5  | BF, spleen & thymus                             |
| Total | 200          |                                                               |                                                  |                                | 20                          | 20 | 20 | 20 | 20 | 20 | 11 |                                                 |

KEY: BF = Bursa of Fabricius, dph = Days post hatch, vvIBDV = very virulent infectious bursal disease virus, zp = Zero probiotics, ow = Ordinary water, a = Antox<sup>®</sup> Probiotic , b = Bactofort<sup>®</sup> Probiotic

### **3.3.2 Inoculation of chicks with infectious bursal disease virus**

Each of the chicks per group was inoculated with 0.05 ml of the vIBDV suspension via conjunctival instillation at 28 days of age.

### **3.3.3 Collection of blood**

One to two millilitres of blood were collected through the heart from each bird using a plastic disposable 5 mL syringe and 25 x 7 mm gauge needle from day-old to six weeks of age at weekly interval. The blood was emptied into plain and heparinised universal bottles to obtain a serum and whole blood, respectively.

The blood samples collected in the plain universal bottle were allowed to clot at room temperature (25-26 °C, then centrifuged at 1,200 x g for 15 min using a Hermle® centrifuge (Model No.: Z 364 B. HERMLE GmbH & CO., Germany) to obtain sera, which were harvested into clean plastic sample bottles, labeled and stored in a freezer until examined for IBD antibodies, the activities of antioxidant enzymes and oxidative stress biomarkers while the whole blood samples were processed for haematology.

### **3.3.4 Determination of packed cell volume**

The packed cell volume (PCV) was determined using standard technique as described by Rehman *et al.* (2003). Non-heparinised capillary tube was filled up to about  $\frac{3}{4}$  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 machine. Then the proportion of cells in the total

volume of blood was measured and recorded as a percentage using the Hawksley® Micro-haematocrit Reader.

### **3.3.5 Determination of haemoglobin concentration**

Blood haemoglobin concentration was assayed colorimetrically as cyanomethhaemoglobin (Drakin, 1945). Five millilitre of HICN (Drabkin) reagent was measured using a 5 ml syringe into plastic test tubes. Twenty microlitre of blood were measured using a micropipette and added to the Drabkin solution in the test tube and properly mixed by gently shaking the test tube (Appendix 3). The serum was centrifuged at  $1,509 \times g$  for 15 min so as to separate the empty RBCs from interfering with the reading. The supernatant was separated into a sample bottle. The mixture was absorbed into Audiocomb Serum Auto analyser (Bayer Express Plus, Bayer Germany and Serial Number 15950). After the wiggling pump stopped working, the value displayed on the screen was recorded in g/dl as the haemoglobin concentration.

### **3.3.6 Determination of red blood cells and leucocyte counts**

Red blood cell (RBC) and total leucocyte (TWBC) counts were determined with the Natt-Herrick solution (1:200 dilutions) and the improved Neubauer haemocytometer (Campbell and Ellis, 2007) as both counts can be prepared directly from the same sample placed in the haemocytometer (Appendix 2).

The heparinised blood samples were slightly agitated and an RBC diluting pipette was used to pipette the blood to the 0.5 marking. The tip of the pipette was cleaned properly using a tissue paper without touching the distal opening of the pipette tip with tissue, as this will

cause capillary shift of blood into the tissue paper. The diluting solution (Natt-Herrick) was also prepared to the 101 marking (1:200) without entirely immersing the pipette tip into the diluting fluid. The mixture was well shaken for 1 min to obtain equal distribution then emptied into a clean sample scientific cuvette. The Neubauer haemocytometer and cover slip were cleaned using a dry, lint free cloth Appendix 4). The cover slip was properly placed on the haemocytometer the mixture was then agitated a little and a capillary tube was use to withdraw a small aliquot. Both sides of the haemocytometer were filled up (charged) by gently touching the intersection between the cover slip and haemocytometer with the loaded capillary tube avoiding air bubbles and under-filling or over-filling, then left for 5 min for cells to settle down. The light microscope (Olymopus-XSZ-107BN), at low power magnification ( $\times 40$ ) was used to view the cells and counting was done using a tally counter. For total leucocyte count, the leucocyte in the four outer large squares of the haemocytometer were counted and calculated using the formula (1):

$$N/20 = \text{WBC} \times 10^9/\text{L} \quad (1)$$

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

For RBC count, the cells contained in the four corner and central squares in the mid-section of the haemocytometer were counted. Following the “L” rule: cells that touch the center triple lines of the ruling on the left and the bottom sides were counted but cells that touch the center triple lines of the ruling on the right and the top sides were not counted. The RBC count was calculated using the formula (2):

$$N/100 = \text{RBC} \times 10^{12}/\text{L} \quad (2)$$

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.

### **3.3.7 Differential leucocyte counts**

In the entire group sampled, pair of smears for each sample was made. A small drop (about 2  $\mu$ L) of blood was immediately used for the preparation of blood smears each using the standard slide-to-slide technique. The air dried smears were properly labeled using a pencil and then fixed in 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 of 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 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 were wiped with tissue paper to remove the excess stain and allowed to air dry. These stained slides were neatly packed into a slide box until viewed (Appendix 3).

Examination of the blood smears was done using a light microscope (Olympus-XSZ-107BN) under high-power magnification with oil immersion (X 100). One hundred WBC 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 (3):

$$\frac{\text{Percentage of WBC Counted X Total WBC}}{100} = \text{Absolute Number} \times 10^9/\text{L} \quad (3)$$

### 3.3.8 Determination of erythrocytic indices

The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated using the following standard formulae (Campbell and Ellis, 2007) (4-6):

$$\text{MCV} = (\text{PCV} \times 10)/\text{RBC} = \text{MCV in femto litre (fl)} \quad (4)$$

$$\text{MCH} = (\text{Hb} \times 10)/\text{RBC} = \text{MCH in pictogram (pg)} \quad (5)$$

$$\text{MCHC} = (\text{Hb} \times 100)/\text{PCV} = \text{MCHC in grammes per litre (g/l)} \quad (6)$$

### 3.3.9 Determination of oxidative stress biomarkers

Serum samples were diluted (1:100 v/v) in phosphate buffered saline (PBS) (pH 7.3 ± 0.1) before being used for determination of the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and malondialdehyde (MDA). Standard protocol of the North West Life Science Specialist, Vancouver, Canada was used to measure the SOD, GPx, MDA concentrations (Martin *et al.*, 1987). The method for SOD assay were based on monitoring the autoxidation rate of haematoxylin as originally described by Martin *et al.* (1987), with modification to increase robustness and reliability. Catalase kits, was purchased from Abcam Plc, Cambridge, United Kingdom, and

preparation of the reagents' and the test procedure were carried out strictly according to manufacturer's instruction. The method for GPx determination was also a standard procedure for microplate assay and all the reagents were brought to room temperature. Diluted sample (50  $\mu$ L) was added to each well of microplate, 50  $\mu$ L working nicotinamide adenine dinucleotide phosphate and working H<sub>2</sub>O<sub>2</sub> (50  $\mu$ L). Then, the mixture were allowed to stay for 1 min and microplate was then placed in a plate reader and read at the wavelength of 340 nm using a spectrophotometer (Spectrumlab<sup>®</sup> 23A; Q/SEEK2: Model No.: 23A07025 Huangwei, China) (Flohê and Gunzler, 1984). The MDA concentration was assayed after mixing in a micro-centrifuge vial, containing butylated hydroxytoluene reagent, serum sample, phosphoric acid and 2-thiobarbituric acid. The mixtures were then incubated for 60 min at 60°C using a GallenRamp<sup>®</sup> incubator (Model: IH-100; Made in England), and centrifuged at 10,000 x g for 2-3 min using a Hermle<sup>®</sup> centrifuge (Model No.: Z 364, B. HERMLE GmbH & CO., Germany). The reacted mixture were transfered into a cuvette and the absorbance of the test sample was read at 548 nm using a spectrophotometer (Spectrumlab<sup>®</sup> 23A; Q/SEEK2: Model No.: 23A07025 Huangwei, China) (Botsoglou, 1994).

### **3.3.10 Enzyme-linked immunosorbent assay**

Enzyme-linked immunosorbent assays (ELISA) was carried out according to the methods described by IDEXX Laboratories, Incorporate, Westbrook, Maine 04092, USA. Briefly, the antigen coated plates and the ELISA kit reagents were adjusted to room temperature. The test sample was diluted to five hundred folds (1:500) with sample diluents. A 100  $\mu$ L of diluted serum sample was then poured into each well of the plate. This was followed by 100  $\mu$ L of undiluted negative control serum sample into well A1 and A2, 100  $\mu$ L of undiluted

positive control serum sample into well A3 and A4. The plate was incubated for 30 min at room temperature. Each well was then washed with 350  $\mu$ L of distilled water three times. Goat anti-chicken conjugate (100  $\mu$ L) was dispensed into each well. The plate was incubated at room temperature for 30 min, followed by washing each well with 350  $\mu$ L of distilled water three times. Tetramethylbenzidine (TBM) solution (100  $\mu$ L) was dispensed into each well. The plate was then incubated at room temperature for 15 min. Finally, 100  $\mu$ L of stop solution was dispensed into each well to stop the reaction. The absorbance values were measured and recorded at 650 nm using 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 or equal to 0.2 were considered negative. Ratios above 0.2 (titres higher than 396) were considered positive as indicated in the IDEXX ELISA Kit manual.

### **3.3.11 Determination of organ body weight and organ body weight ratios**

From day-old to 42 days of age, five birds from each group were randomly selected weekly and euthanised. Their body (BD), bursal Fabricius (BF), spleen (SPL) and thymus (THY) weights were taken using weighing scale. For each bird the organ weight in grammes was divided by body weight in grammes then multiplied by 1000 (Etteradossi *et al.*, 2004). To obtain the mean organ BD weight ratio per group, the organ BD weight ratio for bird in that group was added and divided by five.

### 3.3.12 Determination of morbidity and mortality rate

Morbidity recorded in different groups after inoculation with vvIBDV at the end of the experiment (6 weeks) was calculated as number of chicks sick divided by the number of chicks inoculated multiplied by one hundred percent (Babiker *et al.*, 2008) (7):

$$\text{MDR} = \frac{\text{NCD}}{\text{NCI}} \times 100 \% \quad (7)$$

Where NCD = number of chicks sick, NCI = number of chicks inoculated

Mortality recorded in different groups after challenge with vvIBDV at the end of the experiment was calculated as number of chicks dead divided by number of chicks inoculated multiplied by 100 (Babiker *et al.* (2008) (8):

$$\text{MR} = \frac{\text{NCD}}{\text{NCI}} \times 100 \% \quad (8)$$

Where NCD = number of chicks dead, NCI = number of chicks inoculated.

### 3.3.13 Histopathology

Pieces of tissue of the BF, SPL and THY, were obtained and fixed in 10% phosphate-buffered neutral formalin solution. The tissue were cut into blocks, identified and dehydrated through a series of graded alcohols (70%, 80%, 90% and 100%). Then after blocks were cleared in xylene and then infiltrated with molten paraffin wax. Sections of 5 microns ( $\mu\text{m}$ ) were cut from embedded tissue. The tissues were mounted on grease free clean glass slides and kept at room temperature then stained alternatively with Haematoxylin and Eosin (H and E). The processed and stained slides were examined using microscope at  $\times 4$ ,  $\times 10$  and  $\times 40$  power objectives less for histopathological changes (Hair-Bejo *et al.*, 2000; Babiker *et al.*, 2008).

### **3.3.14 Data analyses**

For statistical analysis Tukey's multiple comparison *post-hoc* tests, using GraphPad Prism 4.0 for windows (GraphPad Software, San Diego, California USA) were used. Data of haematological parameters, serum biochemical, antioxidant enzymes, BD, BF, SPL and THY weight, BF/BD ratio, SPL/BD ratio and THY/BD weight ratio and antibody titre level obtained were expressed as mean  $\pm$  standard error of the mean (Mean  $\pm$  SEM), Chi-Square were used to analysed proportion. Values were subjected to one-way analysis of variance (ANOVA), to compare the differences between the means, obtained from the control and treated birds. Values of  $P < 0.05$  were considered significant, while descriptive statistics was used to express the morbidity and mortality rate. Results were presented in tables.

## CHAPTER FOUR

### RESULTS

#### 4.1 Haematological Parameters of ISA Brown Chicks Treated with Antox® and Bactofort® and Inoculated with a Very Virulent Infectious Bursal Disease Virus

##### 4.1.1 Packed cell volume

PCV in groups (A and B) treated with probiotics and inoculated with vvIBDV decreased from initial mean value of  $23.40 \pm 2.11$  %,  $26.80 \pm 0.58$  at 0 dpi to  $18.30 \pm 0.37$  %,  $17.00 \pm 0.35$  % but the decrease was less severe when compared to positive control  $24.80 \pm 1.69$  % and  $15.20 \pm 0.37$  at 7 dpi respectively . There was no statistical significant difference ( $P > 0.05$ ) of PCV between Antox®, Bactofort® and positive control (Table 4.1).

##### 4.1.2 Haemoglobin concentration

The mean Hb concentration of groups (A and B) treated with probiotics and inoculated with vvIBDV at 0 dpi and 7 dpi decreased from initial mean value of  $7.7 \pm 0.71$  g/dl,  $9.02 \pm 1.19$  g/dl and  $6.16 \pm 0.14$  g/dl,  $6.00 \pm 0.27$  g/dl, respectively, when compared to positive control ( $8.26 \pm 0.56$  g/dl and  $5.20 \pm 0.25$  g/dl). There was no statistical significant difference ( $P > 0.05$ ) in the mean Hb value between Antox®, Bactofort® and positive control (Table 4.2).

##### 4.1.3 Red blood cell count

The RBC count of the vvIBDV inoculated and probiotics treated groups (A and B) decreased from  $7.55 \pm 0.62 \times 10^{12}/L$  and  $6.31 \pm 0.16 \times 10^{12}/L$  at 0 dpi to  $5.40 \pm 0.29 \times 10^{12}/L$  and  $4.10 \pm 0.43 \times 10^{12}/L$  at 7 dpi but the decreased was significantly lower when compared to positive control ( $5.48 \pm 0.63 \times 10^{12}/L$  and  $3.40 \pm 0.43 \times 10^{12}/L$ ). There was very high

statistical significant difference ( $P < 0.001$ ) in RBC count between Antox<sup>®</sup>, Bactofort<sup>®</sup> and positive control (Table 4.3).

#### **4.1.4 White blood cell count**

The total WBC count of the vvIBDV inoculated and probiotics treated groups (A and B) decreased from  $14.88 \pm 1.24 \times 10^9/L$  and  $14.58 \pm 0.32 \times 10^9/L$  at 0 dpi to  $8.90 \pm 0.29 \times 10^9/L$  and  $7.00 \pm 0.42 \times 10^9/L$  at 7 dpi when compared to positive control ( $12.70 \pm 0.54 \times 10^9/L$  and  $4.30 \pm 0.37 \times 10^9/L$ ). There was very high statistical significant difference ( $P < 0.001$ ) in RBC count between Antox<sup>®</sup>, Bactofort<sup>®</sup> and positive control (Table 4.4).

