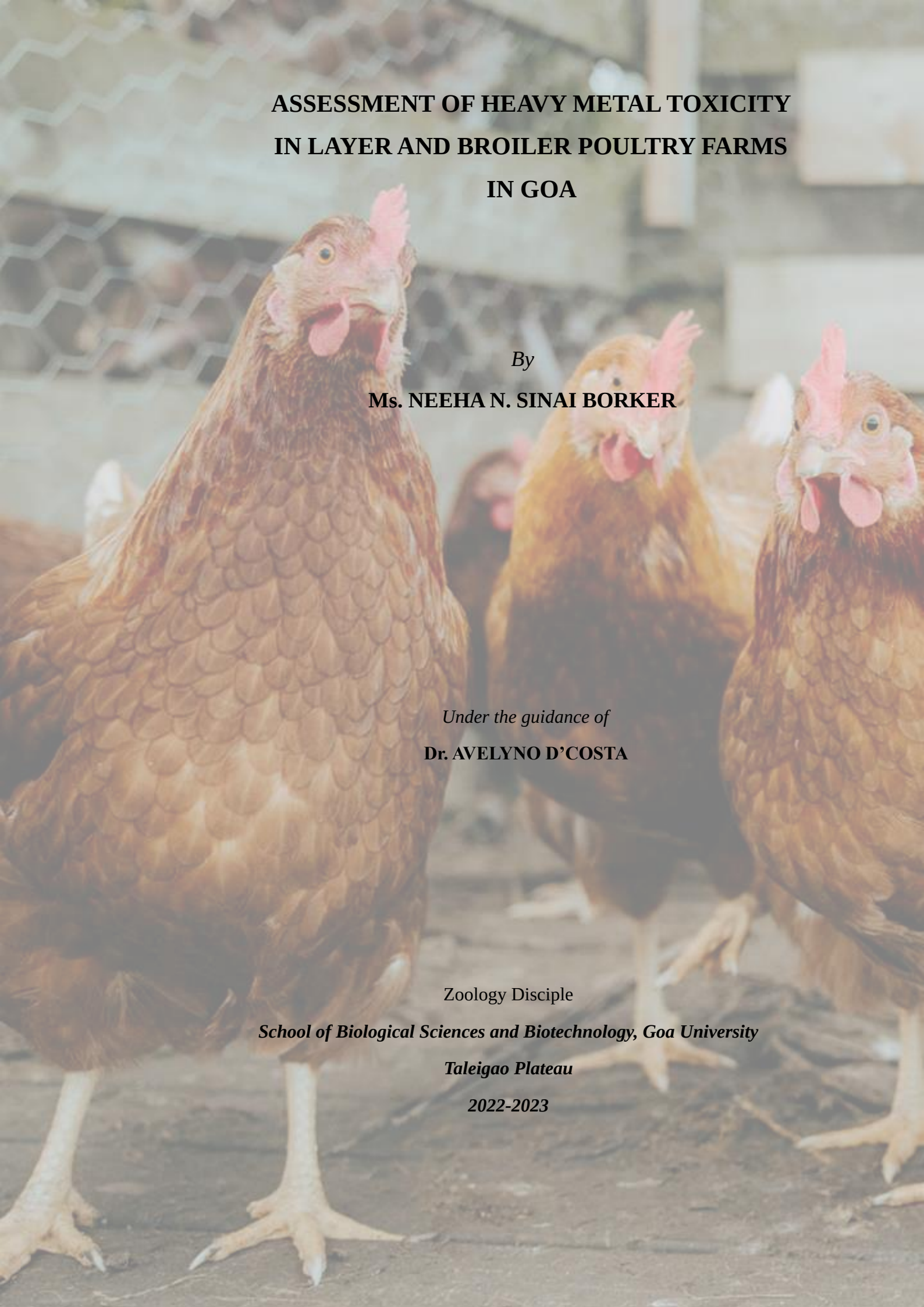

ASSESSMENT OF HEAVY METAL TOXICITY IN LAYER AND BROILER POULTRY FARMS IN GOA

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Assessment of heavy metal toxicity in Layer and Broiler poultry farms in Goa

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation entitled, **"Assessment of heavy metal toxicity in Layer and Broiler poultry farms in Goa"** is based on the results of investigations carried out by me in the **Zoology Discipline** at the **School of Biological Sciences and Biotechnology**, Goa University under the Supervision of **Dr. Avelyno H. D'Costa** and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given in the dissertation.

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- **Neeha Sinai Borker**

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LIST OF ABBREVIATIONS

- AAS – Atomic Absorption Spectroscopy
- As -Arsenic
- Cd- Cadmium
- C:N- Carbon: nitrogen ratio
- COD- Chemical oxygen demand
- Cr -Chromium
- Cu – Copper
- DW- Distilled water
- EDTA – Ethylenediaminetetraacetic acid
- Fe -Iron
- g- gram
- HM – Heavy metal
- Hb- Hemoglobin
- ICP-OES -Inductively Coupled Plasma Optical Emission Spectroscopy
- kg -kilogram
- ml- millilitre
- mM – millimolar
- Mn – Manganese
- MNi - Micronuclei
- Pb -Lead
- ppb – parts per billion
- ppm- part per million
- PM- Poultry manure

- Rpm – Revolutions per minute
- TS- Total solids
- TOC- Total organic carbon
- TP- Total phosphorus
- μl – microlitre
- VS- Volatile solids
- Zn – Zinc

PREFACE

Heavy metals are those metals and metalloids which have a high relative density of greater than 3 g/cm^3. They are incorporated in trace amounts in the diet of poultry animals to enhance feeding efficiency and cater to micronutrient deficiencies. Due to higher density and non-biodegradable nature, a part of it is retained in the body whereas the rest enters the environment through defecation. Accumulation of this litter in poultry farms can have two major consequences: first, exposure to this litter can be harmful to poultry workers and poultry birds in the long run, second, direct application of this chicken litter as manure may increase the heavy metal levels more than the permissible limits of soil, amounting to ecotoxicity. Moreover, the non-essential heavy metals like Arsenic, Lead and Cadmium To date, most studies have determined the heavy metal content in composted chicken manure, however, very few studies pertaining to the quantification of heavy metals in fresh chicken litter exist. To our best knowledge, no study has been conducted in determining the heavy metal content of fresh chicken litter in Goa. Additionally, broiler breed *Vencobb 400* has not been studied for genotoxicity. Hence, this thesis is aimed at filling these lacunae by estimating the content of heavy metals in chicken feed and manure. It is further aimed at assessing the genotoxicity in broiler chicken using micronucleus test and comet assay.

The thesis is divided into five chapters. The first chapter is introduction, which details about poultry farming, immense contribution of chicken in poultry

sector, chicken litter and chicken feed as possible sources of heavy metals and the consequences of improper disposal of chicken litter.

The second chapter is the review of literature on the studies conducted in heavy metal toxicity on account of poultry litter, feed and litter studies and genotoxicity in broiler chicken and identifies gaps.

The third chapter is materials and methods which consists of selection criteria of study areas, methods used for estimating the physicochemical parameters and heavy metal content in chicken litter and feed.

The fourth chapter is results where the concentration of each metal in feed and litter was estimated and subsequently correlated using statistical analysis.

The fifth chapter is discussion, where the possible reasons for the high or low values of heavy metal content are discussed and validated with other research work.

CHAPTER 1: INTRODUCTION

1.1 Poultry farming industry

Poultry farming refers to the act of rearing domesticated birds for the production of meat and egg. It is one of the fastest-growing sub-agricultural sectors, with a rate of 8% increase per annum. This industry has become one of the most efficient utilities of animal husbandry, providing nutritional security to a significant number of people across the globe. Chicken, ducks, turkeys, quails, gees,e and pigeons are some of the predominantly reared poultry birds (Gržinić et al., 2023).

1.2 Chicken species

Gallus Gallus domesticus, (Linnaeus, 1758) also known as "domestic chicken", is the most widely reared poultry bird. According to Food and Agricultural Organisation, this species constitutes 94% of the world's poultry population, contributing to 90% of world's meat production and 93% of world's egg production (FAO, 2023). Belonging to Order Galliformes and Family Phasianidae, chicken is an excellent, relatively cheaper source of protein than other meat products. Chicken eggs are wholesome, rich sources of vitamins, iron, folate, selenium, zinc, lutein and zeaxanthin whereas chicken meat, offers low caloric value and low content of trans- fats due to which it is preferred over red meat. The innumerable benefits offered by these by-products, has resulted in spark rise in the consumption of chicken meat from 9 to 133.8 million tonnes and chicken eggs from 15 to 93 million tonnes from 1961-2020 (MoA & FW, 2017).

Depending on the purpose of consumption, chicken breeds can be classified into 3 types **(*Table 1.1)***.

Table 1.1: Types of chicken breed

Characteristics	**Dual Purpose (Mueller et al., 2018)**	**Egg Layers (TNAU, 2015)**	**Meat Broilers (MoA & FW, 2017)**
Purpose	Female chicks reared for eggs while male chicks are fattened for meat	Reared for eggs	Reared for meat
Gender	Young chicks of both gender	Young chicks of gender	Male chicks
Type of housing system	**Rural backyard poultry farming:** Litter containing leaf material, husk, wood scrappers and scrap wire netting walls.	**Battery-caged system:** Battery cages constructed with Nipple system of feed and water supply.	**Deep litter system:** Housed on litter containing husk and wood shavings with automatic feeders and water provision.
Rearing period	Reared for 18-78 weeks, with male chicks culled at 32 days.	Reared from 18 to 78 weeks.	Reared for 6 to 8 weeks to attain 1.8-2.2kg slaughter weight
Culling	Male chicks culled at 32 days.	Sold after 78 weeks to backyard poultry or culled.	Culled between 39-42 days.
Commercial strains	*Walesby Specials, Novogen Dual, Speckled sussex*.	*BV-300, Bowans, Hyline and Dekalb Lohman*	*Cobb, Hubbard, Anak, Avian-34*

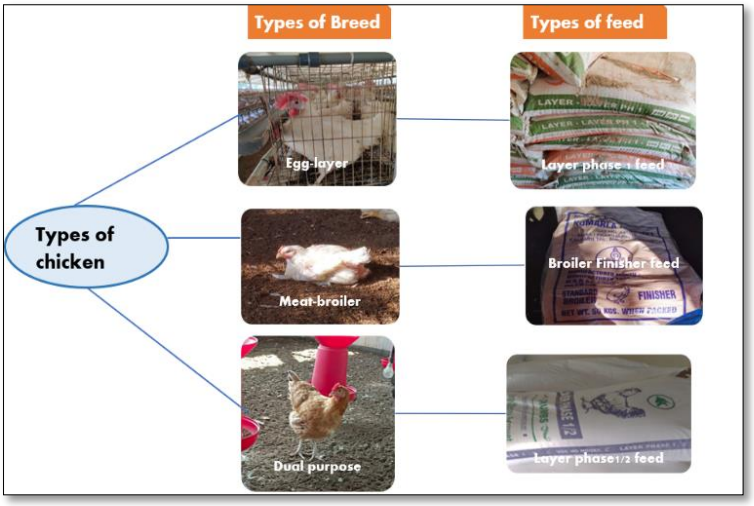

Fig. 1.1: Types of chicken breeds

1.3 Evolution of broiler chicken

The present-day broiler chicken is a result of intentional selection pressure and genetic hybridisation. This species is the descendant of a sole ancestor called 'red jungle fowl' (*Gallus gallus)* in the early 5400 BC in Southeast Asia (West & Zhou, 1988). Historically, poultry farming was a small-scale domestic activity with lack of commercialisation and practised with the intention of egg production. Back then, male and female chicken could not be differentiated until 7 weeks of age, so they were grown to their full lifespan of 96 weeks upon which they were culled for poultry meat. However, with increasing commercialization and the advent of genetic studies, this activity turned into a mass-scale intensive production where optimising egg production and obtaining chicken meat at a faster rate was the prime focus. The crossbreed between male broad-breasted Cornish strain and female broad-breasted White Plymouth Rocks in 1930's was the very first attempt that resulted in a broiler strain. This strain however had problems associated with disease susceptibility and availability of low meat. Subsequently, modern strains were developed to tackle these problems and

attain faster growth as compared to chicken layer breeds. These breeds are derived from stocks of White leghorn stock and Cobb. In particular, Cobb breed accounts for majority of the broilers in India which was developed from white feathered female line (Griffin & Goddard, 1994).

The modern-day broiler strains have successfully reduced the lifespan of chicken to 39 days, attaining a slaughter weight of 2.2kg in such a short span of time. There has also been an enormous increase in broiler growth by almost 400% from 1957 to 2005 (Zuidhof et al., 2014). While this change has been successful on an economic scale, this genetic pressure has resulted in skeletal deformities, compromised the immune system functioning and resulted in metabolic disorders. To tackle this, chicken feed, is supplemented with minerals which may contribute to DNA damage in the long run (Ogunwale et al., 2021). In addition to this, the recurrent genetic manipulation, may increases the risk of DNA damage. Given this fact, it can be hypothesised that there is possible DNA damage in chicken, owing to the abuse of feed additives.

1.4 Chicken litter

The rapid expansion of layer and broiler industry has also contributed to the large-scale generation of tonnes of waste in the poultry industry. These waste products include chicken litter, dead birds, feathers, sawdust, excess feed and spilled water, among which, chicken litter constitutes a major portion (Muduli et al., 2018).

Chicken litter refers to the faecal matter mixed with floor constituents like wasted feed and bedding material. It is produced through a cloacal passage, which includes the urethra, ejaculation canal and the anal canal. Due to this

unique excretory organ, the faecal and urine matter is excreted together (Zhu et al., 2020). The production of chicken litter is massive across the globe, with India contributing to at least 38.33 million tonnes of poultry litter and US contributing to 14 million tonnes of chicken litter annually (Z. Chen & Jiang, 2014; Prabakaran & Valavan, 2021) .

Though chicken litter is a foul odour producing contaminant, human beings have managed to utilise it as a useful resource. For centuries, chicken litter has been the cheapest and rich source of manure for crop production due to the excess amount of Nitrogen (N), Phosphorus (P) and Potassium (K). Currently, it is being utilised as a low-cost protein feed for dairy and beef cattle, biodiesel and biogas production (Jeffrey et al., 1998; Prabakaran & Valavan, 2021).

While chicken litter is an excellent source of manure, it is a time-consuming process for the manure to undergo complete composting. In most poultry farms, it is observed that there is direct application of untreated chicken manure to agricultural land, which results in two major repercussions: -

Microbial contamination: In battery-cage poultry system, the chicken litter is accumulated in heaps and piles of open landfills. The top surface is usually filled with fresh chicken excreta which provides breeding grounds for coliform bacteria like *Salmonella sp.*, *Staphylococci* , *Enterobacteriaceae* and fungal species like *Histoplasma capsulatum* which may contribute to infections in chicken and poultry workers (Elasri & El amin Afilal, 2016; Kim et al., 2012; Mohamed el Amin, 2014).

Trace elemental toxicity: Trace elements are those elements present at low concentrations (mg kg^{-1} or less) in agroecosystems (He et al., 2005). They are

incorporated in later stages of poultry feed to enhance feeding efficiency and cater to the micronutrient deficiencies. Once ingested, they enter the environment through excrement. In battery-cage systems, these elements pile up and accumulate in the open landfills amounting to ecotoxicity. One class of trace elements which is a rising matter of concern is "heavy metals.

1.5 Heavy metal toxicity in chicken litter

Heavy metals are those elements having high densities of ≥ 3 g/cm^3(Bánfalvi, 2011). According to Raychaudhuri et al., (2021), Heavy metals (HM) are defined as those metals and metalloids which have an atomic number greater than 20 and relative density greater than 5 g/cm^3. They are naturally occurring minerals in trace concentrations (ppb to <10ppm) in the earth's crust due to which they are also termed as 'trace metals.' A few examples of heavy metals include Copper (Cu), Lead (Pb) and Zinc (Zn) (Singh et al., 2011).

1.6 Sources of heavy metals in chicken litter

Heavy metals enter the environment through anthropogenic activities like application of fertilisers, smelting industries and emissions through municipal waste (Suganya et al., 2016). The poultry industry contributes to their release in the environment through chicken litter. Few studies have suggested that chicken feed is the major source of heavy metals in chicken litter ***(Fig.1.3)*** (Ogunwale et al., 2021; Oyewale et al., 2019; Ravindran et al., 2017). The National Research Council 1994 also stated that layer and broiler chicken have varying requirements of trace minerals in their diet (Dale, 1994). Heavy metals like Arsenic (As), Cobalt (Co), Copper (Cu), Iron (Fe), Manganese (Mn), Selenium (Se), and Zinc (Zn) are added in different formulations to enhance feeding

efficiency and egg production. This is evident particularly in broiler chicken, where As, Cd and Pb are added in the broiler feed at higher levels for faster weight gain. Though the input is high, chicken manage to ingest as little as 5-15% while the remaining amount is excreted in chicken litter hence contributing to the heavy metal toxicity in the environment (Kyakuwaire et al., 2019).

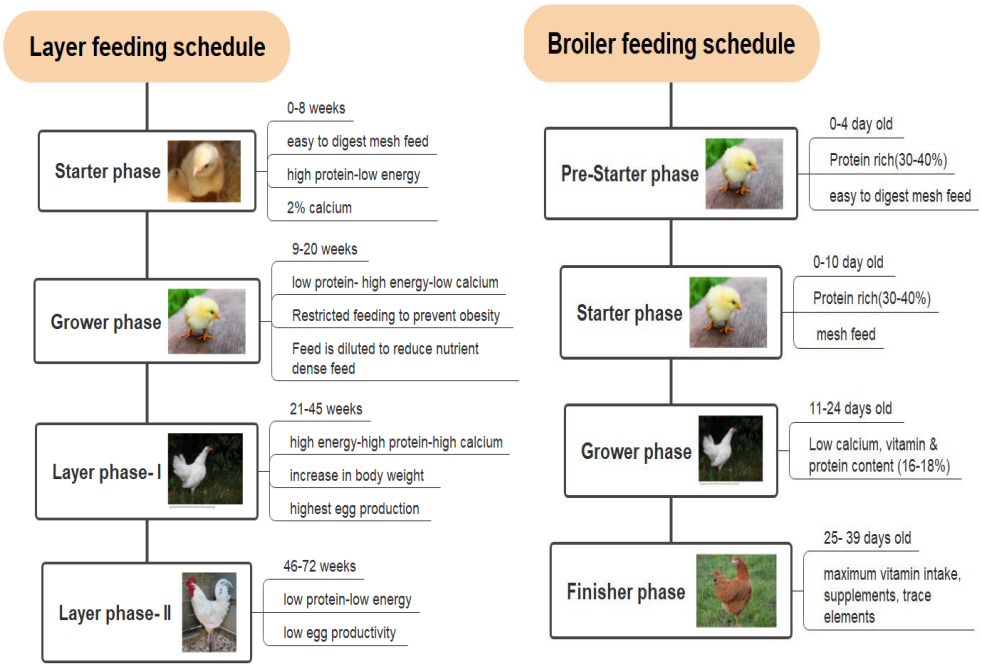

Fig. 1.2 Feeding schedules of Broiler and Layer chicken

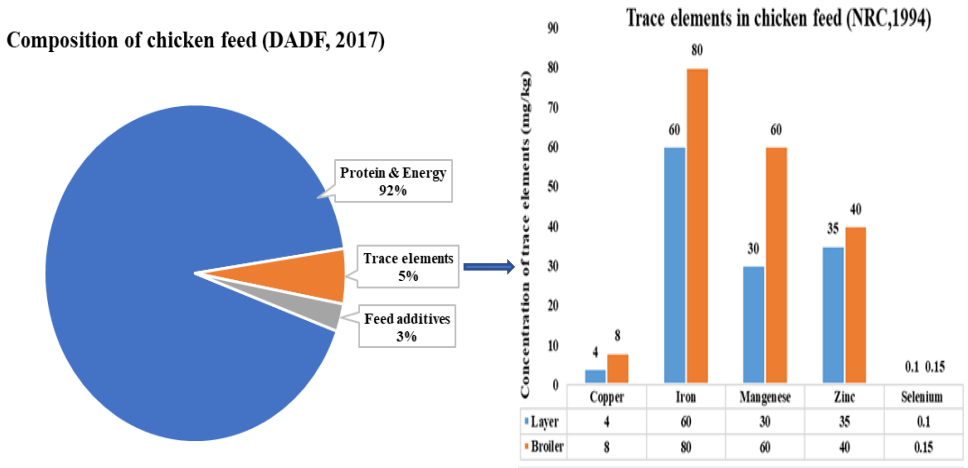

Fig. 1.3 Composition of chicken feed (NRC,1994)

Based on the requirement of organisms, heavy metals can be classified into two categories : -

Essential heavy metals: These heavy metals are required by organisms for fundamental processes like cellular metabolism and physiological functions. They are also known as 'micronutrients', which are needed by organisms in trace concentrations like 10-15 ppm. They elicit toxic effects at higher concentrations. E.g., Cu, Fe, Zn, Mn, Co and Ni (Raychaudhuri et al., 2021).

Non-essential heavy metals: These heavy metals are not required by an organism for any metabolic process. They elicit toxic effects in animals and plants at extremely low concentrations and are also known as 'toxic heavy metals'. E.g., As, Cd, Pb, Hg, Cr and Al (He et al., 2005).

1.7 Effects of Heavy Metal toxicity

While heavy metals do not exhibit detrimental effects when present in trace amounts, exposure to higher concentrations for a prolonged period of time can have adverse effects. Untreated and excessive application of chicken litter to the soil, is a matter of concern as heavy metals like As and Zn may exceed their permissible limit in the soil amounting to ecotoxicity. Owing to their high density, atomic mass and non-biodegradability, they accumulate over extended period of time, get absorbed in the vegetable tissues amounting to phytotoxicity and bioaccumulate in the food chain (Ravindran et al., 2017; R. K. Sharma et al., 2009). Multiple studies have suggested the risk of groundwater contamination and pollution of surface runoff owing to the use of inadequately composted chicken manure (Giddens & Barnett, 1980; Williams, 2013).

CHAPTER 2:
LITERATURE REVIEW

2.1 Studies on Heavy Metal Toxicity

Chicken has served as a useful bioindicator species for environmental monitoring. This is witnessed by multiple studies which have demonstrated presence of toxicants in the tissues, feed, blood and litter samples of both the breeds. Given below are a list of studies that have detected prominent heavy metals in litter and feed and its devastating effects in the environment:-

A plethora of studies have shown the bioaccumulation of heavy metals in various chicken tissues like liver, feathers, muscles, kidney as well as consumable products like eggs and meat (Chowdhury et al., 2022; Korish & Attia, 2020; Zhuang et al., 2014).

A multitude of studies have reported the recurrent use of the following feed supplements in the diet which happen to be heavy metals : Iron (Fe) and Copper (Cu) are added for the growth and development and to reduce anaemia, Selenium (Se) is added to prevent oxidative cell damage, Zinc (Zn) and Manganese (Mn) for strengthening eggshell formation and feather growth, preventing coccidial infections, increasing the body weight and improving the feeding efficiency (Bolan et al., 2004; Imtiaz et al., 2020; Nicholson et al., 1999; Sager, 2007). However, these metals are ultimately excreted in the form of poultry litter, which is released in the environment through chicken manure.

A study conducted in England and Wales proved the accumulation of heavy metals in animal manure on account of feed supplements. His study assessed different types of feed and animal manure like swine manure, dairy manure, cattle manure and poultry manure. His findings revealed that layer feed contained higher level of heavy metals as compared to broiler feed, with an

average concentration of 28±4030 mg Zn/kg and 5±234 mg Cu/kg. This contributed to 180 mg Zn/kg dm and 50 mg Cu/kg dm in poultry manure. (Nicholson et al., 1999)

Sager (2007) in his study in Austria stated the poultry farms produce at least 20 x 10^6 tonnes of excrement annually. This study revealed intense loads of Cu in pig manure (282 mg kg^{-1}) along with Zn and Se, which exceeded their threshold concentration in soil, amounting to heavy metal toxicity.

Banton et al., (1987) in his study, had reported the presence of 620 to 920 ppm of Cu in the poultry manure, which was used as a cattle feed amounting to accumulation of heavy metals in the body of organisms.

A study conducted in China assessed 388 animal feeds and manures which revealed the following concentrations ; Cu (10–1800 mg kg^{-1}), Zn (50–6300mg kg^{-1}), Cr (0.1–340mg kg^{-1}), Pb (1.0–310 mg kg^{-1}), As (UDL to 280 mg kg^{-1}), Cd (UDL to 10 mg kg^{-1}), and Hg (0.01–2.5 mg kg^{-1}). His study raised concerns over the growing levels of these heavy metals from 1990 to 2010 on account of feed additives (Wang et al., 2014).

In a study in Virginia, fresh chicken litter samples contained 30-50 µg/kg of As which contributed to heavy metal pollution in water on account of chicken manure (American Chemical Society, 2001). In poultry farms, As has been traditionally used in chicken feed in its organic form called “Roxarsone” (3-nitro-4-hydroxyphenyl arsonic acid) to control microbial infections and increase weight of the bird. As this compound is excreted through chicken litter, it ends up in the soil or contaminates the ground water levels.

Zhang et al., (2012) in his study reported high levels of Cu (65.6 mg/kg), As (3.3 mg/kg) and Cd (1.6 mg /kg). His study also proved that poultry feed was the major source, as highest content of Cu (0.88–98.08 mg Cu/kg dm) and As (0.02–6.42 mg /kg) was found, in the feed.

Similarly, a review conducted by Kyakuwaire et al., (2019) , enlisted the various contaminants like bacterial species, heavy metals, and antibiotics present in the layer and broiler litter to prove that in its natural form (without composting), chicken litter was deemed to be inefficient as an organic fertiliser. She further suggested that high levels of As, Cd and Pb found in the chicken litter samples contributed to heavy metal toxicity in the soil, where litter was used as manure.

Recently in Saudi Arabia, an experiment was conducted to test the quality of chicken consumed by human beings by studying heavy metal accumulation in 60 table eggs, 45 frozen broiler meat and 30 feed samples. In addition to this, the layer and broiler chicken litter was also tested. This study demonstrated that higher levels of Fe (183.3 ppm), Cu (16.61ppm) and Mn (42.5ppm) was detected in the layer feed, whereas Zn was higher quantities in the starter feed (60.3 ppm). On the other hand, Pb (3.37 ppm) was the highest in the broiler litter and As (0.00071), Cd (0.629 ppm), Ni (11.4 ppm) and B (0.461 ppm) were highest in the layer litter (Korish & Attia, 2020).