#### **4.1.5 Erythrocytic indices**

The highest MCV ( $33.48 \pm 0.43$  fl), MCH ( $11.28 \pm 0.17$  pg) and MCHC ( $33.64 \pm 0.42$  g/l) were recorded group treated with Bactofort<sup>®</sup> and inoculated with vvIBDV and positive control ( $33.74 \pm 0.68$  fl,  $11.20 \pm 0.23$  pg,  $33.26 \pm 0.05$  g/l) at 0 dpi when compared to group treated with Antox<sup>®</sup> and inoculated with vvIBDV ( $32.92 \pm 0.59$  fl,  $10.90 \pm 0.24$  pg,  $33.14 \pm 0.17$  g/l). The lowest MCV ( $12.70 \pm 76.06$  fl) and MCH ( $09.26 \pm 21.75$  pg) were recorded in positive control at 7 dpi. There was no statistical significant difference ( $P > 0.05$ ) in erythrocytic indices between Antox<sup>®</sup>, Bactofort<sup>®</sup> and positive control at 7 (Table 4.5, 4.6 and 4.7)

**Table 4.1: Mean ( $\pm$  SE) packed cell volume (%) of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group**

| Age in days | Group                       |                             |                             |                             |
|-------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|             | A                           | B                           | C                           | D                           |
| 1 (n = 20)  | 22.40 $\pm$ 2.44            | 23.40 $\pm$ 0.68            | 19.80 $\pm$ 0.58            | 25.00 $\pm$ 1.87            |
| 7 (n = 20)  | 22.40 $\pm$ 2.44            | 22.80 $\pm$ 0.97            | 20.10 $\pm$ 0.40            | 22.40 $\pm$ 0.75            |
| 14 (n = 20) | 23.20 $\pm$ 0.66            | 23.00 $\pm$ 1.34            | 21.60 $\pm$ 1.54            | 23.60 $\pm$ 3.78            |
| 28 (n = 20) | 23.40 $\pm$ 2.11            | 26.8 $\pm$ 0.58             | 24.80 $\pm$ 1.69            | 25.8 $\pm$ 3.56             |
| 35 (n = 20) | 18.30 $\pm$ 0.37            | 17.00 $\pm$ 0.35            | 15.20 $\pm$ 0.37            | 26.60 $\pm$ 0.93            |
| 42          | 21.00 $\pm$ 0.58<br>(n = 3) | 20.00 $\pm$ 0.00<br>(n = 1) | 18.50 $\pm$ 0.50<br>(n = 2) | 27.40 $\pm$ 1.60<br>(n = 5) |

**Key: n = total number of birds sampled, Mean  $\pm$  SE = standard error of the means, no statistical significant difference, P > 0.05**

**Table 4.2: Mean ( $\pm$  SE) haemoglobin concentration (g/d) of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group**

| Age in days | Group                      |                            |                            |                            |
|-------------|----------------------------|----------------------------|----------------------------|----------------------------|
|             | A                          | B                          | C                          | D                          |
| 1 (n = 20)  | 7.44 $\pm$ 0.81            | 7.76 $\pm$ 0.23            | 6.80 $\pm$ 0.17            | 8.30 $\pm$ 0.61            |
| 7 (n = 20)  | 7.44 $\pm$ 0.81            | 7.76 $\pm$ 0.23            | 6.36 $\pm$ 0.29            | 6.08 $\pm$ 1.55            |
| 14 (n = 20) | 7.60 $\pm$ 0.30            | 7.62 $\pm$ 0.44            | 7.16 $\pm$ 0.52            | 7.76 $\pm$ 1.29            |
| 28 (n = 20) | 7.76 $\pm$ 0.71            | 9.02 $\pm$ 1.19            | 8.26 $\pm$ 0.56            | 8.46 $\pm$ 1.21            |
| 35 (n = 20) | 6.16 $\pm$ 0.14            | 6.00 $\pm$ 0.27            | 5.20 $\pm$ 0.25            | 7.86 $\pm$ 0.31            |
| 42          | 7.37 $\pm$ 0.52<br>(n = 3) | 7.00 $\pm$ 0.00<br>(n = 1) | 5.75 $\pm$ 0.25<br>(n = 2) | 8.42 $\pm$ 0.53<br>(n = 5) |

**Key: n = total number of birds sampled, Mean  $\pm$  SE = standard error of the means, no statistical significant difference, P > 0.05**

**Table 4.3: Mean ( $\pm$  SE) red blood cells ( $\times 10^{12}/l$ ) count of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group**

| Age in days | Group                       |                             |                             |                            |
|-------------|-----------------------------|-----------------------------|-----------------------------|----------------------------|
|             | A                           | B                           | C                           | D                          |
| 1 (n = 20)  | 4.34 $\pm$ 0.48             | 4.32 $\pm$ 0.86             | 4.22 $\pm$ 0.54             | 4.32 $\pm$ 0.29            |
| 7 (n = 20)  | 7.10 $\pm$ 0.62             | 6.50 $\pm$ 0.35             | 5.40 $\pm$ 0.29             | 5.30 $\pm$ 0.25            |
| 14 (n = 20) | 7.34 $\pm$ 0.27             | 6.15 $\pm$ 0.19             | 5.40 $\pm$ 0.51             | 5.50 $\pm$ 0.94            |
| 28 (n = 20) | 7.55 $\pm$ 0.62             | 6.31 $\pm$ 0.16             | 5.48 $\pm$ 0.63             | 5.90 $\pm$ 0.60            |
| 35 (n = 20) | 5.40 $\pm$ 0.29***          | 4.10 $\pm$ 0.43***          | 3.40 $\pm$ 0.43***          | 7.30 $\pm$ 0.12            |
| 42          | 7.00 $\pm$ 0.29*<br>(n = 3) | 6.00 $\pm$ 0.00*<br>(n = 1) | 4.25 $\pm$ 0.25*<br>(n = 2) | 7.52 $\pm$ 0.37<br>(n = 5) |

**Key: n = total number of birds sampled, Mean  $\pm$  SE = standard error of the means, Means with asterics (\*)-(P < 0.05) or (\*\*\*)-(P < 0.001) in the same row differed significantly.**

**Table 4.4: Mean ( $\pm$  SE) total white blood cells ( $\times 10^9/l$ ) count of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group**

| Age in days | Group                         |                             |                               |                             |
|-------------|-------------------------------|-----------------------------|-------------------------------|-----------------------------|
|             | A                             | B                           | C                             | D                           |
| 1 (n = 20)  | 10.38 $\pm$ 1.61              | 10.32 $\pm$ 1.59            | 10.06 $\pm$ 0.94              | 10.06 $\pm$ 1.38            |
| 7 (n = 20)  | 12.54 $\pm$ 0.94              | 12.41 $\pm$ 0.74            | 11.16 $\pm$ 1.69              | 11.12 $\pm$ 0.58            |
| 14 (n = 20) | 13.86 $\pm$ 0.74              | 13.50 $\pm$ 0.35            | 12.44 $\pm$ 0.73              | 12.92 $\pm$ 1.54            |
| 28 (n = 20) | 14.88 $\pm$ 1.24              | 14.58 $\pm$ 0.32            | 12.70 $\pm$ 0.54              | 12.98 $\pm$ 1.44            |
| 35 (n = 20) | 8.90 $\pm$ 0.29***            | 7.00 $\pm$ 0.42**           | 4.30 $\pm$ 0.37***            | 15.20 $\pm$ 0.25            |
| 42          | 10.00 $\pm$ 0.29**<br>(n = 3) | 8.00 $\pm$ 0.00*<br>(n = 1) | 7.00 $\pm$ 0.50***<br>(n = 2) | 14.70 $\pm$ 1.07<br>(n = 5) |

**Key: n = total number of birds sampled, Mean  $\pm$  SE = standard error of the means, means with asterics (\*)-(P < 0.05), (\*\*)-(P < 0.01) or (\*\*\*)-(P < 0.001) in the same row differed significantly**

**Table 4.5: Mean ( $\pm$  SE) mean corpuscular volume (fl) of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group**

| Age in days | Group                       |                             |                             |                             |
|-------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|             | A                           | B                           | C                           | D                           |
| 1 (n = 20)  | 48.26 $\pm$ 4.24            | 54.14 $\pm$ 1.43            | 40.16 $\pm$ 5.18            | 57.70 $\pm$ 1.49            |
| 7 (n = 20)  | 31.28 $\pm$ 0.86            | 30.46 $\pm$ 1.13            | 28.72 $\pm$ 1.76            | 26.98 $\pm$ 0.74            |
| 14 (n = 20) | 29.02 $\pm$ 0.56            | 32.38 $\pm$ 1.82            | 24.40 $\pm$ 5.49            | 31.26 $\pm$ 2.09            |
| 28 (n = 20) | 32.92 $\pm$ 0.59            | 33.48 $\pm$ 0.43            | 33.74 $\pm$ 0.68            | 33.24 $\pm$ 2.16            |
| 35 (n = 20) | 22.26 $\pm$ 2.81            | 23.08 $\pm$ 3.51            | 12.7 $\pm$ 76.06            | 28.38 $\pm$ 0.99            |
| 42          | 30.70 $\pm$ 0.40<br>(n = 3) | 32.30 $\pm$ 0.00<br>(n = 1) | 34.25 $\pm$ 0.75<br>(n = 2) | 33.68 $\pm$ 0.77<br>(n = 5) |

**Key: n = total number of birds sampled, Mean  $\pm$  SE = standard error of the means, no statistical significant difference, P > 0.05**

**Table 4.6: Mean ( $\pm$  SE) mean corpuscular haemoglobin (pg) of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group**

| Age in days | Group                       |                            |                             |                             |
|-------------|-----------------------------|----------------------------|-----------------------------|-----------------------------|
|             | A                           | B                          | C                           | D                           |
| 1 (n = 20)  | 16.00 $\pm$ 1.39            | 17.92 $\pm$ 0.46           | 13.74 $\pm$ 1.67            | 51.46 $\pm$ 37.32           |
| 7 (n = 20)  | 10.36 $\pm$ 0.30            | 10.38 $\pm$ 0.47           | 8.56 $\pm$ 0.32             | 8.96 $\pm$ 0.25             |
| 14 (n = 20) | 9.46 $\pm$ 0.19             | 10.70 $\pm$ 0.59           | 9.64 $\pm$ 0.31             | 10.20 $\pm$ 0.68            |
| 28 (n = 20) | 10.90 $\pm$ 0.24            | 11.28 $\pm$ 0.17           | 11.20 $\pm$ 0.23            | 10.88 $\pm$ 0.83            |
| 35 (n = 20) | 11.16 $\pm$ 0.82            | 10.12 $\pm$ 4.78           | 09.26 $\pm$ 21.75           | 9.42 $\pm$ 0.35             |
| 42          | 10.47 $\pm$ 0.29<br>(n = 3) | 11.6 $\pm$ 0.00<br>(n = 1) | 10.95 $\pm$ 0.05<br>(n = 2) | 11.12 $\pm$ 0.27<br>(n = 5) |

**Key: n = total number of birds sampled, Mean  $\pm$  SE = standard error of the means, no statistical significant difference, P > 0.05**

**Table 4.7: Mean ( $\pm$  SE) mean corpuscular haemoglobin concentration (g/l) of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group**

| Age in days | Group                        |                             |                              |                             |
|-------------|------------------------------|-----------------------------|------------------------------|-----------------------------|
|             | A                            | B                           | C                            | D                           |
| 1 (n = 20)  | 33.18 $\pm$ 0.06             | 33.14 $\pm$ 0.14            | 34.36 $\pm$ 0.69             | 33.18 $\pm$ 0.06            |
| 7 (n = 20)  | 33.18 $\pm$ 0.06             | 34.12 $\pm$ 0.85            | 31.58 $\pm$ 1.05             | 33.34 $\pm$ 0.86            |
| 14 (n = 20) | 32.68 $\pm$ 0.45             | 33.06 $\pm$ 0.02            | 33.32 $\pm$ 0.15             | 32.62 $\pm$ 0.48            |
| 28 (n = 20) | 33.14 $\pm$ 0.17             | 33.64 $\pm$ 0.42            | 33.26 $\pm$ 0.05             | 32.70 $\pm$ 0.58            |
| 35 (n = 20) | 23.62 $\pm$ 0.38             | 25.18 $\pm$ 1.10            | 25.10 $\pm$ 0.92             | 31.40 $\pm$ 1.85            |
| 42          | 32.97 $\pm$ 1.51*<br>(n = 3) | 31.00 $\pm$ 0.00<br>(n = 1) | 31.00 $\pm$ 0.50*<br>(n = 2) | 33.12 $\pm$ 0.06<br>(n = 5) |

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

#### **4.1.6 Differential leucocyte count**

The heterophil and lymphocyte counts of the vvIBDV inoculated and probiotics treated groups (A and B) decreased from  $3.31 \pm 0.21 \times 10^9/l$ ,  $14.09 \pm 1.17 \times 10^9/l$  and  $3.23 \pm 0.25 \times 10^9/l$ ,  $13.20 \pm 0.16 \times 10^9/l$  at 28 dph to  $2.2 \pm 0.26$ ,  $7.42 \pm 0.47$  and  $1.88 \pm 0.29$ ,  $13.20 \pm 0.42$  at 7 dpi when compared to positive control  $2.11 \pm 0.35 \times 10^9/l$ ,  $1.48 \pm 0.08 \times 10^9/l$  and  $10.73 \pm 0.83 \times 10^9/l$ ,  $2.79 \pm 0.12 \times 10^9/l$ . There was very high statistical significant difference ( $P < 0.001$ ) in heterophil and lymphocyte counts between group A, B and C (Tables 4.8 and 4.9).

#### **4.1.7 Heterophil/lymphocyte ratios**

There were increases in heterophil/lymphocyte ratios in groups (A, B, and C) inoculated with vvIBDV. However, the increase was significantly lower in the groups (A and B) treated with probiotics when compared to positive control. There was statistical significant difference ( $P < 0.05$ ) in heterophil/lymphocyte between group A, B and C (Table 4.10).

#### **4.2 Activities of Antioxidant Enzymes of ISA Brown Chicks Treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> and Inoculated with a Very Virulent Infectious Bursal Disease Virus**

The superoxide dismutase and catalase activity in groups (A and B) treated with probiotics and inoculated with vvIBDV decreased from the initial mean value of  $23.87 \pm 40.22 \mu\text{g/ml}$ ,  $6.03 \pm 0.56 \mu\text{g/ml}$  and  $22.71 \pm 1.20 \mu\text{g/ml}$ ,  $7.94 \pm 0.25 \mu\text{g/ml}$  at 0 dpi to  $10.72 \pm 1.51 \mu\text{g/ml}$ ,  $5.83 \pm 0.66 \mu\text{g/ml}$  and  $8.70 \pm 0.99 \mu\text{g/ml}$ ,  $6.99 \pm 0.25 \mu\text{g/ml}$  but the decrease was significantly lower when compared to positive control ( $6.17 \pm 1.95 \mu\text{g/ml}$  and  $3.88 \pm 4.15 \mu\text{g/ml}$  at 7 dpi, respectively). There was no statistical significant difference ( $P > 0.05$ ) in superoxide dismutase activity and catalase activity between group A, B and C (Table 4.11 and 4.12).

**Table 4.8: Mean ( $\pm$  SE) heterophils ( $\times 10^9/l$ ) count of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group**

| Age in days | Group                       |                              |                               |                            |
|-------------|-----------------------------|------------------------------|-------------------------------|----------------------------|
|             | A                           | B                            | C                             | D                          |
| 1 (n = 20)  | 3.66 $\pm$ 0.61*            | 3.62 $\pm$ 0.43**            | 3.27 $\pm$ 0.24               | 3.52 $\pm$ 0.21            |
| 7 (n = 20)  | 4.11 $\pm$ 0.50             | 3.98 $\pm$ 0.71              | 3.31 $\pm$ 0.36               | 3.40 $\pm$ 0.35            |
| 14 (n = 20) | 3.31 $\pm$ 0.31**           | 3.24 $\pm$ 0.31*             | 2.18 $\pm$ 0.28               | 2.20 $\pm$ 0.26            |
| 28 (n = 20) | 3.12 $\pm$ 0.21***          | 3.23 $\pm$ 0.25              | 2.11 $\pm$ 0.35**             | 2.21 $\pm$ 0.60            |
| 35 (n = 20) | 2.22 $\pm$ 0.26*            | 1.83 $\pm$ 0.05*             | 1.88 $\pm$ 0.29***            | 2.72 $\pm$ 0.37            |
| 42          | 2.44 $\pm$ 0.71*<br>(n = 3) | 3.30 $\pm$ 0.10**<br>(n = 1) | 1.48 $\pm$ 0.08***<br>(n = 2) | 3.14 $\pm$ 0.23<br>(n = 5) |

**Key: n = total number of birds sampled, Mean  $\pm$  SE = standard error of the means, Means with asterics (\*)-(P < 0.05), (\*\*)-(P < 0.01) or (\*\*\*)-(P < 0.001) in the same row differed significantly**

**Table 4.9: Mean ( $\pm$  SE) lymphocytes ( $\times 10^9/l$ ) count of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with A very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group**

| Age in days | Group                        |                             |                              |                             |
|-------------|------------------------------|-----------------------------|------------------------------|-----------------------------|
|             | A                            | B                           | C                            | D                           |
| 1 (n = 20)  | 10.32 $\pm$ 1.05             | 10.29 $\pm$ 1.24            | 10.28 $\pm$ 0.72             | 10.38 $\pm$ 1.26            |
| 7 (n = 20)  | 12.71 $\pm$ 0.70             | 11.64 $\pm$ 0.44            | 10.32 $\pm$ 1.34             | 10.48 $\pm$ 0.73            |
| 14 (n = 20) | 13.47 $\pm$ 0.92             | 12.75 $\pm$ 0.17            | 10.42 $\pm$ 0.62             | 10.56 $\pm$ 1.51            |
| 28 (n = 20) | 14.09 $\pm$ 1.17             | 13.20 $\pm$ 0.46            | 10.73 $\pm$ 0.83             | 10.82 $\pm$ 1.16            |
| 35 (n = 20) | 7.42 $\pm$ 0.47***           | 6.99 $\pm$ 0.42***          | 2.79 $\pm$ 0.12***           | 12.54 $\pm$ 0.25            |
| 42          | 9.53 $\pm$ 0.03**<br>(n = 3) | 6.70 $\pm$ 0.00*<br>(n = 1) | 4.63 $\pm$ 1.07**<br>(n = 2) | 12.92 $\pm$ 0.81<br>(n = 5) |

**Key: n = total number of birds sampled, Mean  $\pm$  SE = standard error of the means, Means with asterics (\*)-(P < 0.05), (\*\*)(P < 0.01) or (\*\*\*)(P < 0.001) in the same row differed significantly**

**Table 4.10: Mean ( $\pm$  SE) heterophils/ lymphocytes ratio of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group**

| Age in days | Group                      |                            |                            |                            |
|-------------|----------------------------|----------------------------|----------------------------|----------------------------|
|             | A                          | B                          | C                          | D                          |
| 1 (n = 20)  | 0.38 $\pm$ 0.024           | 0.26 $\pm$ 0.04            | 0.32 $\pm$ 0.00            | 0.31 $\pm$ 0.03            |
| 7 (n = 20)  | 0.38 $\pm$ 0.04            | 0.26 $\pm$ 0.07            | 0.42 $\pm$ 0.04            | 0.31 $\pm$ 0.05            |
| 14 (n = 20) | 0.11 $\pm$ 0.35            | 0.13 $\pm$ 0.03            | 0.18 $\pm$ 0.03            | 0.23 $\pm$ 0.03            |
| 28 (n = 20) | 0.18 $\pm$ 0.35            | 0.21 $\pm$ 0.03            | 0.26 $\pm$ 0.06            | 0.33 $\pm$ 0.06            |
| 35 (n = 20) | 0.54 $\pm$ 0.10*           | 0.52 $\pm$ 0.08*           | 0.76 $\pm$ 0.09*           | 0.21 $\pm$ 0.03            |
| 42          | 0.31 $\pm$ 0.10<br>(n = 3) | 0.33 $\pm$ 0.00<br>(n = 1) | 0.57 $\pm$ 0.01<br>(n = 2) | 0.31 $\pm$ 0.04<br>(n = 5) |

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

Glutathione peroxidase activity and malondialdehyde concentration in groups (A and B) treated with probiotics and inoculated with vvIBDV increased from the initial mean value of  $19.81 \pm 30.71 \mu\text{g/ml}$ ,  $485.80 \pm 22.52 \text{ nmols/mg}$  and  $23.78 \pm 2069 \mu\text{g/ml}$ ,  $554.80 \pm 22.65 \text{ nmols/mg}$  at 0 dpi to  $45.97 \pm 60.90 \mu\text{g/ml}$ ,  $826.22 \pm 17.24 \text{ nmols/mg}$  and  $55.59 \pm 40.99 \mu\text{g/ml}$ ,  $873.10 \pm 24.22 \text{ nmols/mg}$  at 7 dpi but the increase was significantly lower when compared to positive control ( $79.80 \pm 40.63 \mu\text{g/ml}$  and  $1406.86 \pm 24.90 \text{ nmols/mg}$  at 7 dpi, respectively). There was very high statistical significant difference ( $P < 0.001$ ) in glutathione peroxidase activity and malondialdehyde concentration between Antox<sup>®</sup>, Bactofort<sup>®</sup> and positive control (Table 4.13 and 4.14).

#### **4.3 Body, Bursal, Spleen and Thymus Weights of ISA Brown Chicks Treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> and Inoculated with a Very Virulent Infectious Bursal Disease Virus**

The body weight in groups (A and B) treated with probiotics and inoculated with vvIBDV decreased from the initial mean value of  $230.50 \pm 50.69 \text{ g}$ , and  $200.24 \pm 21.42 \text{ g}$ , at 0 dpi to  $200.40 \pm 70.13 \text{ g}$ , and  $170.40 \pm 9010 \text{ g}$ , but the decrease was significantly lower when compared to positive control ( $140.62 \pm 10.03 \text{ g}$  at 7 dpi respectively). There was high statistical significant difference ( $P < 0.01$ ) in body weight between the groups (A, B and C). The bursa of Fabricius weight in groups (A and B) treated with probiotics and inoculated with vvIBDV increased from the initial mean value of  $0.90 \pm 0.17 \text{ g}$ , and  $0.80 \pm 0.23 \text{ g}$  at 0 dpi to  $1.13 \pm 0.14 \text{ g}$  and  $1.30 \pm 0.12 \text{ g}$  at 7 dpi when compared to positive control ( $0.39 \pm 2.3 \text{ g}$ ,  $0 \pm 0.20 \text{ g}$ ) but the increase was significantly lower when compared to positive control and no statistical significant different in BF between the group inoculated with vvIBDV.

**Table 4.11: Mean ( $\pm$  SE) superoxide dismutase ( $\mu\text{g/ml}$ ) activity of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group**

| Age in days | Group                       |                             |                             |                             |
|-------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|             | A                           | B                           | C                           | D                           |
| 1 (n = 20)  | 19.33 $\pm$ 0.65            | 15.26 $\pm$ 0.25            | 15.98 $\pm$ 0.35            | 19.09 $\pm$ 0.48            |
| 7 (n = 20)  | 20.43 $\pm$ 0.75            | 19.57 $\pm$ 0.21            | 16.40 $\pm$ 0.29            | 18.60 $\pm$ 1.78            |
| 14 (n = 20) | 22.94 $\pm$ 0.50            | 21.59 $\pm$ 1.16            | 20.59 $\pm$ 1.16            | 20.46 $\pm$ 0.79            |
| 28 (n = 20) | 23.87 $\pm$ 40.22           | 22.71 $\pm$ 1.20            | 21.70 $\pm$ 1.03            | 21.03 $\pm$ 0.64            |
| 35 (n = 20) | 10.72 $\pm$ 1.51**          | 8.70 $\pm$ 0.99             | 6.17 $\pm$ 1.95**           | 22.47 $\pm$ 0.77            |
| 42          | 15.84 $\pm$ 0.88<br>(n = 3) | 14.13 $\pm$ 0.00<br>(n = 1) | 10.08 $\pm$ 5.00<br>(n = 2) | 20.71 $\pm$ 0.53<br>(n = 5) |

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

**Table 4.12: Mean ( $\pm$  SE) catalase ( $\mu\text{g}/\text{mg}$ ) activity of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group**

| Age in days | Group                      |                            |                             |                            |
|-------------|----------------------------|----------------------------|-----------------------------|----------------------------|
|             | A                          | B                          | C                           | D                          |
| 1 (n = 20)  | 6.22 $\pm$ 0.33            | 6.86 $\pm$ 0.49            | 8.86 $\pm$ 0.36             | 19.32 $\pm$ 0.35           |
| 7 (n = 20)  | 6.53 $\pm$ 0.38            | 7.76 $\pm$ 0.15            | 6.09 $\pm$ 0.40             | 7.83 $\pm$ 0.48            |
| 14 (n = 20) | 7.62 $\pm$ 0.29            | 8.67 $\pm$ 2.39            | 8.67 $\pm$ 2.39             | 17.49 $\pm$ 1.83           |
| 28 (n = 20) | 6.03 $\pm$ 0.56            | 7.94 $\pm$ 0.25            | 6.14 $\pm$ 1.17             | 7.38 $\pm$ 2.27            |
| 35 (n = 20) | 5.83 $\pm$ 0.66*           | 6.99 $\pm$ 0.25            | 3.88 $\pm$ 4.15*            | 8.33 $\pm$ 3.85            |
| 42          | 8.30 $\pm$ 0.55<br>(n = 3) | 7.35 $\pm$ 0.00<br>(n = 1) | 10.67 $\pm$ 2.46<br>(n = 2) | 7.35 $\pm$ 5.69<br>(n = 5) |

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

**Table 4.13: Mean ( $\pm$  SE) glutathione peroxidase ( $\mu\text{g/ml}$ ) activity of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group**

| Age in days | Group                          |                               |                                |                              |
|-------------|--------------------------------|-------------------------------|--------------------------------|------------------------------|
|             | A                              | B                             | C                              | D                            |
| 1 (n = 20)  | 31.33 $\pm$ 50.33              | 30.83 $\pm$ 20.66             | 32.37 $\pm$ 20.13              | 31.84 $\pm$ 30.38            |
| 7 (n = 20)  | 23.44 $\pm$ 30.67              | 26.46 $\pm$ 30.26             | 43.99 $\pm$ 10.51              | 41.44 $\pm$ 30.72            |
| 14 (n = 20) | 20.89 $\pm$ 20.09              | 22.32 $\pm$ 20.31             | 44.32 $\pm$ 20.30              | 42.80 $\pm$ 30.59            |
| 28 (n = 20) | 19.81 $\pm$ 30.71***           | 23.78 $\pm$ 20.69             | 46.54 $\pm$ 00.31***           | 43.45 $\pm$ 20.15            |
| 35 (n = 20) | 45.97 $\pm$ 60.90***           | 55.59 $\pm$ 40.99**           | 79.80 $\pm$ 40.63***           | 44.80 $\pm$ 20.63            |
| 42          | 35.79 $\pm$ 30.38**<br>(n = 3) | 49.09 $\pm$ 30.38*<br>(n = 1) | 78.28 $\pm$ 30.03**<br>(n = 2) | 45.39 $\pm$ 40.48<br>(n = 5) |

**Key:** n = total number of birds sampled, Mean  $\pm$  SE = standard error of the means, Means with asterics (\*)-(P < 0.05), (\*\*)(P < 0.01) or (\*\*\*)(P < 0.001) in the same row differed significantly

**Table 4.14: Mean ( $\pm$  SE) malondialdehyde (nmols/mg) concentration of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group**

| Age<br>days | in | Group                          |                                |                                 |                               |
|-------------|----|--------------------------------|--------------------------------|---------------------------------|-------------------------------|
|             |    | A                              | B                              | C                               | D                             |
| 1 (n = 20)  |    | 610.54 $\pm$ 22.49             | 673.16 $\pm$ 29.53             | 604.8 $\pm$ 22.06               | 677.54 $\pm$ 22.49            |
| 7 (n = 20)  |    | 621.36 $\pm$ 24.28             | 677.40 $\pm$ 91.05             | 775.40 $\pm$ 91.05              | 821.36 $\pm$ 24.28            |
| 14 (n = 20) |    | 610.30 $\pm$ 40.32             | 625.8 $\pm$ 24.58              | 829.79 $\pm$ 26.23              | 855.30 $\pm$ 40.32            |
| 28 (n = 20) |    | 485.80 $\pm$ 22.52             | 554.80 $\pm$ 22.65*            | 884.26 $\pm$ 13.74**            | 745.80 $\pm$ 22.52            |
| 35 (n = 20) |    | 826.22 $\pm$ 17.24***          | 873.10 $\pm$ 24.22***          | 1406.86 $\pm$ 24.90*            | 756.22 $\pm$ 17.24            |
| 42          |    | 898.13 $\pm$ 31.68*<br>(n = 3) | 922.10 $\pm$ 31.68*<br>(n = 1) | 110.34 $\pm$ 20.00**<br>(n = 2) | 698.13 $\pm$ 31.68<br>(n = 5) |

**Key: n = total number of birds sampled, Mean  $\pm$  SE = standard error of the means, Means with asterics (\*)-(P < 0.05), (\*\*)(P < 0.01) or (\*\*\*) (P < 0.001) in the same row differed significantly**

The spleen and thymus weights in groups (A and B) treated with probiotics and inoculated with vvIBDV increased from the initial mean value of  $0.79 \pm 0.07$  g,  $2.19 \pm 0.08$  g, and  $0.80 \pm 0.25$  g,  $0.74 \pm 0.09$  g,  $1.81 \pm 0.18$  g at 0 dpi to  $1.80 \pm 0.03$  g,  $3.15 \pm 0.15$  g and  $1.95 \pm 0.15$  g,  $2.25 \pm 0.09$  g at 7 dpi when compared to positive control ( $2.80 \pm 0.06$  g and  $3.70 \pm 0.17$  g). There was statistical significant difference ( $P < 0.05$ ) in spleen and thymus weights between the Antox<sup>®</sup>, Bactofort<sup>®</sup> and positive control (Tables 4.15, 4.16, 4.17 and 4.18).

#### **4.4 Bursa to Body, Spleen to Body and Thymus to Body Weight Ratios of ISA Brown Chicks Treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> and Inoculated with a Very Virulent Infectious Bursal Disease Virus**

The bursa/body weight ratios in groups (A and B) treated with probiotics and inoculated with vvIBDV increase from the initial mean value of  $0.90 \pm 0.17$  and  $0.80 \pm 0.23$  at 0 dpi to  $1.13 \pm 0.14$  and  $1.30 \pm 0.12$  at 7 dpi but the increased was significantly lower when compared to positive control. There was no statistical significant difference ( $P < 0.05$ ) in bursa/body weight ratio between between the Antox<sup>®</sup>, Bactofort<sup>®</sup> and positive control.

The spleen/body and thymus/body weight ratios in groups (A and B) treated with probiotics and inoculated with vvIBDV increase from the initial mean value of  $0.79 \pm 0.07$ ,  $2.19 \pm 0.08$  and  $0.74 \pm 0.09$ ,  $1.81 \pm 0.18$  at 0 dpi to  $0.79 \pm 0.07$ ,  $3.15 \pm 0.15$  and  $0.74 \pm 0.09$ ,  $2.25 \pm 0.09$  at 7 dpi but the increased was significantly lower when compared to positive control ( $2.80 \pm 0.06$  and  $3.70 \pm 0.17$ ) at 7 dpi, respectively. There was statistical significant difference ( $P < 0.05$ ) in spleen/body and thymus/body weight ratios between Antox<sup>®</sup>, Bactofort<sup>®</sup> and positive control (Tables 4.19, 4.20 and 4.21).