In Bangladesh, a comparative study was made between different types of poultry feeds to assess the heavy metal supplements, which revealed Mn (3.021 ± 0.003mg/kg), Fe (108.392 ± 0.002 mg/kg), Cu (1.307 ±0.002 mg/kg) and Zn (2.223 ± 0.002 mg/kg) was higher in grower phase whereas Cr (0.470 ±0.003

mg/kg) levels were higher in Grower phase. Though all the supplements were under permissible limits (Chowdhury et al., 2022).

A study conducted in China, compared the accumulation of heavy metals in swine manure, chicken manure and swine manure organic fertiliser which revealed that Zn and Cu were highest in concentration among all types of manure, suggesting heavy metal toxicity in manure (46.5 to 843mg/kg) (Xue et al., 2021).

Morocco, the first country for initiating broiler breeding in North Africa, produces 5,19,000 tonnes of broiler droppings every year, out of which, 95% is directly used as crop fertilisers. A study conducted by Mohamed el Amin, A. (2014) had reported an increase in the concentration of heavy metals like Pb (2,37 mg/kg), Zn (196,35 mg/kg), Cu (70,90 mg/kg) and K (32,86 mg/kg) in the nearby water sources due to this practise.

Ogunwale et al., (2021) in his study explored the accumulation of heavy metals among crops cultivated at a poultry farm in Nigeria. His study showed the levels of As (9.88-32.33 µg/g) and Fe (4.40-250.05 µg/g) were way above the permissible limit of plant intake, amounting to bioaccumulation in the food chain due to the use of poultry manure.

Another study conducted in Nigeria suggested the seasonal variation of heavy metals in water reservoirs owing to the leachate of heavy metals from poultry waste, with concentration of iron showing significant increase (0.104±0.401 mg/L) (Oyewale et al., 2019).

However, one study conducted in South Africa showed heavy metals in poultry manure were at lower limits. This study was conducted by Ravindran et al.,

(2017) where he assessed ten poultry manure samples for heavy metals (Cr, Cu, Ni, Pb and Zn) and found that heavy metal levels were below the permissible limits.

In a comparative study conducted in Malaysia, between the chemical characteristics of fresh and composted chicken manure, high content of proteins (27%), carbohydrates (31%) and a low C:N ratio of 7.19 in both types of manure on account of overfeeding practise of chicken to attain the slaughter weight at a faster pace (Sarbjit et al., 2018).

A solution to the problem of heavy metal leachate is increasing the efficacy of chicken manure composting. Through the findings of Zhang et al., (2021) , it was evident that co-composting the chicken manure with a combination of plant residues and wheat/rice decreased ammonia emissions by 50-80%, As by 0-53%, and Cd by 5-28%, thus reducing their release in the environment.

In India, few studies have been conducted to investigate and optimise the use of poultry manure and its effects.

A study conducted near the Rakha mines in Jharkhand, evaluated the effects of chicken manure on revegetation in the areas. Findings of this study revealed, an increase in plant biomass growth as well as an increase in heavy metals like Mn and Zn to a permissible limit (Das & Maiti, 2009).

Similarly, in New Delhi, a study was conducted to estimate the efficacy of manure on account of poultry droppings, leaf supplements and fungal species like *Aspergillus awamori* in alleviating C:N ratio. This study suggested that proper composting time and fungal species escalated and optimised the use of manure (Gaind et al., 2009)

Kar et al., (2018) , in their study in West Bengal, determined the concentrations of Cadmium(Cd), Lead(Pb), Copper (Cu) and Cobalt (Co) in the tissues as well as droppings of backyard chickens from polluted and unpolluted sites. His study revealed that concentrations of Cd and Pb were significantly higher in the polluted site as compared to the unpolluted one ($p < 0.05$).

The poultry sector in Goa is a small-scale sector, limited to domestic farming and catering to domestic needs. In 2009, a study was conducted by ICAR-CCARI, Ela Goa, in 2009, to identify the constraints faced by poultry farmers by surveying 100 poultry farms in Goa. Back then, 90% of the poultry farms were broiler while the remaining 10% were layer farms. High cost of chicken feed, competition with outside farmers, high labour cost, high cost of chicks and non-availability of health services were cited as major challenges back then (Swain et al., 2009). No other study has been published in this regard.

2.2 Studies on Genotoxicity

A review conducted by Cotelle and Férard, (1999), had discussed the applications of comet assay to determine genotoxicity in plants, amphibians, fishes and mammals. However, the application of this assay in avian species was not discussed.

Subsequently, Sokolovic et al., (2007) developed a protocol to inculcate the use of comet assay parameters to detect DNA damage on account of feed additives, mycotoxins and other parameters.

Awad et al., (2014) in his study, performed a comet assay to evaluate DNA damage caused by Deoxynivalenol (DON) present in the chicken feed. His findings revealed that there was a significant increase in blood lymphocytes and

tail of the comet (31.99±0.89%, p=0.001) which confirmed that the compound induced genotoxicity in broiler chicken.

A study conducted in US investigated the effects of T-2 and HT-2 toxin, one of the most toxic classes of trichothecenes, in twenty *Cobb 540* broiler chicken using comet assay. Findings of the study revealed noticeable DNA damage witnessed by the decrease in DNA % in the tail (Szabó et al., 2019).

Micronucleus test is another method used to determine genotoxicity by examining the micronuclei erythrocytes (MNE) present in the peripheral blood smears. This method was used to assess the genotoxicity of broiler chicken in Saudi Arabia. Findings of this study revealed MNE was 7-8 times high on account of feed supplements and predicted that this may also cause clastogenic effects in the long run (Saleh & Sarhan, 2007).

A study in Brazil assessed the effects of antigenotoxic effects of Piperine against aflatoxins and carcinogens using comet and micronucleus assay. There was significant reduction in Micronuclei erythrocytes and DNA % in tails of comets, which suggest this agent is suitable for antigenotoxic effects against feed supplements (Da Silva Cardoso et al., 2016).

In addition to mycotoxins contributing to genotoxicity, one study in China also investigated the genotoxic potential of Roxarsone (Arsenic compound), a prominent feed additive in broiler chicken in V_{79} cells , using micronucleus and comet assay. This study revealed a significant increase in the comet parameters ($p<0.05$) and increase in the frequency of MNi as compared to the control, indicating roxarsone is a potential genotoxic agent (Y. Zhang et al., 2012).

In Egypt, a study proved that DNA damage was seen on account of feed supplementation like $CuSO_4$ in the broiler chicken *Cobb 500,* as evidenced by the decrease in mean comet tail, by performing comet assay (Hashem et al., 2021).

Moreover, some studies have also suggested the incorporation of antibiotics in the poultry feed to boost the growth performance and reduce the growth of pathogenic bacteria like *Clostridium perfringens* among poultry animals. However, sometimes these antibiotics may give rise to cytotoxic effects (Dahiya et al., 2006; Mehdi et al., 2018).

2.3 Lacunae in study

A plethora of exposure-based studies have been conducted to quantify the presence of heavy metals in broiler meat, eggs, liver, and other organs of chicken. Few studies have also highlighted bioaccumulation of heavy metals in plants through the soil enriched in poultry manure. However, there is a lacuna of information with regards to the presence of heavy metals in fresh chicken litter, which is produced on a large scale in the few poultry farms present in Goa and used directly as manure in vegetable farms. Similarly, there have been numerous studies conducted on the safety concerns regarding consumption of broiler chicken meat, feed formulations to optimise the fattening of meat and use of antibiotics in chicken feed. However, no published literature has studied the genotoxicity in *Vencob400* broiler chicken on account of heavy metals present in the feed. Thus, the present study was aimed at fulfilling this literature gap by analysing the chicken feed, litter and blood samples collected from poultry farms across Goa.

2.4 Objectives of the study

To our best knowledge, no study has been conducted in the poultry farms of Goa to assess the heavy metal toxicity nor genotoxicity. Considering the above scenario, the present study was undertaken to achieve the following objectives:

1. To determine the physicochemical characteristics of fresh chick litter from layer and broiler farms in Goa.
2. To estimate and compare the levels of heavy metals present in chicken litter in layer and broiler poultry farms of Goa.
3. To analyse and compare the contents of layer and finisher feeds as a potential source of heavy metals.
4. To determine the genotoxicity in broiler chicken using Micronucleus and Comet Assay.

CHAPTER 3:

MATERIALS AND METHODS

3.1 Study animal

Gallus Gallus domesticus, (Linnaeus, C 1758), also known as "domestic chicken" was chosen for the present study due to easy availability of chicken litter and procurement of chicken blood. The local name of this species is 'Kombi.'

Fig. 3.1 Breeds of study animal A). Cari devendra B) BV 300 C) Vencobb 400

3.2 Classification of the study animal

Kingdom: Animalia

Phylum: Chordata

Sub-Phylum: Vertebrata

Class: Aves

Order: Galliformes

Family: Phasianidae

Genus: *Gallus*

Species: *gallus*

Sub-species: *domesticus*

3.3 Study area

Goa is a small state located along the western coast of the Indian peninsula (28° 38′ N latitude and 72° 12′ E longitude) covering an area of 3,700 km^2. Spanning across the Eastern boundary are the Western Ghats whereas flanking its western coast is the Arabian sea. Goa has a tropical climate with an average temperature of 28-33^oC, with humid weather, receiving an annual rainfall of 330cm. Fishing, Agriculture, and Tourism are the major industrial activities. Poultry farming, on the other hand, is a small-scale activity in Goa, with less than ten farms working in good conditions. This is insufficient to cater to the high demand of chicken meat and eggs, hence Goans are dependent on the neighbouring states for the supply of broiler meat and eggs.

Selection criteria: A preliminary questionnaire-based survey was conducted across poultry farms in Goa to study the working conditions prevalent in these areas. The poultry farm workers were interviewed about the following parameters: type of farming (broiler, layer, or rural backyard poultry farming), size of the farm, housing system (deep-litter or batter cage system), type of chicken feed (pre-starter, starter, grower, layer, finisher), water source, vaccination status, supply chain of day-old chicks, chicken litter production and disposal ***(Table 3.1).*** Based on the data collected, three poultry farms were chosen as the study sites ***(Fig. 3.1)***.

Study site 1: This study site was located at 15°29'42.75"N latitude, 73°55'4.61"E longitude, where rural-backyard poultry farming was practised in deep-litter housing system. *Cari devendra* and *Grama priya* were the chicken breeds reared exclusively for dual-purpose (egg and meat), genetic studies and optimizing the breed. The criteria for selecting this site as a reference was as

follows: its dual-purpose nature, chicken coop being cleaned on a regular basis and chicken litter being buried under landfill for composting.

Study Site 2: This study site was located at 15°26'47.86"N latitude, 74° 4'38.88"E longitude, where layer farming was practised in battery-cage housing system. The chicken breeds were reared for the sole purpose of egg production. This farm had a stocking capacity of 38,000 adult and juvenile chicks of *BV300* layer strain. The day-old chicks were obtained from supply chains in Pune. Here, the chicken litter was stockpiled in open landfills under the cages, for about one and a half year after which it was sold off to vegetable farms as manure.

Study Site 3: This study site was located at 15°0'25.74"N latitude and 74°4'0.62"E longitude, where broiler farming is practised in deep-litter housing system. The chicken breeds were reared for the sole purpose of broiler meat production. This farm had about 30,000 adult and juvenile chicks of *Vencobb400* broiler strain. The day-old chicks were obtained from Mandovi Hatcheries in Goa hence the entire farming process was Indigenous. The chicken litter was cleaned from each shed, once every two weeks and compiled in an open landfill. It was given to vegetable farms after three months as manure.

In addition to this, chicken blood was procured from the reference site having dual-purpose breed (*Cari Devendra)* and study site 3 having *Vencobb400* broiler strain (one of the most popular indigenous supply chains of broiler chicken) from North and South Goa, respectively.

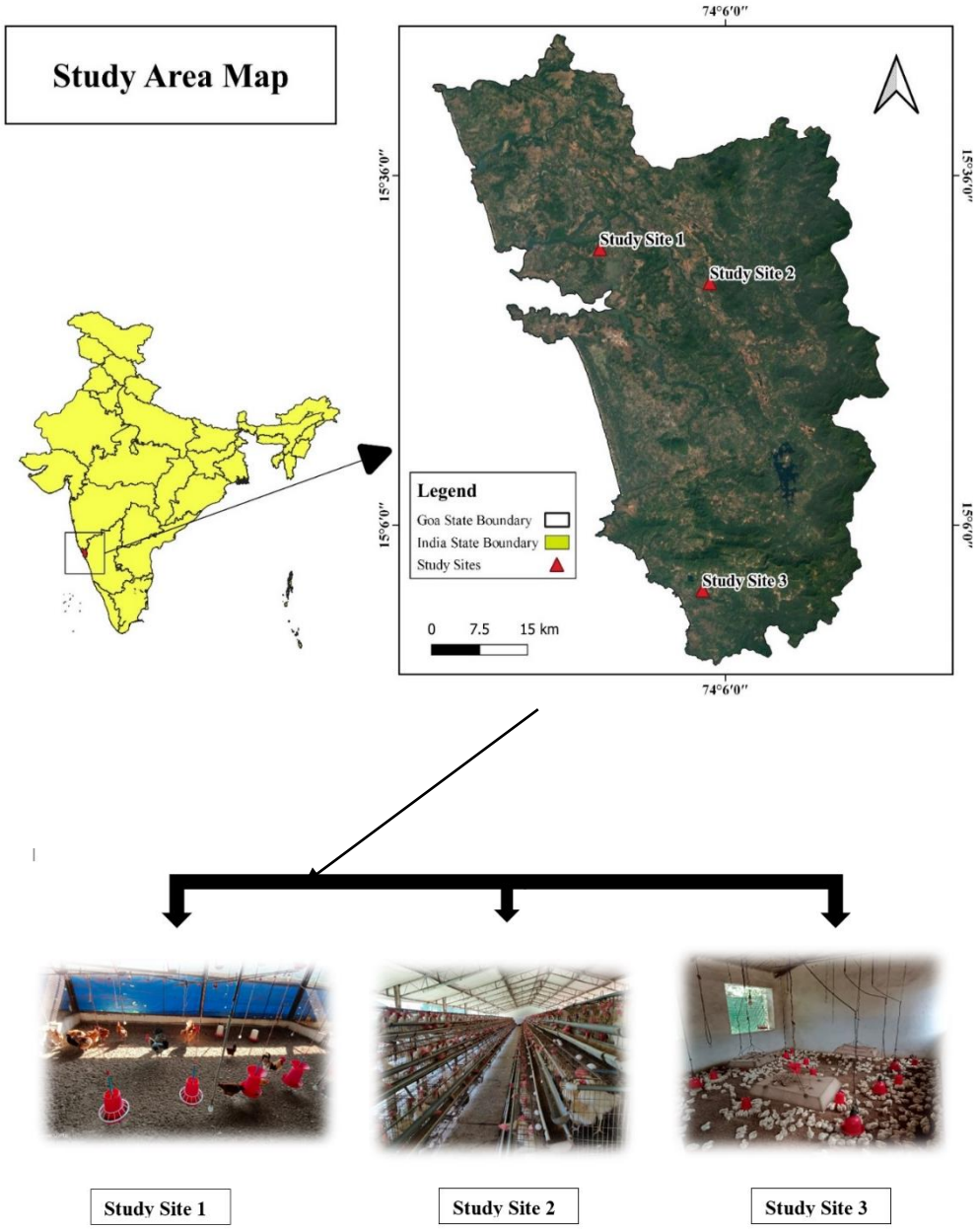

Fig. 3.2 Study area

Table 3.1 Characteristics of Poultry farms in Goa

Name	Type of farming	Breed	Source of Breed	Size of poultry (No.)	Feed	Chick Litter
Farm 1	Day-old chick hatchery	*Vencobb 400*	Venketeshwara Hatcheries,Pune (Day-old chick)	35,000		Sold off to vegetable farms
Farm 2	Broiler	*Vencobb 400*	Mandovi Hatchery (Day-old chicks)	30,000 6 sheds	Starter feed Grower feed Finisher feed	Sold after 38 days to vegetable farms
Farm 3	Broiler	*Vencobb 400*	Mandovi Hatchery (Day old chicks)	8,000 8 sheds		Directly used as chicken manure for the vegetables grown in their farm
Farm 4	Layer	*BV-300*	Balkrishna Hatchery (Day-old chicks) Sangli Vita (>12weeks old juvenile hen)	38,000 7 and ½ sheds 1shed= 5000	Chick mesh Grower mesh Layer mesh	Collected in open landfill and sold off as manure after 1.5 years
Farm 5	Layer	*BV-300*	Miraj (Day-old chick)	30,000 1shed= 10,000		4 tonnes collected every 8 weeks or 2 months and given vegetable farms
Farm 6	Dual purpose	*Grama Priya Cari devendra*	Directorate of Poultry Research, Hyderabad	2 sheds *Grama Priya =313 Cari Devendra=187*	Starter feed Grower feed Layer feed	Collected every morning with approx. 10-15kg daily shed cleaned every 2 days

Note: The rows highlighted in indicate layer farms, indicate broiler farms and depicts dual-purpose farm

Table 3.2 Characteristics of study sites

Study site	Type of Breed	Strains	Age of the bird	Type of feed	Number of birds	Chick Litter
Study Site 1	Dual-purpose	*Cari devendra*	>12 weeks	Layer phase 1/2	187 birds per shed	10-15 kg of chicken litter is collected on a daily basis.
Study Site 2	Egg-Layer	*BV300*	>12 weeks	Layer phase 1	500 layers per shed	Collected in an open landfill and sold off as manure after 1.5 years
Study Site 3	Meat-Broiler	*Vencobb400*	>25 days	Finisher phase	500 broilers per shed	Collected in an open landfill and given after 38 days to vegetable farms

Note: The rows highlighted in indicate layer farms, indicate broiler farms and depicts dual-purpose farm

3.4 Sample collection and preservation

For the present study, two types of samples were used: **chicken litter** samples were collected for the chemical characterisation and assessment of heavy metals and **chicken blood** samples were collected for studying the genotoxicity. In addition to this, **chicken feed** samples were also obtained to check the source of heavy metals and possible DNA damage. The baseline characteristics of each study site are indicated in ***Table 3.2*.**

1. **Chicken litter**: Chicken litter samples were collected from a single shed at each study site, once a month for a period of two months (October to December 2022). Twenty fresh chicken litter pellets, weighing 150g were collected from each poultry shed in plastic containers and mixed thoroughly. The homogenised sample mixture was labelled and stored in plastic zipper bags at -20^{o}C until further analysis (except for the analysis of pH, total solid and moisture content, where the homogenised sample was processed

immediately). Due care was taken during the time of sampling to ensure that the sample was fresh and not contaminated by external sources of heavy metals.

Fig. 3.3 Fresh chicken droppings from each study site

2. **<u>Chicken feed</u>**: From the chicken sheds chosen for sampling the chicken litter, 50g of layer and finisher chicken feeds were procured, labelled, and stored in plastic zipper bags at room temperature until further analysis.

Fig. 3.4 Different Types of chicken feeds (A) Layer phase 1/2, (B) Layer phase 1, (C) Finisher phase

3. **<u>Chicken blood</u>**: Three blood samples, each of 2ml blood volume, were obtained from the chosen study sites. The blood samples were collected at the time of culling of broiler chicken, in EDTA-coated blood collection tubes (Ethylenediaminetetraacetic acid). Due care was taken to prevent blood clotting by continuously shaking the EDTA tubes at the time of sampling. The samples were processed within 4 hours of collection.

3.5 Quality Assurance

It is necessary to maintain sterile conditions and prevent cross-contamination by other heavy metals to prevent an inaccurate reading. To avoid this, the glassware and crucibles were thoroughly cleaned, sterilized, and placed in hot air oven incubator. Prior to the conduct of the experiment, they were washed using concentrated hydrochloric acid to clean the surface. Experiments were conducted in clean and sterile environment, wearing clean lab coats, gloves, and mask. Any acid digestion was performed in fume hood. Samples were constantly covered using aluminium foil to avoid contamination.

3.6 Apparatus and instruments

General laboratory wares like measuring cylinders, beakers, test tubes, glass rods, micropipettes, reagent bottles, mortar and pestle, vertical coupling jars, porcelain crucibles, Erlenmeyer flasks, volumetric flasks, Whatman No.1 filter papers, hemocytometer and microscopic slides were used. Special Frosted slides were used for comet assay. Instruments like pH meter (TMP 3) Muffle furnace (i-therm AI-7981), Hot air oven (MIC-165), Hot plate, weighing balance (PGB, 200), Centrifuge(R-24), UV-Visible spectrophotometer(BL 1073), Orbital shaker, Cyclo mixer (CM, 101) , and Agarose Gel Electrophoresis unit were also used. Olympus Light Microscope(BX40) & Olympus Fluorescence Microscope (BX53) were used for analysing slides for micronucleus and comet assay. Heavy metal analysis was carried out using Atomic Absorption Spectrophotometer novAA series at Italab Pvt. Ltd., Margao, Goa and Soil Science Department of ICAR-CCARI, Ela, Old Goa, Goa and Sadekar's Envio Engineers Private Limited Lab.

3.7 Physicochemical analyses

All the chemicals and reagents used in the present study are of analytical grade. The following physicochemical parameters of the chicken litter samples were studied:

i) pH
ii) Total solids (TS)
iii) Volatile solids (VS)
iv) Moisture content
v) Total Organic Carbon (TOC)
vi) Total Phosphorus (TP)
vii) Total Potassium (TK)

Total Potassium was estimated using AAS along with other heavy metals.

3.7.1 Estimation of pH

The estimation of pH was carried out according to the protocol of Irshad et al., (2013). A suspension of fresh chicken litter and distilled water was prepared in the ratio of 1:10, using a pH meter (TMP3). The pH meter measures the voltage produced by the suspension and compares it with the known neutral solution reference voltage, the difference of which will give the pH value.

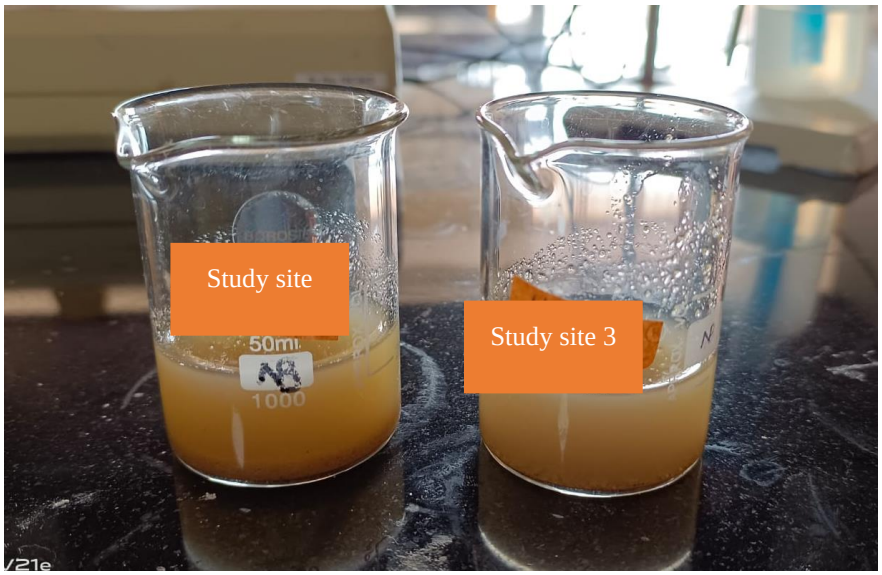

Fig. 3.5 Suspension of chicken litter

3.7.2 Estimation of Total solids (TS)

The estimation of Total solids present in the chicken litter was conducted using a gravimetric method, given by APHA (1999). Total solids refer to the material left in the crucible after evaporation of the moisture content and drying of the sample in an oven, at a defined temperature. This method helps to estimate the total suspended solids (portion retained by filter paper) and total dissolved solids (portion that passes through the filter paper).15 g of fresh chicken litter sample was air dried at 105°C to a constant weight for 16 hours. The TS was calculated as per the formula given below:

$$TS = \frac{M_f \times 100}{M_i}$$

where,

TS = Total Solids (g TS/ g Fresh matter)

M_f = weight of fresh chicken litter after drying (g)

M_i = weight of fresh chicken litter (g)

3.7.3 Estimation of Volatile solids (VS)

The determination of volatile solids was conducted using a gravimetric method given by APHA (2005). Volatile solids is the organic matter from the solid portion that is easily volatised during ignition. In order to estimate the mineral content of dry sample from TS, air dried chicken litter sample was converted into ash by placing it in the muffle furnace at 500°C for 5 hours. The VS from this ash was calculated as follows:

$$\boldsymbol{VS = \frac{M_i - M_f}{M_i} \times 100}$$

where,

VS = Volatile Solids (g VS/ g TS)

M_i = weight of dried chicken litter (g)

M_f = weight of fresh chicken litter calcined at 550°C (g)

3.7.4 Estimation of Moisture

The estimation of moisture content was done using a gravimetric method given by AOAC 985.01 Guidelines (Peters et al., 2003). Moisture content gives an estimation of retention of water by the manure, thus allowing sufficient gaseous exchange and growth of microbes. It was estimated as follows:-

$$\%\ \boldsymbol{Moisture} = [(\boldsymbol{W_1} + \boldsymbol{C}) - (\boldsymbol{W_2} + \boldsymbol{C})] \times \frac{\boldsymbol{100}}{[(\boldsymbol{W_1} + \boldsymbol{C}) - \boldsymbol{C_0}]}$$

where,

W_1 = Weight of the undried chicken litter sample [g]

W_2 = Weight of the dried chicken litter sample [g]

C = weight of the crucible [g]

C_o = weight of the empty crucible [g]

3.7.5 Estimation of Total Organic Carbon (TOC)

The estimation of Total Organic Carbon (TOC) was carried out according to the protocol of Heanes, (1984).

Reagents:

1. **1N Potassium Dichromate solution:** 49.0 g of $K_2Cr_2O_7$ was mixed in 100 ml distilled water.
2. **Stock sucrose solution (2.0 mg/ml):** 0.475 g of sucrose was dissolved in 100 ml distilled water. A working standard series with concentration 0-24 mg was prepared using this stock solution.

Principle:

This method works on the principle of recovery of TOC present in the chicken litter sample by digesting it in chromic acid ($K_2Cr_2O_7 + H_2SO_4$) and heating it on hot plate. The TOC is then determined as a product of excess Cr^{3+} ions released in the mixture by spectrophotometry at 600nm with calibration against sucrose standards in solution.

Procedure:

Fresh chicken litter samples were air dried in hot air oven at 100^{o}C for two hours. The air-dried samples were grinded into thin powder using mortar and pestle. 1

ml of 1N $K_2Cr_2O_7$ solution and 2 ml of H_2SO_4 was gradually added in 0.20 g of the dried chicken litter. The mixture was heated on hot plate for over 20 minutes at 135^oC and allowed to cool. The mixture was diluted to volume (10 ml) and centrifuged at 3000 rpm for 15 minutes. The absorbance was measured against a blank at 600 nm. The TOC was estimated using standard curves and expressed as % dry weight.