#### **4.5 Clinical signs of ISA Brown Chicks Treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> and Inoculated with a Very Virulent Infectious Bursal Disease Virus**

At 2 dpi, clinical signs of IBD such as depression and ruffled feathers appeared in positive control group C (17.65) only. At 3 dpi, the groups (A and B) treated with Antox<sup>®</sup> (11.80 %) and Bactofort<sup>®</sup> (11.80 %) started showing clinical signs of IBD. Five dpi, the clinical signs increased to highest percentage in group A (23.56 %), B (64.71 %) and C (47.06 %) for the groups treated with Antox<sup>®</sup>, Bactofort<sup>®</sup> and positive control, respectively. Severe depression, ruffled feathers, anorexia and diarrhoea characterised by greenish yellow watery faeces were observed in vvIBDV inoculated groups (A, B and C) after the onset of clinical signs. Clinical signs declined gradually and by 9 dpi, all survivors appeared apparently healthy (Table 4.22).

#### **4.6 Mortality and Morbidity Rates of ISA Brown Chicks Treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> and Inoculated with a Very Virulent Infectious Bursal Disease Virus**

Morbidity was observed 2 dpi in group C and 3 dpi in groups A and B. The morbidity rate lasted 6 dpi and 9 dpi in groups A, B and C, respectively. The highest morbidity rates were recorded 5 dpi in all the groups inoculated with vvIBDV (A, 14.39 %; B, 15.04 % and C, 19.18 %). Mortality rate was observed 2 dpi in group C and 3 dpi in group A and B. The mortality rates lasted for 6, 7 and 9 dpi in group A, B and C respectively. The overall mortality rates were 34.76 %, 47.60 % and 72.70 % in Antox<sup>®</sup>, Bactofort<sup>®</sup> and positive control, respectively (Table 4.23).

#### **4.7 Enzyme-linked Immuno-sorbent Antibody Titre level of ISA Brown Chicks Treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> and Inoculated with a Very Virulent Infectious Bursal Disease Virus**

The enzymes-linked immune-sorbent antibody titre level in groups (A and B) treated with probiotics and inoculated with vvIBDV were increases from the initial mean value of  $4.72 \pm 0.78$  and  $4.29 \pm 0.89$  at 28 dph to  $9.12 \pm 0.52$  and  $8.12 \pm 1.58$  but the increased was significantly lower when compared to positive control group (C)  $4.42 \pm 1.87$  at 7 dpi respectively. There was high statistical significant difference ( $P < 0.01$ ) in enzymes-linked immune-sorbent antibody titre level between between Antox<sup>®</sup>, Bactofort<sup>®</sup> and positive control (Table 4.24).

#### **4.8 Gross Lesions of ISA Brown Chicks Treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> and Inoculated with a Very Virulent Infectious Bursal Disease Virus**

The BF becomes severely oedematous, hyperaemic, with a gelatinous yellowish exudate covering the serosal surface (Plate II). There were less gross lesions in the groups (A and B) treated with probiotics and inoculated with vvIBDV when compared to positive control group (C) at 7 dpi (Appendix 5). On the mucosal surfaces, the BF showed petechial haemorrhages in groups inoculated (A, B and C) with vvIBDV. The BF haemorrhages were extensive in positive control (Plate II) when compared to groups (A and B) treated with probiotics. There were petecchial and ecchymotic haemorrhages in the thigh and breast muscles of the chicks inoculated with vvIBDV (A, B and C). Lesions were less severe in the groups (A and B) treated with probiotics when compared to positive control (Plate I). The THY and SPL were congested in chicks inoculated with vvIBDV (A, B and C) but congestion was less severe in the groups (A and B) treated with probiotic when compared to positive control (Plate III). (Appendix 5, 6 and 7).

**Table 4.15: Mean ( $\pm$  SE) body weight (g) of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from 1 day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group**

| Age in days | Group                           |                               |                                  |                               |
|-------------|---------------------------------|-------------------------------|----------------------------------|-------------------------------|
|             | A                               | B                             | C                                | D                             |
| 1 (n = 20)  | 31.76 $\pm$ 10.41               | 28.31 $\pm$ 00.76             | 28.68 $\pm$ 10.05                | 28.41 $\pm$ 10.59             |
| 7 (n = 20)  | 58.05 $\pm$ 00.76               | 40.44 $\pm$ 30.36             | 36.49 $\pm$ 30.46                | 36.68 $\pm$ 40.57             |
| 14 (n = 20) | 177.79 $\pm$ 60.98              | 129.19 $\pm$ 40.63            | 81.48 $\pm$ 40.88                | 82.52 $\pm$ 60.30             |
| 28 (n = 20) | 230.50 $\pm$ 50.69              | 200.24 $\pm$ 21.42            | 182.60 $\pm$ 30.83               | 188.65 $\pm$ 24.24            |
| 35 (n = 20) | 200.4 $\pm$ 70.13***            | 170.40 $\pm$ 90.10**          | 140.60 $\pm$ 10.03***            | 293.40 $\pm$ 29.66            |
| 42          | 305.33 $\pm$ 17.25**<br>(n = 3) | 281.0 $\pm$ 00.00*<br>(n = 1) | 230.50 $\pm$ 00.50***<br>(n = 2) | 377.40 $\pm$ 16.44<br>(n = 5) |

**Key: n = total number of birds sampled, Mean  $\pm$  SE = standard error of the Means, Means with asterics (\*)-(P < 0.05), (\*\*)-(P < 0.01) or (\*\*\*)-(P < 0.001) in the same row differed significantly**

**Table 4.16: Mean ( $\pm$  SE) bursa of Fabricius weight (g) of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group**

| Age in days | Group                       |                             |                             |                            |
|-------------|-----------------------------|-----------------------------|-----------------------------|----------------------------|
|             | A                           | B                           | C                           | D                          |
| 1 (n = 20)  | 0.30 $\pm$ 0.01             | 0.29 $\pm$ 0.01             | 0.29 $\pm$ 0.01             | 0.29 $\pm$ 0.00            |
| 7 (n = 20)  | 0.33 $\pm$ 0.01             | 0.33 $\pm$ 0.01             | 0.31 $\pm$ 0.02             | 0.31 $\pm$ 0.02            |
| 14 (n = 20) | 0.59 $\pm$ 0.03             | 0.60 $\pm$ 0.03             | 0.47 $\pm$ 0.02             | 0.47 $\pm$ 0.04            |
| 28 (n = 20) | 0.90 $\pm$ 0.17             | 0.80 $\pm$ 0.23             | 0.39 $\pm$ 0.12             | 0.43 $\pm$ 0.16            |
| 35 (n = 20) | 1.13 $\pm$ 0.14             | 1.30 $\pm$ 0.12             | 2.30 $\pm$ 0.20             | 0.32 $\pm$ 0.36            |
| 42          | 0.96 $\pm$ 0.19*<br>(n = 3) | 0.86 $\pm$ 0.00*<br>(n = 1) | 2.18 $\pm$ 0.06*<br>(n = 2) | 0.73 $\pm$ 0.11<br>(n = 5) |

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

**Table 4.17: Mean ( $\pm$  SE) spleen weight (g) of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group**

| Age in days | Group                        |                              |                              |                            |
|-------------|------------------------------|------------------------------|------------------------------|----------------------------|
|             | A                            | B                            | C                            | D                          |
| 1 (n = 20)  | 0.27 $\pm$ 0.01              | 0.26 $\pm$ 0.01              | 0.26 $\pm$ 0.00              | 0.26 $\pm$ 0.01            |
| 7 (n = 20)  | 0.47 $\pm$ 0.00              | 0.37 $\pm$ 0.01              | 0.28 $\pm$ 0.00              | 0.27 $\pm$ 0.01            |
| 14 (n = 20) | 0.57 $\pm$ 0.01              | 0.46 $\pm$ 0.02              | 0.35 $\pm$ 0.02              | 0.34 $\pm$ 0.01            |
| 28 (n = 20) | 0.79 $\pm$ 0.07              | 0.74 $\pm$ 0.09              | 0.60 $\pm$ 0.03              | 0.55 $\pm$ 0.03            |
| 35 (n = 20) | 1.80 $\pm$ 0.03*             | 1.95 $\pm$ 0.15*             | 2.80 $\pm$ 0.06*             | 0.57 $\pm$ 0.36            |
| 42          | 0.89 $\pm$ 0.13**<br>(n = 3) | 0.95 $\pm$ 0.00**<br>(n = 1) | 2.39 $\pm$ 0.00**<br>(n = 2) | 0.65 $\pm$ 0.16<br>(n = 5) |

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

**Table 4.18: Mean ( $\pm$  SE) thymus weight (g) of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive (C) and negative control group (D)**

| Age in days | Group                        |                              |                              |                            |
|-------------|------------------------------|------------------------------|------------------------------|----------------------------|
|             | A                            | B                            | C                            | D                          |
| 1 (n = 20)  | 0.35 $\pm$ 0.03              | 0.35 $\pm$ 0.03              | 0.34 $\pm$ 0.01              | 0.29 $\pm$ 0.02            |
| 7 (n = 20)  | 0.59 $\pm$ 0.01              | 0.42 $\pm$ 0.03              | 0.31 $\pm$ 0.02              | 0.35 $\pm$ 0.03            |
| 14 (n = 20) | 0.72 $\pm$ 0.07              | 0.81 $\pm$ 0.05              | 0.67 $\pm$ 0.05              | 0.68 $\pm$ 0.06            |
| 28 (n = 20) | 2.19 $\pm$ 0.08              | 1.81 $\pm$ 0.18              | 1.64 $\pm$ 0.07              | 1.74 $\pm$ 0.15            |
| 35 (n = 20) | 3.15 $\pm$ 0.15 *            | 2.25 $\pm$ 0.09*             | 3.70 $\pm$ 0.17*             | 1.51 $\pm$ 0.16            |
| 42          | 2.76 $\pm$ 0.28**<br>(n = 3) | 2.21 $\pm$ 0.00**<br>(n = 1) | 3.30 $\pm$ 0.27**<br>(n = 2) | 1.46 $\pm$ 0.16<br>(n = 5) |

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

**Table 4.19: Mean ( $\pm$  SE) bursa of Fabricius/body weight ratio of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group**

| Age in days | Group                       |                             |                             |                            |
|-------------|-----------------------------|-----------------------------|-----------------------------|----------------------------|
|             | A                           | B                           | C                           | D                          |
| 1 (n = 20)  | 10.60 $\pm$ 0.59            | 10.42 $\pm$ 0.42            | 10.2 $\pm$ 0.25             | 10.25 $\pm$ 0.42           |
| 7 (n = 20)  | 8.51 $\pm$ 0.44             | 8.09 $\pm$ 0.54             | 7.89 $\pm$ 0.45             | 8.97 $\pm$ 1.11            |
| 14 (n = 20) | 6.91 $\pm$ 0.42             | 6.75 $\pm$ 0.22             | 6.40 $\pm$ 0.51             | 6.02 $\pm$ 0.51            |
| 28 (n = 20) | 7.79 $\pm$ 0.59             | 5.85 $\pm$ 0.73             | 6.47 $\pm$ 0.61             | 7.62 $\pm$ 0.67            |
| 35 (n = 20) | 5.36 $\pm$ 0.56**           | 3.36 $\pm$ 0.39**           | 2.01 $\pm$ 1.29**           | 7.65 $\pm$ 0.76            |
| 42          | 6.81 $\pm$ 0.71*<br>(n = 3) | 4.57 $\pm$ 0.00*<br>(n = 1) | 2.18 $\pm$ 0.19*<br>(n = 2) | 7.21 $\pm$ 0.51<br>(n = 5) |

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

**Table 4.20: Mean ( $\pm$  SE) spleen/body weight ratio of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group**

| Age in days | Group                       |                             |                             |                            |
|-------------|-----------------------------|-----------------------------|-----------------------------|----------------------------|
|             | A                           | B                           | C                           | D                          |
| 1 (n = 20)  | 9.54 $\pm$ 0.29             | 9.28 $\pm$ 0.31             | 9.09 $\pm$ 0.32             | 9.30 $\pm$ 0.59            |
| 7 (n = 20)  | 7.15 $\pm$ 0.16             | 6.98 $\pm$ 0.48             | 6.17 $\pm$ 0.51             | 7.80 $\pm$ 0.96            |
| 14 (n = 20) | 4.25 $\pm$ 0.26             | 4.05 $\pm$ 0.11             | 4.19 $\pm$ 0.27             | 4.19 $\pm$ 0.26            |
| 28 (n = 20) | 3.51 $\pm$ 0.24             | 3.61 $\pm$ 0.54             | 3.44 $\pm$ 0.15             | 3.90 $\pm$ 0.25            |
| 35 (n = 20) | 2.32 $\pm$ 0.13*            | 2.25 $\pm$ 0.53*            | 1.23 $\pm$ 0.14*            | 4.71 $\pm$ 1.40            |
| 42          | 2.88 $\pm$ 0.31*<br>(n = 3) | 3.47 $\pm$ 0.00*<br>(n = 1) | 2.36 $\pm$ 0.01*<br>(n = 2) | 3.75 $\pm$ 0.35<br>(n = 5) |

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

**Table 4.21: Mean ( $\pm$  SE) thymus /body weight ratio of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group**

| Age in days | Group                        |                              |                              |                            |
|-------------|------------------------------|------------------------------|------------------------------|----------------------------|
|             | A                            | B                            | C                            | D                          |
| 1 (n = 20)  | 10.81 $\pm$ 0.76             | 12.32 $\pm$ 0.99             | 11.86 $\pm$ 0.35             | 10.22 $\pm$ 0.54           |
| 7 (n = 20)  | 10.28 $\pm$ 0.47             | 10.65 $\pm$ 0.91             | 10.73 $\pm$ 0.27             | 9.86 $\pm$ 0.78            |
| 14 (n = 20) | 8.37 $\pm$ 0.62              | 9.17 $\pm$ 0.34              | 8.61 $\pm$ 0.47              | 8.62 $\pm$ 1.01            |
| 28 (n = 20) | 9.61 $\pm$ 0.51              | 9.07 $\pm$ 0.31              | 8.02 $\pm$ 0.51              | 8.31 $\pm$ 1.44            |
| 35 (n = 20) | 6.95 $\pm$ 0.51*             | 5.33 $\pm$ 0.22*             | 4.69 $\pm$ 0.57*             | 8.70 $\pm$ 0.72            |
| 42          | 6.71 $\pm$ 0.58**<br>(n = 3) | 6.67 $\pm$ 0.00**<br>(n = 1) | 5.48 $\pm$ 0.91**<br>(n = 2) | 9.20 $\pm$ 0.42<br>(n = 5) |

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

**Table 4.22: Mean (%) clinical signs of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group**

|       |                                  | Days post-inoculation with a very very virulent infectious bursal disease virus |         |          |          |          |          |          |          |          |          |
|-------|----------------------------------|---------------------------------------------------------------------------------|---------|----------|----------|----------|----------|----------|----------|----------|----------|
|       |                                  | 0                                                                               | 1       | 2        | 3        | 4        | 5        | 6        | 7        | 8        | 9        |
| Group | No. of observable clinical signs | No. (%) of clinical signs observed                                              |         |          |          |          |          |          |          |          |          |
| A     | 17                               | 0(0.00)                                                                         | 0(0.00) | 0(0.00)  | 2(11.80) | 4(23.56) | 4(23.56) | 1(5.88)  | 2(11.80) | 0(0.00)  | 0(0.00)  |
| B     | 17                               | 0(0.00)                                                                         | 0(0.00) | 0(0.00)  | 2(11.80) | 9(52.94) | 6(35.29) | 6(35.29) | 3(17.65) | 0(0.00)  | 0(0.00)  |
| C     | 17                               | 0(0.00)                                                                         | 0(0.00) | 3(17.65) | 5(29.41) | 4(23.25) | 8(47.06) | 9(52.94) | 5(29.41) | 4(23.52) | 4(23.52) |
| D     | 17                               | 0(0.00)                                                                         | 0(0.00) | 0(0.00)  | 0(0.00)  | 0(0.00)  | 0(0.00)  | 0(0.00)  | 0(0.00)  | 0(0.00)  | 0(0.00)  |

**Table 4.23: Mean (%) morbidity and mortality of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group**

| <b>% Morbidity</b>                                                                     |                    |          |          |          |          |          |          |          |          |          |                               |
|----------------------------------------------------------------------------------------|--------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|-------------------------------|
| <b>Days post-inoculation with a very very virulent infectious bursal disease virus</b> |                    |          |          |          |          |          |          |          |          |          |                               |
| <b>Group</b>                                                                           | <b>No.of birds</b> | <b>1</b> | <b>2</b> | <b>3</b> | <b>4</b> | <b>5</b> | <b>6</b> | <b>7</b> | <b>8</b> | <b>9</b> | <b>Highest morbidity rate</b> |
| A                                                                                      | 23                 | 0.00     | 0.00     | 11.04    | 12.04    | 14.39    | 8.04     | 0.00     | 0.00     | 0.00     | 5 (14.39)                     |
| B                                                                                      | 21                 | 0.00     | 0.00     | 12.28    | 13.04    | 15.04    | 9.52     | 0.00     | 0.00     | 0.00     | 5 (15.04)                     |
| C                                                                                      | 22                 | 0.00     | 12.63    | 15.18    | 18.63    | 19.18    | 13.63    | 9.09     | 6.09     | 4.54     | 5 (19.18)                     |
| D                                                                                      | 25                 | 0.00     | 0.00     | 0.00     | 0.00     | 0.00     | 0.00     | 0.00     | 0.00     | 0.00     | 0.00                          |

| <b>% Mortality</b>                                                                     |                     |          |          |          |          |          |          |          |          |          |                               |
|----------------------------------------------------------------------------------------|---------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|-------------------------------|
| <b>Days post-inoculation with a very very virulent infectious bursal disease virus</b> |                     |          |          |          |          |          |          |          |          |          |                               |
| <b>Group</b>                                                                           | <b>No. of birds</b> | <b>1</b> | <b>2</b> | <b>3</b> | <b>4</b> | <b>5</b> | <b>6</b> | <b>7</b> | <b>8</b> | <b>9</b> | <b>Overall mortality rate</b> |
| A                                                                                      | 23                  | 0.00     | 0.00     | 4.34     | 8.69     | 13.04    | 8.69     | 0.00     | 0.00     | 0.00     | 34.76                         |
| B                                                                                      | 21                  | 0.00     | 0.00     | 4.76     | 14.28    | 14.28    | 9.52     | 4.76     | 0.00     | 0.00     | 47.60                         |
| C                                                                                      | 22                  | 0.00     | 4.54     | 9.09     | 18.18    | 13.63    | 13.63    | 9.09     | 0.00     | 4.54     | 72.70                         |
| D                                                                                      | 25                  | 0.00     | 0.00     | 0.00     | 0.00     | 0.00     | 0.00     | 0.00     | 0.00     | 0.00     | 0.00                          |

**Table 4.24: Mean ( $\pm$  SE) changes in enzyme linked immunosorbent assay antibody titre level of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group**

| Age in days | Group                       |                             |                             |                            |
|-------------|-----------------------------|-----------------------------|-----------------------------|----------------------------|
|             | A                           | B                           | C                           | D                          |
| 1 (n = 20)  | 0.64 $\pm$ 0.27             | 0.59 $\pm$ 0.13             | 0.37 $\pm$ 0.06             | 0.23 $\pm$ 0.15            |
| 7 (n = 20)  | 3.46 $\pm$ 0.52             | 2.25 $\pm$ 0.79             | 1.08 $\pm$ 0.67             | 0.84 $\pm$ 0.31            |
| 14 (n = 20) | 6.23 $\pm$ 1.63             | 6.57 $\pm$ 0.80             | 4.04 $\pm$ 1.27             | 5.15 $\pm$ 1.38            |
| 28 (n = 20) | 4.72 $\pm$ 0.78             | 4.29 $\pm$ 0.89             | 3.06 $\pm$ 0.73             | 3.72 $\pm$ 0.91            |
| 35 (n = 20) | 9.12 $\pm$ 0.52**           | 8.12 $\pm$ 1.58*            | 4.42 $\pm$ 1.87**           | 3.12 $\pm$ 0.83            |
| 42          | 6.12 $\pm$ 0.52*<br>(n = 3) | 3.98 $\pm$ 0.00*<br>(n = 1) | 3.21 $\pm$ 0.83*<br>(n = 2) | 2.17 $\pm$ 1.52<br>(n = 5) |

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

## **4.9 Microscopic Lesions of ISA Brown Chicks Treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> and Inoculated with a Very Virulent Infectious Bursal Disease Virus**

### **4.9.1 Bursa of Fabricius**

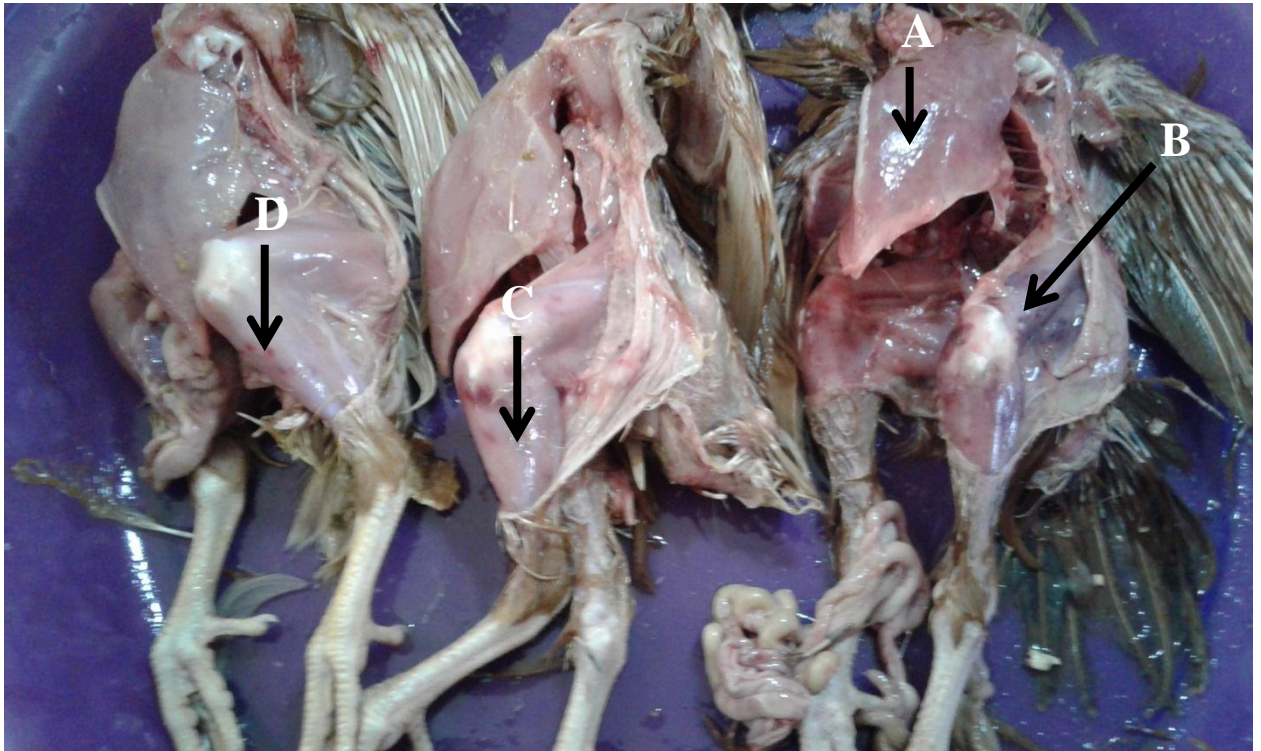
The observable microscopic lesions of BF in the groups (A and B) treated with probiotics were depletion of lymphocytes in the medullar of the bursal follicles but less severe when compared to positive control, while in positive control group, there was depletion of lymphocyte in the medullar, congestion of blood cells in the blood vessels and vaculation of lymphoid cells in the cortex and medullar. Necrosis has become more pronounced in the modullar of bursal follicles in positive control and foci of lymphocytes necrosis have spread to the cortex. The interfollicular spaces also increased in the positive control at 7 dpi respectively (Plate IV, V, VI and VII).

### **4.9.2 Spleen**

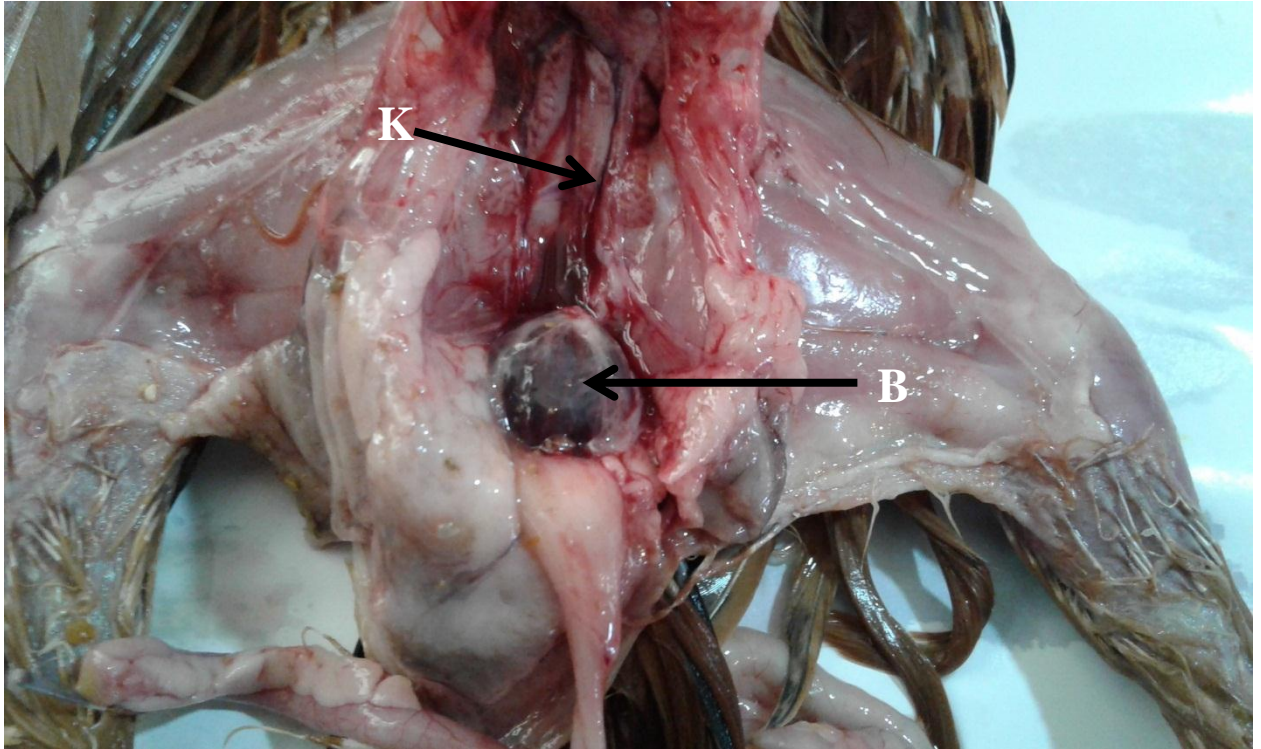
The observable microscopic lesions of SPL in groups (A and B) treated with probiotics and inoculated with vvIBDV was depletion of lymphocytes in the white pulp but less severe when compared to positive control while in positive control group, there was depletion of lymphoid cells and perivascular lymphoid cells infiltration in the white pulp. Lymphoid cells necrosis was observed in the white pulp in all the groups (A, B and C) inoculated with vvIBDV but necrosis was less severe in groups treated with probiotics at 7 dpi (Plate VIII, IX, X and XI).

### **4.9.3 Thymus**

The observable microscopic lesions in the THY of chicks in groups (A and B) treated with probiotics was depletion of lymphocytes in the medullary, congestion of blood cells in blood vessels but the groups treated with probiotics had less severe microscopic lesions when compared to positive control while in positive control too, there was depletion of lymphocytes in the medullary, congestion of blood cells in the blood vessels and vaculation of lymphoid cells in the cortex at 7 dpi (Plate XII, XIII XIV and XV).



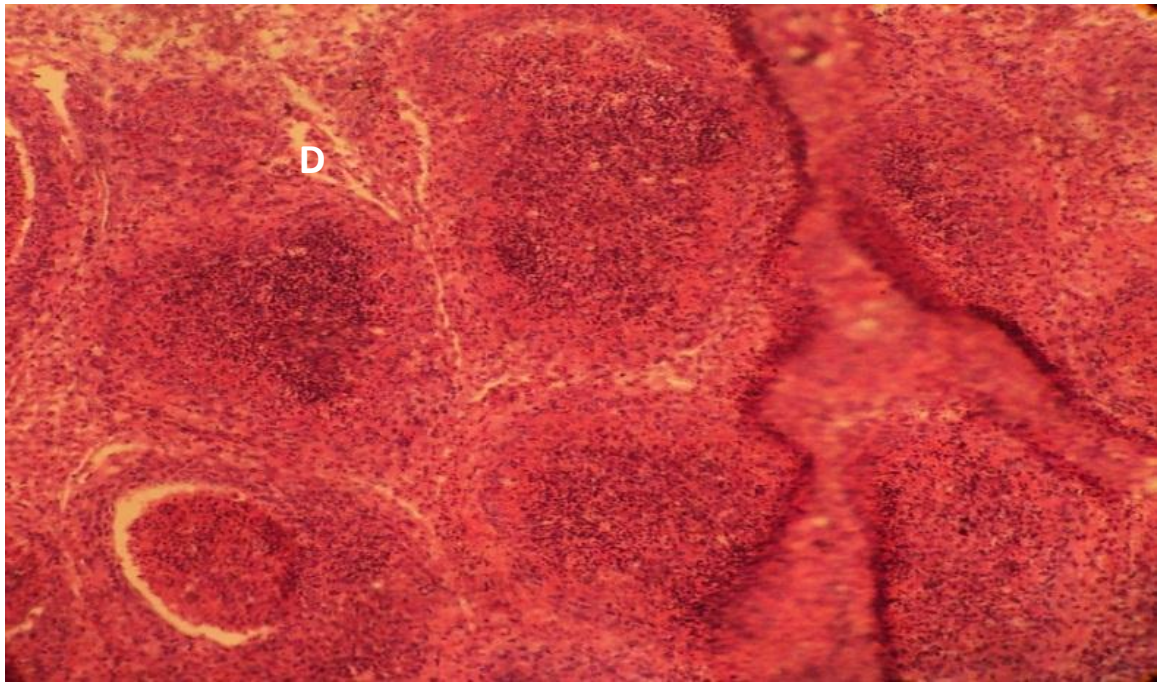
**Plate I: Gross pathology of breast, thigh and leg muscles, arrows showed; pale breast muscles (arrow A), petechial haemorrhages on the thigh muscles (arrow B), and leg muscles (arrow C & D) in the groups inoculated with a very virulent infectious bursal diseases virus**