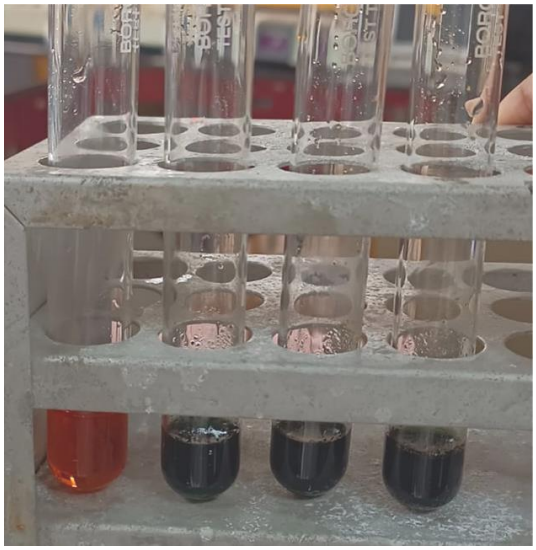

Fig. 3.6 Estimation of TOC

3.7.6 Estimation of Total Phosphorus (TP)

The estimation of Total Phosphorus (TP) was carried out according to Olsen method (Trinchera & Baratella, 2019).

Reagents:

1. **Solution of sulphuric acid (R1**): 14 ml of sulphuric acid ($H_2 SO_4$) [96%] was added in 5.0 ml of distilled water and diluted to 100 ml volume.

2. **Ammonium molybdate solution (R2):** 4.0 g of ammonium molybdate [$(NH_4).6Mo_7\ O_{24}.4H_2O$] was dissolved in 100 ml distilled water and stored in a dark container.
3. **Solution of ascorbic acid(R3):** 1.76 g of ascorbic acid ($C_6H_4O_6$) was dissolved in 100 ml distilled water and prepared fresh.
4. **Potassium tartrate antimony solution (R4**): 0.03 g of potassium antimony tartrate [$(K(SbO).C_4H_4O_6.\ ½\ H_2O$] was dissolved in 100 ml of distilled water.
5. **Sulphomolybdic Reagent (Mixed reagent):** 5 ml of the R1 solution, 1.5 ml of the R2 solution, 3 ml of the R3 solution and 0.5 ml of the R4 solution was mixed at the time of use.
6. **Sodium hydroxide solution (R5):** 4.0 g of sodium hydroxide (NaOH) was dissolved in 100 ml distilled water.
7. **Extraction solution / Sodium bicarbonate solution**: 4.20 g of sodium bicarbonate ($NaHCO_3$) was dissolved in 90 ml distilled water. The pH was adjusted to 8.5 by adding R5 solution drop by drop and diluted to 100 ml volume.
8. **Standard Phosphorus solution(0.005mg/ml):** 0.044 g of potassium dihydrogen phosphate (KH_2PO_4) was air dried at 40^oC for an hour and dissolved in 100ml distilled water. 1 ml of this stock solution was diluted to 10 ml to prepare a series of working standards of 0-5mg/L concentration.

Principle:

Ammonium molybdate and antimony potassium tartrate reacted in an acidic medium to form antimony-phospho-molybdate complex, which was reduced to an intensely blue-coloured compound by ascorbic acid that was proportional to the orthophosphate concentration, determined by colorimetry at 882 nm.

Procedure:

Sample extraction: 2.50 g of air-dried fresh chicken litter sample (at 100^{o}C for two hours) was mixed in 50 ml Extraction solution and 0.25 g of activated charcoal was added to it. The mixture was stirred for 45 minutes at 180-200 oscillations per minute using orbital shaker. The mixture was filtered twice using Whatman filter paper No. 1 and used for colorimetric estimation.

Colorimetry: 1.5 ml of the sample and 1.5 ml of Mixed Reagent was mixed in cyclo mixer for 1 minute and allowed to stand for an hour until blue colour formation. The absorbance was estimated at 882 nm. The TP was estimated using standard curves and expressed as mg/kg of chicken litter using the formula given below:-

$$\boldsymbol{C = \frac{(A - B) \times V_1 \times 50}{V_2 \times m}}$$

where,

C= litter extractable P content [mg kg^{-1}]

A= P concentration in the sample solution [mg L^{-1}]

B= P concentration in the blank sample solution [mg L^{-1}]

V_1= volume of the extract [50 mL]

V_2= volume of the sample solution used for colorimetric determination [ml]

M= soil mass [g]

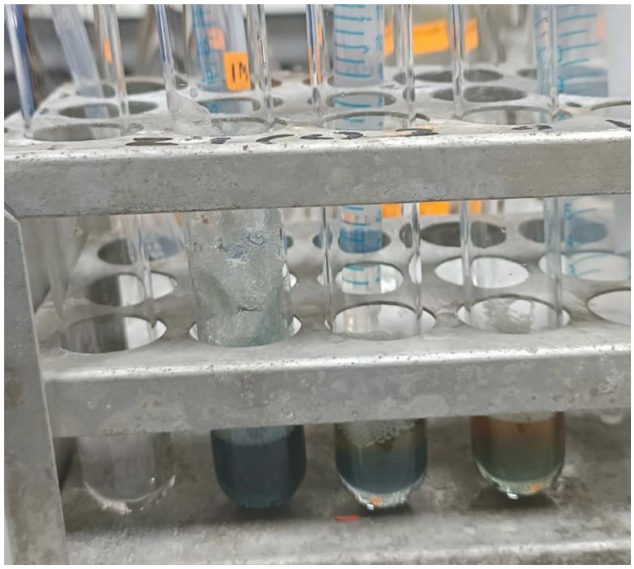

Fig. 3.7 Estimation of TP

3.8 Estimation of Heavy Metals

Atomic Absorption Spectrophotometry is based on the principle of absorption of light by metallic ions, at a specific wavelength which helps in determining the concentration of an element in the given sample. This analytical method was used to detect specific elements like Potassium (K) and heavy metals like Arsenic (As), Cadmium (Cd), Lead (Pb), Chromium (Cr), Iron (Fe), Copper (Cu), Zinc (Zn) and Manganese (Mn).

Principle:

In the present study, the sample was heated to oxidise the carbon structures and evaporate the volatile substances like Nitrogen, Phosphorus and Potassium. The ash was acidified to dissolve any other elements, leaving behind the inorganic mineral elements which were detected using Atomic Absorption Spectrophotometry.

Sample preparation for chicken litter

The sample preparation of Heavy metals was performed according to the AOAC 985.01 Guidelines (Peters et al., 2003) and the heavy metal content was determined using Atomic Absorption Spectrophotometer. The sample preparation of detection of Potassium and heavy metals was performed according to the AOAC 985.01 Guidelines (Peters et al., 2003). 15g of fresh chicken litter sample was oven dried at 100^{o}C for 2 hours to remove the moisture content. 0.5g of the dry weight was placed in porcelain crucible and converted to ash in muffle furnace at 500^{o}C for 4 hours. The ash was digested in 10ml HCl pure (1+1), filtered using Whatman filter paper No.1 and transferred in 100ml Volumetric flask, diluting to the volume. They were stored at 4^{o}C until sample analysis.

Fig. 3.8 Procedure for heavy metal analysis

Sample preparation for chicken feed

The estimation of heavy metals in chicken feed samples was performed according to the protocol given by Dahri et al., (2020). 2 g of layer feed and finisher feed was ground into fine powder using mortar and pestle and digested in 10ml of HNO_3 (65%) and 4ml of H_2O_2 (30%). The mixture was thoroughly mixed in Fume Hood and heated on a hotplate at 250^oC for 20 minutes until the appearance of white fumes. The digested samples were kept for cooling and filtered using a Whatman filter paper No.1, dissolved in 20ml distilled water and filtered. The filtrates were poured individually into a 50ml volumetric flask and diluted to volume.

Fig. 3.9 Sample preparation for heavy metal analysis of chicken feed

<u>Detection of heavy metals</u>

Both, chicken litter samples and feed samples were analysed in duplicate for the following heavy metals: Iron (Fe), Zinc (Zn), Copper (Cu), Manganese (Mn), Arsenic (As), Cadmium (Cd), Lead (Pb), Chromium (Cr). The determination of heavy metals in each sample was carried out using Atomic Absorption Spectrophotometry in fully automated Atomic Absorption Spectrometer (novAA 400P model) and ICP-OES (Inductively Coupled Plasma- Optical Emission Spectroscopy). The machine was calibrated using the standard for each metal. 25 ml of each sample was used for detection of As, Cd, Cr and Pb. As was detected using the Flame AAS technique after adding 5ml of Potassium iodide (KI) whereas Pb was detected using the Vapour technique. Fe, Mn, Cu, Zn and K were detected after diluting the sample to 25ml using Flame AAS. The final detection was made at individual wavelengths of 259 nm (Fe), 213.9 nm(Zn), 324.8 nm (Cu), 279.5 nm (Mn), 357.8 nm (Cr),193.7(As), 217 nm(Pb) and 228.8 nm(Cd).

Further a transfer factor (TF) ,also known as “accumulation factor” was computed to estimate the heavy metals transferred into the litter on account of feed (El-Amier et al., 2018). It was calculated as follows :-

$$\boldsymbol{TF = \frac{Ci}{Cf}}$$

where,

TF = Transfer factor

Ci = metal concentration in feed *(mg/kg of feed)*

Cf = metal concentration in litter (mg/kg of dry weight)

3.9 Analysis of Chicken blood

The following haematological parameters were tested prior to subjecting the blood samples to genotoxicity.

i) Total Red Blood Cell Count
ii) Differential White Blood Cell count
iii) Haemoglobin (Hb)

Prior to testing the genotoxicity, a total Erythrocyte count was performed using the blood samples from both the study sites to ensure sufficient RBC cells were present. The following tests were performed to detect genotoxicity using chicken erythrocytes:

i) Micronucleus test
ii) Single-cell gel electrophoresis / Comet Assay

3.9.1 Total Red Blood Cell Count

The procedure estimating the Red Blood Cell count was according to the guidelines given by Samour (2006). Manual Total RBC count was performed using Improved Neubauer hemocytometer.

Reagents:

0.85% Saline: 0.85 g of NaCl was mixed in 100 ml distilled water.

Principle:

In order to count the innumerable red blood cells, the blood sample was diluted using a diluting fluid (1:200) and fixed on the hemocytometer to count

erythrocytes. The Neubar's Chamber present on the hemocytometer has a fixed set of squares which makes the counting easier and efficient.

Procedure:

10µl of fresh blood sample was mixed with 1.9ml of 0.85% Saline in an Eppendorf tube to achieve a dilution factor of 1:200. The contents of the mixture were mixed using cyclo mixer for 1 minute and allowed to rest. The initial 3-4 drops of blood were discarded and 20 µl of this mixture was pipetted into an initially fixed hemocytometer. The erythrocytes were observed under 40x Olympus light microscope and the RBCs were counted in the Neubauer's chamber using the "L rule" wherein, those cells in the triple lining on the left and bottom region of the larger chamber are counted, excluding the cells present on the triple lining on the right and top region, to give an accurate estimation of RBC.

Characteristics of RBC:

Morphologic characters: The matured erythrocytes were medium sized of average length of 11.9 µm, and a width of 7.1µm, ellipsoidal in shape and showed presence of small prominent nucleus.

Staining characters: The matured erythrocytes were characterised by pale uniform cytoplasm and condensed, darkly stained nucleus when observed under microscope.

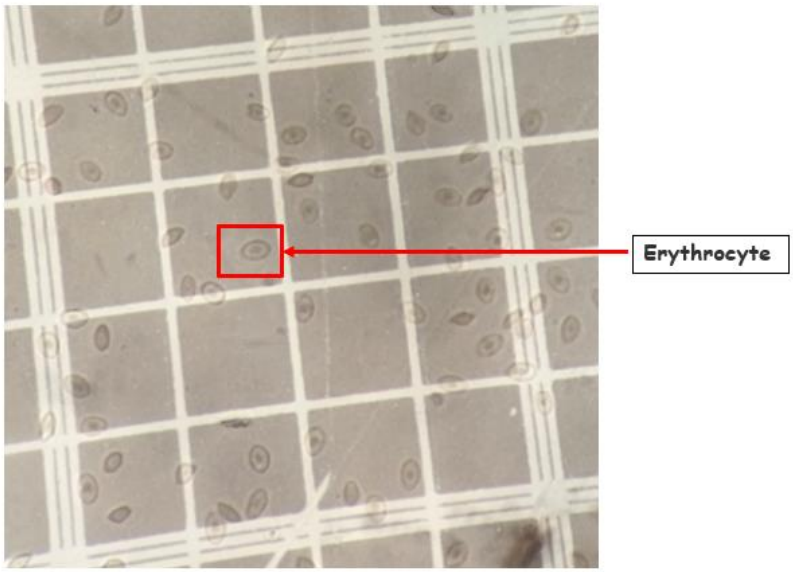

Fig. 3.10 Erythrocyte count in Hemocytometer

3.9.2 Differential White Blood Cell Count

The differential WBC Count was performed using Giemsa staining method. Air dried blood smears were rinsed in absolute methanol for 5 minutes and stained using Giemsa stain for 15-20 minutes. The stain was washed in Phosphate buffer for 10 minutes and the final slide was observed under Olympus light microscope at magnification of 100x. The different types of leucocytes seen within 100 cells was counted.

3.9.3 Haemoglobin Estimation

The haemoglobin estimation was performed using Saheli's method. The graduated tube of hemoglobinometer was filled with 0.1N HCl upto 10 mark and rinsed with 20uL chicken blood. The mixture was allowed to settle for 5 minutes for the conversion of haemoglobin to dark-brown acid-hematin. The mixture was diluted using distilled water until it had same intensity of color and tint as that of the standard tubes, when observed against sunlight. When an exact color

match was obtained, the lower meniscus was observed and the percentage figures corrssponding to it was noted. The hemoglobin level was reported in g/dL

3.9.4 Micronucleus test

Micronucleus test is a simple, rapid and indirect measure of induced, structural and numerical chromosome aberrations. It is used to detect genotoxicity in peripheral red blood cells. The present test was carried out according to the protocol given by Nagpure et al., (2007).

Reagents:

a. **Giemsa stock solution:** 0.50 g of Giemsa Powder was dissolved in 33 ml glycerol and incubated at 60^{o}C in water bath for 4 hours and cooled to room temperature. 50 ml of methanol was added to the solution and filtered using Whatman Filter Paper No. 1 and stored in amber bottle.

b. Giemsa working solution (5%): 5ml of Giemsa stock solution was mixed with 45ml of Phosphate Buffer (pH 6.8).

c. Phosphate Buffer (pH 6.8): A stock solution of 0.5M Potassium Dihydrogen Phosphate (6.80 g of KH_2PO_4 dissolved in 100 ml distilled water) and 0.5M Disodium Hydrogen Phosphate (8.90 g Na_2HPO_4 of dissolved in 100 ml distilled water) was mixed. From this, 31.30 KH_2PO_4 ml of and 22.80 ml of 0.5M Na_2HPO_4 was added in 490 ml distilled water, upon which the pH was adjusted using Sodium Hydroxide Flakes. Finally, the mixture was diluted to 500ml volume to prepare the working solution.

Principle:

Giemsa stain is a differential stain comprising of azure, methylene blue and eosin dye, which stain different components of the blood smear. The azure and eosin

stain are acidic dyes which stain basic components like cytoplasm and granules whereas methylene blue acts as a basic dye which stains the acidic component such as the nucleus. Methanol acts as a fixative and cellular stain which does not allow further change in staining properties.

Procedure:

10µl of anticoagulated blood sample was used to make a thin blood film, immediately after blood sampling. The air-dried blood smears were fixed in absolute methanol for 5 minutes and labelled. The slides were stained using 5% Giemsa working solution for 15-20 minutes in vertical coupling jars, washed in Phosphate buffer (pH 6.8) and further analysed for micronuclei (MNi).

Characteristics of Micronuclei (MNi):

Micronuclei (MNi) were identified through their appearance as a spherical extra nuclear bodies in the cytoplasm having a diameter of one-third of the main nucleus and similar color and texture as that of the nucleus.

Scoring of Micronuclei:

All the stained slides were examined under Olympus light microscope under bright field illumination [objective: 40X and 100X (oil immersion)]. Approximately 2000 erythrocytes with or without micronuclei were scored from each slide.

3.9.5 Single-Cell Gel Electrophoresis (Comet Assay)

Single Cell Gel Electrophoresis, also known as comet assay, is a reliable and rapid method for detection of single and double stranded DNA breaks in

eukaryotic cells. The Cometa assay was performed as per the guidelines given Nagpure et al., (2007).

Principle

In this technique, cell suspension of eukaryotic blood cells embedded in low-melting-point agarose, lysed by detergents and high salt treatment to remove cell content except DNA, and the liberated DNA is electrophoresed under alkaline conditions to unwind from breakage sites. Thus, cells with higher level of DNA damage display increased migration of the DNA from the nucleus towards the anode under an electrical current, giving the appearance of a "comet tail" , when viewed under fluorescent dye (ethidium bromide).

Reagents

All the reagents were freshly prepared for the present study.

1. **0.5% Low Melting Agarose:** 0.25 g of Low Melting Agarose was dissolved in 50ml of 1X PBS.
2. **1% Normal Melting Agarose:** 0.50 g of Normal Melting Agarose was dissolved in 50ml of 1X PBS.
3. **1X Phosphate Buffered Saline (pH=7.4):** 8.0 g of Sodium Chloride (NaCl) , 0.20 g of Potassium Chloride (KCl), 1.40 g Na_2HPO_4 and 0.27 g of KH_2PO_4 were dissolved in 100 ml distilled water after adjusting the pH using Sodium Hydroxide flakes. This stock solution of 10X PBS was diluted to 1X PBS and used.
4. **Lysis buffer (pH 10):** 14.6 g of Sodium Chloride (2.5M NaCl), 3.74 g of Disodium salt of EDTA (100mM $C_{10}H_{14}N_2O_8.2Na_2H_2O$), 0.12 g of Tris HCl was mixed in 100ml distilled water. 89 ml of this stock solution was mixed

in 0.1 ml of 1% Triton X and 10 ml of 10% DMSO (dimethyl sulfoxide) was added after adjusting the pH to using Sodium Hydroxide flakes.

5. **1% Triton X:** 1ml of Triton X(10mM) liquid was diluted to 100ml using distilled water.
6. **10% DMSO:** 10ml of DMSO was diluted to 100ml using distilled water.
7. **Neutralisation buffer (pH 7.5):** 4.84g of Tris Base (400mM) was added in 100 ml distilled water after adjusting the pH to 7.5. It was stored at room temperature and chilled right before use.
8. **Unwinding/ Electrophoresis buffer:** 20.0 g of Sodium Hydroxide (NaOH) was mixed in 50 ml in an exothermic reaction. Likewise, 1.49 g of Disodium salt of EDTA ($C_{10}H_{14}N_2O_8.2Na._2H_2O$) was mixed in 20 ml distilled water after adjusting the pH to 10. To prepare the working solution, 27 ml of NaOH, 4.5ml Disodium salt of EDTA ($C_{10}H_{14}N_2O_8.2Na_2H_2O$) and 1.8 ml of DMSO was diluted to 1000 ml distilled water.
9. **Ethidium Bromide solution:** 0.01 g of Ethidium Bromide was added in 50 ml distilled water and stored in Amber Bottles. From this stock solution, 100 µl was diluted to 1 ml distilled water to prepare the working solution in an Eppendorf tube, covered with aluminium foil. Due care was ensured while handling ethidium bromide with the use of gloves.

Procedure:

All the steps were performed under dim-light to prevent any photo-oxidation. A layer of 500 µl of 1% Normal Melting Agarose was smeared on clean frosted slides and covered with a coverslip. After the gel solidified, a layer of 200 µl of anticoagulated blood sample (double dilution using 1X PBS) was mixed with 600 µl of 0.5% Low Melting Agarose and spread over the previous layer. Once

the gel suspension was solidified, a third layer of 500 µl of0.5% Low Melting Agarose was added and allowed to solidify. The coverslip was removed, and the frosted slides were placed in Lysis buffer (pH 10) at 4^{o}C, overnight. Following lysis, the slides were placed in unwinding buffer (electrophoresis buffer, pH 10) for 30 minutes to unwind the DNA and Electrophoresis was performed subsequently for 20 minutes at 280mA and 25V. The slides were placed in pre-cooled Neutralisation buffer (pH 7.5) for 5 minutes and the slides were cleaned off excess buffer. The slides were stained with 100 ml 1X Ethidium Bromide solution, covered with a coverslip and observed under fluorescence microscope (Olympus BX53) under 20x magnification using a red filter.

Screening of comets: The slide was carefully assessed to check for presence of comets and were analysed using CASP software to compute the % tail DNA.

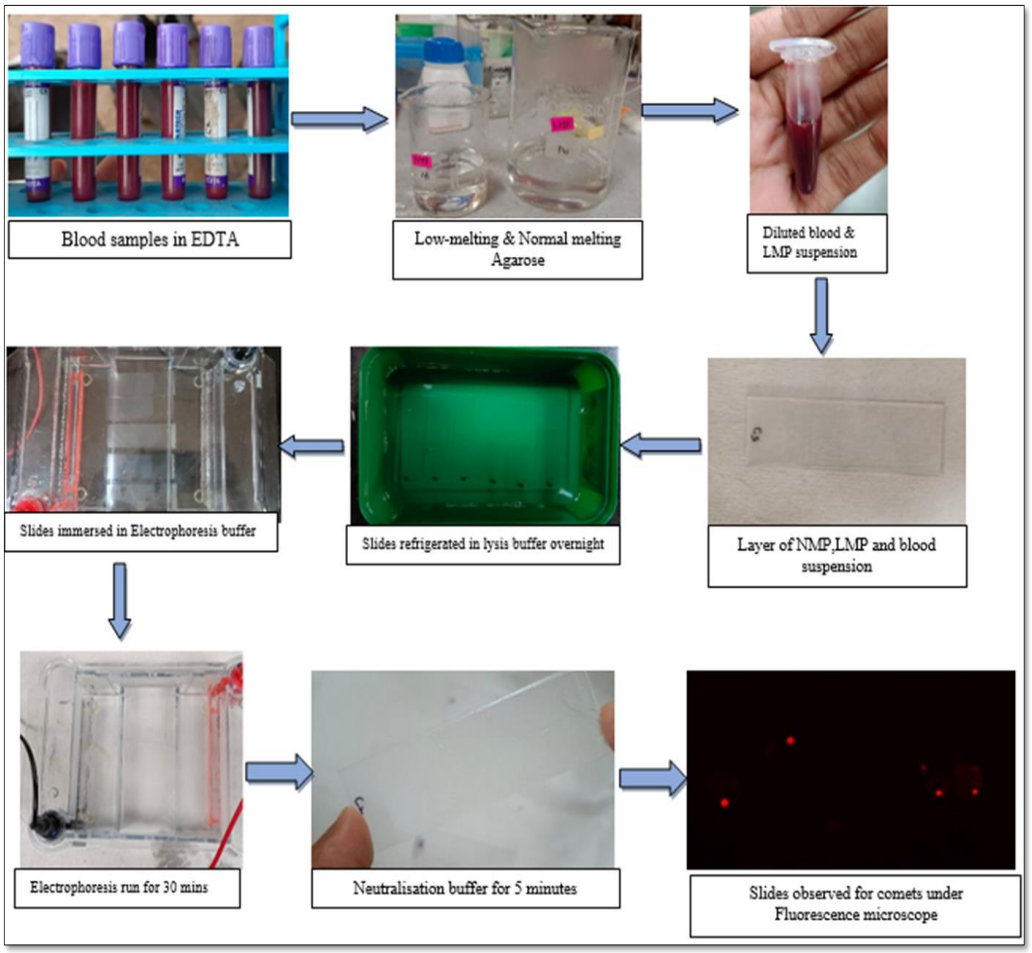

Fig. 3.11 Protocol for Comet assay

3.10 Statistical analysis

The data collected from the study was subjected to statistical analysis using RStudio 4.2.1 and IBM SPSS Statistics 26 software. All the samples were analysed in duplicate and were expressed as Mean ± SD (Standard Deviation), wherever necessary. The data was subjected to a test of normality using Kolmogorov-Smirnov test and Shapiro-Wilk test, followed by Levene test of homogeneity to check if the samples have equal variance. When the normality and homogeneity assumption was satisfied, parametric test called one-way ANOVA was used to estimate the difference in mean concentrations of heavy

metals in both samples. When F values of the ANOVA test were significant, ($P<0.05$), the means were compared by post-hoc Tukey's Honest Significant Difference test (Tukey-HSD) for pairwise comparison across each site. When assumptions of normality and homoscedasticity were not met, a non-parametric test called Kruskal–Wallis test was used, followed by Wilcoxon signed-rank test. The chicken blood samples were analysed in triplicates for micronucleus test and Student t test was used to compute the mean differences between micronuclei frequency across both the sites. Pearson's correlation analysis was used to check association between non-essential heavy metals in feed, hematological and genotoxicity parameters.

CHAPTER 4: RESULTS

To this date, there has been no published study that has estimated the heavy metal content in fresh and untreated chicken litter which is bioaccumulated in landfills or used directly as field manure. Similarly, no genotoxicity study was conducted in the *Vencobb 400* broiler breed. The present study was aimed at estimating the heavy metal content in chicken litter and as well as chicken feed samples across poultry farms in Goa. It was further aimed at analysing the genotoxicity in broiler chicken owing to the presence of toxic heavy metals in the feed.

4.1 Physicochemical characterization of fresh chicken litter

The physicochemical characteristics of the litter samples in each farm are summarised in ***Table 4.1.*** These properties were tested to find out the overall chemical composition of fresh chicken litter in its natural state. There was significant variation noticed in the physicochemical parameters across layer and broiler farms. The pH reported in the broiler farm was found to be acidic as compared to the layer farm (5.35, p=0.0001, f= 132.597). The percentage of TOC was significantly higher in the layer farm as compared to the broiler farm (p = 0.022). TP and TK, the major organic components, were significantly higher across the both the poultry farms (p = 0.0042 and p = 0.027). However, the differences in the moisture content and VS across the farms were statistically insignificant (p = 0.061 and p=0.558).

4.2 Heavy metal content in chicken litter samples

Results of the present study revealed that heavy metals were indeed present in the chicken litter samples across all the farms. On an average, these metals were found to be highest in the broiler farms as compared to the layer farms (868.58±21.18, p=0.638, f=0.454). The distribution of heavy metals across each

farm are demonstrated in ***Table 4.2***. The concentrations of Fe (938 mg kg^{-1}), Zn (348 mg kg^{-1}) and Mn (994 mg kg^{-1}) were significantly higher in the broiler litter in comparison to the reference site ($p=0.00752$ and $p<0.001$ respectively) ***(Fig. 4.1)***.