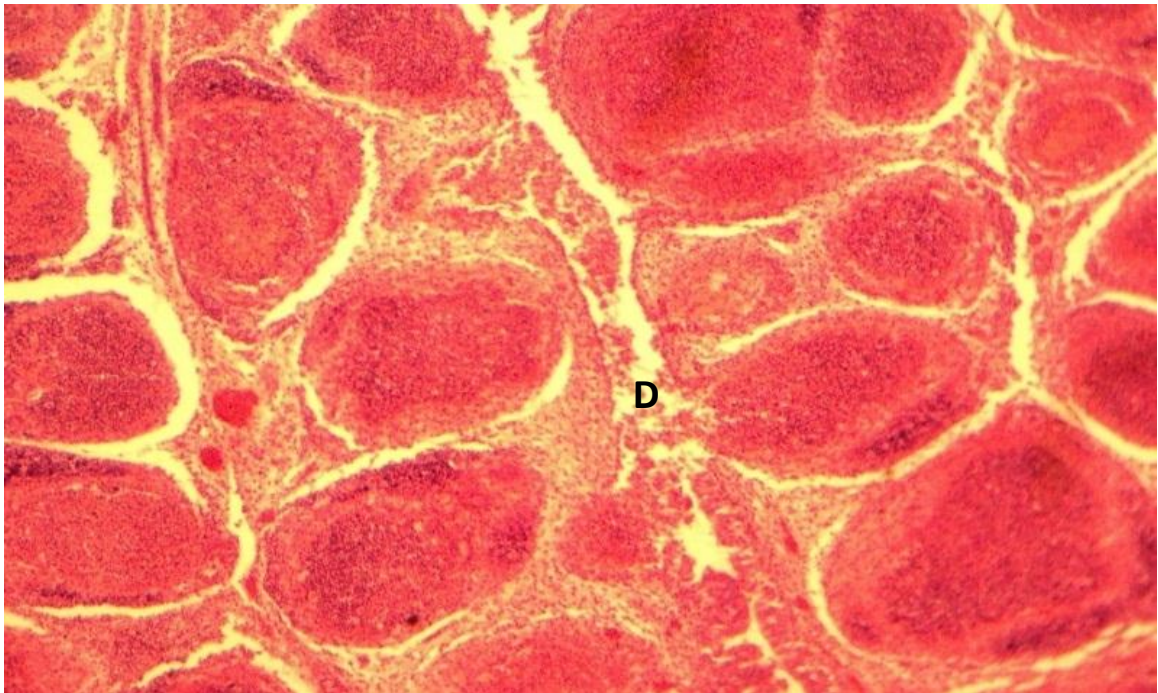
**Plate II: Gross pathology of kidneys and bursa of Fabricius, arrow showed pale and enlarged kidneys (arrow K) with prominent tubules and diffusely haemorrhagic bursa of Fabricius (arrow B) of ISA Brown chicks in the groups inoculated with a very virulent infectious bursal disease virus**



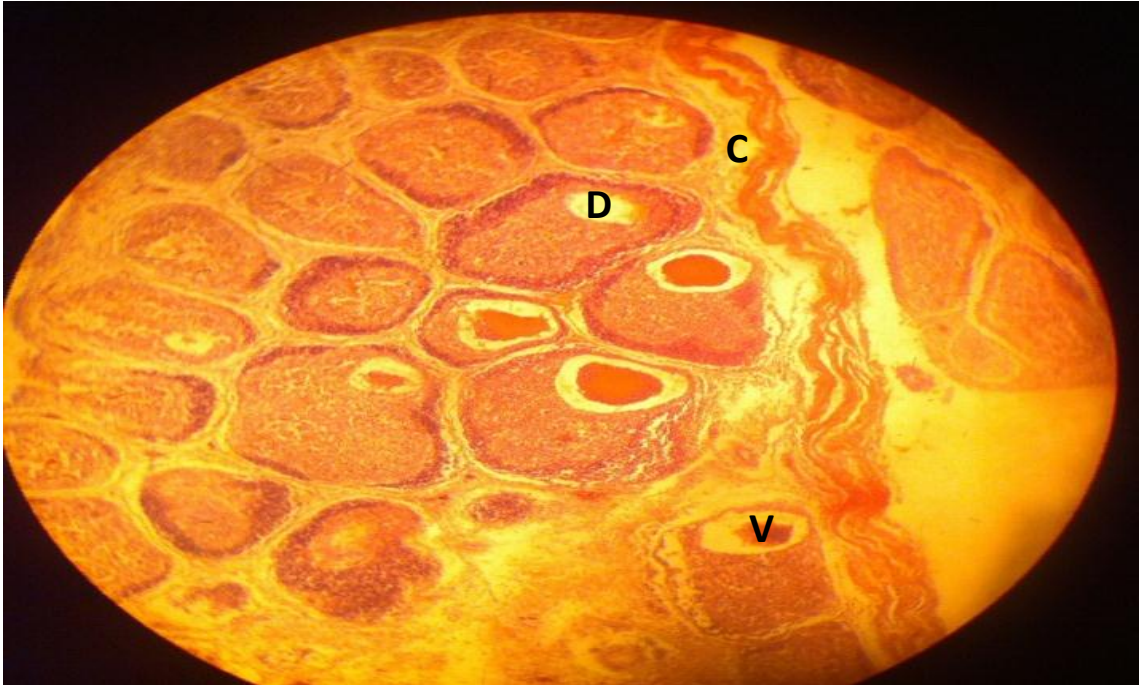
**Plate III: Grss pathology of spleen, showed enlarged and congested spleen of ISA  
Brown chicks in the groups inoculated with a very virulent infectious bursal  
disease virus**



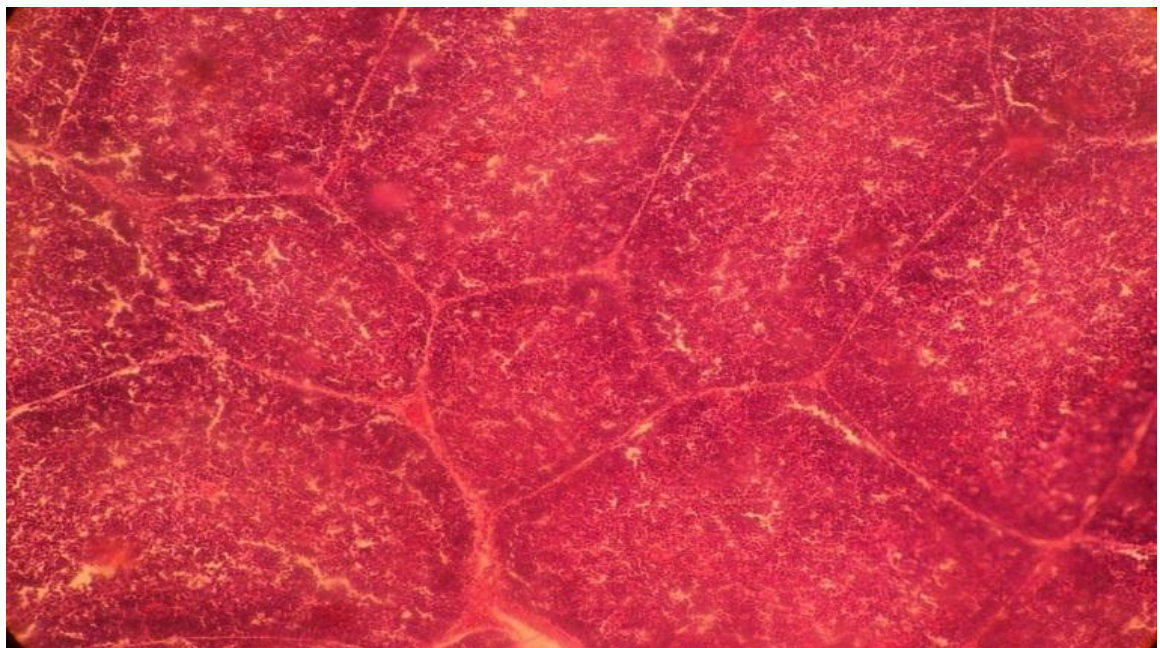
**Plate IV: Section of the bursa of Fabricius of ISA Brown chicks treated with Antox<sup>®</sup> (group A) and inoculated with a very virulent Infectious bursal disease virus. Note: showing depleted lymphoid cells (D) at 7 dpi (H and E, M 200)**



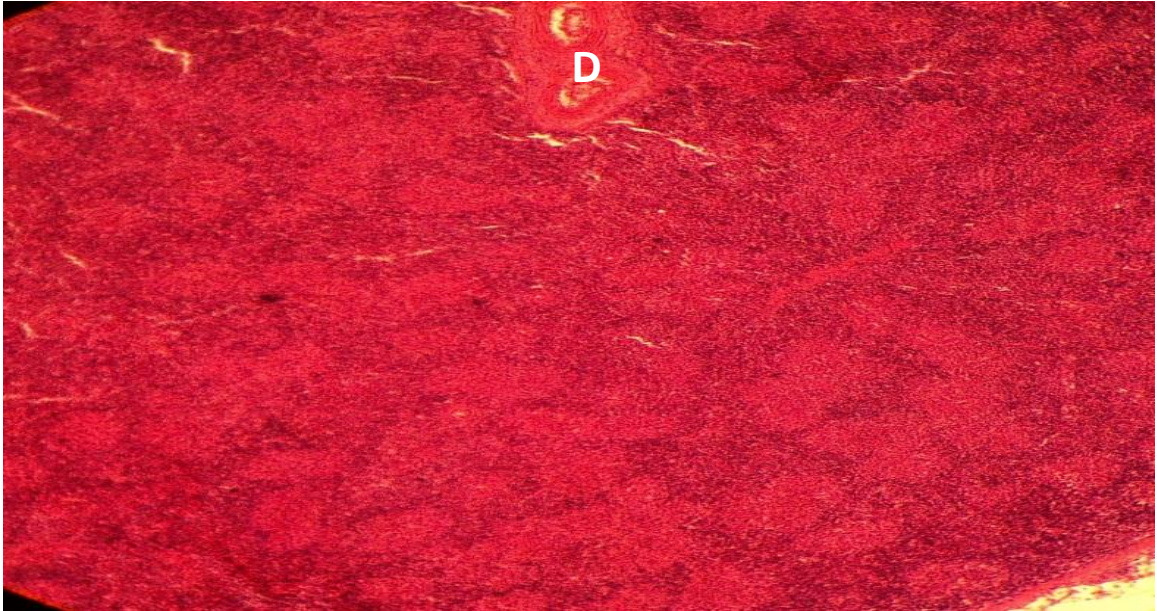
**Plate V: Section of the bursa of Fabricius of ISA Brown chicks treated with Bactofor<sup>®</sup> (group B) and inoculated with a very virulent infectious bursal disease virus. Note: showing depleted lymphoid cells (D) at 7 dpi (H and E, M 200)**



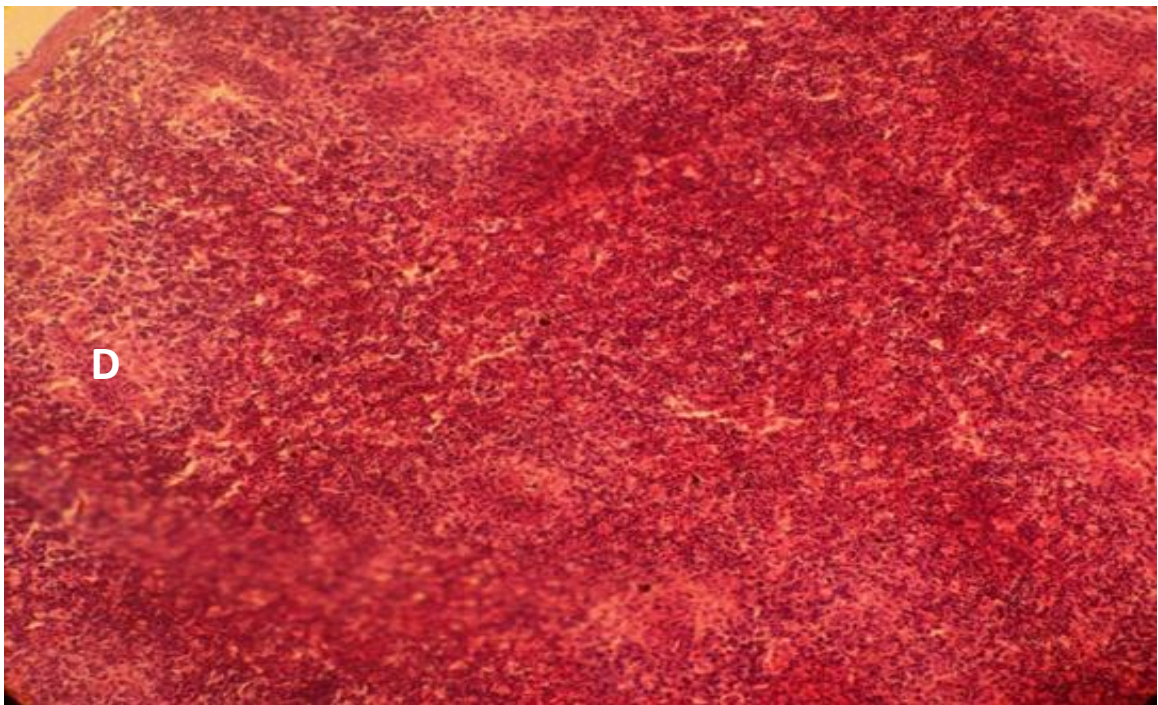
**Plate VI:** Section of the bursa of Fabricius of ISA Brown chicks (group C) infected with a very virulent infectious bursal disease virus. Note: showing congestion of blood cells (C), vacuolation in the cortex and medulla (V) and depleted lymphoid cells (D) at 7 dpi (H and E, M 200)



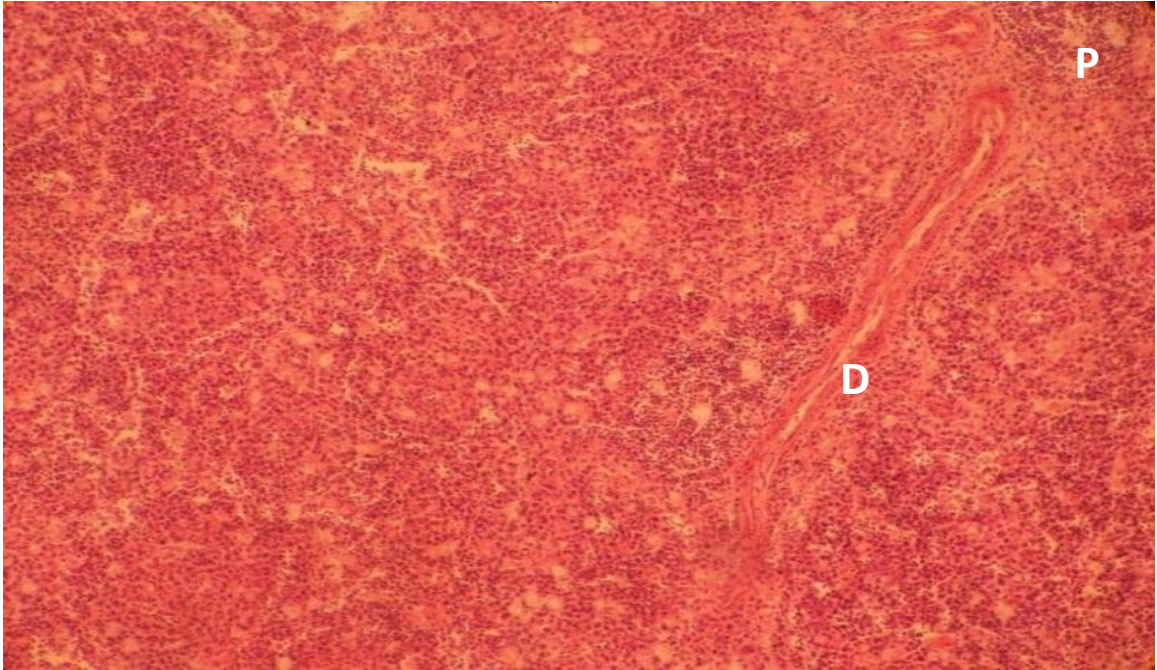
**Plate VII:** Section of the bursa of Fabricius of ISA Brown chicks (group D). Note: showing no significant histopathological findings, (B) bursal follicles at 35 dph (H and E, M 200)



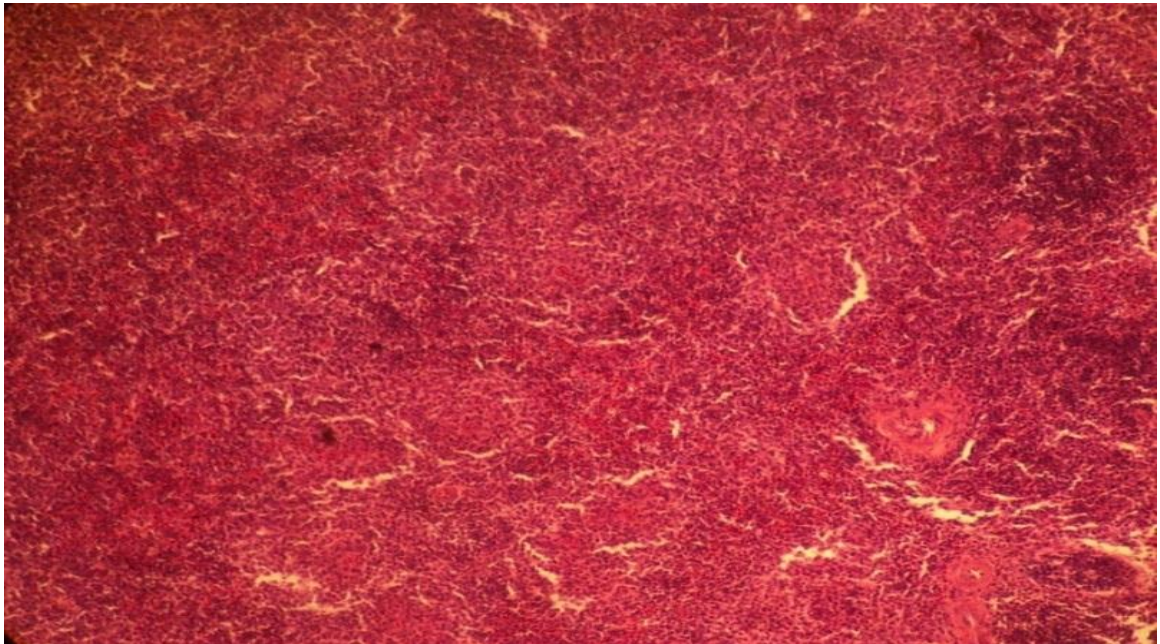
**Plate VIII:** Section of the spleen of ISA Brown chicks treated with Antox<sup>®</sup> (group A) and inoculated with a very virulent infectious bursal disease virus. Note: showing depleted lymphoid cells (D) at 7 dpi (H and E, M 200)



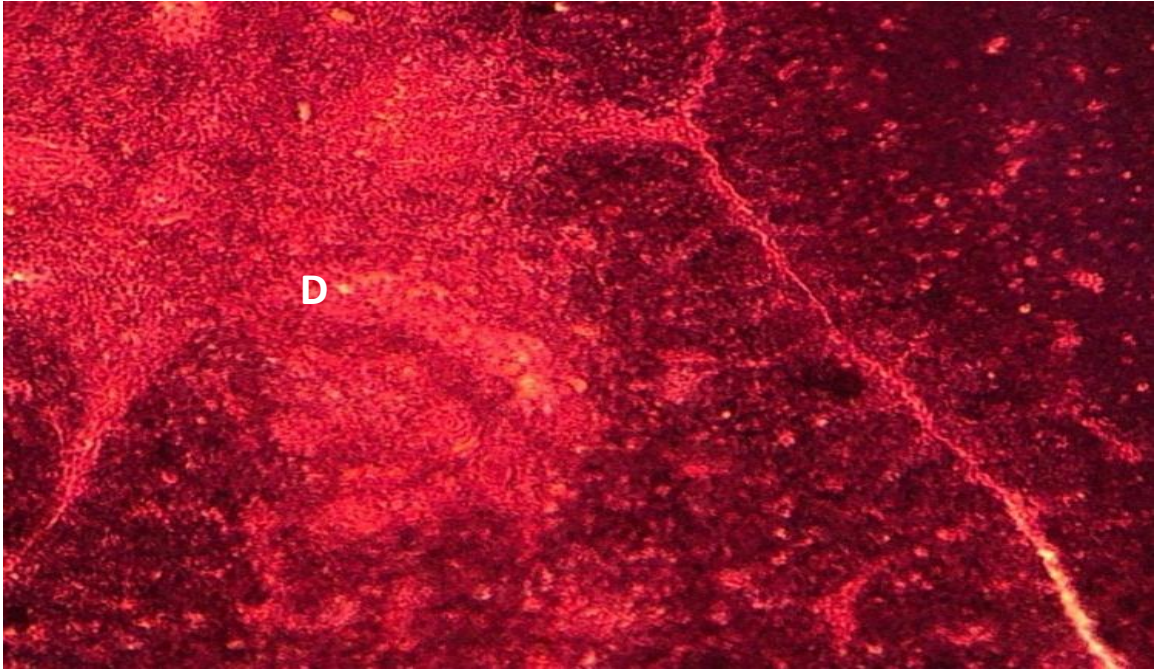
**Plate IX:** Section of the spleen of a ISA Brown chick treated with Bactofort<sup>®</sup> (group B) and inoculated with a very virulent infectious bursal disease virus. Note: showing depleted lymphoid cells (D) at 7 dpi (H and E, M 200)



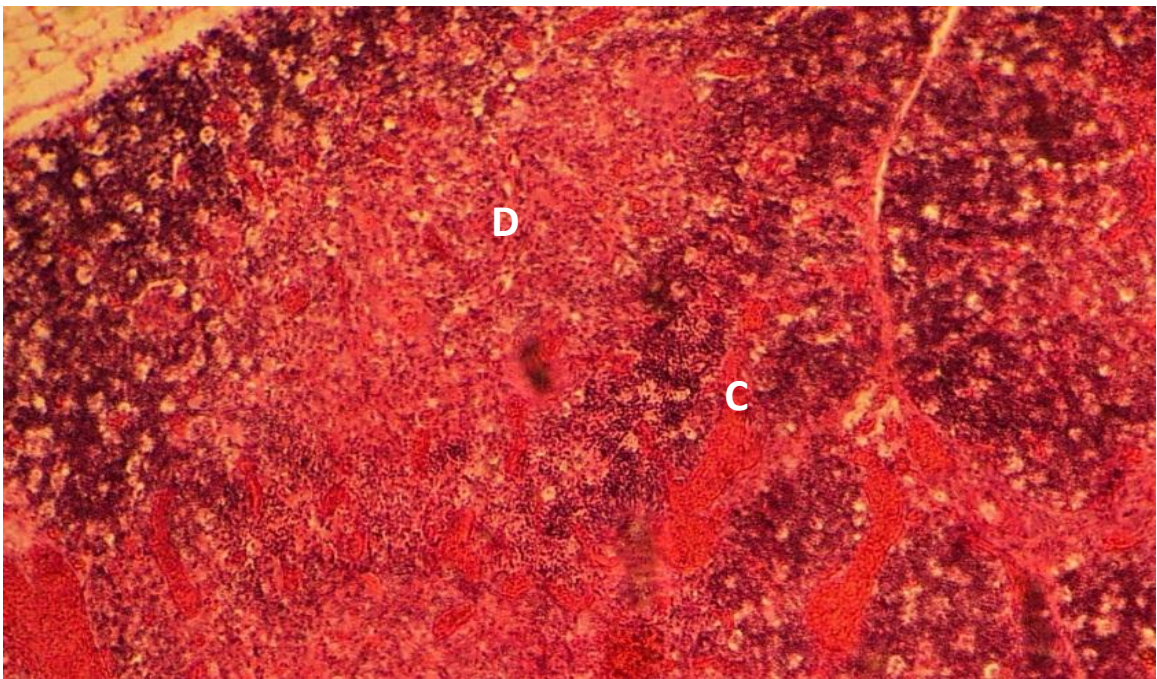
**Plate X: Section of the spleen of ISA Brown chicks (group C) inoculated with a very virulent infectious bursal disease virus. Note: showing depletion of lymphoid cells (D) and perivascular lymphoid cell infiltration (P) at 7 dpi (H and E, M 200)**



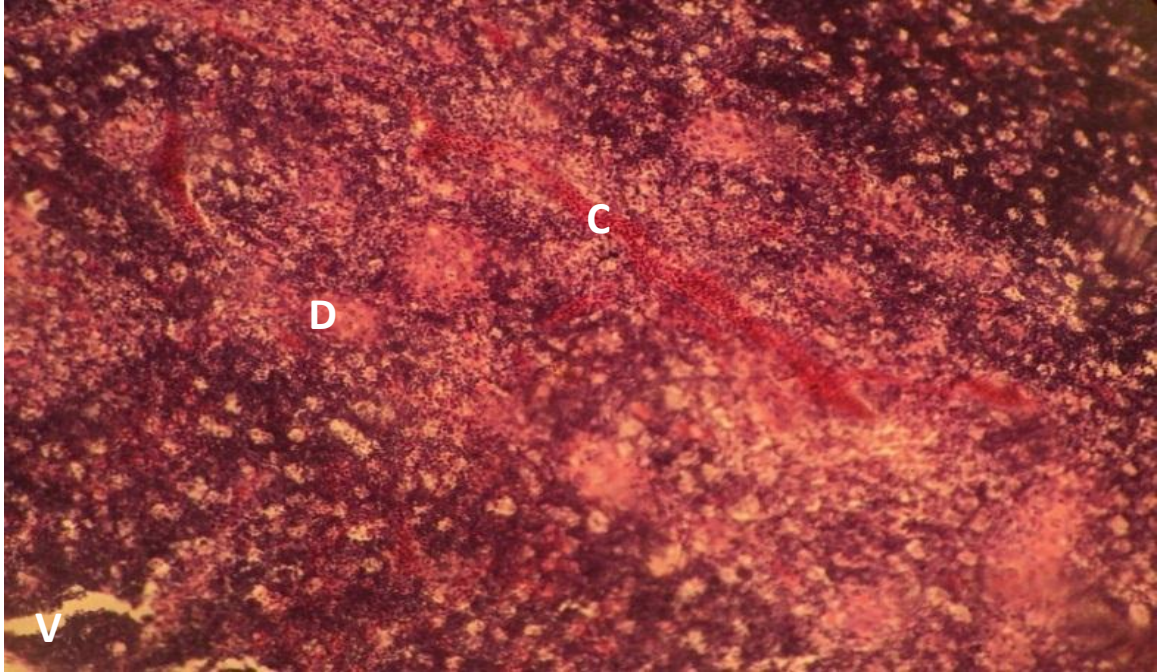
**Plate XI: Section of the of ISA Brown spleen (group D). Note: showing no significant histopathological findings, (M) medullary at 35 dph (H and E, M 200)**



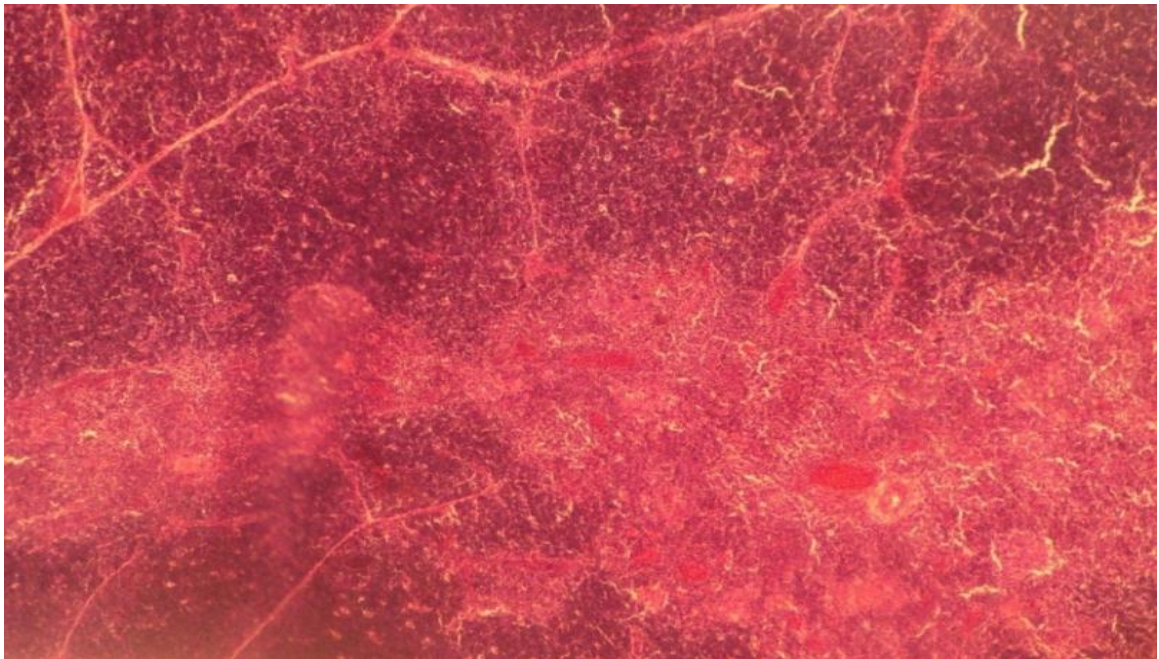
**Plate XII: Section of the thymus of ISA Brown chicks treated with Antox<sup>®</sup> (group A) and inoculated with a very virulent infectious bursal disease virus. Note: showing depleted lymphoid cells in the medullary region (D) at 7 dpi (H and E, M 200)**



**Plate XIII: Section of the Thymus of ISA Brown chicks treated with Bactofort<sup>®</sup> and inoculated with a very virulent Infectious bursal disease virus. Note: showing congestion of blood cells (C) and depleted lymphoid cells (D) at 7 dpi (H and E, M 200)**



**Plate XIV:** Section of the thymus of ISA Brown chicks (group C) inoculated with a very virulent infectious bursal disease virus. Note: showing congestion of blood cells (C) depleted lymphoid cells (D) and vacuolation in the cortex and medulla (V) at 7 dpi (H and E, M 200)



**Plate XV:** Section of the thymus of ISA Brown chicks (group D). Note: showing no significant histopathological findings at 35 dph (H and E, M 200)

## CHAPTER FIVE

### DISCUSSION

The morbidity rate of up to 85 per cent and mortality rate of up to 65 per cent as observed in positive control was highly suggestive of IBD. This indicates that the chicks were highly susceptible to IBDV at the time of inoculation. Antox<sup>®</sup> and Bactofort<sup>®</sup> were able to delay the onset of clinical signs by 2 dpi when compared to positive control in which the clinical signs 1 day were observed postinoculation. The highest percentage of clinical signs was recorded 4, 5 and 6 dpi in Antox<sup>®</sup>, Bactofort<sup>®</sup> and positive control group respectively.

The mortality rate was lower in the groups treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> when compared to positive control. Antox<sup>®</sup> and Bactofort<sup>®</sup> were able to delay the onset of morbidity and mortality when compared to positive control. Antox<sup>®</sup> and Bactofort<sup>®</sup> either reduced the destruction of body tissues or reduced the multiplication of vvIBDV and thus shorten course of morbidity and mortality due to IBD.

Anaemia in birds treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> were less severe, when compared to that in positive control. Antox<sup>®</sup> and Bactofort<sup>®</sup> probably enhanced production of erythropoietin, prevented the destruction of precursor cells in the bone marrow or haemorrhages in tissues usually seen in IBD (Kabir *et al.*, 2004).

Decrease in TWBC, heterophil and lymphocyte counts was significantly lower in chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> when compared to positive control. Antox<sup>®</sup> and Bactofort<sup>®</sup> possibly elicited the production of significant amount of immunoglobulins that

neutralised vvIBDV thereby reduced destruction of leucocytes as observed by Midilli *et al.* (2008).