4.3 Heavy metal content in feed samples

Analysis of the feed samples revealed presence of all the tested trace metals, indicating the use of feed supplements across all the farms. Layer feed showed the highest concentration of heavy metals (868.58±21.18, $p=0.455$, $f=0.801$), as compared to the finisher feed. The mean concentration of these heavy metals is given in ***Table 4.3.*** The concentration of Pb (0.175 mg kg^{-1}) and Cu (413.75 mg kg^{-1}), was significantly higher in the finisher feed as compared to the other feeds ($p=0.03097$ and $p=0.0280$) ***Fig. 4.2.*** Presence of non-essential toxic heavy metals like As, Pb and Cd, detected in both the chicken feeds is a matter of concern.

4.4 Transfer factor of heavy metals

From ***Table 4.4,*** it was evident that chicken feed is the major source of heavy metals in the litter. The overall TF of heavy metals was highest in the Layer farm (0.49, $p=0.118$, $f=2.245$), which was confirmed by the high values of Fe (0.44, $p=0.003$) and Mn (0.17, $p=0.026$). A higher TF implies that heavy metal bioaccumulation is mainly from the feed. This indicates a high health risk of toxic metal exposure to poultry workers as well as chicken. On the other hand, TF of all the heavy metals in Broiler farm was lower, indicating a possible retention of these heavy metals in their body ($p=0.740$) ***(Fig. 4.3).*** As broiler

chicken is widely consumed by human beings, these metals can enter in our body leading to dire consequences.

4.5 Haematological Analysis

The haematological parameters were assessed to review the health status of broiler chicken. All the parameters were found to be in normal range (***Table 4.5)***

4.6 Micronucleus test

The low levels of TF indicate a possible retention of heavy metals in the broiler chicken, which may cause DNA damage. To prove our hypothesis, micronucleus test was performed to estimate the DNA damage in erythrocytes. Results of the present study indicate that there was DNA damage in the *Vencobb 400* broiler breed ***(Fig. 4.4),*** witnessed by the significantly higher values of % MNi in the broiler farm (1.32%, p= 0.0001) as compared to the reference site (0.57%, p=0.001).

4.7 Comet Assay

Due to the presence of non-essential heavy metals like Cd, As and Pb, we tested the blood samples using Comet assay. To prove our hypothesis, comet assay was performed to check any DNA damage on account of single and double stranded breaks. Results of the present study revealed there was no DNA damage seen due to the absence of comets ***(Plate 2).***

4.8 Correlation analysis

The results of heavy metal analysis were correlated with genotoxicity and hematological analysis as shown in ***Table 4.6 & Table 4.7.*** The Pearson's correlation analysis showed significant positive relationship between lead and

micronuclei in the dual-purpose farm(r_s=0.834, p=0.039). On the other hand both lead and arsenic, were significantly negatively correlated with haematological parameters (r_s = -0.825, p=0.043 and r_s = -821, p=0.045). This suggests DNA damage increases on account of heavy metals in feed.

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Table 4.1 Physicochemical characteristics of fresh chicken litter

Parameters	Units	Poultry farms			p value
		Dual-purpose	Layer	Broiler	
pH	-	6.45[a]	6.88[b]	5.35[c]	0.001***
TS	%	35.97 ±0.96 [a]	26.63 ±0.78 [b]	26.14 ±0.63 [c]	0.001***
VS	%	64.35±0.11 [a]	64.23±0.36 [a]	64.23±0.01 [a]	0.558
Moisture	%	64.03±0.96 [a]	73.37±0.78 [a]	73.86±0.63 [a]	0.061
TOC	%	24.89 ±0.22 [ab]	28.1 ±0.24 [a]	16.25 ±0.25 [b]	0.027**
TP	mg kg^{-1}	1362.89 ±0.18 [a]	1339.8 ±0.76 [b]	1300.46 ±0.16 [b]	0.011*
TK	mg kg^{-1}	570 ±0.76 [a]	940 ± 0.15 [ac]	621 ±0.45 [c]	0.027*

Different letters indicate significant differences at p< 0.05 as per Tukey's test. Values are significant at *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001

Table 4.2 Heavy metal content in fresh chicken litter (mg kg^{-1})

Heavy metals	Poultry farms			Mean Concentration	p value
	Dual-purpose	Layer	Broiler		
Fe	650±42.43 [a]	925±18.38 [b]	1050±70.21 [b]	875.00±21.39	0.00826**
Zn	240±0.00 [a]	354±8.49 [b]	390±14.4 [b]	328.00±5.81	0.00115***
Mn	910±70.71 [a]	998±5.66 [a]	1490±27.28 [b]	1132.67±49.69	0.0117*
Cu	2430±14.14 [a]	2668±45.25 [a]	3980±28.28 [a]	3026.00±12.72	0.1017
Cr	4.4±0.57 [a]	9±1.41 [a]	6±2.83 [a]	6.47±0.93	0.182
As	0.01±0.01 [a]	0.4±0.28 [a]	BDL	0.14±0.13	0.1229
Cd	0.4±0.28 [a]	0.4±0.28 [a]	0.6±0.57 [a]	0.47±0.13	0.854
Pb	2.2±0.28 [a]	4±2.83 [a]	24±2.83 [a]	10.07±1.20	0.1651

BDL: Below Detection Limit, Different letters indicate significant differences at p< 0.05 as per Tukey's test. Values are significant at *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001

Table 4.3 Heavy metal content in different types of chicken feed (mg kg^{-1})

Heavy metals	Poultry farms			Mean Concentration	p value
	Dual purpose	Layer feed	Finisher feed		
Fe	78.75 ±6.25 [a]	406.87 ±12.13 [a]	125 ±5 [a]	203.54 ±13.02	0.1017
Zn	33.75 ±6.25 [a]	63.75 ±25.13 [a]	41.25 ±3.75 [a]	46.25 ±6.01	0.191
Mn	115 ±12.5 [a]	173.87 ±13.75 [b]	160 ±5 [ab]	149.63 ±4.43	0.047*
Cu	257.5 ±15 [a]	295 ±10.13 [ab]	413.75 ±31.35 [b]	322.08 ±11.76	0.0282*
Cr	1.12 ±0.125 [c]	3.75 ±12.5 [b]	1.37 ±0.38 [c]	2.08 ±0.14	0.011*
As	BDL	0.06 ±0.01 [a]	0.04 ±0.01 [a]	0.03 ±0.01	0.223
Cd	BDL	0.04 ±0.01 [a]	0.18 ±0.05 [a]	0.07 ±0.03	0.0518
Pb	0.5 ±0.25 [a]	6.75 ±0.5 [b]	3.0 ±0.25 [c]	3.42 ±0.17	0.00254**

BDL: Below Detection Limit, Different letters indicate significant differences at p< 0.05 as per Tukey's test. Values are significant at *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001

Table 4.4 Transfer factor of heavy metals

Heavy metals	Poultry farms			Mean value
	Dual-purpose	Layer	Broiler	
Fe	0.12	0.44	0.12	0.23
Zn	0.14	0.18	0.11	0.14
Mn	0.13	0.17	0.11	0.14
Cu	0.11	0.11	0.10	0.11
As	0.00	0.18	0.00	0.06
Pb	0.22	2.33	0.13	0.89
Cr	0.26	0.42	0.28	0.32
Cd	0.01	0.10	0.63	0.25

Table 4.5 Haematological Parameters

Blood samples	Total RBC count (cells/L)	Differential count (cells/L)				Haemoglobin (g/dL)
		Heterophil	Basophil	Eosinophil	Lymphocyte	
Dual-purpose farm	3.72 x 10^{12}	1 x 10^9	1 x 10^9	2 x 10^9	1 x 10^9	13.9
Broiler farm	4.35 x 10^{12}	1.25 x 10^9	1.50 x 10^9	2.25 x 10^9	0.50 x 10^9	14.22

Normal range for the haematological parameters are as follows : Total RBC: 2.5 - 4.0 x 10^{12} cells/L , Heterophil count : 0.5-7.6 x 109 cells/L , Basophil count : 0-1 x 109 cells/L , Eosinophil count : 0.0-1.80 109 cells/L , Lymphocyte: 1.26-4.2 x 109 cells/L, Hemoglobin : 10.2 – 15.1 g/dL

Table 4.6 Correlation matrix between association of DNA damage parameters in response to heavy metal exposure at Dual-purpose farm (N=6)

	Cadmium (Cd)	Lead (Pb)	Hemoglobin (Hb)	Total RBC count	Micronuclei (MNi)	% tail DNA
Cadmium (Cd)	-					
Lead (Pb)	0.757	-				
Haemoglobin (Hb)	0.158	0.736	-			
Total RBC count	0.232	-0.151	-0.403	-		
Micronuclei (MNi)	0.504	0.834*	0.633	-0.57	-	
% tail DNA	-0.094	0.289	0.297	-0.062	0.408	-

**** Correlation is significant at 0.05 level (2-tailed)***

Table 4.7 Correlation matrix between association of DNA damage parameters in response to heavy metal exposure at broiler farm (N=6)

	Arsenic (As)	**Cadmium (Cd)**	**Lead (Pb)**	**Hemoglobin (Hb)**	**Total RBC count**	**Micronuclei (MNi)**	**% tail DNA**
Arsenic (As)	-						
Cadmium (Cd)	-0.850*	-					
Lead (Pb)	-0.159	0.144	-				
Hemoglobin (Hb)	-0.821*	-0.757	0.278	-			
Total RBC count	0.520	-0.518	-0.825*	0.022	-		
Micronuclei (MNi)	0.098	-0.331	-0.780	-0.337	0.845*	-	
% tail DNA	0.160	-0.526	-0.026	0.451	-0.050	0.079	-

**** Correlation is significant at 0.05 level (2-tailed).***

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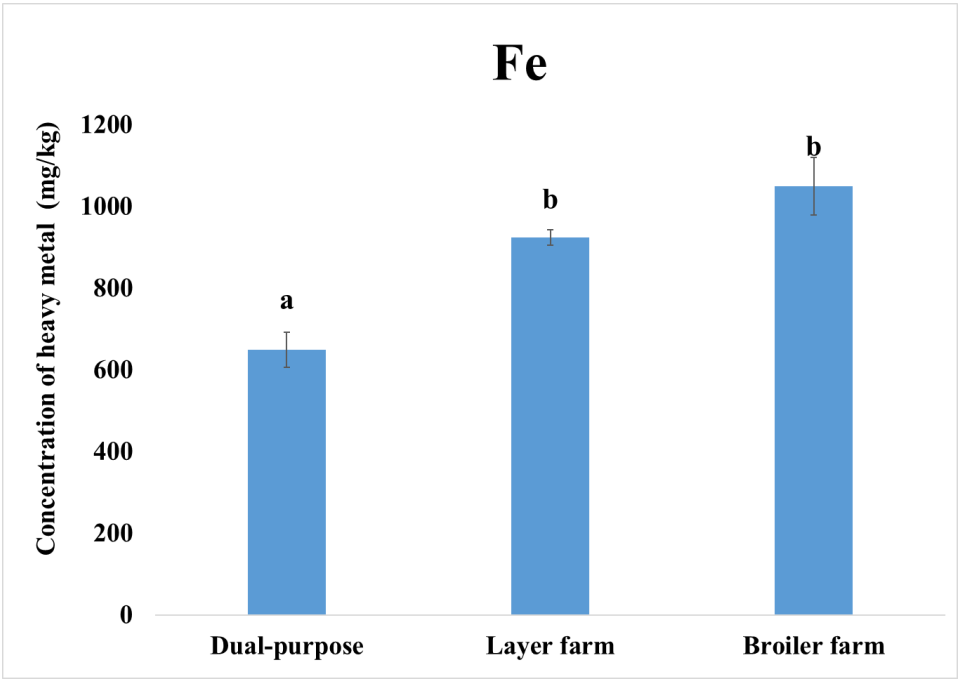
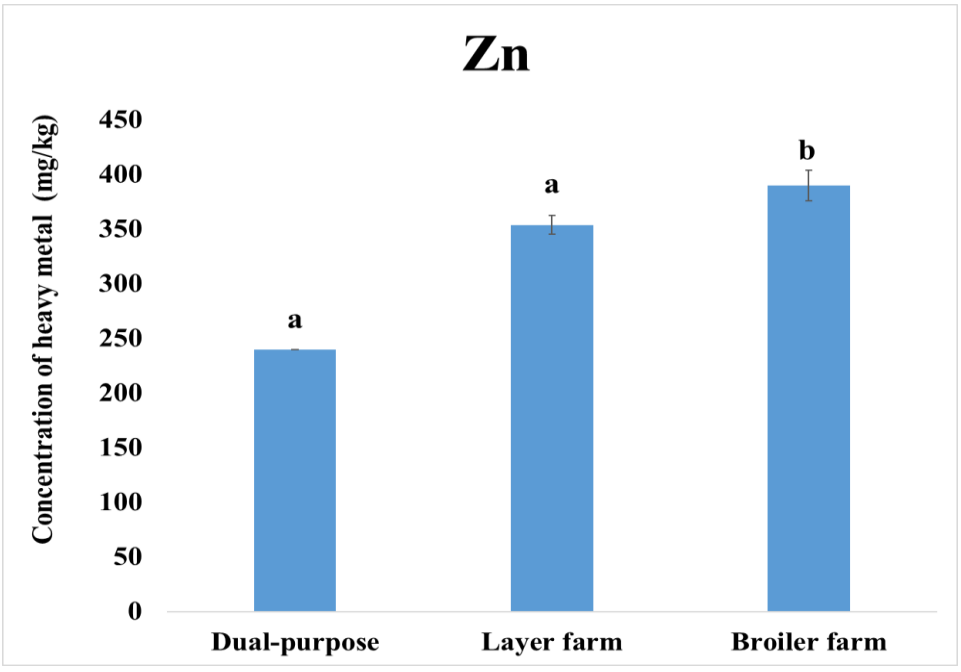
Fig.4.1 Heavy metal content in chicken litter (A -B)

Different letters indicate significant differences at p< 0.05 as per Tukey's test.



(A)



(B)

Fig.4.1 Heavy metal content in chicken litter (C-D)

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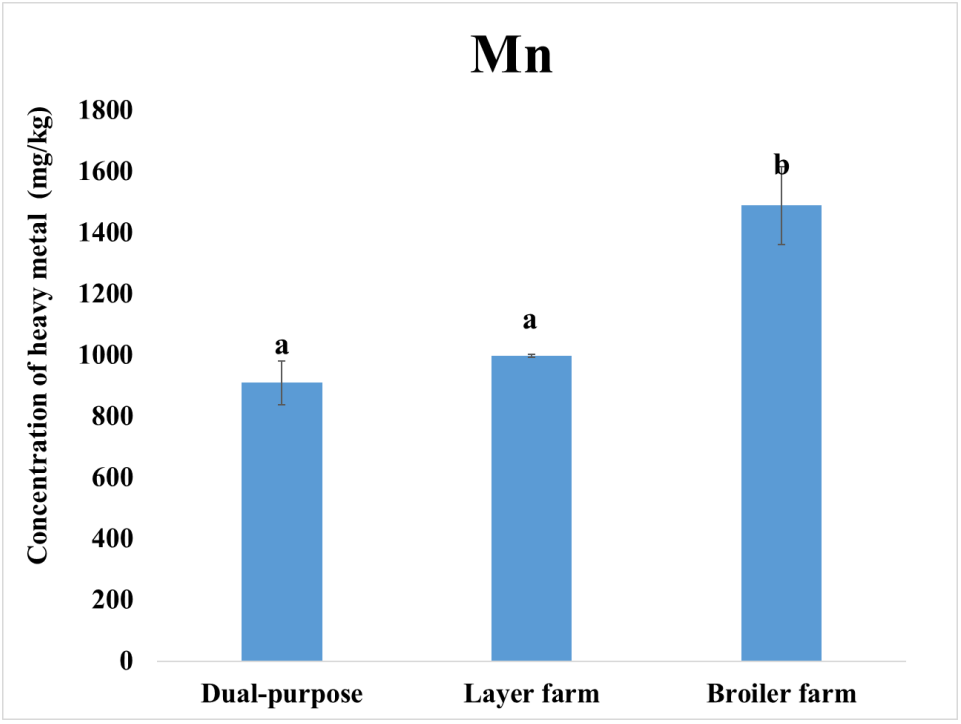
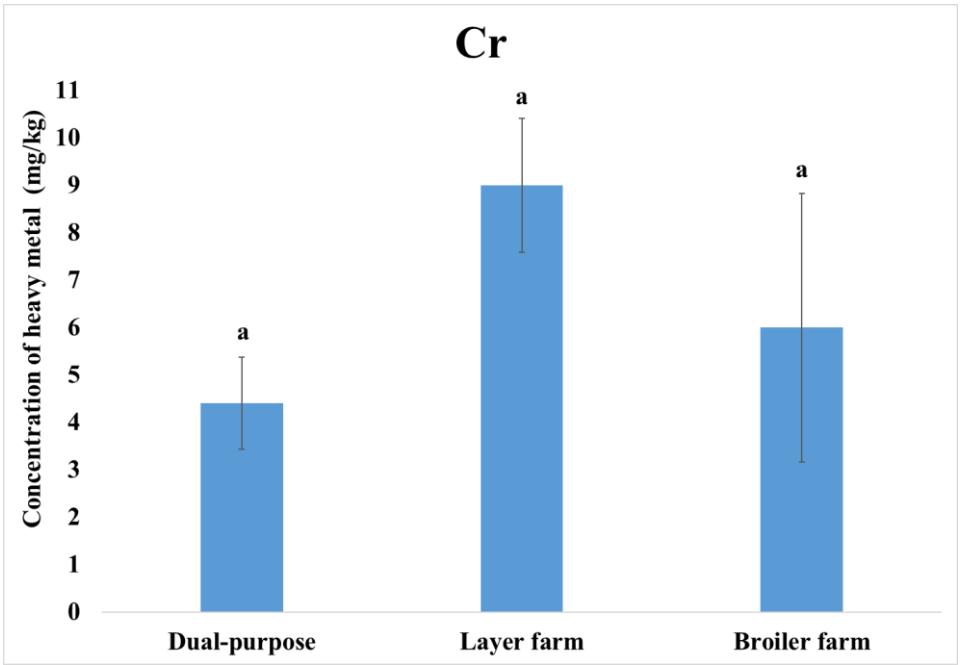


(C)



(D)

Fig.4.1 Heavy metal content in chicken litter (E-F)

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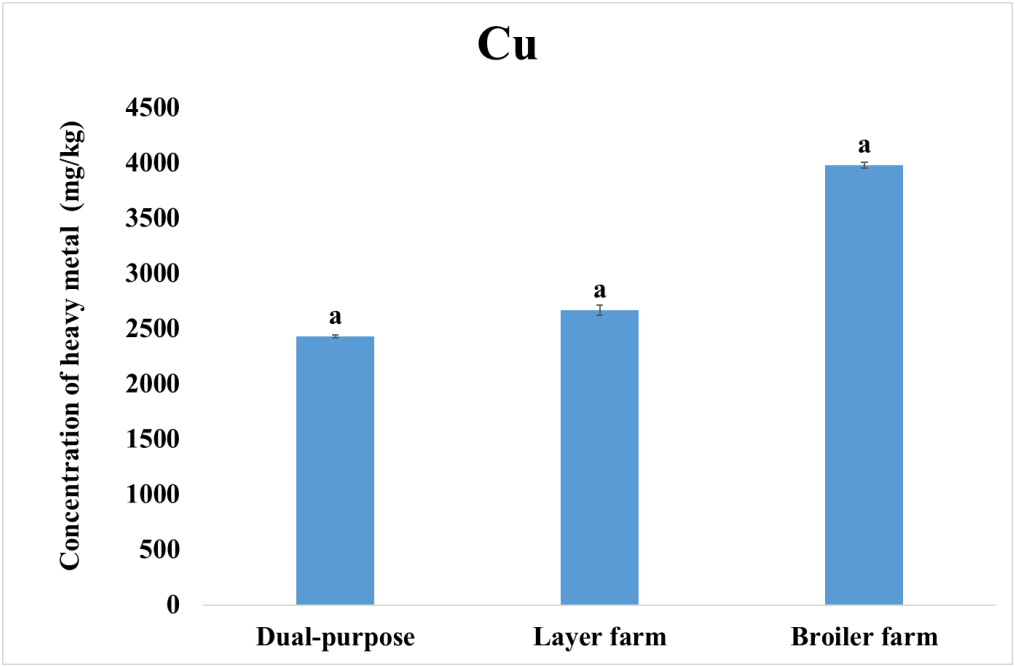
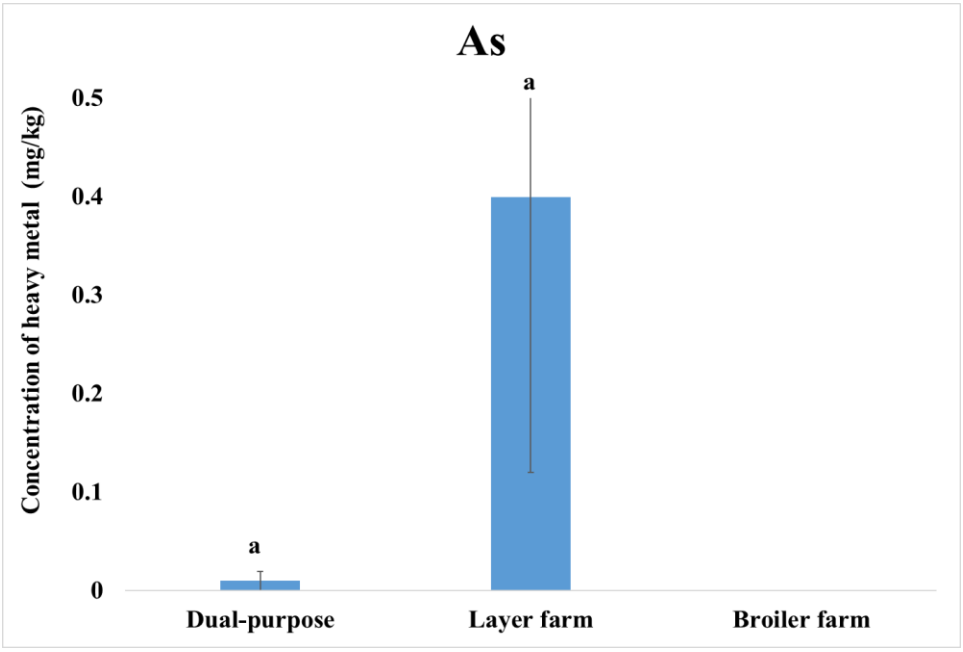


(E)



(F)

Fig.4.1 Heavy metal content in chicken litter (G-H)

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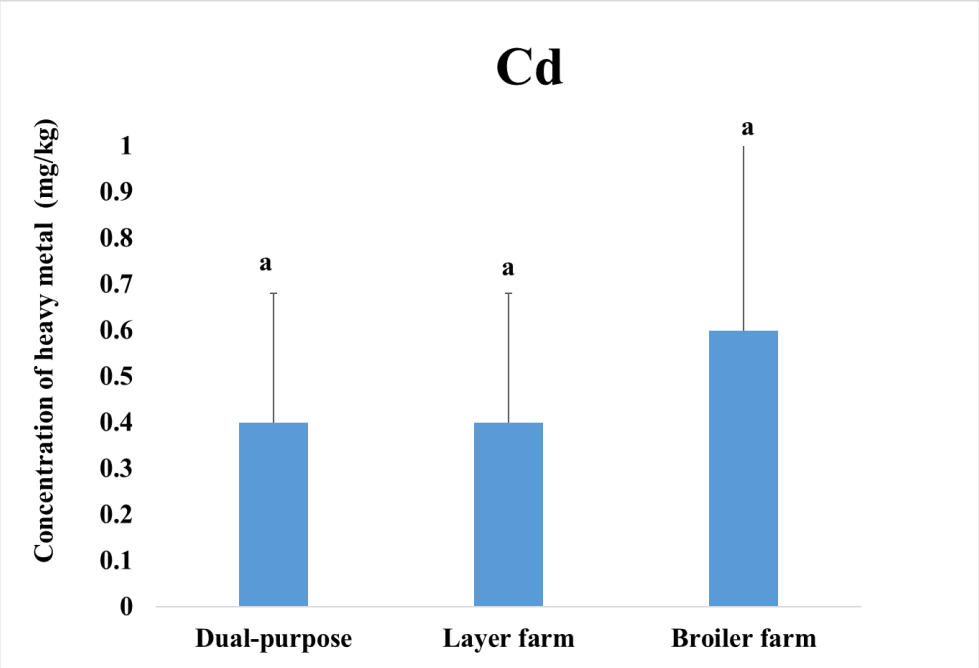
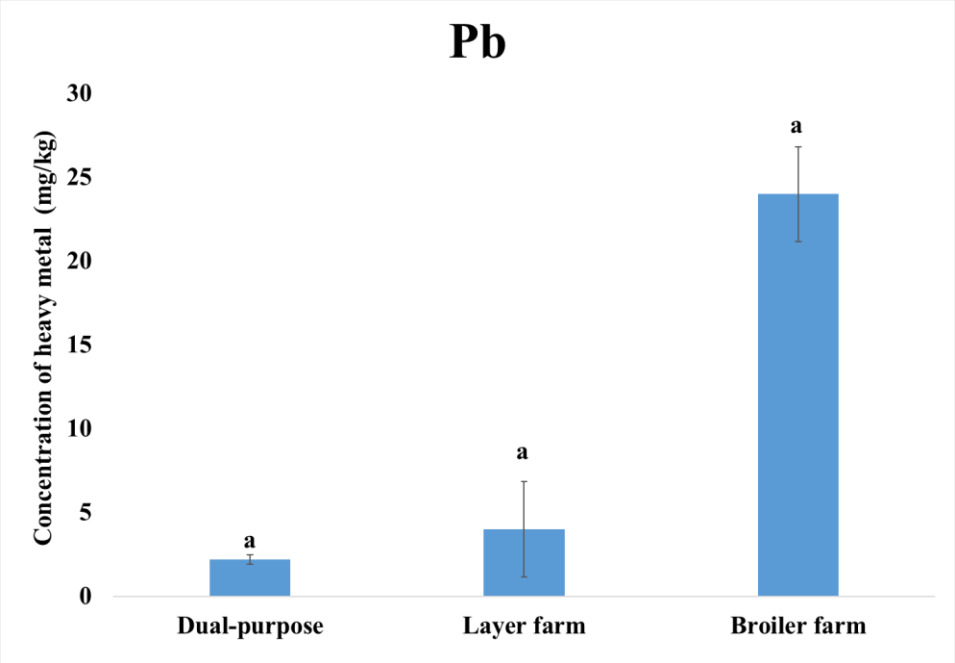
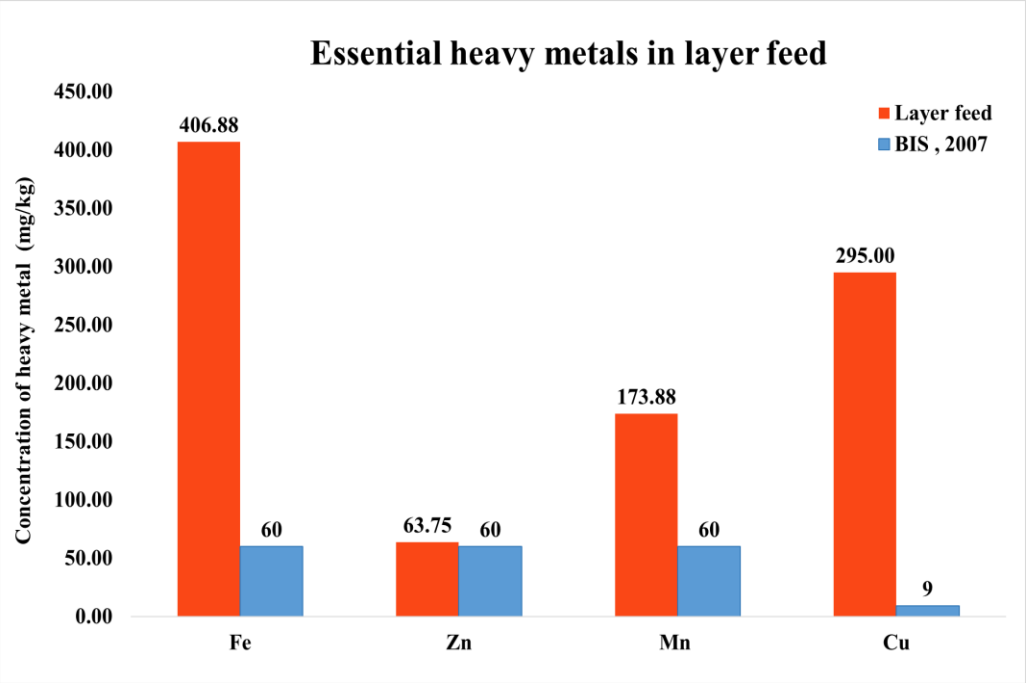


(G)

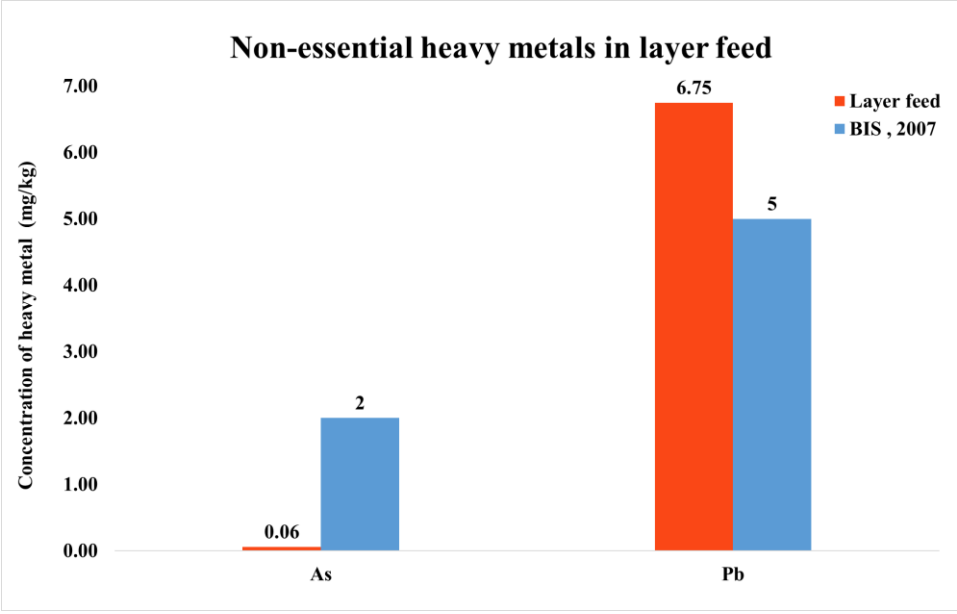


(H)

Fig.4.2 Heavy metal content in different types of chicken feed in comparison with BIS standards for layer feed (A -B)

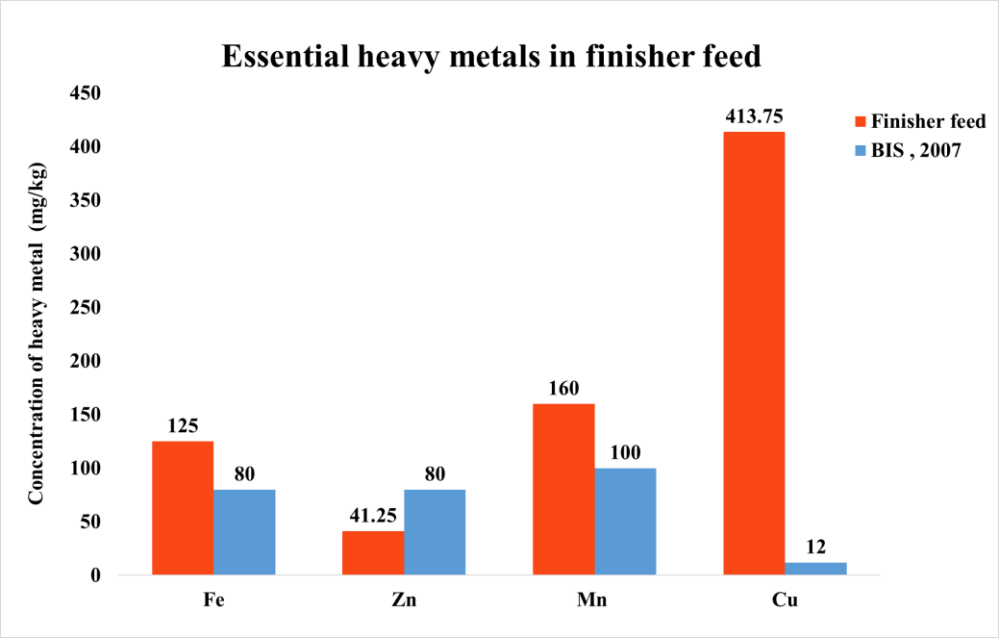


(A)

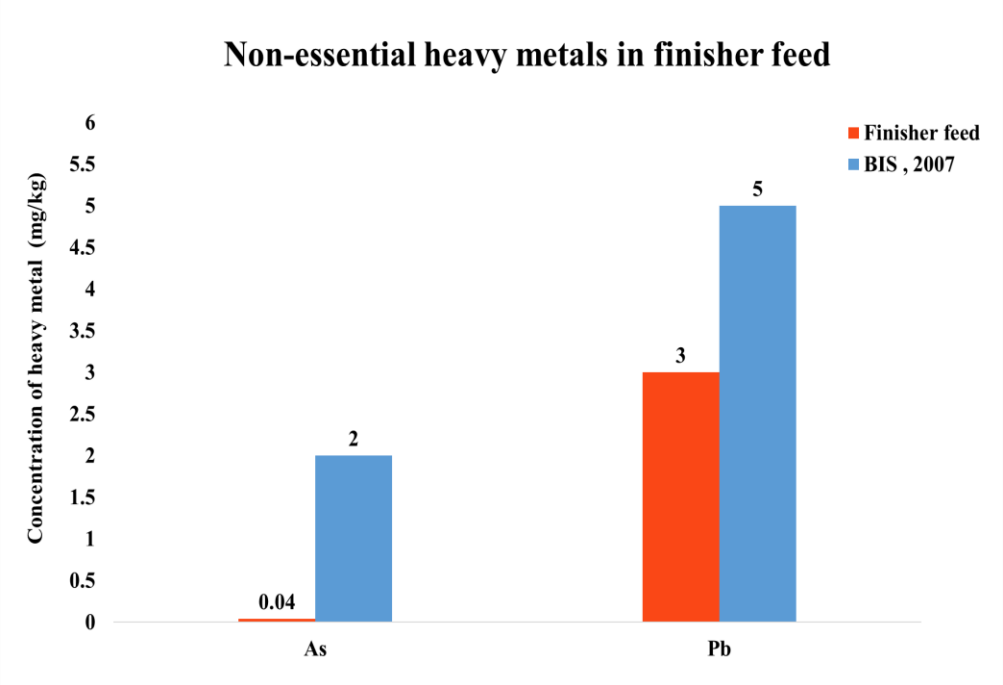


(B)

Fig.4.2 Heavy metal content in different types of chicken feed in comparison with BIS standards for finisher feed (C-D)



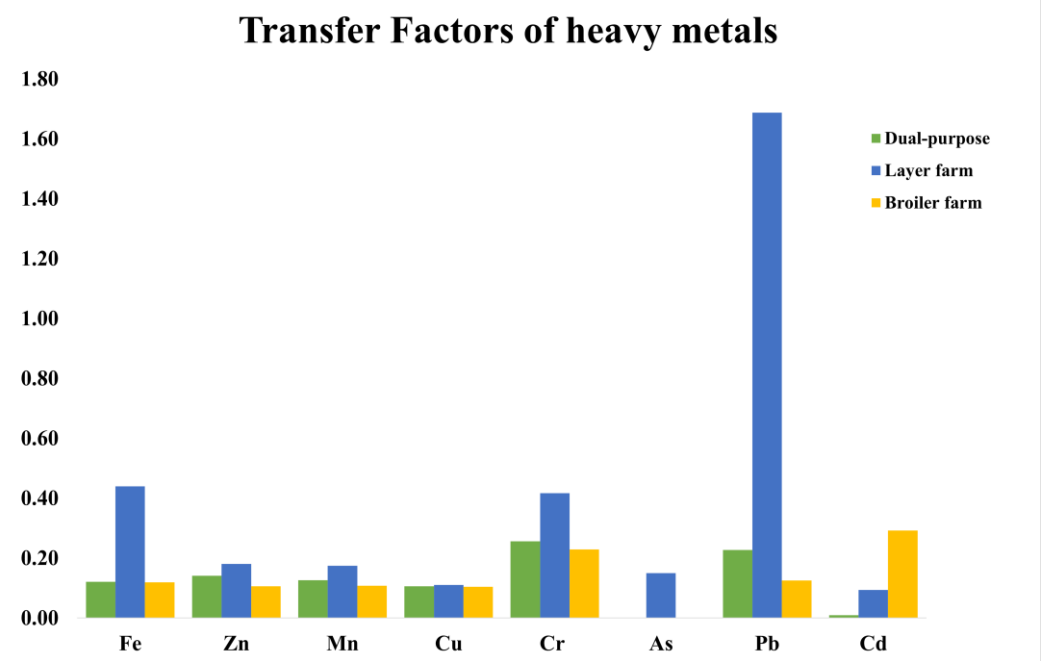
(C)



(D)

Fig.4.3 Transfer factor of heavy metals across different farms

Different letters indicate significant differences at p< 0.05 as per Tukey's test.



LIST OF PLATES

Plate 1: Micronucleated Erythrocyte(MNi) observed under 100x immersion oil in (A) Reference site (B) Broiler farm

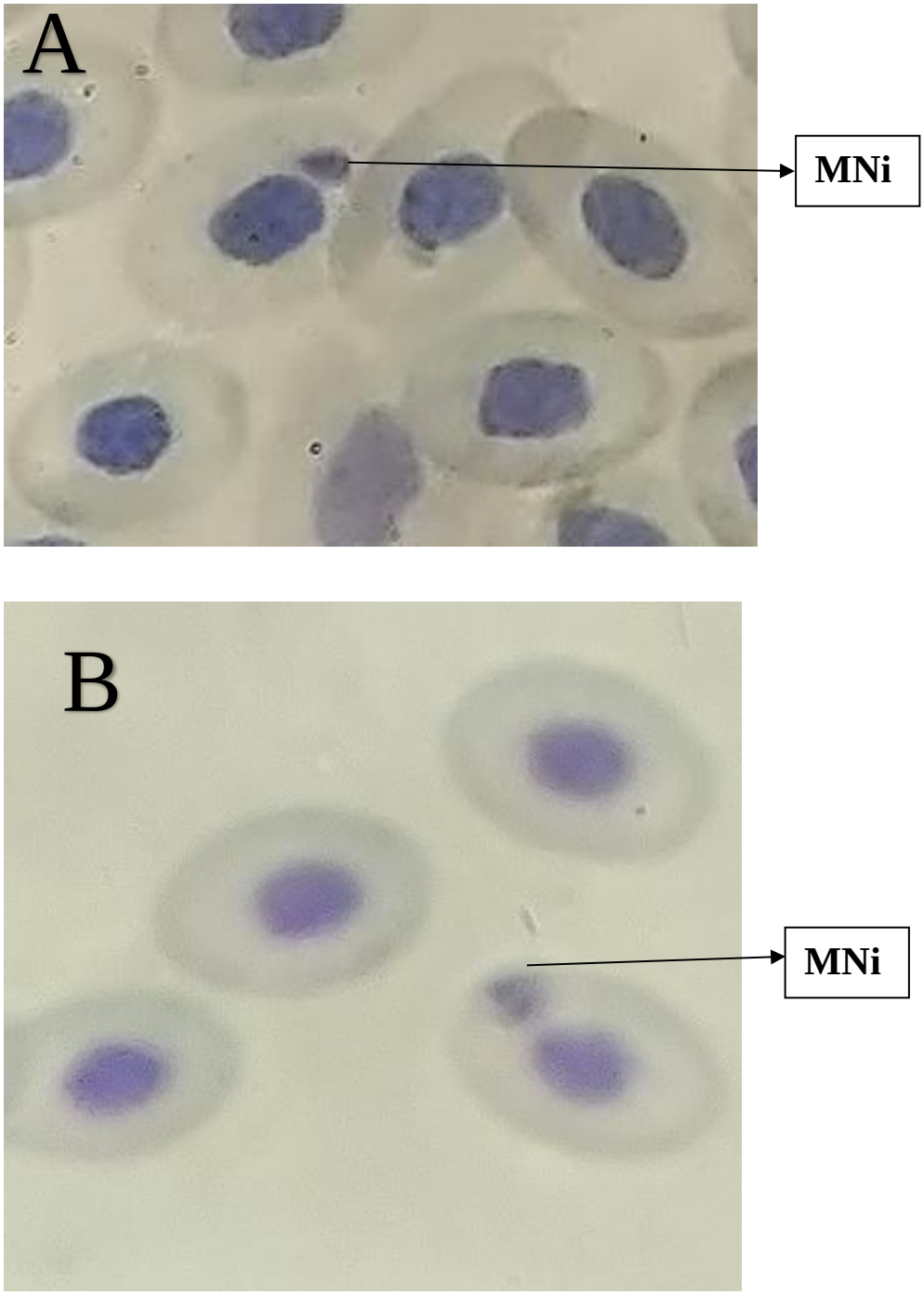
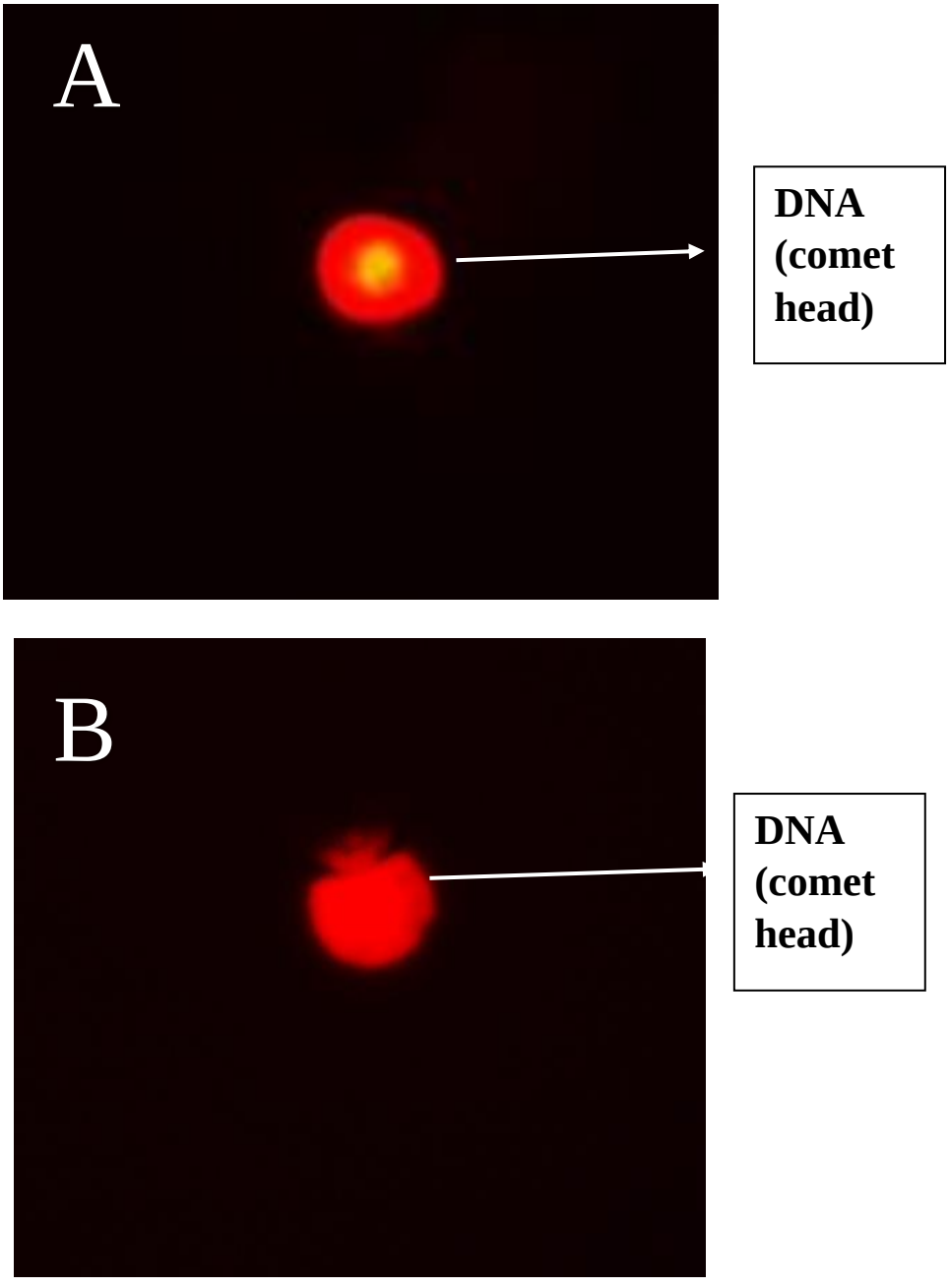


Plate 2: Analysis of peripheral blood cells using comet assay observed using Fluorescence microscope (Olympus BX 53) with a red filter under 200x magnification(A) Reference site (B) Broiler farm.



CHAPTER 5: DISCUSSION

To this date, there has been no published study that estimated the heavy metal content in fresh and untreated chicken litter in poultry farms, which is bioaccumulated in landfills or used directly as field manure in Goa. The present study revealed the following findings: first, there was presence of all the tested heavy metals in chicken litter and feed samples across both the sites, indicating the use of mineral metals as feed supplements in the diet of chicken. Broiler litter contained high levels of Fe, Zn and Mn, as compared to the layer litter. Second, toxic heavy metals like As, Cd and Pb were also detected though As and Cd were below permissible limits. However, Pb was detected above the permissible limits of feed, which is a huge matter of concern, considering Pb does not have any physiological role in the poultry feed ($p=0.00254$). Third, genotoxicity tests revealed that broiler farm contained significantly higher number of MNi than the references site, indicating presence of long-term DNA damage ($p =0.001$). However, no visible comets (DNA tails) were seen in the blood samples tested , implying that DNA damage on account of single and double stranded breaks were not prominent.

As of 2021, Goa has a total of 0.349 million poultry birds (MoEFCC, 2021) concentrated in less than ten functionally working poultry farms. Out of these, three poultry farms, which are the major supply chains in the poultry industry, were chosen for this study. These farms are classified as “medium” according to MoEFCC, (2021) with 30,000-38,000 chicks, juvenile, and adults in the layer and broiler farms. With such high numbers, it is suffice to say that these farms would produce tonnes of chicken waste (3-6 tonnes per year at site 1,100 tonnes per year at site 2 and 25-40 tonnes per year at site 3). Through a preliminary interview with the poultry workers, it was found that poultry litter would get

accumulated for months to one and a half year until it was sufficient to be discarded. We hypothesised that this litter had some sort of toxicants which can cause harm to the poultry workers and chicken who are exposed to it. It was also a common practise for poultry workers to apply chicken litter samples as manure in local farms or sell the discarded manure. Hence, we also hypothesised that application of this untreated manure would result in bioaccumulation of nutrients and metals in the soil, more than the required limits. Through published studies it was established that there are certain essential minerals like Fe, Mn, Cu and Zn, added in trace amounts at different stages of life to suffice the mineral needs. However, instances have shown a possibly higher concentration added in later stages i.e. layer and finisher phases (Chowdhury et al., 2022; Korish & Attia, 2020). Hence, chicken litter samples were collected from layer (> 12 weeks of age) and finishers (> 25 days) from the study sites to find out whether there were any metals incorporated in these stages. Due to this assumption, we also tested the hypothesis of chicken feed being the main source of heavy metals in the litter.

5.1 Physicochemical characteristics of chicken litter

Prior to the use of any manure, it is essential to determine its physicochemical parameters to ensure the sufficient availability of nutrients to the plants. Hence, a few physicochemical parameters like pH, Total Solids (TS), Volatile Solids (VS) and Moisture content were assessed to find out the overall chemical composition and preferable use of fresh chicken litter in its natural state (Kaur et al., 1997)These parameters greatly influence the quality of manure composition. Phosphorus (P) and Potassium (K) are considered to be primary macronutrients due to their relatively larger requirement and influence on growth and development of plants. (Sheikh & Dwivedi, 2020).

5.1.1 pH

pH is an important component of the litter as it influences the pH of soil. pH of chicken litter varies according to the age, diet of the bird and moisture content of the litter. In the present study, pH of the layer litter was around 6.7 (p=0.0001, f=132.597), similar to the values obtained in other studies (Faissal et al., 2017; Rizzo et al., 2020; Sarbjit et al., 2018). This alkaline pH is owing the protein-rich diet (nitrogen-based compound) in the layer farm. Though this pH is optimum for the growth of most plants, it is also favours the growth of bacteria and retention of ammonia levels (Ravindran et al., 2017). On the other hand, an acidic pH of 5.35 was observed in the broiler litter, which is much acidic and unsuitable for the use as manure. This acidic pH could be due to the composition of finisher feed which is intense in carbohydrate rich diet.

5.1.2 Total Solids (TS)

Another equally important parameter is the Total solids. TS is required to estimate the levels of suspended and dissolved solids in the litter samples. The % of TS was significantly lower across both the farms in comparison to the reference site (26.63±0.78, p= 0.001, f=47.843) indicating a relatively lower nutrient and organic content. Similar values have been reported from studies conducted in Japan and Nigeria (25% & 28.6 ± 0.7 %) (Abouelenien et al., 2009; Rizzo et al., 2020). Chicken manure is known for its containing higher levels of organic solids as compared to the other animal manures and hence it is preferred over other manure. However, in comparison to the composted manure, the fresh chicken litter has a much higher % of TS, and this becomes a matter of concern due to a possible risk of extensive nutrient loading in the soil.

Sometimes, these nutrient levels cross the permissible limits and cause ecotoxicity, though the distribution of nutrients cannot be estimated.

5.1.3 Total Organic Carbon (TOC)

The Total Organic Carbon is a measure of the carbon available for energy source. This carbon is usually deposited in chicken litter on account of feed. The % of TOC reported in the present study was found to be significantly lower in the in the broiler litter (16.25%, p=0.022) as compared to the layer litter (28.1%). These values coincides with studies conducted by Sarbjit Singh et al., (2018) and Ravindran et al., (2017) (21.12% and 30-40% respectively).Though a higher TOC implies a better energy source and conversion to CO_2 during composting, it is also an implication of overfeeding practise as stated by Sheikh and Dwivedi, (2020). Based on the feeding schedule, chicks are fed with carbohydrate rich grains in their diet to gain sufficient weight at a faster rate. Due to this, and inability of the chicks to digest enough carbohydrates, sometimes it may exceed the limits of digestion and may be excreted out thus contributing to the TOC of chicken litter. As layer hen require a relatively less carbohydrate rich diet, they prioritise egg-laying overweight gain, hence the excess carbohydrates excreted through faces and get deposited in the form of TOC. This could be a plausible reason for the higher levels of TOC in layer litter in the present study.

5.1.4 Moisture and Volatile Solids

Moisture content, also known as the extent of drying a material, is a crucial component when it comes to the use of manure. From the above result, it was evident that a majority of the TS comprises of volatile and moisture rich content (64%). There was not significant difference noticed in their levels across the

different farms in the current study (p=0.558). Similar values were obtained in studies conducted in Spain and Canada (74.53% and 73 to 80 %) (Fernandes et al., 1994; Quiroga et al., 2010). Most studies have also reported the values of composted chicken manure to be 58 % , 55-65% and 45.4±2.5% respectively (Abouelenien et al., 2009; Dalkılıc & Ugurlu, 2015; Rizzo et al., 2020). A moisture content of greater than 75% is considered to be high, deemed unfit for manure, as it hampers the process of efficient composting. It can harbour bacterial and fungal manifestations and increase the release of ammonia contributing to the bad odour (Zhu et al., 2020). Rynk et al., (1991) suggested a value between 40-60% is deemed for receiving major benefits of poultry manure. Hence, the chicken litter from both the farms could be deemed to be fit.

5.1.5 Total Phosphorus (TP)

Total Phosphorus (TP) is one of the prerequisites for the chicken litter to be an excellent source of manure. Phosphorus is relatively immobile element which can penetrate shallow waters and cause excessive loading in the upper profiles of the soil, it is important to quantify the levels prior to the use of manure (Kelleher et al., 2002). In the present study, the phosphorus levels in the fresh chicken litter were reported to be 1.3-1.36 g kg^{-1} across both the farms (p=0.011, f= 10.544). In comparison to the values obtained by Sager (2007) (19.1 g kg^{-1}) and Li et al., (2014) (15.8 g/kg) in poultry droppings, these values seem to be low. These studies have also reported higher levels of phosphorus in cattle manure (8.4 g kg^{-1}, 7g/kg) and swine dung (29.9 g kg^{-1} ,32g/kg). A study conducted in India had also reported the higher values 1.9 to 2.6 g/kg (Sheikh & Dwivedi, 2020). The phosphorus levels are generally higher in the manure due to its utilisation by microorganisms in the soil through decomposition process.

This releases more phosphorus content in the soil. In the present study, fresh chicken litter is most likely deficient in phosphorus content in its natural form and does not contribute to excessive nutrient loading when used as a manure.

5.1.6 Total Potassium (TK)

Total Potassium (TK) is one of the major constituents of animal manure. The present study reported a relatively higher level of TK in layer litter (940 mg/kg, p=0.027) as compared to broiler litter (621 mg/kg, p=0.180). Studies conducted elsewhere have reported varying values 1,305g kg^{-1} (Faissal et al., 2017) and 34.4 mg g^{-1} (Rizzo et al., 2020). Potassium is essential for plant growth and development however, it can lead to harmful effects if present in toxic levels. Studies have reported that composted manure usually have a high K content due to the decomposition process by animals (Sarbjit et al., 2018). However, almost 89% of the ingested potassium is usually excreted in the litter which could explain the higher levels of TK in the present study.

5.2 Heavy metal toxicity in litter

In the current study, we have analysed eight heavy metals in fresh chicken litter and feed samples. They are as follows: Iron (Fe), Manganese (Mn), Copper (Cu), Zinc (Zn) and Chromium (Cr) being the essential heavy metals and Arsenic (As), Cadmium (Cd) and Lead (Pb) being non-essential toxic heavy metals. The overall distribution of heavy metals across the poultry farm was as follows : Cu > Mn > Fe > Zn > Cr > Pb > Cd > As ***(Table 4.1).*** Broiler litter showed higher concentration of heavy metals as compared to the layer farms (p=0.638, f=0.454).

To discern this fact, it is necessary to consider the following factors.

Chicken excretes urine and faeces in the form of a singular pellet; thus, constituents of litter would be directly dependent on the feed constituents. The feed is enriched with mineral supplements, which are not retained and excreted through the litter. Both the breeds are reared for different purpose and in order to achieve that purpose, their feed content is variable. On an average, broilers need extra nutrient ingredients, protein and fat-rich diet to achieve faster growth rates and slaughter weight as compared to the layer hens. Hence the average consumption amounts to approximately 1.5 kg of feed intake during the finisher phase. Layers, on the other hand, do not need fattening diet and hence they have a lower protein intake, with a diet rich in micronutrients and essential minerals, with an average consumption of 100 – 150 g during layer phase (TNAU, 2015). Hence, broiler litter production is more as compared to layer on a daily basis.

As stated earlier, broiler was housed in deep litter system where the droppings are accumulated and cleaned after few weeks. There is relative movement of the broiler chicken within the litter system On the other hand, layers are housed in battery-cage system , where the litter is collected beneath the cage and piled up for many months. Here there is no particular movement of the chicken, and they undergo more stress in such a system. To meet up with the humongous demand, this battery cage system is used which can rear 5000 birds at a stretch and more modifications are made to the feed. This could also explain the higher levels

The dietary needs of broiler and layer chicken differ based on the purpose of production. Factors like age, sex, housing system, stress, stocking density and management system also affect the average intake by chicken. A closer look at the feeding schedule of chicks reveals the variation of nutrient intake at various stages of their life. On an average, broilers need extra nutrient ingredients,

protein and fat-rich diet to achieve faster growth rates and slaughter weight as compared to the layer hens. Hence the average consumption amounts to approximately 1.5 kg of feed intake during the finisher phase. Layers, do not need fattening diet and hence they have a lower protein intake, with a diet rich in micronutrients and essential minerals, with an average consumption of 100 – 150 g during layer phase (TNAU, 2015). Considering age as a prime factor, protein, carbohydrate and calcium rich diet is essential in the initial stages of life, so as to attain sufficient size and calcification of egg-shell. Once the protein–carbohydrate demand decreases, possibilities of micronutrient deficiencies may arise which are catered through incorporation of minerals in the diet.

Currently, there was no data available which stated the permissible limits of using fresh chicken litter samples as manure. Hence, we looked at a series of studies which reported the heavy metals in layer and broiler manure samples to compare each metal :-

5.2.1 Iron (Fe)

Iron is one of the major essential elements required for the growth and development. It is added in trace quantities in the poultry feed to decrease instances of anaemia (Ghaedi et al., 2007). According to the Bureau of Indian Standards 2007, the permissible limit of Fe in the layer feed and finisher is 60 mg/kg and 80 mg/kg respectively (BIS, 2007). The values of Fe reported in the layer feed were found to be 6 times higher and that in finisher feed were 1.5 times higher than the maximum limits. A study conducted in Bangladesh however, stated that Fe was within the permissible limits of 500 mg/kg as per the NRC 1994 guide (Chowdhury et al., 2022; Dale, 1994; National Research

Council (U.S.), 1994). The present study reported a higher concentration of Fe in the finisher feed (125mg/kg , p =0.33) and layer feeds (406.87 mg/kg, p=0.33), with layer feed having a higher concentration as compared to the broiler feed. Higher content of Iron may be added to boost the growth and to prevent the instances of anaemia. In addition to this, it also helps in prevention of any bacterial infection, which are common considering the tropical climates of Goa. The average concentration of Fe in chicken litter was reported to be 0.875 g/kg which was relatively lower to values reported in chicken manure samples in Austria ; 1.25 g/kg (Sager, 2007). The Fe content in broiler litter was significantly higher (1.05 g/kg, p =0.00752) as compared to the layer litter (0.925 g/kg, p=0.02258). However, multiple studies conducted in Bangladesh and Austria reported lower values ; broiler litter (0.7 g/kg) (Li et al., 2014), 0.1 g/kg Fe in broiler litter and 0.18 g/kg in layer litter (Chowdhury et al., 2022). According to a study in US, the critical toxicity levels of Fe in the soil is between 680 to 850 mg/kg (Foust et al., 2018). Our study reported higher values which suggests that in its natural form, fresh chicken litter will contribute to increased iron load in the soil. The transfer factor (TF) of Fe in the layer litter was high (0.44), indicating lower retention of this heavy metal in the body. Similarly, a lower TF of Fe in broiler litter (0.12) indicated higher retention in the broiler chicken, which is frequently consumed by humans.

5.2.2 Zinc (Zn)

Zinc is an essential element added as Zinc oxide in poultry feed to improve the reproductive health of the bird (Park et al., 2004). The Bureau of Indian Standards 2007, has stated the permissible limit of Zn in the layer feed to be 60 mg/kg and in the broiler feed to be 80 mg/kg (BIS, 2007). The concentration of

Zn reported in the present study was within the permissible limits in the finisher feed (41.25 mg/kg , p =0.835) and slightly higher in the layer feed (63.75 mg/kg, p=0.189). However, studies conducted in England and China have reported values higher than the acceptable limits ; 135 mg/kg (Nicholson et al., 1999), and 103mg/kg (F. Zhang et al., 2012). On an average, chicken litter contained 338 mg/kg of Zn which was similar to the values obtained by Sager (2007) (314 mg/kg) and Zhang et al., (2012) (384.15 mg/kg) in chicken manure. However one study conducted in New Zealand reported higher values of 2300 mg/kg (Bolan et al., 2004). The Zn content was higher in broiler litter (390 mg/kg , p <0.001) as compared to the layer litter (354 mg/kg, p=0.00265). These values are slightly above the critical toxicity limits in the soil (100-300 mg/kg) which suggests, a necessary prior treatment before the use of fresh chicken litter as manure (Foust et al., 2018). The TF of Zn was comparatively similar in both the litter samples (0.11- 0.18) which suggest higher retention levels of Zn in the chicken.

5.2.3 Manganese (Mn)

Manganese is an essential element which is required in the diet of the birds for its critical role in lipid and carbohydrate metabolism. It is required for the development of bone density, increasing the egg shell quality and improving performance of poultry (Olgun, 2017). As per the BIS, the permissible limit of Mn in the layer feed and finisher feed is 60 mg/kg and 100 mg/kg respectively (BIS, 2007). Results of the present study reveal Mn content was higher than the permissible limit in the layer feed (173.87 mg/kg) as well as finisher feed (160 mg/kg).

On an average, chicken litter contained 1132.67 mg/kg of Mn with broiler litter (1490 mg/kg , p =0.0125) having a higher value as compared to the layer litter (998 mg/kg, p=0.6030). The values obtained were somewhere in between those obtained by Sager (2007) (339 mg/kg) and Bolan et al., (2004) (1800 mg/kg) in the chicken litter. According to the US standards, the Mn levels were within the normal range for uptake by the soil (200-3500 mg/kg) hence fresh chicken litter would not be detrimental for the soil during land application (Foust et al., 2018). The TF of Mn was similar to that of Zn suggesting accumulation in the body.

5.2.4 Copper (Cu)

Copper is one of the most important trace metal used in the poultry feed as copper sulphate ($CuSO_4$), which is involved in numerous physiological processes like growth, activation of the immune system and biochemical processes by acting as a prominent cofactor (El Sabry et al., 2021). The present study reported a relatively higher concentration of Cu in finisher feed (413.75 mg/kg, p =0.028) as compared to the layer feed (295 mg/kg, p=0.5088). These values are at least 32 times higher than the ones recommended by BIS, (2007) (9 mg/kg in layer feed and 12 mg/kg in the finisher feed). Cu is also known for its bactericidal effects, whereby it inhibits the growth of bad bacterial colonies in the gut and is responsible for improving the feeding efficiency of chicken. Owing to the tropical climatic conditions and instances of recurrent avian bacterial infections, poultry farmers may use such higher levels to prevent infections in the birds. Reiterating to the previously reported feeding schedules of broiler chicken, which have high nutrient intake, this protects them on a larger scale.

Results of the present study reveal a higher level of average concentration of Cu (3026 mg/kg) with a relatively higher concentration in broiler litter (3980 mg/kg, p =0.33) as compared to the layer litter (2668 mg/kg). These high values suggest the minimum retention of Cu in the body as most of it is excreted in the litter. Most studies have reported relatively lower levels of Cu ranging from 66-400 mg/kg (Bolan et al., 2004; Sager, 2007; F. Zhang et al., 2012). The high levels of Cu content in the chicken litter is way above the critical toxicity limits of the soil (20-30 mg/kg) making the litter unfit for direct application in soil (Foust et al., 2018). This may increase and mobilise the copper present in the soil and overshoot it's the limits in the soil as suggested by a review study (Korish & Attia, 2020). As Cu showed similar values of TF, this also suggests lower accumulation in the body.

5.2.5 Chromium (Cr)

Chromium (Cr) is added as a supplement in poultry feed for glucose, lipid, and protein metabolism especially among birds experiencing stressful conditions (White & Vincent, 2019). Though the level of Cr intake is not explicitly stated in BIS, India, critical levels of Cr as per the EU was 0.01 mg/kg (European Commission, 2003). The Cr content in the feed was above these limits, with a significantly lower concentration in finisher feed (1.375 mg/kg , p =0.8032) as compared to the layer feed (3.75 mg/kg, p=0.0129). These values were higher than those in a study conducted in Wales, in layer feed (0.76 mg/kg) and finisher feed (0.22 mg/kg). Such high supplementation is sometimes needed, especially in tropical climates where birds tend to have reduced feed intake and lower performance. In such instances Chromium helps in utilisation and uptake of Glucose and helps in boosting the immunity of cells by significantly reducing

glucocorticoids (A. Sharma et al., 2020). The overall concentration of Cr was 6.47 mg/kg, with a relatively lower concentration in broiler litter (6 mg/kg , p=0.696) as compared to the layer litter (9 mg/kg, p =0.171). These values were lower as compared to those obtained in different studies ; 10.7 mg/kg (Sager, 2007) and 23.71mg/kg (F. Zhang et al., 2012). Cr forms various insoluble compounds in the soil and thus inhibits the uptake of essential nutrients in the soil as stated by a study conducted in Amritsar. Hence it is not beneficial to the soils, and hence there are non-established critical limits for the soil. However, critical limits exist for the uptake by variety of plants with a varying range of less than 1mg/kg (A. D. Sharma et al., 2005). The TF of by Cr was 0.28 in broiler litter as compared to the layer (0.42) , which suggests a higher retention in the layer breed.

5.2.6 Arsenic (As)

Arsenic (As) is a naturally occurring, mobile metalloid element, which exists in both organic and inorganic forms which can easily be absorbed by the plants and metabolised inside human body. This element is naturally present in the earth's crust, however, anthropogenic activities may increase its levels and cause toxicity (Q. Y. Chen & Costa, 2021; Rehman et al., 2021). In poultry farms, As has been used in chicken feed in its organic form called "Roxarsone" (3-nitro-4-hydroxyphenyl arsonic acid) to control microbial infections like coccidiosis and increase weight of the bird. As this compound is excreted through chicken litter, it ends up in the soil or contaminates the ground water levels. According to the Bureau of Indian Standards 2007, As is considered to be toxic metal and should not be introduced in the feed. However , if present, it must be below the permissible limit of less than 2 mg/kg in both types of feed (BIS, 2007). The

present study reported the presence of As, at relatively lower concentration of As in finisher feed (0.04 mg/kg, p =0.426) as compared to the layer feed (0.06 mg/kg, p=0.210), both of which were under the maximum limit. This suggests that As was added as feed supplement in the diet. One such study reported higher values of 0.05 mg/kg in finisher feed and 0.10 mg/kg of As in the layer feed. The present study reported 0.4 mg/kg As in the layer litter (p=0.62). Few studies have suggested the following concentrations in the diet 0.12 mg/kg (Sager, 2007) and 3.79 mg/kg (F. Zhang et al., 2012). A shocking result was that As was Below Detectable Limits (BDL) in the litter samples. This implies that it was retained in the body of broiler chicken. Such a toxic heavy metal in the broiler chicken which is consumed by people will amount to bioaccumulation in the human beings and elicit ill effects. According to the US standards, the As levels were within the normal range for uptake by the soil (<2 to 80 mg/kg) hence fresh chicken litter would be considered safe during land application (Foust et al., 2018).

5.2.7 Cadmium (Cd)

Cadmium is a non-essential heavy metal, added in the poultry feed to enhance eggshell quality in lower doses (<10 mg/kg). The present study reported a relatively higher concentration of Cd in finisher feed (0.18 mg/kg, p =0.0536) as compared to the layer feed (0.04 mg/kg, p=0.7079). However, these were under the permissible limits. Higher values were obtained in a study conducted in Wales 0.12 mg/kg in finisher feed and 0.39 mg/kg in layer feed (Nicholson et al., 1999). The present study reported a relatively higher concentration of Cd in broiler litter (0.6 mg/kg, p=0.01) as compared to the layer litter (0.4 mg/kg, p =0.50). Similar values were obtained in studies conducted in Bangladesh and

Austria ; 0.485 mg /kg in layer feed and 0.499 mg/kg in broiler feed (Chowdhury et al., 2022) and 0.27 mg/kg (Sager, 2007). However a study conducted in China reported higher levels of 4.05mg/kg Cd in the chicken manure (F. Zhang et al., 2012). The Cd content in fresh chicken litter is below the critical toxicity levels of Cd in the soil (less than 8 mg/kg), suggesting the litter is safe for application in the soil. However, the TF reported in layer litter was very higher 2.33, indicating a higher retention in the body. Higher accumulation of Cd is known to cause toxic effects, are known to reduce the egg quality, feeding efficiency of chicken and growth performance of chicken (Kar et al., 2018).

5.2.8 Lead (Pb)

Lead is a naturally occurring toxic heavy metal found in the earth's crust within a range of 72.4 ppm to 251.5 ppm (Kabir et al., 2019). According to the Bureau of Indian Standards 2007, the permissible limit of Pb in the layer feed and finisher feed is 5 mg/kg (BIS, 2007). The present study reported a significantly higher concentration of Pb in layer feed (6.75 mg/kg, p=0.00235) as compared to the finisher feed (3.0 mg/kg , p =0.03097). A study conducted in Wales, however reported much lower values <1mg/kg in both types of feeds (Nicholson et al., 1999). The layer feed had a Pb content higher than the permissible limits which can get accumulated in their body as well as get deposited in the eggs . As lead is a dense heavy metal, it persists in the gastrointestinal tract of hen, get absorbed into the blood stream and interfere with calcium resorption (Sobhakumari et al., 2019). These eggs are widely consumed by humans hence they can also bioaccumulate in our body. The present study reported an average Pb concentration of 10.07 mg/kg, significantly higher concentration in broiler litter (24 mg/kg) as compared to the layer litter (4 mg/kg, p >0.05) Studies have

reported similar values ; 3.62 mg/kg (Bolan et al., 2004), 4.44 mg/kg (F. Zhang et al., 2012) and 5.4mg/kg (Sager, 2007). The higher levels of TF 2.33 in layer litter indicate higher accumulation in the litter, whereas a TF of 0.13 in broiler litter indicates accumulation in broiler chicken which may cause Pb toxicity in the long run.

5.3 Genotoxicity in Broiler chicken

Prior to commencement of Genotoxicity testing, Haematological parameters were assessed to evaluate health status of the bird. Since, they were in the normal range, it can be ascertained broiler breeds at both sites were healthy. Non-essential heavy metals like As and Pb were found in the layer and finisher feeds, which have no biological role and must have been incorporated accidentally during the preparation of feed, transport and storage. Due to this finding, we assessed genotoxicity using Micronucleus test and comet assay. Micronucleus test revealed that lower DNA damage was seen . However, values reported in other studies were significantly higher than the present study (Da Silva Cardoso et al., 2016; Saleh & Sarhan, 2007). Micronucleus is a small nucleus formed during the anaphase after failure of incorporating the chromosome in daughter cells , which is surrounded by nuclear membrane to form micronucleus. It indicates DNA damage which cannot be repaired as it has passed the cell cycle checkpoints. Correlation analysis also revealed that a possible reason behind the DNA damage could be on account of non-essential heavy metals, however, further sample testing is needed to conclusively ascertain the Mni result. Single Cell Gel Electrophoresis, also known as comet assay, is used to reveal short term DNA damage like single strand DNA breaks, in an individual cell, which can be repaired through DNA repair mechanisms before passing the cell cycle check

points. The present study showed an intact DNA head, with no visible comets which suggests the broiler breeds can repair the short term DNA damage. Most studies however, showed presence of higher DNA damage through prominent % tail DNA (Arooj et al., 2023). Hence, further sample testing is needed to conclusively ascertain this finding.

CONCLUSION

CONCLUSION

The present study is a pilot study undertaken to address the growing concern of disposing chicken litter without prior treatment in the soil. Findings of this study reveal that essential heavy metals like Fe, Zn, Mn, Cu and Cr as well as non-essential heavy metals like As, Pb and Cd were present in chicken litter across different poultry farms in Goa. In particularly, broiler litter had higher heavy metal content as compared to layer litter indicating higher toxicity in broiler farm. The source of these heavy metals was found to be chicken feed, where essential HM were incorporated in trace amounts to cater to micronutrient deficiencies . However, Fe, Mn and Cu were found to be much above the permissible limits, which can lead to increased antibacterial resistance in birds and make them more susceptible to bacterial infections. Non-essential heavy metals such as Pb and Cd, that have no biological role to play, were found in the both types of feed, in particular, the Pb content was higher than permissible limits in the layer feed. This is a matter of concern since these metals can cause carcinogenic effect, increased diarrhoeal instances, neurotoxicity and hepatotoxicity in the birds. One such effect tested was genotoxicity which revealed lower DNA damage on account of presence of Micronuclei. Hence stringent measures must be taken to monitor their levels in the feed as well as their source. Despite being a non-essential heavy metal, As was incorporated in feed to decrease coccidial infections, but was banned in most countries because it would accumulate in the litter and contaminate soil and groundwater. However, it was found in both types of feed but retained in the broiler chicken which we consume, once again raising safety concerns about consumption of this chicken. The direct consequence of these non-biodegradable and highly

dense elements is using the untreated litter as manure, which would increase heavy metal content in the soil amounting to ecotoxicity.

LIMITATIONS

Findings of the present study needs to be substantiated with more sample analysis to detect heavy metals in litter and feed samples which will make our study more conclusive.

FUTURE PROSPECT

The present study has successfully proven that heavy metals exist in chicken litter samples hence, it would be interesting to study the effects of these heavy metal exposure in poultry workers and poultry birds and studying the bioaccumulation of these heavy metals in vegetables, grown in untreated chicken manure.

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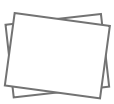

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Poultry farming refers to the act of rearing domesticated birds for the production of meat and egg. It is one of the fastest-growing sub-agricultural sectors, with a rate of 8% increase per annum. This industry has become one of the most efficient utilities of animal husbandry, providing nutritional security to a
significant number of people across the globe. Chicken, ducks, turkeys, quails, gees,e and pigeons are some of the predominantly reared poultry birds. Gallus Gallus domesticus, (Linnaeus, 1758) also known as “domestic chicken”, is the most widely reared poultry bird. According to Food and Agricultural
Organisation, this species constitutes 94% of the world’s poultry population, contributing to 90% of world’s meat production and 93% of world’s egg
production (FAO, 2023). Belonging to Order Galliformes and Family Phasianidae, chicken is an excellent, relatively cheaper source of protein than
other meat products. Chicken eggs are wholesome, rich sources of vitamins, iron, folate, selenium, zinc, lutein and zeaxanthin whereas chicken meat, offers
low caloric value and low content of trans- fats due to which it is preferred over red meat. The innumerable benefits offered by these by-products, has resulted in spark rise in the consumption of chicken meat from 9 to 133.8 million tonnes and chicken eggs from 15 to 93 million tonnes from 1961-2020 Depending on the purpose of consumption, chicken breeds can be classified into 3 types. The present-day broiler chicken is a result of intentional selection pressure and genetic hybridisation. This species is the descendant of a sole ancestor called ‘red jungle fowl’ (Gallus gallus) in the early 5400 BC in Southeast Asia . Historically, poultry farming was a small-scale domestic activity with lack of commercialisation and practised with the intention of egg
production. Back then, male and female chicken could not be differentiated until 7 weeks of age, so they were grown to their full lifespan of 96 weeks upon which they were culled for poultry meat. However, with increasing commercialization and the advent of genetic studies, this activity turned into a mass-scale intensive production where optimising egg production and obtaining chicken meat at a faster rate was the prime focus. The crossbreed between male broad-breasted Cornish strain and female broad-breasted White Plymouth Rocks in 1930’s was the very first attempt that resulted in a broiler strain. This strain however had problems associated with disease susceptibility and availability of low meat. Subsequently, modern strains were developed to tackle these problems and attain faster growth as compared to chicken layer breeds. These breeds are derived from stocks of White leghorn stock and Cobb. In particular, Cobb breed accounts for majority of the broilers in India which was developed from white feathered female line (Griffin & Goddard, 1994). The modern-day broiler strains have successfully reduced the lifespan of chicken to 39 days, attaining a slaughter weight of 2.2kg in such a short span of time. There has also been an enormous increase in broiler growth by almost 400% from 1957 to 2005. While this change has been successful on an economic scale, this genetic pressure has resulted in skeletal deformities, compromised the immune system functioning and resulted in metabolic disorders. To tackle this, chicken feed, is supplemented with minerals which may contribute to DNA damage in the long run .In addition to this, the recurrent genetic manipulation, may increases the risk of DNA damage. Given this fact, it can be hypothesised that there is possible DNA damage in chicken, owing to the abuse of feed additives. The rapid expansion of layer and broiler industry has also contributed to the large-scale generation of tonnes of waste in the poultry industry. These waste products include chicken litter, dead birds, feathers, sawdust, excess feed and spilled water, among which, chicken litter constitutes a major portion

Chicken litter refers to the faecal matter mixed with floor constituents like wasted feed and bedding material. It is produced through a cloacal passage, which includes the urethra, ejaculation canal and the anal canal. Due to this unique excretory organ, the faecal and urine matter is excreted together. The production of chicken litter is massive across the globe, with India contributing to at least 38.33 million tonnes of poultry litter and US contributing to 14 million tonnes of chicken litter annually Though chicken litter is a foul odour producing contaminant, human beings have
managed to utilise it as a useful resource. For centuries, chicken litter has been the cheapest and rich source of manure for crop production due to the excess amount of Nitrogen (N), Phosphorus (P) and Potassium (K). Currently, it is being utilised as a low-cost protein feed for dairy and beef cattle, biodiesel and biogas production (Jeffrey et al., 1998; Prabakaran & Valavan, 2021). While chicken litter is an excellent source of manure, it is a time-consuming process for the manure to undergo complete composting. In most poultry farms, it is observed that there is direct application of untreated chicken manure to agricultural land, which results in two major repercussions: -Microbial contamination: In battery-cage poultry system, the chicken litter is accumulated in heaps and piles of open landfills. The top surface is usually filled with fresh chicken excreta which provides breeding grounds for coliform bacteria like Salmonella sp., Staphylococci , Enterobacteriaceae and fungal species like Histoplasma capsulatum which may contribute to infections in chicken and poultry workers Trace elemental toxicity: Trace elements are those elements present at low concentrations (mg kg−1 or less) in agroecosystems. incorporated in later stages of poultry feed to enhance feeding efficiency and cater to the micronutrient deficiencies. Once ingested, they enter the environment through excrement. In battery-cage systems, these elements pile up and accumulate in the open landfills amounting to ecotoxicity. One class of trace elements which is a rising matter of concern is “heavy metals. Heavy metals are those elements having high densities of ≥ 3 g/cm3. According to Raychaudhuri et al., (2021), Heavy metals (HM) are defined as those metals and metalloids which have an atomic number greater
than 20 and relative density greater than 5 g/cm3.

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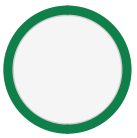

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Similarly, a review conducted by Kyakuwaire et al., (2019) , enlisted the various contaminants like bacterial species, heavy metals, and antibiotics present in the layer and broiler litter to prove that in its natural form (without composting), chicken litter was deemed to be inefficient as an organic fertiliser. She further suggested that high levels of As, Cd and Pb found in the chicken litter samples contributed to heavy metal toxicity in the soil, where litter was used as manure. Recently in Saudi Arabia, an experiment was conducted to test the quality of chicken consumed by human beings by studying heavy metal accumulation in 60 table eggs, 45 frozen broiler meat and 30 feed samples. In addition to this, the layer and broiler chicken litter was also tested. This study demonstrated that higher levels of Fe (183.3 ppm), Cu (16.61ppm) and Mn (42.5ppm) was detected in the layer feed, whereas Zn was higher quantities in the starter feed (60.3 ppm). On the other hand, Pb (3.37 ppm) was the highest in the broiler litter and As(0.00071), Cd (0.629 ppm), Ni (11.4 ppm) and B (0.461 ppm) were highest in the layer litter (Korish & Attia, 2020). In Bangladesh, a comparative study was made between different types of poultry feeds to assess the heavy metal supplements, which revealed Mn (3.021 ± 0.003mg/kg), Fe (108.392 ± 0.002 mg/kg), Cu (1.307 ±0.002 mg/kg) and Zn (2.223 ± 0.002 mg/kg) was higher in grower phase whereas Cr (0.470 ±0.003). mg/kg) levels were higher in Grower phase. Though all the supplements were under permissible limits (Chowdhury et al., 2022). A study conducted in China, compared the accumulation of heavy metals in swine manure, chicken manure and swine manure organic fertiliser which revealed that Zn and Cu were highest in concentration among all types of manure, suggesting heavy metal toxicity in manure (46.5 to 843mg/kg) (Xue et al., 2021).Morocco, the first country for initiating broiler breeding in North Africa, produces 5,19,000 tonnes of broiler droppings every year, out of which, 95% is directly used as crop fertilisers. A study conducted by Mohamed el Amin, A.(2014) had reported an increase in the concentration of heavy metals like Pb (2,37 mg/kg), Zn (196,35 mg/kg), Cu (70,90 mg/kg) and K (32,86 mg/kg) in the nearby water sources due to this practise. Ogunwale et al., (2021) in his study explored the accumulation of heavy metals among crops cultivated at a poultry farm in Nigeria. His study showed the levels of As (9.88-32.33 µg/g) and Fe (4.40-250.05 µg/g) were way above the permissible limit of plant intake, amounting to bioaccumulation in the food chain due to the use of poultry manure. Another study conducted in Nigeria suggested the seasonal variation of heavy metals in water reservoirs owing to the leachate of heavy metals from poultry waste, with concentration of iron showing significant increase (0.104±0.401 mg/L) (Oyewale et al., 2019). However, one study conducted in South Africa showed heavy metals in poultry manure were at lower limits. This study was conducted by Ravindran et al., (2017) where he assessed ten poultry manure samples for heavy metals (Cr, Cu, Ni, Pb and Zn) and found that heavy metal levels were below the permissible limits. In a comparative study conducted in Malaysia, between the chemical characteristics of fresh and composted chicken manure, high content of proteins (27%), carbohydrates (31%) and a low C:N ratio of 7.19 in both types of manure on account of overfeeding practise of chicken to attain the slaughter weight at a faster pace (Sarbjit et al., 2018).A solution to the problem of heavy metal leachate is increasing the efficacy of chicken manure composting. Through the findings of Zhang et al., (2021) , it was evident that co-composting the chicken manure with a combination of plant residues and wheat/rice decreased ammonia emissions by 50-80%, As by 0-53%, and Cd by 5-28%, thus reducing their release in the environment. In India, few studies have been conducted to investigate and optimise the use of poultry manure and its effects.A study conducted near the Rakha mines in Jharkhand, evaluated the effects of chicken manure on revegetation in the areas. Findings of this study revealed, an
increase in plant biomass growth as well as an increase in heavy metals like Mn and Zn to a permissible limit (Das & Maiti,

2009).Similarly, in New Delhi, a study was conducted to estimate the efficacy of manure on account of poultry droppings, leaf supplements and fungal species like Aspergillus awamori in alleviating C:N ratio. This study suggested that proper composting time and fungal species escalated and optimised the use of manure (Gaind et al., 2009). Kar et al., (2018) , in their study in West Bengal, determined the concentrations of Cadmium(Cd), Lead(Pb), Copper (Cu) and Cobalt (Co) in the tissues as well as droppings of backyard chickens from polluted and unpolluted sites. His study revealed that concentrations of Cd and Pb were significantly higher in the polluted site as compared to the unpolluted one ($p < 0.05$).The poultry sector in Goa is a small-scale sector, limited to domestic farming and catering to domestic needs. In 2009, a study was conducted by ICAR┐CCARI, Ela Goa, in 2009, to identify the constraints faced by poultry farmers by surveying 100 poultry farms in Goa. Back then, 90% of the poultry farms were broiler while the remaining 10% were layer farms. High cost of chicken feed, competition with outside farmers, high labour cost, high cost of chicks and non-availability of health services were cited as major challenges back then (Swain et al., 2009). No other study has been published in this regard.2.2 Studies on GenotoxicityA review conducted by Cotelle and Férard, (1999), had discussed the applications of comet assay to determine genotoxicity in plants, amphibians, fishes and mammals. However, the application of this assay in avian species was not discussed. Subsequently, Sokolovic et al., (2007) developed a protocol to inculcate the use of comet assay parameters to detect DNA damage on account of feed additives, mycotoxins and other parameters.

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Awad et al., (2014) in his study, performed a comet assay to evaluate DNA damage caused by Deoxynivalenol (DON) present in the chicken feed. His
findings revealed that there was a significant increase in blood lymphocytes and tail of the comet (31.99±0.89%, p=0.001) which confirmed that the compound induced genotoxicity in broiler chicken.A study conducted in US investigated the effects of T-2 and HT-2 toxin, one of the most toxic classes of trichothecenes, in twenty Cobb 540 broiler chicken using comet assay. Findings of the study revealed noticeable DNA damage witnessed by the decrease in DNA % in the tail (Szabó et al., 2019).Micronucleus test is another method used to determine genotoxicity by examining the micronuclei erythrocytes (MNE) present in the peripheral blood smears. This method was used to assess the genotoxicity of broiler chicken in Saudi Arabia. Findings of this study revealed MNE was 7-8 times high on account of feed supplements and predicted that this may also cause clastogenic effects in the long run (Saleh & Sarhan, 2007).A study in Brazil assessed the effects of antigenotoxic effects of Piperine against aflatoxins and carcinogens using comet and micronucleus assay. There was significant reduction in Micronuclei erythrocytes and DNA % in tails of comets, which suggest this agent is suitable for antigenotoxic effects against feed supplements (Da Silva Cardoso et al., 2016). In addition to mycotoxins contributing to genotoxicity, one study in China also
investigated the genotoxic potential of Roxarsone (Arsenic compound), a prominent feed additive in broiler chicken in V79 cells , using micronucleus and
comet assay. This study revealed a significant increase in the comet parameters (p

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Gallus Gallus domesticus, (Linnaeus, C 1758), also known as “domestic chicken” was chosen for the present study due to easy availability of chicken
litter and procurement of chicken blood. The local name of this species is ‘Kombi.’ Goa is a small state located along the western coast of the Indian peninsula (28° 38′ N latitude and 72° 12′ E longitude) covering an area of 3,700 km2. Spanning across the Eastern boundary are the Western Ghats whereas flanking its western coast is the Arabian sea. Goa has a tropical climate with an average temperature of 28-33oC, with humid weather, receiving an annual rainfall of 330cm. Fishing, Agriculture, and Tourism are the major industrial activities. Poultry farming, on the other hand, is a small-scale activity in Goa, with less than ten farms working in good conditions. This is insufficient to cater to the high demand of chicken meat and eggs, hence Goans are dependent on the neighbouring states for the supply of broiler meat and eggs.Selection criteria: A preliminary questionnaire-based survey was conducted
across poultry farms in Goa to study the working conditions prevalent in these areas. The poultry farm workers were interviewed about the following
parameters: type of farming (broiler, layer, or rural backyard poultry farming), size of the farm, housing system (deep-litter or batter cage system), type of
chicken feed (pre-starter, starter, grower, layer, finisher), water source, vaccination status, supply chain of day-old chicks, chicken litter production and
disposal (Table 3.1). Based on the data collected, three poultry farms were chosen as the study sites (Fig. 3.1).Study site 1: This study site was located at 15°29'42.75"N latitude, 73°55'4.61"E longitude, where rural-backyard poultry farming was practised in deep-litter housing system. Cari devendra and Grama priya were the chicken breeds reared exclusively for dual-purpose (egg and meat), genetic studies and optimizing the breed. The criteria for selecting this site as a reference was as follows: its dual-purpose nature, chicken coop being cleaned on a regular basis and chicken litter being buried under landfill for composting. Study Site 2: This study site was located at 15°26'47.86"N latitude, 74° 4'38.88"E longitude, where layer farming was practised in battery-cage housing system. The chicken breeds were reared for the sole purpose of egg production. This farm had a stocking capacity of 38,000 adult and juvenile chicks of BV300 layer strain. The day-old chicks were obtained from supply chains in Pune. Here, the chicken litter was stockpiled in open landfills under the cages, for about one and a half year after which it was sold off to vegetable farms as manure. Study Site 3: This study site was located at 15°0'25.74"N latitude and 74°4'0.62"E longitude, where broiler farming is practised in deep-litter housing system. The chicken breeds were reared for the sole purpose of broiler meat production. This farm had about 30,000 adult and juvenile chicks of Vencobb400 broiler strain. The day-old chicks were obtained from Mandovi Hatcheries in Goa hence the entire farming process was Indigenous. The chicken litter was cleaned from each shed, once every two weeks and compiled in an open landfill. It was given to vegetable farms after three months as manure.In addition to this, chicken blood was procured from the reference site having dual-purpose breed (Cari Devendra) and study site 3 having Vencobb400 broiler strain (one of the most popular indigenous supply chains of broiler chicken) from North and South Goa, respectively For the present study, two types of samples were used: chicken litter samples were collected for the chemical characterisation and assessment of heavy metals and chicken blood samples were collected for studying the enotoxicity. In addition to this, chicken feed samples were also obtained to check the source of heavy metals and possible DNA damage. The baseline characteristics of each study site are indicated in Table 3.2.1. Chicken litter: Chicken litter

samples were collected from a single shed at each study site, once a month for a period of two months (October to December 2022). Twenty fresh chicken litter pellets, weighing 150g were collected from each poultry shed in plastic containers and mixed thoroughly. The homogenised sample mixture was labelled and stored in plastic zipper bags at -20oC until further analysis (except for the analysis of pH, total solid and moisture content, where the homogenised sample was processed. immediately). Due care was taken during the time of sampling to ensure that the sample was fresh and not contaminated by external sources of heavy metals. Chicken feed: From the chicken sheds chosen for sampling the chicken litter, 50g of layer and finisher chicken feeds were procured, labelled, and stored in plastic zipper bags at room temperature until further analysis.Chicken blood: Three blood samples, each of 2ml blood volume, were obtained from the chosen study sites. The blood samples were collected at the time of culling of broiler chicken, in EDTA-coated blood collection tubes (Ethylenediaminetetraacetic acid). Due care was taken to prevent blood clotting by continuously shaking the EDTA tubes at the time of sampling. The samples were processed within 4 hours of collection. It is necessary to maintain sterile conditions and prevent cross-contamination by other heavy metals to prevent an inaccurate reading. To avoid this, the glassware and crucibles were thoroughly cleaned, sterilized, and placed in hot air oven incubator. Prior to the conduct of the experiment, they were washed using concentrated hydrochloric acid to clean the surface. Experiments were conducted in clean and sterile environment, wearing clean lab coats, gloves, and mask. Any acid digestion was performed in fume hood. Samples were constantly covered using aluminium foil to avoid contamination. 3.6 Apparatus and instruments. General laboratory wares like measuring cylinders, beakers, test tubes, glass rods, micropipettes, reagent bottles, mortar and pestle, vertical coupling jars, porcelain crucibles, Erlenmeyer flasks, volumetric flasks, Whatman No.1 filter papers, hemocytometer and microscopic slides were used. Special Frosted slides were used for comet assay. Instruments like pH meter (TMP 3) Muffle furnace (i-therm AI-7981), Hot air oven (MIC-165).

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Hot plate, weighing balance (PGB, 200), Centrifuge(R-24), UV-Visible spectrophotometer(BL 1073), Orbital shaker, Cyclo mixer (CM, 101) , and Agarose Gel Electrophoresis unit were also used. Olympus Light Microscope(BX40) & Olympus Fluorescence Microscope(BX53) were used for analysing slides for micronucleus and comet assay. Heavy metal analysis was carried out using Atomic Absorption Spectrophotometer novAA series at Italab Pvt. Ltd., Margao, Goa and Soil Science Department of ICAR-CCARI, Ela, Old Goa, Goa and Sadekar's Envio Engineers Private Limited Lab. All the chemicals and reagents used in the present study are of analytical grade. The following physicochemical parameters of the chicken litter samples were studied: i) pH ii) Total solids (TS) iii) Volatile solids (VS) iv) Moisture content v) Total Organic Carbon (TOC) vi) Total Phosphorus (TP) vii)Total Potassium (TK) Total Potassium was estimated using AAS along with other heavy metals. The estimation of pH was carried out according to the protocol of Irshad et al., (2013). A suspension of fresh hicken litter and distilled water was prepared in the ratio of 1:10, using a pH meter (TMP3). The pH meter measures the voltage produced by the suspension and compares it with the known neutral solution reference voltage, the difference of which will give the pH value. The estimation of Total solids present in the chicken litter was conducted using a gravimetric method, given by APHA (1999). Total solids refer to the material left in the crucible after evaporation of the moisture content and drying of the sample in an oven, at a defined temperature. This method helps to estimate the total suspended solids (portion retained by filter paper) and total dissolved solids (portion that passes through the filter paper).15 g of fresh chicken litter sample was air dried at 105°C to a constant weight for 16 hours. The TS was calculated as per the formula given below:Estimation of Volatile solids (VS)The determination of volatile solids was conducted using a gravimetric method given by APHA (2005). Volatile solids is the organic matter from the solid portion that is easily volatised during ignition. In order to estimate the mineral content of dry sample from TS, air dried chicken litter sample was converted into ash by placing it in the muffle furnace at 500°C for 5 hours. The VS from this ash was calculated as follows: where,VS = Volatile Solids (g VS/ g TS)Mi = weight of dried chicken litter (g)Mf = weight of fresh chicken litter calcined at 550°C (g)3.7.4 Estimation of Moisture The estimation of moisture content was done using a gravimetric method given by AOAC 985.01 Guidelines (Peters et al., 2003). Moisture content gives an estimation of retention of water by the manure, thus allowing sufficient gaseous exchange and growth of microbes. It was estimated as follows:-Weight of the undried chicken litter sample [g]W2 = Weight of the dried chicken litter sample [g]C = weight of the crucible [g]Co = weight of the empty crucible [g]The estimation of Total Organic Carbon (TOC) was carried out according to the protocol of Heanes, (1984). Reagents: 1. 1N Potassium Dichromate solution: 49.0 g of K2Cr2O7 was mixed in 100ml distilled water.2. Stock sucrose solution (2.0 mg/ml): 0.475 g of sucrose was dissolved in 100 ml distilled water. A working standard series with concentration 0-24 mg was prepared using this stock solution.Principle: This method works on the principle of recovery of TOC present in the chicken litter sample by digesting it in chromic acid (K2Cr2O7 + H2SO4) and heating it on hot plate. The TOC is then determined as a product of excess Cr3+ions released in the mixture by spectrophotometry at 600nm with calibration against sucrose standards in solution.Procedure: Fresh chicken litter samples were air dried in hot air oven at 100oC for two hours. The air-dried samples were grinded into thin powder using mortar and pestle. ml of 1N K2Cr2O7 solution and 2 ml of H2SO4 was gradually added in 0.20 g of the dried chicken litter. The mixture was heated on hot plate for over 20 minutes at 135oC and allowed to cool. The mixture was diluted to volume (10 ml) and centrifuged at 3000 rpm for 15 minutes. The absorbance was measured against a blank at 600 nm. The TOC was estimated using standard curves and expressed as % dry weight. Estimation of Total Phosphorus (TP)The estimation of Total Phosphorus (TP) was carried out

according to Olsen method (Trinchera & Baratella, 2019).Reagents: 1. Solution of sulphuric acid (R1): 14 ml of sulphuric acid (H2 SO4) [96%] was added in 5.0 ml of distilled water and diluted to 100 ml volume. Ammonium molybdate solution (R2): 4.0 g of ammonium molybdate [(NH4).6Mo7 O24.4H2O] was dissolved in 100 ml distilled water and stored in a dark container.3. Solution of ascorbic acid(R3): 1.76 g of ascorbic acid (C6H4O6) was dissolved in 100 ml distilled water and prepared fresh.4. Potassium tartrate antimony solution (R4): 0.03 g of potassium antimony tartrate [(K(SbO).C4H4O6. ½ H2O] was dissolved in 100 ml of distilled water.5. Sulphomolybdic Reagent (Mixed reagent): 5 ml of the R1 solution, 1.5 ml of the R2 solution, 3 ml of the R3 solution and 0.5 ml of the R4 solutionwas mixed at the time of use.6. Sodium hydroxide solution (R5): 4.0 g of sodium hydroxide (NaOH)was dissolved in 100 ml distilled water. 7. Extraction solution / Sodium bicarbonate solution: 4.20 g of sodium bicarbonate (NaHCO3) was dissolved in 90 ml distilled water. The pH was adjusted to 8.5 by adding R5 solution drop by drop and diluted to 100 ml volume. 8. Standard Phosphorus solution(0.005mg/ml): 0.044 g of potassium dihydrogen phosphate (KH2PO4) was air dried at 40oC for an hour and dissolved in 100ml distilled water. 1 ml of this stock solution was diluted to 10 ml to prepare a series of working standards of 0-5mg/L concentration Principle: Ammonium molybdate and antimony potassium tartrate reacted in an acidic medium to form antimony-phospho-molybdate complex, which was reduced to an intensely blue-coloured compound by ascorbic acid that was proportional to the orthophosphate concentration, determined by colorimetry at 882 nm.

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This complex is reduced to an intensely blue-coloured complex by ascorbic acid which proportional to the orthophosphate concentration, determined by colorimetry at 882 nm.Procedure: Sample extraction: 2.50 g of air-dried fresh chicken litter sample (at 100oC for two hours) was mixed in 50 ml Extraction solution and 0.25 g of activated charcoal was added to it. The mixture was stirred for 45 minutes at 180-200 oscillations per minute using orbital shaker. The mixture was filtered twice using Whatman filter paper No. 1 and used for colorimetric estimation.Colorimetry: 1.5 ml of the sample and 1.5 ml of Mixed Reagent was mixed in cyclo mixer for 1 minute and allowed to stand for an hour until blue colour formation. The absorbance was estimated at 882 nm. The TP was estimated using standard curves and expressed as mg/kg of chicken litter using the formula given below:-where,C= litter extractable P content [mg kg-1]A= P concentration in the sample solution [mg L-1]B= P concentration in the blank sample solution [mg L-1] V1= volume of the extract [50 mL] V2= volume of the sample solution used for colorimetric determination [ml]M= soil mass [g]Atomic Absorption Spectrophotometry is based on the principle of absorption of light by metallic ions, at a specific wavelength which helps in determining the concentration of an element in the given sample. This analytical method was used to detect specific elements like Potassium (K) and heavy metals like Arsenic (As), Cadmium (Cd), Lead (Pb), Chromium (Cr), Iron (Fe), Copper (Cu), Zinc (Zn) and Manganese (Mn).Principle: In the present study, the sample was heated to oxidise the carbon structures and
evaporate the volatile substances like Nitrogen, Phosphorus and Potassium. The ash was acidified to dissolve any other elements, leaving behind the inorganic mineral elements which were detected using Atomic Absorption Spectrophotometry.Sample preparation for chicken litter The sample preparation of Heavy metals was performed according to the AOAC 985.01 Guidelines (Peters et al., 2003) and the heavy metal content was
determined using Atomic Absorption Spectrophotometer. The sample preparation of detection of Potassium and heavy metals was performed
according to the AOAC 985.01 Guidelines (Peters et al., 2003). 15g of fresh chicken litter sample was oven dried at 100oC for 2 hours to remove the moisture
content. 0.5g of the dry weight was placed in porcelain crucible and converted to ash in muffle furnace at 500oC for 4 hours. The ash was digested in 10ml HClpure (1+1), filtered using Whatman filter paper No.1 and transferred in 100ml Volumetric flask, diluting to the volume. They were stored at 4oC until sample analysis. Sample preparation for chicken feed The estimation of heavy metals in chicken feed samples was performed according to the protocol given by Dahri et al., (2020). 2 g of layer feed and finisher feed was ground into fine powder using mortar and pestle and digested in 10ml of HNO3 (65%) and 4ml of H2O2 (30%). The mixture was thoroughly mixed in Fume Hood and heated on a hotplate at 250oC for 20 minutes until the appearance of white fumes. The digested samples were kept for cooling and filtered using a Whatman filter paper No.1, dissolved in 20ml distilled water and filtered. The filtrates were poured individually into a 50ml volumetric flask and diluted to volume. Detection of heavy metalsBoth, chicken litter samples and feed samples were analysed in duplicate for the following heavy metals: Iron (Fe), Zinc (Zn), Copper (Cu), Manganese (Mn), Arsenic (As), Cadmium (Cd), Lead (Pb), Chromium (Cr). The determination of heavy metals in each sample was carried out using Atomic Absorption Spectrophotometry in fully automated Atomic Absorption Spectrometer (novAA 400P model) and ICP-OES (Inductively Coupled Plasma- Optical Emission Spectroscopy). The machine was calibrated using the standard for each metal. 25 ml of each sample was used for detection of As, Cd, Cr and Pb. As was detected using the Flame AAS technique

after adding 5ml of Potassium iodide (KI) whereas Pb was detected using the Vapour technique. Fe, Mn, Cu, Zn and K were detected after diluting the sample to 25ml using Flame AAS. The final detection was made at individual wavelengths of 259 nm (Fe), 213.9 nm(Zn), 324.8 nm (Cu), 279.5 nm (Mn), 357.8 nm (Cr),193.7(As), 217 nm(Pb) and 228.8 nm(Cd).Further a transfer factor (TF) ,also known as "accumulation factor" was computed to estimate the heavy metals transferred into the litter on account of feed (El-Amier et al., 2018). It was calculated as follows :-where,TF = Transfer factorCi = metal concentration in feed (mg/kg of feed) Cf = metal concentration in litter (mg/kg of dry weight) Analysis of Chicken blood The following haematological parameters were tested prior to subjecting the blood samples to genotoxicity. i) Total Red Blood Cell Countii) Differential White Blood Cell count iii) Haemoglobin (Hb)Prior to testing the genotoxicity, a total Erythrocyte count was performed using the blood samples from both the study sites to ensure sufficient RBC cells were present. The following tests were performed to detect genotoxicity using chicken erythrocytes: i) Micronucleus testii) Single-cell gel electrophoresis / Comet Assay3.9.1 Total Red Blood Cell CountThe procedure estimating the Red Blood Cell count was according to the
guidelines given by Samour (2006). Manual Total RBC count was performed using Improved Neubauer hemocytometer.Reagents: 0.85% Saline: 0.85 g of NaCl was mixed in 100 ml distilled water.Principle:In order to count the innumerable red blood cells, the blood sample was diluted using a diluting fluid (1:200) and fixed on the hemocytometer to counterythrocytes. The Neubar's Chamber present on the hemocytometer has a fixed set of squares which makes the counting easier and efficient. Procedure:10µl of fresh blood sample was mixed with 1.9ml of 0.85% Saline in an Eppendorf tube to achieve a dilution factor of 1:200. The contents of the mixture were mixed using cyclo mixer for 1 minute and allowed to rest. The initial 3-4 drops of blood were discarded and 20 µl of this mixture was pipetted into an initially fixed hemocytometer.

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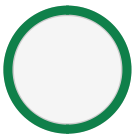

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The erythrocytes were observed under 40x Olympus light microscope and the RBCs were counted in the Neubauer's chamber using the "L rule" wherein, those cells in the triple lining on the left and bottom region of the larger chamber are counted, excluding the cells present on the triple lining on the right and top region, to give an accurate estimation of RBC. Characteristics of RBC: Morphologic characters: The matured erythrocytes were medium sized of average length of 11.9 µm, and a width of 7.1µm, ellipsoidal in shape and showed presence of small prominent nucleus. Staining characters: The matured erythrocytes were characterised by pale uniform cytoplasm and condensed, darkly stained nucleus when observed under microscope. Differential White Blood Cell CountThe differential WBC Count was performed using Giemsa staining method. Air dried blood smears were rinsed in absolute methanol for 5 minutes and stained using Giemsa stain for 15-20 minutes. The stain was washed in Phosphate buffer for 10 minutes and the final slide was observed under Olympus light microscope at magnification of 100x. The different types of leucocytes seen within 100 cells was counted.3.9.3 Haemoglobin Estimation The haemoglobin estimation was performed using Saheli's method. The graduated tube of hemoglobinometer was filled with 0.1N HCl upto 10 mark and rinsed with 20uL chicken blood. The mixture was allowed to settle for 5 minutes for the conversion of haemoglobin to dark-brown acid-hematin. The mixture was diluted using distilled water until it had same intensity of color and tint as that of the standard tubes, when observed against sunlight. When an exact color match was obtained, the lower meniscus was observed and the percentage figures corrssponding to it was noted. The hemoglobin level was reported in g/dL3.9.4 Micronucleus testMicronucleus test is a simple, rapid and indirect measure of induced, structuraland numerical chromosome aberrations. It is used to detect genotoxicity in peripheral red blood cells. The present test was carried out according to the protocol given by Nagpure et al., (2007).Reagents: a. Giemsa stock solution: 0.50 g of Giemsa Powder was dissolved in 33 ml glycerol and incubated at 60oC in water bath for 4 hours and cooled to room temperature. 50 ml of methanol was added to the solution and filtered using Whatman Filter Paper No. 1 and stored in amber bottle.b. Giemsa working solution (5%): 5ml of Giemsa stock solution was mixed with 45ml of Phosphate Buffer (pH 6.8).c. Phosphate Buffer (pH 6.8): A stock solution of 0.5M Potassium Dihydrogen Phosphate (6.80 g of KH2PO4 dissolved in 100 ml distilled water) and 0.5M Disodium Hydrogen Phosphate (8.90 g Na2HPO4 of dissolved in 100 ml distilled water) was mixed. From this, 31.30 KH2PO4 ml of and 22.80 ml of 0.5M Na2HPO4 was added in 490 ml distilled water, upon which the pH was adjusted using Sodium Hydroxide Flakes. Finally, the mixture was diluted to 500ml volume to prepare the working solution.Principle:Giemsa stain is a differential stain comprising of azure, methylene blue and eosin dye, which stain different components of the blood smear. The azure and eosin stain are acidic dyes which stain basic components like cytoplasm and granules whereas methylene blue acts as a basic dye which stains the acidic component such as the nucleus. Methanol acts as a fixative and cellular stain which does not allow further change in staining properties. Procedure:10µl of anticoagulated blood sample was used to make a thin blood film, immediately after blood sampling. The air-dried blood smears were fixed in absolute methanol for 5 minutes and labelled. The slides were stained using % Giemsa working solution for 15-20 minutes in vertical coupling jars, washed in Phosphate buffer (pH 6.8) and further analysed for micronuclei (MNi).Micronuclei (MNi) were identified through their appearance as a spherical extra nuclear bodies in the cytoplasm having a diameter of one-third of the main nucleus and similar color and texture as that of the nucleus.Scoring of Micronuclei: All the stained slides were examined under Olympus light microscope under bright field illumination [objective: 40X and 100X (oil immersion)]. Approximately 2000 erythrocytes with or without micronuclei were scored from each slide.

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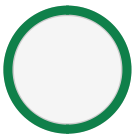

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3.9.5Single-Cell Gel Electrophoresis (Comet Assay)Single Cell Gel Electrophoresis, also known as comet assay, is a reliable and rapid method for detection of single and double stranded DNA breaks in eukaryotic cells. The Cometa assay was performed as per the guidelines given Nagpure et al., (2007).Principle In this technique, cell suspension of eukaryotic blood cells embedded in low-melting-point agarose, lysed by detergents and high salt treatment to remove cell content except DNA, and the liberated DNA is electrophoresed under alkaline conditions to unwind from breakage sites. Thus, cells with higher level of DNA damage display increased migration of the DNA from the nucleus towards the anode under an electrical current, giving the appearance of a “comet tail” , when viewed under fluorescent dye (ethidium bromide).ReagentsAll the reagents were freshly prepared for the present study.1.0.5% Low Melting Agarose: 0.25 g of Low Melting Agarose was dissolved in 50ml of 1X PBS.2. 1% Normal Melting Agarose: 0.50 g of Normal Melting Agarose was dissolved in 50ml of 1X PBS.3. 1X Phosphate Buffered Saline (pH=7.4): 8.0 g of Sodium Chloride (NaCl) , 0.20 g of Potassium Chloride (KCl), 1.40 g Na2HPO4 and 0.27 g of KH2PO4 were dissolved in 100 ml distilled water after adjusting the pH using Sodium Hydroxide flakes. This stock solution of 10X PBS was diluted to 1X PBS and used. 4. Lysis buffer (pH 10): 14.6 g of Sodium Chloride (2.5M NaCl), 3.74 g of Disodium salt of EDTA (100mM $C_{10}H_{14}N_2O_8$.2Na2H_2O), 0.12 g of Tris HCl was mixed in 100ml distilled water. 89 ml of this stock solution was mixed in 0.1 ml of 1% Triton X and 10 ml of 10% DMSO (dimethyl sulfoxide) was added after adjusting the pH to using Sodium Hydroxide flakes.5. 1% Triton X: 1ml of Triton X(10mM) liquid was diluted to 100ml using distilled water.6. 10% DMSO: 10ml of DMSO was diluted to 100ml using distilled water.7. Neutralisation buffer (pH 7.5): 4.84g of Tris Base (400mM) was added in 100 ml distilled water after adjusting the pH to 7.5. It was stored at room temperature and chilled right before use.8. Unwinding/ Electrophoresis buffer: 20.0 g of Sodium Hydroxide (NaOH) was mixed in 50 ml in an exothermic reaction. Likewise, 1.49 g of Disodium salt of EDTA ($C_{10}H_{14}N_2O_8$.2Na.2H_2O) was mixed in 20 ml distilled water after adjusting the pH to 10. To prepare the working solution, 27 ml of NaOH, 4.5ml Disodium salt of EDTA ($C_{10}H_{14}N_2O_8$.2Na2H_2O) and 1.8 ml of DMSO was diluted to 1000 ml distilled water.9. Ethidium Bromide solution: 0.01 g of Ethidium Bromide was added in 50 ml distilled water and stored in Amber Bottles. From this stock solution, 100 µl was diluted to 1 ml distilled water to prepare the working solution in an Eppendorf tube, covered with aluminium foil. Due care was ensured while handling ethidium bromide with the use of gloves.Procedure: All the steps were performed under dim-light to prevent any photo-oxidation. A layer of 500 µl of 1% Normal Melting Agarose was smeared on clean frosted slides and covered with a coverslip. After the gel solidified, a layer of 200 µl of anticoagulated blood sample (double dilution using 1X PBS) was mixed with 600 µl of 0.5% Low Melting Agarose and spread over the previous layer. Once the gel suspension was solidified, a third layer of 500 µl of0.5% Low Melting Agarose was added and allowed to solidify. The coverslip was removed, and the frosted slides were placed in Lysis buffer (pH 10) at 4oC, overnight. Following lysis, the slides were placed in unwinding buffer (electrophoresis buffer, pH 10) for 30 minutes to unwind the DNA and Electrophoresis was erformed subsequently for 20 minutes at 280mA and 25V. The slides were placed in pre-cooled Neutralisation buffer (pH 7.5) for 5 minutes and the slides were cleaned off excess buffer. The slides were stained with 100 ml 1X Ethidium Bromide solution, covered with a coverslip and observed under fluorescence microscope (Olympus BX53) under 20x magnification using a red filter.Screening of comets: The slide was carefully assessed to check for presence of comets and were analysed using CASP software to compute the % tail DNA.3.10 Statistical analysis The data collected from the study was subjected to statistical analysis using RStudio 4.2.1 and IBM SPSS Statistics 26 software. All the samples were analysed in duplicate and were expressed as Mean ± SD (Standard Deviation),

wherever necessary. The data was subjected to a test of normality using Kolmogorov-Smirnov test and Shapiro-Wilk test, followed by Levene test of homogeneity to check if the samples have equal variance. When the normality and homogeneity assumption was satisfied, parametric test called one-way ANOVA was used to estimate the difference in mean oncentrations of heavy metals in both samples. When F values of the ANOVA test were significant, (P

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These properties were tested to find out the overall chemical composition of fresh chicken litter in its natural state. There was significant variation noticed in the physicochemical parameters across layer and broiler farms. The pH reported in the broiler farm was found to be acidic as compared to the layer farm (5.35, p=0.0001, f= 132.597). The percentage of TOC was significantly higher in the layer farm as compared to the broiler farm (p = 0.022). TP and TK, the major organic components, were significantly higher across the both the poultry farms (p = 0.0042 and p = 0.027). However, the differences in the moisture content and VS across the farms were statistically insignificant (p = 0.061 and p=0.558). 4.2 Heavy metal content in chicken litter samples Results of the present study revealed that heavy metals were indeed present in the chicken litter samples across all the farms. On an average, these metals were found to be highest in the broiler farms as compared to the layer farms (868.58±21.18, p=0.638, f=0.454). The distribution of heavy metals across each farm are demonstrated in Table 4.2. The concentrations of Fe (938 mg kg-1), Zn (348 mg kg-1) and Mn (994 mg kg-1) were significantly higher in the broiler litter in comparison to the reference site (p=0.00752 and p

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Through a preliminary interview with the poultry workers, it was found that poultry litter would get accumulated for months to one and a half year until it was sufficient to be discarded. We hypothesised that this litter had some sort of toxicants which can cause harm to the poultry workers and chicken who are exposed to it. It was also a common practise for poultry workers to apply chicken litter samples as manure in local farms or sell the discarded manure. Hence, we also hypothesised that application of this untreated manure would result in bioaccumulation of nutrients and metals in the soil, more than the required limits. Through published studies it was established that there are certain essential minerals like Fe, Mn, Cu and Zn, added in trace amounts at different stages of life to suffice the mineral needs. However, instances have shown a possibly higher concentration added in later stages i.e. layer and finisher phases (Chowdhury et al., 2022; Korish & Attia, 2020). Hence, chicken litter samples were collected from layer (> 12 weeks of age) and finishers (> 25 days) from the study sites to find out whether there were any metals incorporated in these stages. Due to this assumption, we also tested the hypothesis of chicken feed being the main source of heavy metals in the litter.5.1 Physicochemical characteristics of chicken litterPrior to the use of any manure, it is essential to determine its physicochemical parameters to ensure the sufficient availability of nutrients to the plants. Hence, a few physicochemical parameters like pH, Total Solids (TS), Volatile Solids (VS) and Moisture content were assessed to find out the overall chemical composition and preferable use of fresh chicken litter in its natural state (Kaur et al., 1997)These parameters greatly influence the quality of manure composition. Phosphorus (P) and Potassium (K) are considered to be primary macronutrients due to their relatively larger requirement and influence on growth and development of plants. (Sheikh & Dwivedi, 2020). .1.1 pH pH is an important component of the litter as it influences the pH of soil. pH of chicken litter varies according to the age, diet of the bird and moisture content of the litter. In the present study, pH of the layer litter was around 6.7 ($p=0.0001$, $f=132.597$), similar to the values obtained in other studies (Faissal et al., 2017; Rizzo et al., 2020; Sarbjit et al., 2018). This alkaline pH is owing the protein-rich diet (nitrogen-based compound) in the layer farm. Though this pH is optimum for the growth of most plants, it is also favours the growth of bacteria and retention of ammonia levels (Ravindran et al., 2017). On the other hand, an acidic pH of 5.35 was observed in the broiler litter, which is much acidic and unsuitable for the use as manure. This acidic pH could be due to the composition of finisher feed which is intense in carbohydrate rich diet.5.1.2 Total Solids (TS) Another equally important parameter is the Total solids. TS is required to estimate the levels of suspended and dissolved solids in the litter samples. The % of TS was significantly lower across both the farms in comparison to the reference site (26.63 ± 0.78, $p= 0.001$, $f=47.843$) indicating a relatively lower nutrient and organic content. Similar values have been reported from studies conducted in Japan and Nigeria (25% & 28.6 ± 0.7 %) (Abouelenien et al., 2009; Rizzo et al., 2020). Chicken manure is known for its containing higher levels of organic solids as compared to the other animal manures and hence it is preferred over other manure. However, in comparison to the composted manure, the fresh chicken litter has a much higher % of TS, and this becomes a matter of concern due to a possible risk of extensive nutrient loading in the soil. Sometimes, these nutrient levels cross the permissible limits and cause ecotoxicity, though the distribution of nutrients cannot be estimated.5.1.3 Total Organic Carbon (TOC) The Total Organic Carbon is a measure of the carbon available for energy source. This carbon is usually deposited in chicken litter on account of feed. The % of TOC reported in the present study was found to be significantly lower in the in the broiler litter (16.25%, $p=0.022$) as compared to the layer litter (28.1%). These values coincides with studies conducted by Sarbjit Singh et al., (2018) and Ravindran et al., (2017) (21.12% and 30-40% respectively).Though a higher TOC implies a better energy source and conversion to CO_2 during composting, it is also

an implication of overfeeding practise as stated by Sheikh and Dwivedi, (2020). Based on the feeding schedule, chicks are fed with carbohydrate rich grains in their diet to gain sufficient weight at a faster rate. Due to this, and inability of the chicks to digest enough carbohydrates, sometimes it may exceed the limits of digestion and may be excreted out thus contributing to the TOC of chicken litter. As layer hen require a relatively less carbohydrate rich diet, they prioritise egg-laying overweight gain, hence the excess carbohydrates excreted through faces and get deposited in the form of TOC. This could be a plausible reason for the higher levels of TOC in layer litter in the present study.5.1.4 Moisture and Volatile SolidsMoisture content, also known as the extent of drying a material, is a crucial component when it comes to the use of manure. From the above result, it was evident that a majority of the TS comprises of volatile and moisture rich content (64%). There was not significant difference noticed in their levels across the different farms in the current study (p=0.558). Similar values were obtained in studies conducted in Spain and Canada (74.53% and 73 to 80 %) (Fernandes et al., 1994; Quiroga et al., 2010). Most studies have also reported the values of composted chicken manure to be 58 % , 55-65% and 45.4±2.5% respectively (Abouelenien et al., 2009; Dalkılıc & Ugurlu, 2015; Rizzo et al., 2020).

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12. A moisture content of greater than 75% is considered to be high, deemed unfit for manure, as it hampers the process of efficient composting. It can harbour bacterial and fungal manifestations and increase the release of ammonia contributing to the bad odour (Zhu et al., 2020). Rynk et al., (1991) suggested a value between 40-60% is deemed for receiving major benefits of poultry manure. Hence, the chicken litter from both the farms could be deemed to be fit. 5.1.5 Total Phosphorus (TP)Total Phosphorus (TP) is one of the prerequisites for the chicken litter to be an excellent source of manure. Phosphorus is relatively immobile element which can penetrate shallow waters and cause excessive loading in the upper profiles of the soil, it is important to quantify the levels prior to the use of manure (Kelleher et al., 2002). In the present study, the phosphorus levels in the fresh chicken litter were reported to be 1.3-1.36 g kg -1 across both the farms (p=0.011, f= 10.544). In comparison to the values obtained by Sager (2007) (19.1 g kg -1) and Li et al., (2014) (15.8 g/kg) in poultry droppings, these values seem to be low. These studies have also reported higher levels of phosphorus in cattle manure (8.4 g kg -1, 7g/kg) and swine dung (29.9 g kg -1 ,32g/kg). A study conducted in India had also reported the higher values 1.9 to 2.6 g/kg (Sheikh & Dwivedi, 2020). The phosphorus levels are generally higher in the manure due to its utilisation by microorganisms in the soil through decomposition process. This releases more phosphorus content in the soil. In the present study, fresh chicken litter is most likely deficient in phosphorus content in its natural form and does not contribute to excessive nutrient loading when used as a manure. 5.1.6 Total Potassium (TK) Total Potassium (TK) is one of the major constituents of animal manure. The present study reported a relatively higher level of TK in layer litter (940 mg/kg, p=0.027) as compared to broiler litter (621 mg/kg, p=0.180). Studies conducted elsewhere have reported varying values 1,305g kg-1 (Faissal et al., 2017) and 34.4 mg g-1 (Rizzo et al., 2020). Potassium is essential for plant growth and development however, it can lead to harmful effects if present in toxic levels. Studies have reported that composted manure usually have a high K content due to the decomposition process by animals (Sarbjit et al., 2018). However, almost 89% of the ingested potassium is usually excreted in the litter which could explain the higher levels of TK in the present study.5.2 Heavy metal toxicity in litter In the current study, we have analysed eight heavy metals in fresh chicken litter and feed samples. They are as follows: Iron (Fe), Manganese (Mn), Copper (Cu), Zinc (Zn) and Chromium (Cr) being the essential heavy metals and Arsenic (As), Cadmium (Cd) and Lead (Pb) being non-essential toxic heavy metals. The overall distribution of heavy metals across the poultry farm was as follows : Cu > Mn > Fe > Zn > Cr > Pb > Cd > As (Table 4.1). Broiler litter showed higher concentration of heavy metals as compared to the layer farms (p=0.638, f=0.454). To discern this fact, it is necessary to consider the following factors. Chicken excretes urine and faeces in the form of a singular pellet; thus, constituents of litter would be directly dependent on the feed constituents. The feed is enriched with mineral supplements, which are not retained and excreted through the litter. Both the breeds are reared for different purpose and in order to achieve that purpose, their feed content is variable. On an average, broilers need extra nutrient ingredients, protein and fat-rich diet to achieve faster growth rates and slaughter weight as compared to the layer hens. Hence the average consumption amounts to approximately 1.5 kg of feed intake during the finisher phase. Layers, on the other hand, do not need fattening diet and hence they have a lower protein intake, with a diet rich in micronutrients and essential minerals, with an average consumption of 100 – 150 g during layer phase (TNAU, 2015). Hence, broiler litter production is more as compared to layer on a daily basis. As stated earlier, broiler was housed in deep litter system where the droppings are accumulated and cleaned after few weeks. There is relative movement of the broiler chicken within the litter system On the other hand, layers are housed in battery-cage system , where the litter is collected beneath the cage and piled up for many

months. Here there is no particular movement of the chicken, and they undergo more stress in such a system. To meet up with the humongous demand, this battery cage system is used which can rear 5000 birds at a stretch and more modifications are made to the feed. This could also explain the higher levels The dietary needs of broiler and layer chicken differ based on the purpose of production. Factors like age, sex, housing system, stress, stocking density and management system also affect the average intake by chicken. A closer look at the feeding schedule of chicks reveals the variation of nutrient intake at various stages of their life. On an average, broilers need extra nutrient ingredients, protein and fat-rich diet to achieve faster growth rates and slaughter weight as compared to the layer hens. Hence the average consumption amounts to approximately 1.5 kg of feed intake during the finisher phase. Layers, do not need fattening diet and hence they have a lower protein intake, with a diet rich in micronutrients and essential minerals, with an average consumption of 100 – 150 g during layer phase (TNAU, 2015). Considering age as a prime factor, protein, carbohydrate and calcium rich diet is essential in the initial stages of life, so as to attain sufficient size and calcification of egg-shell.

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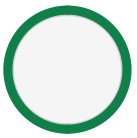

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The present study reported a relatively higher concentration of Cu in finisher feed (413.75 mg/kg, p =0.028) as compared to the layer feed (295 mg/kg, p=0.5088). These values are at least 32 times higher than the ones recommended by BIS, (2007) (9 mg/kg in layer feed and 12 mg/kg in the finisher feed). Cu is also known for its bactericidal effects, whereby it inhibits the growth of bad bacterial colonies in the gut and is responsible for improving the feeding efficiency of chicken.Owing to the tropical climatic conditions and instances of recurrent avian bacterial infections, poultry farmers may use such higher levels to prevent infections in the birds. Reiterating to the previously reported feeding schedules of broiler chicken, which have high nutrient intake, this protects them on a larger scale. Results of the present study reveal a higher level of average concentration of Cu (3026 mg/kg) with a relatively higher concentration in broiler litter (3980 mg/kg, p =0.33) as compared to the layer litter (2668 mg/kg). These high values suggest the minimum retention of Cu in the body as most of it is excreted in the litter. Most studies have reported relatively lower levels of Cu ranging from 66-400 mg/kg (Bolan et al., 2004; Sager, 2007; F. Zhang et al., 2012). The high levels of Cu content in the chicken litter is way above the critical toxicity limits of the soil (20-30 mg/kg) making the litter unfit for direct application in soil (Foust et al., 2018). This may increase and mobilise the copper present in the soil and overshoot it's the limits in the soil as suggested by a review study (Korish & Attia, 2020). As Cu showed similar values of TF, this also suggests lower accumulation in the body. 5.2.5 Chromium (Cr)Chromium (Cr) is added as a supplement in poultry feed for glucose, lipid, and protein metabolism especially among birds experiencing stressful conditions (White & Vincent, 2019). Though the level of Cr intake is not explicitly stated in BIS, India, critical levels of Cr as per the EU was 0.01 mg/kg (European Commission, 2003). The Cr content in the feed was above these limits, with a significantly lower concentration in finisher feed (1.375 mg/kg , p =0.8032) as compared to the layer feed (3.75 mg/kg, p=0.0129). These values were higher than those in a study conducted in Wales, in layer feed (0.76 mg/kg) and finisher feed (0.22 mg/kg). Such high supplementation is sometimes needed, especially in tropical climates where birds tend to have reduced feed intake and lower performance. In such instances Chromium helps in utilisation and uptake of Glucose and helps in boosting the immunity of cells by significantly reducing glucocorticoids (A. Sharma et al., 2020). The overall concentration of Cr was 6.47 mg/kg, with a relatively lower concentration in broiler litter (6 mg/kg , p=0.696) as compared to the layer litter (9 mg/kg, p =0.171). These values were lower as compared to those obtained in different studies ; 10.7 mg/kg (Sager, 2007) and 23.71mg/kg (F. Zhang et al., 2012). Cr forms various insoluble compounds in the soil and thus inhibits the uptake of essential nutrients in the soil as stated by a study conducted in Amritsar. Hence it is not beneficial to the soils, and hence there are non-established critical limits for the soil. However, critical limits exist for the uptake by variety of plants with a varying range of less than 1mg/kg (A. D. Sharma et al., 2005). The TF of by Cr was 0.28 in broiler litter as compared to the layer (0.42) , which suggests a higher retention in the layer breed. 5.2.6 Arsenic (As)Arsenic (As) is a naturally occurring, mobile metalloid element, which exists in both organic and inorganic forms which can easily be absorbed by the plants and metabolised inside human body. This element is naturally present in the earth's crust, however, anthropogenic activities may increase its levels and cause toxicity (Q. Y. Chen & Costa, 2021; Rehman et al., 2021). In poultry farms, As has been used in chicken feed in its organic form called "Roxarsone" (3-nitro-4-hydroxyphenyl arsonic acid) to control microbial infections like coccidiosis and increase weight of the bird. As this compound is excreted through chicken litter, it ends up in the soil or contaminates the ground water levels. According to the Bureau of Indian Standards 2007, As is considered to be toxic metal and should not be introduced in the feed. However , if present, it must be below the permissible limit of less than 2 mg/kg in both types of

feed (BIS, 2007). The present study reported the presence of As, at relatively lower concentration of As in finisher feed (0.04 mg/kg, p =0.426) as compared to the layer feed (0.06 mg/kg, p=0.210), both of which were under the maximum limit. This suggests that As was added as feed supplement in the diet. One such study reported higher values of 0.05 mg/kg in finisher feed and 0.10 mg/kg of As in the layer feed. The present study reported 0.4 mg/kg As in the layer litter (p=0.62). Few studies have suggested the following concentrations in the diet 0.12 mg/kg (Sager, 2007) and 3.79 mg/kg (F. Zhang et al., 2012). A shocking result was that As was Below Detectable Limits (BDL) in the litter samples. This implies that it was retained in the body of broiler chicken. Such a toxic heavy metal in the broiler chicken which is consumed by people will amount to bioaccumulation in the human beings and elicit ill effects. According to the US standards, the As levels were within the normal range for uptake by the soil (

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The present study reported a relatively higher concentration of Cd in finisher feed (0.18 mg/kg, p =0.0536) as compared to the layer feed (0.04 mg/kg, p=0.7079). However, these were under the permissible limits. Higher values were obtained in a study conducted in Wales 0.12 mg/kg in finisher feed and 0.39 mg/kg in layer feed (Nicholson et al., 1999). The present study reported a relatively higher concentration of Cd in broiler litter (0.6 mg/kg, p=0.01) as compared to the layer litter (0.4 mg/kg, p =0.50). Similar values were obtained in studies conducted in Bangladesh and Austria ; 0.485 mg /kg in layer feed and 0.499 mg/kg in broiler feed (Chowdhury et al., 2022) and 0.27 mg/kg (Sager, 2007). However a study conducted in China reported higher levels of 4.05mg/kg Cd in the chicken manure (F. Zhang et al., 2012). The Cd content in fresh chicken litter is below the critical toxicity levels of Cd in the soil (less than 8 mg/kg), suggesting the litter is safe for application in the soil. However, the TF reported in layer litter was very higher 2.33, indicating a higher retention in the body. Higher accumulation of Cd is known to cause toxic effects, are known to reduce the egg quality, feeding efficiency of chicken and growth performance of chicken (Kar et al., 2018).5.2.8 Lead (Pb)Lead is a naturally occurring toxic heavy metal found in the earth's crust within a range of 72.4 ppm to 251.5 ppm (Kabir et al., 2019). According to the Bureau of Indian Standards 2007, the permissible limit of Pb in the layer feed and finisher feed is 5 mg/kg (BIS, 2007). The present study reported a significantly higher concentration of Pb in layer feed (6.75 mg/kg, p=0.00235) as compared to the finisher feed (3.0 mg/kg , p =0.03097). A study conducted in Wales, however reported much lower values 0.05) Studies have reported similar values ; 3.62 mg/kg (Bolan et al., 2004), 4.44 mg/kg (F. Zhang et al., 2012) and 5.4mg/kg (Sager, 2007). The higher levels of TF 2.33 in layer litter indicate higher accumulation in the litter, whereas a TF of 0.13 in broiler litter indicates accumulation in broiler chicken which may cause Pb toxicity in the long run.5.3 Genotoxicity in Broiler chickenPrior to commencement of Genotoxicity testing, Haematological parameters were assessed to evaluate health status of the bird. Since, they were in the normal range, it can be ascertained broiler breeds at both sites were healthy. Non-essential heavy metals like As and Pb were found in the layer and finisher feeds, which have no biological role and must have been incorporated accidentally during the preparation of feed, transport and storage. Due to this finding, we assessed genotoxicity using Micronucleus test and comet assay. Micronucleus test revealed that lower DNA damage was seen . However, values reported in other studies were significantly higher than the present study (Da Silva Cardoso et al., 2016; Saleh & Sarhan, 2007). Micronucleus is a small nucleus formed during the anaphase after failure of incorporating the chromosome in daughter cells , which is surrounded by nuclear membrane to form micronucleus. It indicates DNA damage which cannot be repaired as it has passed the cell cycle checkpoints. Correlation analysis also revealed that a possible reason behind the DNA damage could be on account of non-essential heavy metals, however, further sample testing is needed to conclusively ascertain the Mni result. Single Cell Gel Electrophoresis, also known as comet assay, is used to reveal short term DNA damage like single strand DNA breaks, in an individual cell, which can be repaired through DNA repair mechanisms before passing the cell cycle check points. The present study showed an intact DNA head, with no visible comets which suggests the broiler breeds can repair the short term DNA damage. Most studies however, showed presence of higher DNA damage through prominent % tail DNA (Arooj et al., 2023). Hence, further sample testing is needed to conclusively ascertain this finding.

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CONCLUSION
The present study is a pilot study undertaken to address the growing concern of disposing chicken litter without prior treatment in the soil. Findings of this study reveal that essential heavy metals like Fe, Zn, Mn, Cu and Cr as well as non¬essential heavy metals like As, Pb and Cd were present in chicken litter across
different poultry farms in Goa. In particularly, broiler litter had higher heavy metal content as compared to layer litter indicating higher toxicity in broiler farm. The source of these heavy metals was found to be chicken feed, where essential HM were incorporated in trace amounts to cater to micronutrient deficiencies . However, Fe, Mn and Cu were found to be much above the permissible limits, which can lead to increased antibacterial resistance in birds and make them more susceptible to bacterial infections. Non-essential heavy metals such as Pb and Cd, that have no biological role to play, were found in the both types of feed, in particular, the Pb content was higher than permissible limits in the layer feed. This is a matter of concern since these metals can cause carcinogenic effect, increased diarrhoeal instances, neurotoxicity and hepatotoxicity in the birds. One such effect tested was genotoxicity which revealed lower DNA damage on account of presence of Micronuclei. Hence stringent measures must be taken to monitor their levels in the feed as well as their source. Despite being a non-essential heavy metal, As was incorporated in feed to decrease coccidial infections, but was banned in most countries because it would accumulate in the litter and contaminate soil and groundwater. However, it was found in both types of feed but retained in the broiler chicken which we consume, once again raising safety concerns about consumption of this chicken. The direct consequence of these non-biodegradable and highly

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dense elements is using the untreated litter as manure, which would increase heavy metal content in the soil amounting to ecotoxicity.
LIMITATIONS
Findings of the present study needs to be substantiated with more sample analysis to detect heavy metals in litter and feed samples which will make our study more conclusive.
FUTURE PROSPECT

The present study has successfully proven that heavy metals exist in chicken litter samples hence, it would be interesting to study the effects of these heavy metal exposure in poultry workers and poultry birds and studying the bioaccumulation of these heavy metals in vegetables, grown in untreated chicken manure.

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