The leucopaenia observed in the positive control due to decrease in heterophils and lymphocytes counts at 7 dpi is the consequence of corresponding heteropaenia and lymphopaenia. This result is in agreement with the findings of Cheville (1967), who also reported severe panleukopaenia during the severe inflammatory stage of IBD. The lymphopaenia observed in positive control at 7 dpi is probably due to the multiplication of vvIBDV and subsequent necrosis of bursal lymphocytes as observed by Ley *et al.* (2007).

Empirical evidences have shown that, once inflammation is established, an orchestra of chemical mediators modulates many events (Scope *et al.*, 2002). Cytokines released from local mononuclear cells make their way to the bone marrow, where they increase the rate of release of mature heterophils and the rate of production of stem-cells. Heterophil/lymphocyte (H/L) ratio, considered as providing important information for immune system tension following prolonged stress factors (Moreno *et al.*, 2002; Clinchy *et al.*, 2004), was higher in the positive control when compared to groups treated with Antox<sup>®</sup> and Bactofort<sup>®</sup>. Scope *et al.* (2002) observed a considerable increase in H/L ratio following stress associated with transporting, handling and viral disease of avian species. Therefore, the higher H/L ratio observed in this study could be as a result of the vvIBDV inoculated to the chicks.

There was a significantly higher ELISA antibody titre level in groups treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> when compared to that of positive control at 7 dpi. Antox<sup>®</sup> and Bactofort<sup>®</sup>

were therefore able to elicit stronger antibody response. The probiotics could have substances that stimulated the immune system to produce more immunoglobulins. This result is in agreement with the findings of Khaksefidi and Ghoorchi (2006), who reported that the antibody titre level in probiotics supplemented group was significantly higher at 5 and 10 dpi with vvIBDV when compared to positive control. The ELISA antibody titre level was higher in chicks treated with Antox<sup>®</sup> than Bactofort<sup>®</sup> and positive control group indicating that it was a more potent immune potentiator.

Groups treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> had low of GPx activity and MDA concentration. The lower in GPx activity and MDA concentration in probiotics treated 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). Evidence have shown that activity of GPx and concentration of MDA increase during infections with viral diseases but MDA concentration is an indirect measurement of oxidative stress (Inal *et al.*, 2001).

There was a significant decrease in body weight in vvIBDV inoculated groups. However, the decrease was significantly lower in the groups treated with Antox<sup>®</sup> and Bactofort<sup>®</sup>. The probiotics could have stimulated the appetite of the chicks, thereby enhancing feed intake and slowing decrease in body weight gain. This is in consonance with the findings of Kabir *et al.* (2004) that tested the efficacies of commercial probiotics and found statistical significant increase in the body weights of chicks treated with probiotics and inoculated with *Salmonella*. The significant decrease in the bursal weight of chicks in positive control when compared to the groups treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> can be attributed to

depletion of B-lymphocytes with consequent atrophy of the BF. The bursal body weight ratio was significantly lower in chicks, treated with Antox<sup>®</sup> and Bactofort<sup>®</sup>. The bursal body weight ratio was significantly higher in the chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> compared to chicks in positive control. It appears that the vvIBDV had a more deleterious effect on the BF of chicks in positive control.

The pathology in the BF, SPL and THY was more severe of chicks in positive control when compared to groups treated with Antox<sup>®</sup> and Bactofort<sup>®</sup>. The probiotics therefore protected the integrity of BF, SPL and THY. Treatment with Antox<sup>®</sup> and Bactofort<sup>®</sup> will therefore subsequently protect chicks against diseases prevalent in Nigeria such as Newcastle disease, Marek's disease, avian pox, colibacillosis, fowl cholera, salmonellosis, and coccidiosis.

The oedema, hyperaemia, increase in the interfollicular spaces of BF, SPL and THY observed in positive control inoculated with vvIBDV is as a result of inflammatory process. This re-affirms the findings by Cheville, 1967; Ley *et al.*, 1983; Chineme and Cho, 1984.

## CHAPTER SIX

### CONCLUSION AND RECOMMENDATIONS

#### 6.1 Conclusion

From this study it was concluded that:

1. Antox<sup>®</sup> and Bactofort<sup>®</sup> ameliorated the negative effects of vvIBDV on lymphocyte ( $7.42 \pm 0.47$  and  $6.99 \pm 0.42$ ) and heterophil counts ( $2.22 \pm 0.26$  and  $1.83 \pm 0.05$ ) when compared to positive control ( $2.79 \pm 0.12$  and  $1.48 \pm 0.08$ ) at 7 dpi.
2. Antox<sup>®</sup> and Bactofort<sup>®</sup> ( $9.12 \pm 0.52$  and  $8.12 \pm 1.58$ ) elicited the strongest antibody response against vvIBDV when compared to positive control ( $4.42 \pm 1.87$ ) at 7 dpi.
3. Antox<sup>®</sup> shortens the duration of clinical signs by three days and reduced the percentage of clinical signs observed by 29.23 %.
4. Antox<sup>®</sup> and Bactofort<sup>®</sup> were able to shorten the duration of morbidity by two days.
5. Mortality rates were lower in chicks treated with Antox<sup>®</sup> (34.78 %) and Bactofort<sup>®</sup> (47.60 %) compared to that of positive control (72.70 %).
6. Antox<sup>®</sup> and Bactofort<sup>®</sup> reduced the severity of microscopic lesions in the BF, SPL and THY as compared to that of positive control.
7. Antox<sup>®</sup> and Bactofort<sup>®</sup> prevented the negative effects of vvIBDV on GPx ( $45.97 \pm 6.90$  and  $55.59 \pm 4.99$ ), MDA ( $826.22 \pm 17.24$  and  $873.10 \pm 24.22$ ), when compared to positive controls ( $79.80 \pm 40.63$  and  $1406.86 \pm 24.90$ ) at 7 dpi.

## **6.2 Recommendations**

It was recommended that:

- 1 Antox<sup>®</sup> and Bactofort<sup>®</sup> can be used by farmers and clinicians for the control of IBD in Nigeria.
- 2 Further studies should be carried out to evaluate different dose regimen of Antox<sup>®</sup> Bactofort<sup>®</sup> on IBD.
- 3 Further studies should be carried out to determine the adverse effects of Antox<sup>®</sup> and Bactofort<sup>®</sup> when administered for more than six weeks of age.

## **6.3 Limitations of the Study**

The limitations of this study were as follows:

- 1 Markers of oxidative stress in tissues were not assayed.
2. Feed conversion ratio was not determined.

## **6.4 Contributions of the Study to Knowledge**

The Antox<sup>®</sup> and Bactofort<sup>®</sup>, reduced morbidity and mortality, increased antibody response, increased body weight and reduced oxidative stress and anaemia due to IBD.

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## Appendix 1: Pathotypic variation of infectious bursal disease virus

|                            |                                         |
|----------------------------|-----------------------------------------|
| - Apathogenic (Serotype 2) | no mortality, no bursa lesions          |
| - Pathogenic (Serotype 1)  |                                         |
| 5 Mild                     | no mortality, increasing bursal lesions |
| 6 Intermediate             |                                         |
| 7 Intermediate plus        |                                         |
| 8 Classical                | increasing mortality                    |
| 9 Variant                  |                                         |
| 10 Very or hypervirulent   |                                         |

## Appendix 2: Preparation of Natt-herrick solution

Sodium chloride (NaCl) - 3.88 g

Sodium sulphate (Na<sub>2</sub>SO<sub>4</sub>) - 2.50 g

Sodium Phosphate (Na<sub>2</sub>HPO<sub>4</sub>) - 2.91 g

Potassium phosphate (KH<sub>2</sub>PO<sub>4</sub>) - 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

- Use a standard RBC diluting pipette
- Dilute whole anticoagulated blood with Natt- Herrick's solution at the rate of 1:200
- Allow diluted blood to mix for a minute or two
- Discharge into the haemocytometer counting chamber (using a capillary tube). After charging haemocytometer, allow contents to settle for approximately 3 minutes.

### Performing the Total Erythrocyte Count

- Use high dry (40X) objective of the microscope
- Count total number of RBC (easily recognisable by their nuclei) in the four chambers.
- 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:

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

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

- (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 granularity
- The total leucocyte count is obtained by counting all leukocytes present in the nine large ruled squares of the haemocytometer.
- 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 leucocyte count is as follows:

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

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

### Appendix 3: 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

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

### Appendix 4: Procedure for modified wright- giemsa stain

#### Working Stain

- 3 g Wright stain powder
- 0.3 g Giemsa stain powder
- 5ml glycerol
- To 1000 ml absolute methanol (acetone free)
- 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  $Na_2HPO_4 \cdot 2H_2O$  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

- Prepare thin blood smears
- Place on staining rack
- Flood smear with Wright- Giemsa stain, allow to stand for 3 minutes
- Add equal amount of Sorensen's pH 6.5- 6.8 buffers, depending on batch stain.
- Mix gently by blowing using a pipette until metallic green sheen forms on the surface, allow to stand for 6 minutes
- Rinse with buffer, allowing to stand for 1 minute for differentiation
- Wash copiously with buffer
- Wipe the back of smear with tissue to remove excess stain
- Prop in rack until dry.

**Appendix 5: Gross lesions of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group.**

|       |             | Day post-inoculation                |     |     |     |     |     |     |     |
|-------|-------------|-------------------------------------|-----|-----|-----|-----|-----|-----|-----|
| Organ | Lesion      | 2                                   | 3   | 4   | 5   | 6   | 7   | 8   | 9   |
|       |             | No. birds with lesions/No. examined |     |     |     |     |     |     |     |
|       |             | <b>Group A</b>                      |     |     |     |     |     |     |     |
| Bursa | Enlargement | 0/0                                 | 1/1 | 2/2 | 3/3 | 2/2 | 0/0 | 0/0 | 0/0 |
|       | Congestion  | 0/0                                 | 1/1 | 2/2 | 3/3 | 2/2 | 0/0 | 0/0 | 0/0 |
|       | Oedema      | 0/0                                 | 1/1 | 2/2 | 3/3 | 2/2 | 0/0 | 0/0 | 0/0 |
|       | Haemorrhage | 0/0                                 | 0/1 | 2/2 | 3/3 | 2/2 | 0/0 | 0/0 | 0/0 |
|       | Atrophy     | 0/0                                 | 0/1 | 0/2 | 0/3 | 0/2 | 0/0 | 0/0 | 0/0 |
|       |             | <b>Group B</b>                      |     |     |     |     |     |     |     |
| Bursa | Enlargement | 0/0                                 | 1/1 | 3/3 | 3/3 | 2/2 | 1/1 | 0/0 | 0/0 |
|       | Congestion  | 0/0                                 | 1/1 | 3/3 | 3/3 | 2/2 | 1/1 | 0/0 | 0/0 |
|       | Oedema      | 0/0                                 | 1/1 | 3/3 | 3/3 | 2/2 | 1/1 | 0/0 | 0/0 |
|       | Haemorrhage | 0/0                                 | 0/1 | 3/3 | 3/3 | 2/2 | 1/1 | 0/0 | 0/0 |
|       | Atrophy     | 0/0                                 | 0/1 | 0/3 | 0/3 | 0/2 | 1/1 | 0/0 | 0/0 |
|       |             | <b>Group C</b>                      |     |     |     |     |     |     |     |
| Bursa | Enlargement | 1/1                                 | 2/2 | 4/4 | 3/3 | 3/3 | 2/2 | 0/0 | 1/1 |
|       | Congestion  | 1/1                                 | 2/2 | 4/4 | 3/3 | 3/3 | 2/2 | 0/0 | 1/1 |
|       | Oedema      | 1/1                                 | 2/2 | 4/4 | 3/3 | 3/3 | 2/2 | 0/0 | 1/1 |
|       | Haemorrhage | 1/1                                 | 2/2 | 4/4 | 3/3 | 3/3 | 2/2 | 0/0 | 1/1 |
|       | Atrophy     | 0/1                                 | 0/2 | 0/4 | 0/3 | 0/3 | 2/2 | 0/0 | 1/1 |
|       |             | <b>Group D</b>                      |     |     |     |     |     |     |     |
| Bursa | Enlargement | 0/0                                 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |
|       | Congestion  | 0/0                                 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |
|       | Oedema      | 0/0                                 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |
|       | Haemorrhage | 0/0                                 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |
|       | Atrophy     | 0/0                                 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |

**Appendix 6: Gross Lesions of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group.**

| Organ  | Lesion     | Day post-inoculation                |     |     |     |     |     |     |     |
|--------|------------|-------------------------------------|-----|-----|-----|-----|-----|-----|-----|
|        |            | 2                                   | 3   | 4   | 5   | 6   | 7   | 8   | 9   |
|        |            | No. birds with lesions/No. examined |     |     |     |     |     |     |     |
|        |            | <b>Group A</b>                      |     |     |     |     |     |     |     |
| Thymus | Congestion | 0/0                                 | 1/1 | 2/2 | 3/3 | 2/2 | 0/0 | 0/0 | 0/0 |
|        | Atrophy    | 0/0                                 | 0/1 | 0/2 | 0/3 | 0/2 | 0/0 | 0/0 | 0/0 |
|        |            | <b>Group B</b>                      |     |     |     |     |     |     |     |
| Thymus | Congestion | 0/0                                 | 1/1 | 3/3 | 3/3 | 2/2 | 1/1 | 0/0 | 0/0 |
|        | Atrophy    | 0/0                                 | 0/1 | 0/3 | 0/3 | 0/2 | 1/1 | 0/0 | 0/0 |
|        |            | <b>Group C</b>                      |     |     |     |     |     |     |     |
| Thymus | Congestion | 1/1                                 | 2/2 | 4/4 | 3/3 | 3/3 | 2/2 | 0/0 | 1/1 |
|        | Atrophy    | 0/1                                 | 0/2 | 0/4 | 0/3 | 0/3 | 2/2 | 0/0 | 1/1 |
|        |            | <b>Group D</b>                      |     |     |     |     |     |     |     |
| Thymus | Congestion | 0/0                                 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |
|        | Atrophy    | 0/0                                 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |

**Appendix 7: Gross lesions of ISA Brown chicks treated with Antox<sup>®</sup> and Bactofort<sup>®</sup> from day old and inoculated with a very virulent infectious bursal disease virus at 28 days old as well as positive and negative control group.**

| Organ  | Lesion      | Day post-inoculation                |     |     |     |     |     |     |     |
|--------|-------------|-------------------------------------|-----|-----|-----|-----|-----|-----|-----|
|        |             | 2                                   | 3   | 4   | 5   | 6   | 7   | 8   | 9   |
|        |             | No. birds with lesions/No. examined |     |     |     |     |     |     |     |
|        |             | <b>Group A</b>                      |     |     |     |     |     |     |     |
| Spleen | Congestion  | 0/0                                 | 1/1 | 2/2 | 3/3 | 2/2 | 0/0 | 0/0 | 0/0 |
|        | Enlargement | 0/0                                 | 1/1 | 2/2 | 3/3 | 2/2 | 0/0 | 0/0 | 0/0 |
|        |             | <b>Group B</b>                      |     |     |     |     |     |     |     |
| Spleen | Congestion  | 0/0                                 | 1/1 | 3/3 | 3/3 | 2/2 | 1/1 | 0/0 | 0/0 |
|        | Enlargement | 0/0                                 | 1/1 | 3/3 | 3/3 | 2/2 | 1/1 | 0/0 | 0/0 |
|        |             | <b>Group C</b>                      |     |     |     |     |     |     |     |
| Spleen | Congestion  | 1/1                                 | 2/2 | 4/4 | 3/3 | 3/3 | 2/2 | 0/0 | 1/1 |
|        | Enlargement | 1/1                                 | 2/2 | 4/4 | 3/3 | 3/3 | 2/2 | 0/0 | 1/1 |
|        |             | <b>Group D</b>                      |     |     |     |     |     |     |     |
| Spleen | Congestion  | 0/0                                 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |
|        | Enlargement | 0/0                                 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |