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ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

EVALUATION OF BACTERIAL CONTAMINATIONS AND MINERAL CONTENTS OF EGGSHELL POWDER RECYCLED FROM HATCHERY WASTES AS AN ALTERNATIVE ANIMAL FEED SOURCE IN BISHOFTU, ETHIOPIA

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DEPARTMENT OF PARASITOLOGY, MICROBIOLOGY AND POULTRY HEALTH

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Evaluation of Bacterial Contaminations and Mineral Contents of Eggshell Powder Recycled from Hatchery Wastes as an Alternative Animal Feed Source in Bishoftu, Ethiopia

Addis Ababa University
College of Veterinary Medicine and Agriculture,
Department of Parasitology, Microbiology and Poultry Health

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A Thesis Submitted to College of Veterinary Medicine and Agriculture of Addis Ababa University in the Partial Fulfillment of the Requirements for the Degree of Masters of Science in Poultry Health and Management

By

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Evaluation of Bacterial Contaminations and Mineral Contents of Eggshell Powder Recycled from Hatchery Wastes as an Alternative Animal Feed Source in Bishoftu, Ethiopia

As members of the examining board of the final final MSc thesis open defense, we certify that we have read and evaluated the thesis prepared by Mohammed Beriso on "**Evaluation of Bacterial Contaminations and Mineral Contents of Eggshell Powder Recycled from Hatchery Wastes as an Alternative Animal Feed Source in Bishoftu, Ethiopia"** and recommend it to be accepted as fulfilling the thesis requirement for the degree of **Masters of Science in Poultry Health and Management**.

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DECLARATION

This is to certify that the thesis, entitled: "**Evaluation of Bacterial Contaminations and Mineral Contents of Eggshell Powder Recycled from Hatchery Wastes as an Alternative Animal Feed Source in Bishoftu, Ethiopia''** was accepted in partial fulfillment of the requirements for the award of the degree of Master of Veterinary Science in Poultry Health and Management by the college of veterinary medicine and agriculture, Addis Ababa University conducted by **Mohammed Beriso** was a genuine work carried out by her under my guidance. The assistance and help received during the course of this investigation have been duly acknowledged. Therefore, I recommend that it be accepted as fulfilling the research thesis requirements.

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STATEMENT OF AUTHOR

By my signature below, I declare and affirm that this thesis is my own work. I have prepared, gathered, analyzed, and finished this thesis in accordance with all ethical guidelines. Every academic reference in the thesis has been acknowledged with a citation. I certify that every source I used to create this document has been cited and referenced. This thesis has been prepared with every serious precaution to prevent plagiarism.

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BIOGRAPHICAL SKETCH

Mohammed Beriso was born in Hasassa, Oromia region, Ethiopia, in 1994 G.C. He began his/her/their early education at Tijo kerensa elementary school, where he completed primary and secondary education. He joined Wollo University in 2011 G.C and pursued a DVM degree Veterinary medicine, graduating in 2018 G.C. In 2023, he enrolled in the Master's Program in poultry health and management at AAU, CVMA, where he is currently working on a thesis entitled "Evaluation of Bacterial Contaminations and Mineral Contents of Eggshell Powder Recycled from Hatchery Wastes as an Alternative Animal Feed Source in Bishoftu, Ethiopia" under the supervision of Dr. Olana Merara and Mr. Ewonatu Kebede.

During his/her/their academic journey, Mohammed Beriso has participated in various research projects related animal health. He also gained experience working as a lecturer at Ambo or university, where he contributed to in providing quality education and conducting research related to animal production and health. His main areas of interest include poultry nutrition, poultry health and production, microbial pathogen identification, hatchery management and environmental protection. His current research focuses on hatchery management and environmental protection.

Mohammed Beriso aspires to contribute to the advancement of sustainable livestock production, food safety and animal nutrition through research, innovation, and academic or field engagement. He aims to work with institutions focused on agricultural development, animal health research, or food security.

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LIST OF ABBREVIATIONS

ADARDO	Ada'a District Agricultural And Rural Development Office
ANOVA	Analysis of Variance
APHA	American Public Health Association
bp	base pair
CFU	Colony-Forming Units
DNA	Deoxyribonucleic Acid
EMB	Eosin Methylene Blue
ESM	Eggshell Membrane
ESW	Edible Solid Waste
ICP-OES	Inductively Coupled Plasma Optical Emission Spectroscopy
ISO	International Organization for Standardization
NVI	National Veterinary Institute
PCR	Polymerase Chain Reaction
QGIS	Quantum Geographic Information System
SD	Standard Deviation
SEM	Scanning Electron Microscopy
Stx2	Shiga Toxin 2
TABC	Total Aerobic Bacterial Count
TCC	Total Coliform Count
TSI	Triple Sugar Iron
UV	Ultraviolet
X2	Chi sSquare
XLD	Xylose Lysine Deoxycholate

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ABSTRACT

The rising demand for sustainable and cost-effective animal feed ingredients has sparked interest in using hatchery byproducts, specifically eggshells, as a potential mineral source. The research experiment was conducted from November 2024 to May 2025 in Bishoftu town, Ethiopia, to evaluate the levels of bacterial contaminations and mineral content of hatchery waste recycled eggshell for its potential as an alternative animal feed source. A total 36 eggshell samples of different hatch status and breed origins were collected from four hatchery plants. Microbial analyses were conducted to assess total aerobic bacterial count, coliform count, and *Escherichia coli* count, as well as the presence rate of *Escherichia coli* and *Salmonella spp*. before and after preliminary processing of the eggshell. High contamination level was found with total aerobic bacterial count, total coliform count, and *Escherichia coli* counts averaging 6.80 ± 1.21, 6.23 ± 0.63, and 3.20 ± 2.54 $\log_{10}$ CFU/g, respectively before the processing. Eggshell from unhatched eggs had significantly greater *Escherichia coli* count (4.24 ± 2.33 $\log_{10}$ CFU/g) than eggshell of hatched eggs (2.11 ± 2.34 $\log_{10}$ CFU/g). The study identified that *Escherichia coli* and *Salmonella* contaminated eggshell powder with 69% and 13.8% frequency, respectively. After processing, total aerobic bacterial count and total coliform count levels dropped to 5.32 and 4.53 $\log_{10}$ CFU/g respectively. The frequency of *Escherichia coli* in eggshell samples dropped to 36.1%, whereas *Salmonella* was eliminated. The study found that hatchery waste derived eggshell powder is the best natural source of major and minor minerals, particularly when derived from unhatched eggs. The major minerals like magnesium (2429.8mg/kg ± 266.6), calcium (2078.1 mg/kg ± 0.8), phosphorus (1036.9 mg/kg ± 125.0), and sulfur (1389.2mg/kg ± 139.7) were very prominent than other minerals and trace elements. The study confirms that eggshell powder from hatchery waste can be used as a safe and effective mineral supplement in animal feed, but recommends additional thermal treatment or chemical sanitization steps and improved extraction for safe and effective recycling.

Keywords: *Bacterial contamination; E.coli; Eggshell; Hatchery waste; Mineral; Salmonella*

1. INTRODUCTION

The global poultry industry has expanded rapidly in recent years to meet the rising demand for protein sources like eggs and poultry meat. However, this growth is accompanied by significant waste generation, particularly in the form of eggshells and hatchery by-products (Lipdo *et al.,* 2024). The poultry industry produces large amounts of solid hatchery waste such as eggshells, infertile eggs, dead embryos, late hatchings, dead chickens, low-grade unsalable chicks, male chicks, and a viscous liquid from eggs and decaying tissue (Glatz *et al*., 2011).

Eggs serve a vital function in a variety of products, such as baked goods, salad dressings, and fast food resulting in significant daily production of eggshell waste and considerable disposal costs on a global scale (Verma *et al.,* 2012). This increase in consumption correlates with a rise in egg production. According to the Food and Agriculture Organization global egg production reached approximately 97 million tonnes in 2023 with the estimated eggshell waste approximately 9.7 million tonnes worldwide annually ((FAO, 2024).

China is the preeminent producer of eggshells, with a reported production of 24.8 billion kilograms in 2019, and projections indicated that production would surpass 35 million metric tons by 2020 (Younas *et al.,* 2025). In Africa, approximately 2,367,000 tonnes of eggs are produced annually representing 3.7% of the global output. Nigeria is the leading producer on the continent, with an output of 533,000 tonnes, while the Central African Republic, Comoros, Congo, Gambia, Guinea, and Swaziland each produce less than 1,000 tonnes annually. This substantial level of egg production leads to considerable quantities of eggshell waste, which is predominantly disposed in landfills (Ngayakamo and Onwualu, 2022).

According to Fekadu *et al.* (2023), Ethiopia has significantly expanded its hatchery capacity in recent years. The Ministry of Agriculture set a ten-year plan with a target to increase egg production from 2.85 billion in 2019/20 to 5.55 billion by 2029/30. However, the most recent data already surpasses this projection, with 9.1 billion eggs produced in 2024. Assuming an average eggshell weight of 5.5 grams per egg, Ethiopia produces approximately 50,000 metric tons (50 million kg) of eggshells annually. However, this much eggshell is removed as waste material annually from those hatchery plants (Gulilat *et al.,* 2021). The eggshell wastes are

cumulated on-site without any pre-treatment results an increase in pollution problems (Tsai *et al.,* 2008).

Traditional disposal methods for solid hatchery waste include landfill, composting, and incineration. Most of the hatchery waste is sent to landfill or composting, which costs the chicken industry millions of dollars each year in disposal costs (Das *et al.*, 2002). Also, in view of the environmental odour from biodegradation, waste management is not a pleasant function (Tsai *et al.,* 2008).

According to Urjintseren and Byambaa (2024), the eggshell comprises about 9-12% of an egg's mass and contains 27 essential microelements, primarily calcium carbonate and magnesium carbonate. It also includes trace amounts of sodium, potassium, zinc, manganese, iron, copper, sulfur, water, protein, crude fat, ash, and various amino acids. Recycling this byproduct could help to address mineral deficiencies in livestock linked to reproductive disorders. The processed eggshells may be a viable alternative for producing dicalcium phosphate on animal farms (Mishra and Poonia, 2019).

Calcium deficiency in dairy and poultry diets poses a significant challenge within the agricultural sector. Minerals are critical for various physiological processes, including reproduction, maintenance, metabolism, and growth (Reinhardt *et al.,* 2010). The supplementation of minerals is widely recognized to enhance feed intake and digestibility, thereby improving overall animal performance. However, Ethiopia is currently facing a substantial foreign currency deficit, which limits the importation of commercial mineral sources, such as dicalcium phosphate (Tsegahun *et al.*, 2006).

Eggshell meal, a byproduct of hatchery operations, offers a promising solution for mitigating calcium deficiencies in animal feed. Research indicates that approximately 40% of the calcium content in eggshells is extractable (Lesnierowski and Stangierski, 2018). Additionally, studies show that the bioavailability of calcium from eggshells surpasses that of commercially available calcium carbonate (Singh.*et al.,* 2021).

However, the quality and safety of eggshell powder can be compromised by bacterial contamination and variations in its chemical composition. The surfaces of eggs are susceptible

to microbial contamination. This contamination presents environmental and health risks due to the potential proliferation of pathogenic microorganisms (Brauner *et al.*, 2016). If contaminated eggshell waste is utilized as a feed supplement without appropriate treatment, it may introduce disease-causing microorganisms to poultry and other livestock (De Reu *et al.*, 2006).

Therefore, scrutinizing the chemical makeup and contamination level of eggshell recovered from hatchery waste is very essential to turn eggshell waste into a feed supplement for animals. In Bishoftu Town, where poultry production is expanding, the need for sustainable feed resources is growing. However, there is a scarcity of research on the quality assessment of eggshell powder derived from hatchery waste, particularly in terms of its microbial safety and mineral composition.

General objective:

- To evaluate the suitability of hatchery waste derived eggshell powder as a sustainable and cost effective alternative mineral supplement in animal feed by analyzing its microbial safety and mineral compositions.

Specific Objectives:

- To evaluate bacterial loads and isolate key pathogens, specifically *Escherichia coli* and *Salmonella*, from eggshell samples obtained from selected hatchery plants.
- To compare bacterial contaminations and mineral contents of eggshell powder produced from hatched and unhatched eggs, as well as from eggs of different chicken breeds.
- To evaluate the effect of eggshell preliminary processing on the bacterial load and isolates that impact the safety of eggshell powder produced from hatchery waste

2. LITERATURE REVIEW

2.1. Eggshell Structures, Formation and Composition

Eggshell is the hard outer surface of the egg that protects the inner portion and embryo during incubation. The eggshell is a significant structure for the egg for numerous reasons. Primarily, it acts as an embryonic sac for the chick, providing mechanical support and protection while enabling gas exchange. Additionally, the eggshell serves as a containment structure for eggs that are commercially available to consumers worldwide (King'Ori, 2011). Microscopic studies show that the hen's eggshell is a highly ordered structure. It consists of three parts: cuticle, matrix, and shell membrane. The cuticle is brownish due to the pigment protoporphyrin, while the matrix is whitish and located between the cuticle and shell membrane (Rahman, 2013).

The structure of the eggshell provides both strength and porosity, facilitating gas exchange and insulation for the developing embryo (Gautron *et al.*, 2021). The eggshell, which has an approximate thickness of 400 µm in chickens, consists primarily of two interwoven membrane networks composed of protein fibers. The inner shell membrane, measuring 20 µm in thickness, is situated horizontally beneath the egg white, while the outer shell membrane has a thickness of 50 µm. Mineralization initiates in the outer shell membrane and progresses outward to form the mammillary layer (Hunton, 2005).

The formation of an eggshell involves the deposition of calcium carbonate in the shell gland of birds. About five hours post-ovulation, the egg moves into the red isthmus and uterus, undergoing calcification for 18 to 19 hours. During this time, uterine fluid rich in ionized calcium and bicarbonate, essential for eggshell formation, surround the egg. Calcium carbonate precipitates onto the outer membranes of the eggshell, specifically in the space between the shell membranes, which enclose the hydrated albumen and the uterine wall lining (Gautron *et al.*, 2014). The composition of uterine fluid varies at different stages of eggshell formation, which impacts the growth of calcite crystals in various areas of the calcified shell (Nys *et al.*, 2004).

Calcium carbonate crystallizes during shell formation, and hence the architecture of this protein matrix within the shell, are critical for its mechanical properties (Zhao *et al.*, 2024). Investigations using Scanning Electron Microscopy (SEM) to analyze the microstructure of eggshells have demonstrated that they comprise a complex amalgamation of minerals and proteins, rather than merely consisting of crystalline layers. For example, the shell protein Ovocleidin-17 plays a pivotal role in regulating crystal growth and orientation throughout the shell formation process. These proteins are instrumental in stabilizing amorphous calcium carbonate and facilitating its transformation into calcite crystals, thus influencing the mechanical characteristics of the eggshell (Hincke *et al.*, 2012).

Generally, an avian eggshell, accounting for about 10% of the egg's weight, consists of the shell and the shell membrane. It is predominantly composed of 95.1% inorganic matter and 3.3% protein (Adeyeye, 2009). The shell itself is a calcareous formation primarily consisting of calcium carbonate (CaCO3), which accounts for 95% of its composition, in addition to an organic matrix that encompasses proteins, glycoproteins, and proteoglycans, making up 3.5% of the total composition (Hincke *et al.*, 2010). The eggshell membrane (ESM) is comprised of cross-linked collagens (types I, V, and X), glycosaminoglycans (GAGs), egg white proteins such as ovotransferrin and lysozyme, as well as eggshell matrix proteins including ovocalyxin-36 (Mann *et al.*, 2006).

The mineral composition and structural characteristics of eggshells render them suitable for incorporation into animal feed as nutritional supplements due to their advantageous mechanical properties. One study demonstrated that the inclusion of eggshells in the diet could augment calcium intake and foster improved bone health (Hunton, 2005). Moreover, recent research has revealed antimicrobial properties inherent in eggshells. These defence mechanisms underscore the presence of antimicrobial compounds within the shell, which may strengthen immune defences against microbial agents, enhance infection tolerance, and improve resilience to microbial challenges, thereby ultimately supporting embryonic viability (Moreau *et al.*, 2022).

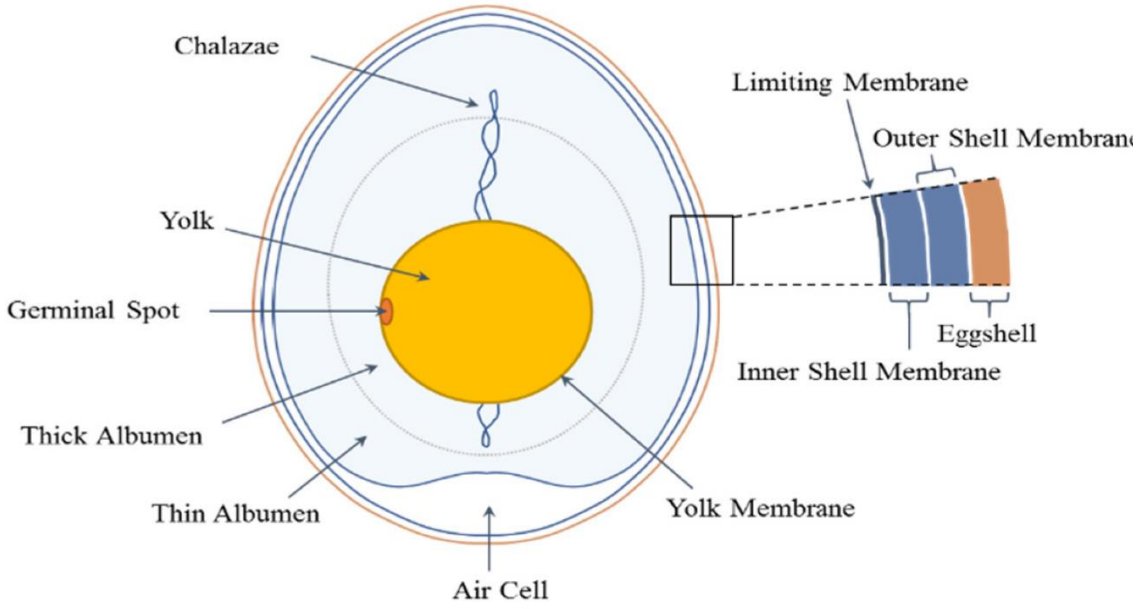

Figure 1: Hen egg structures illustrating the morphology of the eggshell and ESMs.
Source: Shi *et al.*, 2021.

2.2. Physical Properties of Eggshell

The eggshell plays a vital role in both the biological and economic aspects of the poultry industry, and its quality has consistently been a major concern for the safety and quality of egg products (Roberts and Chousalkar, 2014). Key physical properties of the eggshell such as thickness, porosity, and strength greatly influence its functionality and potential use as a supplement in animal feed. Therefore, understanding these properties is essential for improving the utilization of eggshells in agricultural practices (Arif *et al.*, 2022).

Eggshell thickness significantly affects its mechanical strength and protective properties. Typically, chicken eggshells measure around 0.3 mm in thickness, though this can vary depending on genotype and rearing conditions (Ketta and Tumova, 2018). Thicker shells are generally more resistant to physical damage and infection. Research has shown a positive

correlation between shell thickness and resistance to cracks and breaks, with stronger shells being thicker (Kibala *et al.,* 2018). For instance, studies comparing eggs from enriched systems to those from conventional systems have found that the former tend to have cleaner and thicker shells, resulting in greater strength (Marcos, 2023). Additionally, shell thickness impacts overall egg quality; thinner shells are more susceptible to breakage during handling and transportation, leading to potential economic losses for producers (Hunton, 2005).

Porosity, the presence of tiny pores in the eggshell, is a crucial physical property that facilitates gas exchange. A typical chicken eggshell contains between 7,000 and 17,000 pores, which are small enough to allow the movement of oxygen and carbon dioxide while preventing bacterial entry into the egg. The size and distribution of these pores are vital; larger pores increase the likelihood of pathogen penetration. Pore density is influenced by genetics and environmental conditions (Marcos, 2023). While a higher pore density can enhance gas exchange, it also raises the risk of microbial contamination. Therefore, it is essential to achieve an optimal balance between pore size and density to ensure good egg quality while maximizing the shell's effectiveness. Recent studies indicate that increased porosity may improve gas exchange but could compromise the eggshell's barrier properties against pathogens, highlighting the need for careful management of these characteristics (Onagbesan *et al.*, 2007).

Eggshell mechanical strength is determined by its composition, primarily calcium carbonate, and its structural integrity. Several studies have shown that intact eggshells possess significantly higher puncture resistance compared to cracked or leaky shells (Zhao *et al.*, 2024). This strength is crucial for protecting the developing embryo from external forces during incubation. A well-established relationship exists between eggshell strength and thickness; thicker eggshells tend to be stronger. Recent research has confirmed that thicker eggshells offer better mechanical protection and are more capable of withstanding external pressures. However, it is important to note that a shell cannot be deemed stronger if its microstructure is compromised, regardless of its thickness. Factors such as the arrangement of calcite crystals and the presence of organic matrix proteins play a decisive role in the overall integrity of the shells (Gautron *et al.,* 2021).

2.3. Microbial Pathogens in Eggshells

The eggshell acts as a crucial barrier for the developing embryo, protecting it from environmental threats, including microbial pathogens. However, microbial contaminants on eggshells can pose serious health risks to both the embryos and consumers. Contamination can occur at any stage of production, and the porous structure of the eggshell allows harmful pathogens to attach and penetrate (De Reu *et al.*, 2006).

2.3.1. Common microbial contaminants in pre- and post-hatch eggshells

Eggshells can become contaminated with various microorganisms both before and after hatching. During the pre-hatch phase, contamination can occur from exposure to the hen's feces, nesting materials, or contaminated areas within the poultry house (Oviasogie *et al.*, 2016). Common microbial contaminants include *Salmonella spp.*, *Escherichia coli*, *Staphylococcus aureus*, and *Enterobacter spp*. These bacteria typically settle on the eggshell surface, and some can even penetrate the shell through its pores, leading to contamination of the egg's contents (Board and Tranter, 2017).

Post-hatch contamination primarily originates from the hatchery environment. During hatching, eggshells come into direct contact with hatchery debris such as feces, feather dust, and broken eggs resulting in increased exposure to bacteria (Oviasogie *et al.*, 2016). After hatching, eggshells often become contaminated with pathogens like *Salmonella enteritidis*, *Listeria monocytogenes*, *Pseudomonas spp.*, and *Bacillus spp*., which thrive in the warm and moist conditions of the hatchery, thus elevating the risk of contamination (Graham *et al.*, 2022).

Salmonella enteritidis is particularly concerning due to its association with poultry infections and foodborne illnesses in humans. Studies indicate that this pathogen can survive on eggshell surfaces and within the shell membranes for extended periods, posing a risk if eggshells are incorporated into animal feed (Gantois *et al.*, 2009). Additionally, fungi such as *Aspergillus spp*. and *Penicillium spp*. can contaminate eggshells, especially in humid environments, potentially leading to mycotoxin contamination of feed (Tomczyk *et al.*, 2018).

2.3.2. Methods of controlling microbial contamination in eggshells

To ensure the safe use of eggshells in feed supplements, various methods have been developed to manage microbial contamination (Han *et al.,* 2023). These methods include physical, chemical, and biological interventions. Among these, the use of ultraviolet (UV) light has emerged as a particularly effective technique. Research shows that UV-C light irradiation can significantly reduce the bacterial load on eggshells by damaging the DNA of microorganisms, which inhibits their growth (Turtoi and Borda, 2014). In a study that compared different disinfection methods, eggs treated with UV light exhibited a notable decrease in total aerobic mesophilic bacteria compared to untreated controls. This method is beneficial because it does not leave toxic residues and can be seamlessly integrated into existing processing systems without compromising the quality of the eggs (Branco *et al.,* 2021).

Another effective approach to controlling microbial contamination involves the use of chemical disinfectants. Formaldehyde fumigation has been extensively studied and has proven to significantly lower bacterial counts on eggshells (Cadirci, 2009). However, concerns about the toxicity and potential health risks associated with formaldehyde have led researchers to investigate alternative disinfectants like hydrogen peroxide and peracetic acid. These alternatives have shown effectiveness in reducing microbial loads while posing less risk to both humans and the environment (Mahmoud *et al.,* 2022). The selection of disinfectant often hinges on factors such as cost, availability, and regulatory approvals (Bogdanova and Chemykh, 2023).

Biological methods for managing microbial contamination are also gaining attraction. The use of natural antimicrobial agents derived from plant extracts or essential oils has shown potential in lowering microbial loads on eggshells. For instance, certain essential oils have antimicrobial properties that can inhibit the growth of bacteria such as *Escherichia coli* and *Salmonella* (Burt, 2004). Probiotics have also been investigated as a way to boost the natural defenses of eggs against microbial threats. By introducing beneficial bacteria to the eggshell, it could be possible to outcompete harmful microorganisms for resources, which may help lower contamination levels (Abdelqader *et al.,* 2013). These biological methods provide an eco-friendly alternative to conventional chemical disinfectants and could enhance food safety in poultry production (Aziz and Karboune, 2018).

2.4. Eggshell Waste Production from Hatcheries

The global poultry industry has undergone substantial growth in recent years to meet the rising demand for protein sources, including eggs and poultry meat. However, this expansion has resulted in a considerable generation of waste (Lipdo *et al.,* 2024). A primary type of waste produced by the poultry sector is hatchery waste, which is derived from chick hatcheries. Hatchery waste encompasses the byproducts that remain in hatcheries following the completion of the hatching process (Gao and Xu, 2012).

Hatchery waste generated at various stages of the incubation process. It starts with the arrival, reception, and classification of eggs (during which small eggs, those with two yolks, cracked or broken eggs, and eggs with very thin shells are discarded). Another round of discarding takes place at 19 days of incubation, when infertile eggs are eliminated or the times at which early, intermediate and late embryonic mortality are removed. The final stage of waste occurs at 21 days when chicks hatch. At this point, stillborn or malformed chicks, unhatched eggs, and eggshells are discarded (Alves, 2018). Eggshell is produced at large amounts by egg processing industries, and high quantities of this solid residue are still disposed as waste in landfills without any pretreatment being a source of organic pollution (Gao and Xu, 2012, Oliveira *et al.*, 2013).

Global egg production is projected to reach 65.5 million metric tons annually, with China accounting for 45% of this total. Between 2000 and 2010, egg production in Europe increased by 10%, while in China, it experienced a remarkable surge of 50% (Vandeginste, 2021). Over the past three decades, global egg production has risen by more than 150%, with Asia witnessing the most significant growth, expanding its output fourfold (Waheed *et al.,* 2020). In Ethiopia, rural poultry production is vital, contributing to 98.5% of the national egg production and 99.2% of poultry meat production, resulting in an annual output of 72,300 metric tons of meat and 78,000 metric tons of eggs (Emagnaw, 2018).

By 2030, global egg production is projected to reach 90 million tons (Vandeginste, 2021). This substantial quantity of eggs is utilized both domestically and industrially in food manufacturing and processing, making it an essential source of nutrition. However, the

increase in egg production resulted in greater eggshell waste, which is often discarded and sent to landfills (Waheed *et al.,* 2020). Each year, approximately one million tons of waste are generated from discarded eggshells, leading to management challenges related to odor and microbiological growth. Despite these issues, eggshells present potential applications as a solid byproduct and can be repurposed for various industries (Younas *et al.,* 2025).

Figure 2: eggshell waste generated daily at a hatchery farm.
Source: Chuakham *et al.,* 2025.

2.5. Eggshell Waste Disposal Methods and Environmental Implications

Managing large quantities of eggshell waste produced annually become significant challenges, primarily because this material is frequently sent to landfills, which can cause odor problems and promote microbial growth (Mignardi *et al.,* 2020). The Environmental Protection Agency ranks eggshell waste as the 15th most serious pollution issue in the food industry. Improper disposal not only contributes to environmental pollution but also poses health risks due to the potential for fungal growth (Ajala *et al.,* 2018).

Traditional disposal methods for solid hatchery waste include composting, rendering, and incineration. Each method has distinct environmental implications, both positive and negative, and their use varies by region and the scale of egg production. Most hatchery waste is either sent to landfills or composted, which costs the chicken meat industry millions of dollars each year in disposal costs (Das *et al.,* 2002). Additionally, European Union regulations classify eggshells as hazardous waste (Quina *et al.,* 2017). Overall, eggshell waste ranks as the 15th

most significant agro-waste material, with its decay in landfills contributing to adverse environmental impacts (Waheed *et al.,* 2020).

Eggshell waste discarded in landfill sites without pre-treatment releases ammonia (NH3), hydrogen sulphide (H2S), and offensive odors, promoting the growth of harmful insects and bacteria. This can lead to soil, water, and environmental contamination (Mignardi *et al.,* 2020). Burial is another traditional waste disposal method used for infectious agents and during natural disasters; it requires cover material and excavation equipment. Commercial poultry producers often prefer open pit disposal because it is more economical, quicker, cleaner for the environment, and convenient (Ellis, 2001). However, this method exacerbates methane emissions under anaerobic conditions and attracts pests due to the slow decomposition of protein-rich membranes (Adaikalam *et al.,* 2024).

Burning is a common waste disposal method, particularly among small-scale farmers. This process involves incinerating waste at high temperatures using fuels such as wood, tires, or diesel. While effective, burning can contribute to atmospheric pollution, especially in cases of highly infectious diseases like newcastle disease and avian influenza (Singh *et al.,* 2018). During incineration poultry, waste is collected and loaded into an incinerator, where it is heated to achieve complete combustion. Organic materials are burned, producing ash, flue gas, and heat. As organic molecules oxidize, gases are released, and the waste is converted into ash. Before being emitted into the atmosphere, these gases are treated in a secondary chamber to remove harmful pollutants. The resulting residual ash is significantly reduced in volume and is sterile, allowing for safe disposal or specific uses (Nidoni, 2017).

Composting is a commonly used traditional waste disposal method, particularly among households and small farms. Edible solid waste (ESW) is frequently utilized as an organic fertilizer or soil amendment due to its abundance, low cost, and biodegradability (Roy and Mohanty, 2020). One of the key advantages of ESW as a composting additive is its fibrous structure, which enhances ventilation (or aeration) during the composting process (Barthod *et al.,* 2018). This fibrous nature also helps reduce bulk density and increase water-holding capacity in the compost. Additionally, the high surface area of ESW allows it to bind nutrients, minimizing nutrient loss from leaching (Soares *et al.,* 2016).

Eggshell waste is an excellent source of various inorganic and organic materials, such as calcium phosphate, magnesium carbonate, and organic matter. These materials can enhance microbial reproduction and activity during composting, ultimately improving compost quality (Roy and Mohanty, 2020). Globally, studies have demonstrated that co-composting industrial eggshell waste can effectively meet the pathogen-killing criteria of 70ºC for one hour when an N-rich source is included However, its successful implementation depends on the development of efficient operational plans that prioritize both environmental preservation and product quality (Soares, 2016).

Generally, large quantities of eggshells are often disposed of through traditional methods, which can lead to organic pollution (Oliveira *et al.,* 2013). However, eggshells should be viewed as a valuable natural resource rather than waste. They consist of well-organized calcified structures that contain over 96% CaCO3) (Nys and Gautron, 2007). As such, eggshells have significant potential for use as limestone (CaCO3) or lime (CaO) in various applications (Cree and Rutter, 2015). Furthermore, recycling and reusing eggshell waste align with the principles of a circular economy, enhancing resource efficiency by transforming waste or byproducts into valuable resources with both economic and environmental benefits (Ferraz *et al.,* 2018).

2.6. Potential Valorisation Options for Eggshell

Eggshell waste is recognized as a significant contributor to environmental pollution; however, effective applications of eggshells can offer sustainable solutions. In recent years, considerable efforts have been directed towards converting eggshell waste into valuable products (Hamada *et al.,* 2020). Eggshells possess substantial potential for resource recovery and the development of new products. Their high calcium content makes them particularly beneficial as animal feed supplements (Aditya *et al.,* 2021). In aquaculture, powdered eggshells serve as a valuable resource for fish feed and algae fertilization, thereby fostering a symbiotic farming system (Amarnath *et al.,* 2020). Furthermore, eggshells can be integrated into calcium-fortified foods, including cakes, yogurts, sausages, biscuits, and calcium-enriched coffee (Aditya *et al.,* 2021).

Potential applications of eggshells include their use in co-composting mixtures to enhance soil fertility and structure by adding calcium to acidic soils. This is especially beneficial for plants prone to blossom-end rot, such as tomatoes and berries (Lee *et al.,* 2020). Additionally, eggshells can serve as an effective catalyst in biodiesel production (Khemthong *et al.,* 2012). However, to ensure these applications are safe, eggshells must undergo heat treatment to eliminate microbial contamination. Maintaining the safety of final products in both agricultural and industrial practices is essential for hygiene and disease control (Yoo *et al.,* 2009).

Eggshells are used in the preparation of calcium phosphate ceramic materials (Faridi *et al.,* 2018). Research shows that eggshell powder can be incorporated into construction materials such as concrete, cement mortar, bricks, alkali-activated binders, and soil stabilizers, providing an energy-efficient and cost-effective solution for sustainable construction (Sathiparan, 2021). Research was conducted on the use of ground eggshell waste as an adsorbent for removing dyes from aqueous solutions. The findings demonstrate that ground eggshell waste effectively adsorbs anionic dyes (Tsai *et al.* 2008). Eggshells are rich in minerals and serve multiple purposes, including being a pharmaceutical excipient, a base material for medicinal and dental preparations, a food additive and calcium supplement, a diluent in solid dosage forms, an agricultural fertilizer component, and a material for bone implants (Murakami *et al.* 2007).

ESM is a valuable byproduct of hatcheries, rich in collagen that can be extracted and utilized. The collagen derived from eggshells serves multiple purposes in food, pharmaceuticals, and cosmetics. In the food industry, it enhances texture, color, and nutritional value (Kheirabadi *et al.,* 2018). In pharmaceuticals, it is valued for its curative properties, particularly for joint and connective tissue diseases (Ruff *et al.,* 2012). In cosmetics, it improves skin elasticity and moisture content (Marimuthu *et al.,* 2020).

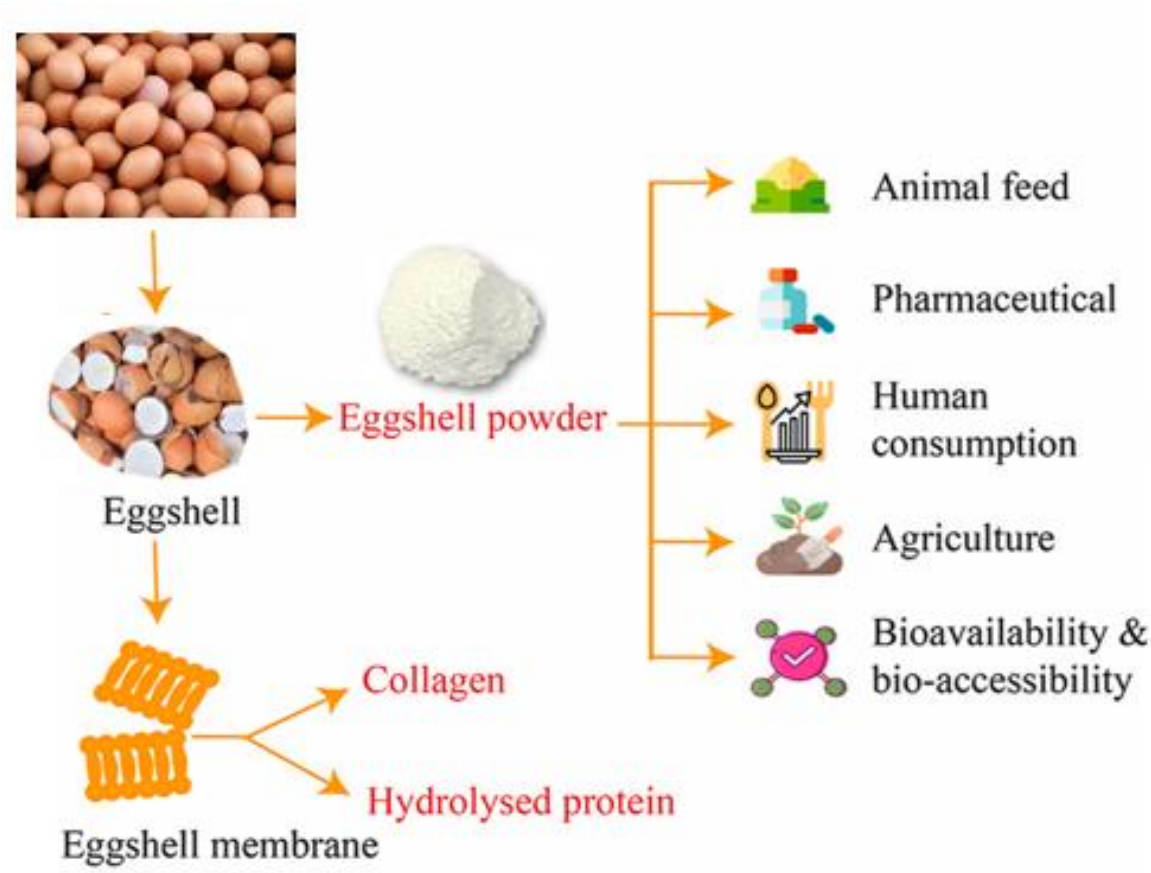

Figure 3: Application of eggshell in various industries.

Source: Aditya *et al.*, 2021.

2.7. Eggshell Meal Production and Factors Influencing its Quality

Waste materials from hatcheries can be transformed into valuable resources for producing animal feed. This alternative method offers several benefits, ultimately helping to address environmental issues (Glatz *et al.,* 2011). Eggshells serve as a calcium-rich supplement in animal feed, enhancing livestock nutrition. Extracting calcium from eggshells is a more convenient and sustainable option compared to other natural sources (Waheed *et al.,* 2019).

The unique physical properties of eggshells make them an attractive option for animal feed supplements. Ground eggshells are rich in calcium carbonate, serving as an excellent source of calcium for livestock and poultry. Incorporating eggshell meal into animal diets can enhance calcium intake, which is essential for bone development and overall health (Arif *et al.,* 2022). Eggshell calcium is relatively easy to extract, making it an excellent alternative to other natural calcium sources (Aditya *et al.,* 2021).

To meet livestock's daily calcium needs, eggshell calcium is recommended as a feed supplement (Quina *et al.,* 2017). Research indicates that adding eggshell meal to animal feed is crucial for chickens to produce eggs (Cordeiro and Hincke, 2012). The Association of American Feed Control Officials has approved eggshell meal as a feed supplement (Ruff *et al.,* 2012). To ensure safety, eggshells must be heated to eliminate microbiological infections. Drying shells at approximately 80 °C removes moisture and converts them into powder (Yoo *et al.,* 2009). The production process involves drying, crushing, and milling at 80 °C while minimizing losses (Quina *et al.,* 2017).

The quality of eggshell powder is influenced by factors such as microbial load, chemical composition, and physical properties. Understanding these factors is essential for optimizing eggshell meal production and ensuring its safety as a feed ingredient. One primary concern is microbial contamination. Pathogens like *Salmonella* and *E. coli* pose health risks to animals and humans (Ketta and Tumova, 2016). Eggs from contaminated environments or mishandled are more likely to harbor these harmful microorganisms. Effective sanitation protocols during collection and processing of eggshells are crucial to mitigate these risks. Research shows that washing and sterilizing eggshells before grinding can significantly reduce microbial loads, enhancing the safety of eggshell meal (Asiedu *et al.,* 2022). Incorporating natural

antimicrobial agents during processing may also help control contamination (Tayel *et al.*, 2018).

The chemical composition of eggshells is crucial for eggshell meal quality. Eggs are primarily composed of calcium carbonate, which makes up about 94% of their structure, along with trace minerals like magnesium, phosphorus, and potassium (Butcher, 1990). These minerals are essential for animal physiology and enhance the nutritional value of eggshell meal. Research shows that calcium content in eggshells varies based on factors such as hen breed, age, and diet (Yasothai and Kavithaa, 2014). Older hens typically produce eggs with thinner shells due to reduced calcium deposition capacity (Park and Sohn, 2018).

Optimizing hen nutrition with adequate dietary calcium and phosphorus is crucial for producing high-quality eggshells for eggshell meal production. The physical characteristics of eggshells, such as thickness and porosity, significantly influence the quality of eggshell meal. Thicker shells generally indicate greater strength and integrity, often associated with higher calcium content (Kibala *et al.,* 2018). Additionally, the structure of eggshells affects their grindability; more porous shells typically yield finer particles during milling (Urjintseren *et al.,* 2024). Assessing the mechanical properties of eggshells is essential to ensure they meet industry standards for feed ingredients. Technological advancements, such as SEM, provide insights into the microstructure of eggshells, allowing producers to refine processing techniques and enhance powder quality (Therdthai *et al.*, 2023).

3. MATERIAL AND METHODS

3.1. Description of Study Area

The study was conducted from November 2024 to May 2025 in Bishoftu town, Ethiopia, which is a hub for poultry production due to its proximity to Addis Ababa and its thriving hatchery industry. This area has several commercial hatcheries, making it ideal for collecting eggshell waste for the study. Bishoftu is located in the East Shewa Zone of the Oromia region, it sits at an elevation of 1,850 meters above sea level (ADARDO, 2007). It is located 45 kilometers southeast of Addis Ababa, at 9°N latitude and 40°E longitude. Bishoftu town comprised more than 50 commercial chicken farms that raise exotic breeds of chickens (Alemu *et al.,* 2022).

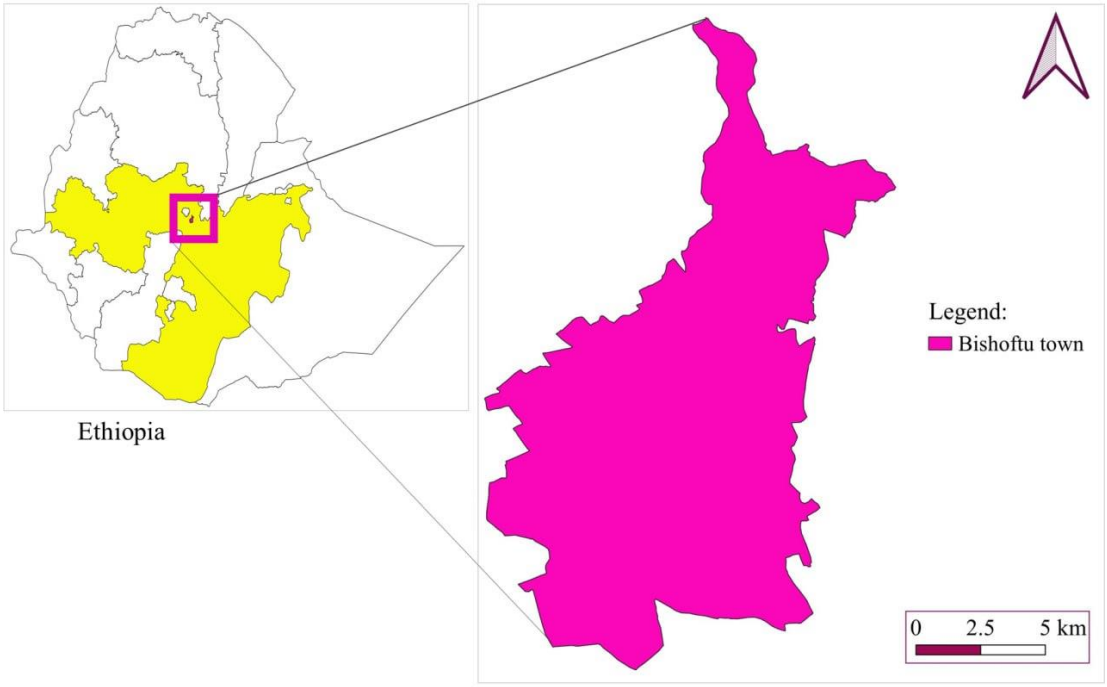

Figure 4: Map of Bishoftu town, East Shewa zone, Ethiopia (Developed by QGIS version 3.3.6).

3.2. Study Design and Sample Size Determination

The study used an experimental design with randomized complete block arrangements. Four commercial hatcheries located in Bishoftu town were purposely selected based on the hatching potential of chicks and hatchery waste handling systems and were considered as a source of

eggshells. Two of the hatcheries (Hatchery A and B) operated both broiler and layer hatchery unit, while the other two (Hatchery C and D) were exclusively layer hatchery unit. Each hatchery unit was considered as a block to control variability among hatchery conditions. From each hatchery unit, six samples were collected, three from hatched eggs and three from unhatched eggs over three randomly selected hatching cycles. Each sample consisted of 500 grams of thoroughly cleaned and air-dried eggshells. Accordingly, 36 samples (18 from hatched and unhatched egg) were collected for this purpose.

3.3. Sample Collection and Transportation

Thoroughly cleaned and air-dried eggshells were collected in sterile polyethylene bags. Each sample was labeled with information such as hatchery plants, breed of chickens of the eggshell source, hatching cycles, and hatch status of eggs (hatched or unhatched). Then all labelled samples were transported by icebox containing ices to the Veterinary Public Health Laboratory of Addis Ababa University, College of Veterinary Medicine and Agriculture for bacteriological analysis. To ensure the integrity of the samples, the samples were maintained at a temperature of 4°C in a refrigerator until analysis was take place when immediate bacteriological culturing is difficult to culture all samples at a time.

3.4. Microbial Analysis

This phase focuses on assessing the microbial contamination on the eggshells of both hatched and unhatched eggs, both before and after preliminary processing. Each samples of eggshells were thoroughly broken in to course powder by hand. Coarsely powdered eggshells of each samples thoroughly mixed and then divided into two groups using sterile aluminum foil to evaluate the presence of bacterial contaminations. The first group (group 1) was evaluated for bacterial contaminations without any further processing while the second group (group 2) underwent thorough washing, drying at 70°C for 3 hours in a hot air dryer, and milling into a fine powder (0.5 micrometers) using an electric grinding machine (generic).

Ten gram of eggshell powder from each group was measured and homogenized in a stomacher bag blender with 90 mL of sterile 0.1% peptone water (1:9 formula). Then, 1 mL of homogenized eggshell powder was used for all bacterial count TABC, coliform count (TCC)

and *E. coli* count). Materials such as petri dishes, test tubes, glass rods, pipettes, measuring cylinders, beakers, and conical flasks required for the investigation were soaked and washed in detergent and rinsed with distilled water. They were wrapped in aluminum foil and dried in the oven in an inverted position at 160°C for 45 min (Oviasogie *et al.,* 2016). All the media were prepared following the manufacturer's instructions and sterilized by autoclaving at 121°C for 20 min (De Reu *et al.,* 2005).

3.4.1. Total aerobic bacterial (TABC), coliform (TCC) and E. coli count

Total aerobic bacterial, coliform and *E. coli* count of eggshell powder samples were determined by the standard method as described by APHA (ISO, 2003). Serial dilutions of the samples were done with buffered peptone water; 1 mL from each dilution (10^{-1} to 10^{-5} or more until countable colony (30-300) was reached poured on standard plate count agar (Himedia, M091A, India) for TABC and Brilliance™ *E. coli*/Coliform Selective Agar (Oxford, England) in duplicates for TCC and E.coli count. The plates were then incubated at 37°C for 24 hr, and plates with colonies from 30 to 300 were used for determining TCC and TABC (De Reu *et al.,* 2005). Bacterial colonies were counted by colony counter (STUART SCIENTIFIC, UK) and record as colony-forming units (CFU) per gram using the formula:

$$\text{CFU per gram} = \frac{\text{Number of colonies on the plate} \times \text{Dilution factor}}{\text{Weight of the sample(g)}}$$

Then CFU per gram were converted into log10 (CFU) per gram to make results easier to interpret (Weldaragay *et al.,* 2012).

3.4.2. Isolation and identification of E. coli and Salmonella from eggshell powder

Eggshell powder samples were analysed for the presence of *Escherichia coli* and *Salmonella spp*. using selective enrichment and differential media, followed by biochemical identification. Homogenized eggshell powder from each groups were prepared by homogenizing 10 grams of eggshell powder in 90 ml of sterile peptone water. Detection of *E. coli* from eggshell samples were carried out according to the protocol of ISO 21150:2015 standard. To isolate *E. coli*, aliquots of the peptone water suspension from each groups were streaked onto MacConkey agar (Oxford, England) and Eosin Methylene Blue (EMB) agar (HIMEDIA, India).

On MacConkey agar), *E. coli* formed bright pink colonies due to lactose fermentation, while on EMB agar agar (Oxford), the colonies displayed a metallic green sheen (Snyder and Atlas, 2006). Then, the bacterial colony identified as *E. coli* in both media was subculture onto nutrient agar (HIMEDIA, India) and incubated at 37°C for 24 hours for biochemical testing (Quinn *et al.,* 2001). Colonies from the nonselective nutrient agar plates were transferred to trypto tryptone soya broth (OXOID CM0129, England), Triple sugar iron(TSI) (OXOID CM0277, England) agar, Simmon's citrate agar (HIMEDIA M099, India), urea broth (HIMEDIA M111A, India), and Methyl red-Voges-Proskauer (HIMEDIA M070, India) broth, followed by incubation for 24 hours at 37°C. *E. coli.* Isolates were identified by their motility, and positive results for indole and methyl red tests, along with negative results for Voges-Proskauer, urease negative, citrate utilization tests and characteristic reactions in TSI agar (Gas production Yellow slant/butt, no H_2S production).

Isolation and identification of *salmonella* from eggshell samples were performed by using techniques ISO 6579-1:2017. The homogenate was then enriched in Rappaport-Vassiliadis broth (Himedia MH1491) to promote the growth of *Salmonella spp* (Quinn *et al.,* 2001). After incubation, the enriched samples were streaked onto Xylose Lysine Deoxycholate (XLD) agar (OXOID CM0469, England), where presumptive *Salmonella* colonies exhibit a clear reddish halo surrounding a black core, indicating hydrogen sulfide production. After identification, four *Salmonella* colonies from both XLD are transferred to nonselective nutrient agar plates for further confirmation via biochemical testing. Colonies from nonselective nutrient agar plates transferred to trypto tryptone soya, TSI agar, Simmon's citrate agar, urea broth, Methyl red-Voges-Proskauer broth and then incubated for 24 hrs at 37o C. *Salmonella* colonies were identified by their motility, characteristic reactions in TSI agar (alkaline(red) slant, acid butt, H_2S production), and positive result of citrate utilization, indole and urease negative, M-R positive, and V-P negative (Schumann *et al.,* 2003).

3.4.3. Molecular confirmation of Salmonella and Escherichia coli isolates by conventional PCR

Biochemically positive isolates of *Salmonella* and *E. coli* were confirmed through molecular detection of the invA gene for *Salmonella* and the Stx2 gene for *E. coli* at molecular biology of National Veterinary Institute (NVI) using conventional Polymerase Chain Reaction (PCR). Twenty percent of the biochemically positive *E. coli* isolates underwent molecular testing, while all biochemically positive *Salmonella* isolates were confirmed. Bacterial isolates were grown overnight in nutrient agar at 37 °C. A loop full of the colonies was added to 100 µl of sterile water. Genomic DNA (Deoxyribonucleic Acid) extraction and purification were performed using the Genomic DNA Purification Kit (Qiagen) at NVI. DNA purity was assessed with a 2% agarose gel (Jothikumar and Griffiths, 2002). Standard methods were utilized for PCR amplification, and the amplified products were analyzed via gel electrophoresis at 120 V for 1hr in a 2% agarose gel containing 1 µl of Gel Red (Biotium). The products were visualized under UV light and documented using a gel documentation system (UVtec 08 100554). The expected sizes of the PCR products were 349 bp for Stx2 and 496 bp for the invA gene (Esquivel-Hernandez *et al.,* 2018).

3.5. Mineral Analyses of Eggshell Powder

Mineral analyses of eggshell powder were done at Horticoop Ethiopia (Horticulture) PLC laboratory, Bishofu. A twelve pooled sample (6 from each hatched and unhatched) was randomly collected from each category separately. Each sample were prepared for by thorough washing (by using distilled water), drying at 70°C for 3 hours in a hot air dryer, and milling into a fine powder (0.5 micrometers) using an electric grinding machine. Minerals such Sodium, Magnesium, Potassium , Calcium, Manganese, Iron, Copper, Zinc, Boron, Phosphorus, Molybdenum, and Silicon were determined by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (Germany), with radial plasma Observation following the De Souza, *et al.* (2008) procedure.

3.6. Statistical Analysis

The raw data collected during the study was filled into Excel spreadsheet 2016, carefully reviewed for errors, coded and subsequently imported into R version 4.3.1 software for data analysis. The count obtained (CFU per gram) for the microbial analysis were first converted to logarithmic form. Means, standard deviations, and ranges were calculated for bacterial counts, and mineral content. The logarithm values of TACC, TCC and *E. coli* count recovered from eggshell powder samples before any treatment and after preliminary processing (washing, drying and grinding) were compared and analyzed by Paired t-test at a 95% confidence ($p < 0.05$) while for *E. coli* and *Salmonella* isolates McNemar's test was used.

Analysis of bacterial loads in eggshell powder among four different hatcheries were compared and analyzed by Kruskal- Wallis Test while for *E. coli* and *Salmonella* isolates, Fisher's exact test was used. Bacterial load analysis of eggshell powder samples from different egg status (Hatched vs. Unhatched) and breeds (layer vs. broiler) were compared and analyzed by Mann-Whitney U test while for *E.coli* and *Salmonella* isolates Fisher's Exact test was used. A p-value of <0.05 was considered statistically significant in all tests. For mineral contents analysis descriptive statistics (mean and standard deviation) and multiple univariate ANOVA were used to compare mineral contents of eggshell among the eggshell powder samples from different egg status (Hatched vs. Unhatched), breeds (layer vs. broiler) and hatchery plants..

3.7. Ethical Clearance

Before the start of this work research ethical clearance, ref. no: VM/ERC/04/17/17/2025 was obtained from the Institutional Animal Care and Use Committee of the College of Veterinary Medicine and Agriculture of Addis Ababa University. Therefore, the research was conducted by keeping the welfare of animals and with complete research ethics of Addis Ababa University (Appendix 5).

4. RESULTS

4.1. Microbial Analysis of Eggshell Powder

4.1.1. Overall bacterial load counts and isolate rates in the eggshell powder

The overall results of the study on bacterial load in eggshell powder showed that eggshells were heavily contaminated with bacteria. The TABC, TCC and *Escherichia coli* counts were found to be high with wide fluctuations between samples as shown in Table 1.

Table 1: Overall Bacterial Loads in the Eggshell Powder

Bacterial Loads	**Mean ± SD**	**SEM**	**Min**	**Max**
TABC (log_{10} CFU/g)	6.80 ± 1.21	0.20	4.14	8.32
TCC (log_{10} CFU/g)	6.23 ± 0.63	0.54	0.10	7.25
E. coli count (log_{10} CFU/g)	3.20 ± 2.54	0.45	0.00	6.84

Apart from the quantitative bacterial load, the finding also determined the presence of certain bacterial pathogens in Eggshell Powder. Of 36 samples that were screened, 25(69%) contained *E. coli*, showing high occurrence of this organism in the hatcheries. Five samples (13.8%) were positive for salmonella, noting a comparatively lower occurrence.

4.1.2. Molecular confirmation results of Salmonella and Escherichia coli isolates by conventional PCR

Multiplex PCR analysis of *E. coli* revealed that out of the 5 biochemically positive isolates subjected to multiplex PCR in order to detect the shiga toxin producing genes (Stx2), only 3 (60%) of them were found to be positive (Figure 6), while for *Salmonella* all 5 sample subjected to multiplex PCR in order to detect the invA gene were found to be positive (Figure 5).

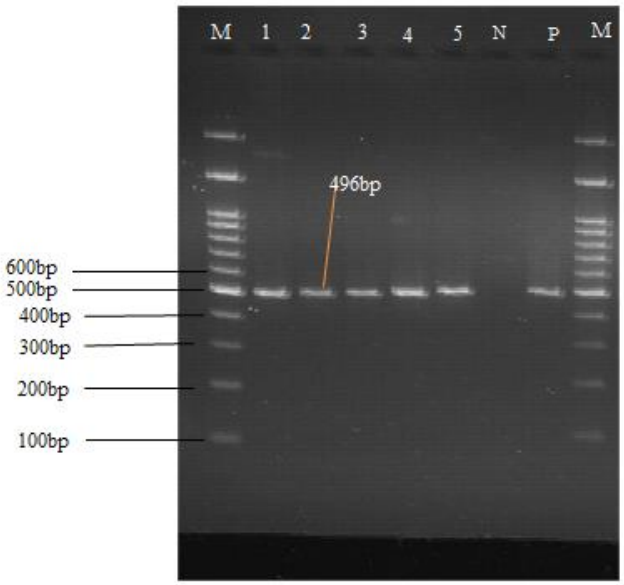

Figure 5: Agarose gel electrophoresis of PCR amplification products of *Salmonella spp* confirmation from the eggshell sample.

Lane M: Molecular ladder 100bp (Bio-rabbit, Germany), Lane 1-5: positive samples 496bp, Lane N: Negative control, Lane P: Positive control.

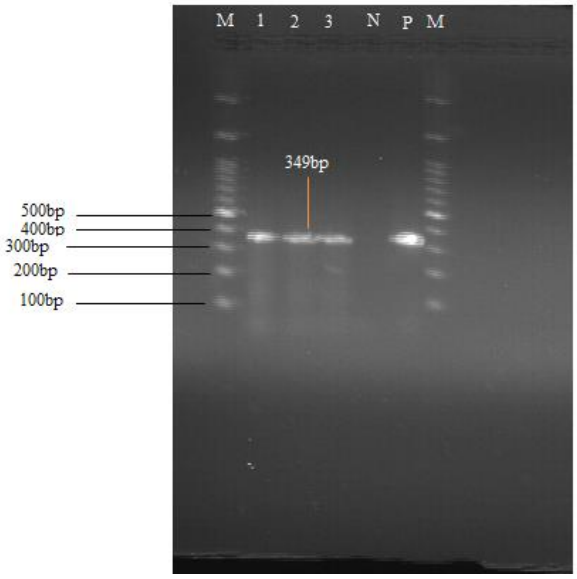

Figure 6: Agarose gel electrophoresis of PCR amplification products for *E. coli* confirmation from the eggshell sample

Lane M: Molecular ladder 100bp (Bio-rabbit, Germany), Lane 1-3: positive samples 349bp, Lane N

4.1.3. Comparative analysis of bacterial load counts and isolate rates among hatcheries

The TABC, TCC, and *E. coli* count of eggshell powder sampled from four hatchery plants (A, B, C, and D) are presented in Table 2. The results show that the mean of each bacterial count reflects moderate variability among hatchery plants. However, the Kruskal-Wallis test returned a p-value of less than 0.05, indicating no statistically significant variation across the hatcheries.

The study examined the frequency of *E. coli* and *Salmonella* isolates in eggshell powder from four hatcheries. The results showed that *E. coli* isolates were predominant in hatchery A and B; while hatchery C and D were lower (Table 2). However, that the differences in *E.coli* frequency between the hatcheries were not statistically significant. The finding results in Table 2 indicated that the frequency of salmonella isolates, was low in all hatcheries when compared to *E.coli*. The statistical test reflects that the differences between hatcheries were not statistically significant either. The findings suggest that although there is apparent difference in bacterial contamination in between hatcheries, it is too minor to become statistically significant.

Table 2: Statistical comparison of bacterial load counts and isolate rates in eggshell powder samples from four different hatcheries

Variables	**Hatchery Plants**					
	A	**B**	**C**	**D**	$\mathbf{X^2}$	***p-value***
Bacterial loads (Mean + SD, $\log_{10}$ CFU/g)						
TABC	6.98 ± 1.54	6.87 ± 1.04	6.53± 1.32	6.62 ± 0.79	0.67	0.68
TCC	6.38 ± 0.67	6.30 ±0.67	6.07± 0.50	5.95 ± 0.49	1.76	0.62
E. coli count	3.46 ± 2.58	3.52 ± 2.24	2.41 ± 3.06	2.69 ± 2.96	1.18	0.76
Bacterial isolates (%)						
E.coli	75	83.3	50	50		0.35
Salmonella	16	8.3	33.3	0		0.39

4.1.4. Comparative analysis of bacterial load and isolates in eggshell powder based on hatch status (hatched vs. unhatched) and breed

Differences in mean of bacterial loads of eggshell powder by breed type of sourced egg (layer vs. broiler) and hatch status of eggs from which eggshell was collected (hatched vs. unhatched) shows some variations (Table 3). As regards the hatch status, TABC and TCC did not show significant difference between hatched. However, a significant difference was observed in the number of *E. coli:* Eggshell of unhatched eggs had immensely greater *E. coli* count than eggshell of hatched eggs (figure 7). Comparative analysis of *E. coli* and *Salmonella* isolates in eggshell powder from hatched and unhatched eggs, and from different breeds (layer vs. broiler). Although the percentage of *E. coli* positive sample is raised in eggshell samples from the unhatched eggs than hatched eggs, the statistical analysis indicated no significance difference. In Salmonella isolates, a notable finding was that none of the eggshell samples from hatched eggs tested positive, while the unhatched samples did, and this difference was statistically significant ($p = 0.04$) (Table 3). These results indicate that unhatched eggshell samples are significantly associated with Salmonella contamination.

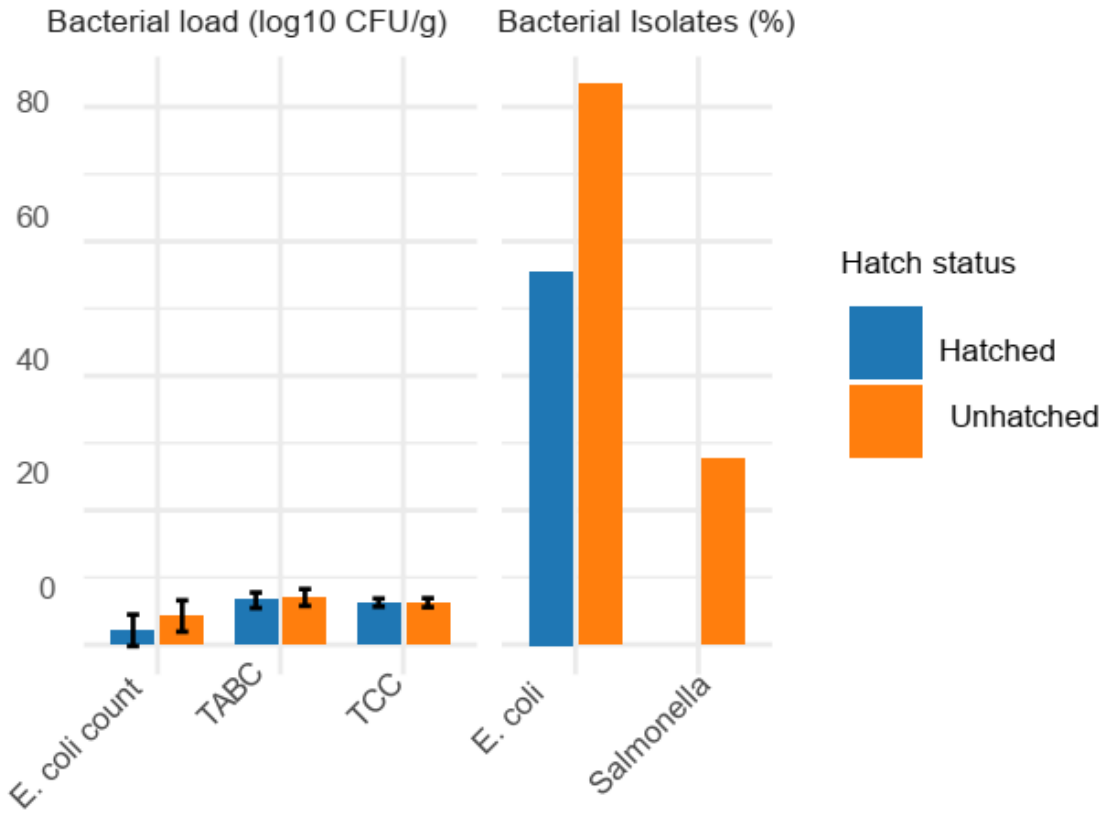

Figure 7: Bacterial load and isolates rate in eggshell powder based on hatch status

When bacterial loads of eggshell powder compared based on breed of origins, the results did not reveal any statistically significant differences in TABC, TCC, or *E. coli* count (Table 3). Despite the numerical differences, none reached statistical significance. When comparing prevalence of *E. coli* on eggshell from layer versus broiler breeds the result indicated higher prevalence in broiler (83.3%) than in layer breeds (62.5%). However, this variation also proved to be statistically nonsignificant ($p = 0.35$). Similarly, no significant difference was observed in *Salmonella* occurrence between layer (2.5%) and broiler (16.6%) eggshell breeds ($p = 1$). This suggests that breed-related factors may not have a significant role to play in Salmonella contamination either.

4.1.5. Comparative analysis of bacterial counts and isolate rates in eggshell powder before and after preliminary processing

Comparative analysis of bacterial load in eggshell powder before and after preliminary processing (washing, drying and grinding) shows radical alterations in all parameters determined (Table 3). All measured bacterial load count (TABC, and TCC and *E. coli* decreased significantly after processing, with the reduction being statistically significant ($p < 0.001$). Similarly, the *E. coli* frequency was sharply decreased after preliminary processing, which was extremely significant ($p < 0.001$). Salmonella isolate was also shows decrease from 13.8% (5of 36) before preliminary processing to complete absence after preliminary processing, even though statistically not significant as shown in the Table 3.

Table 3: Bacterial load and isolate comparison in eggshell powder by hatch status, breed type and processing stages.

Parameters	**Bacterial loads (Mean + SD, log_{10} CFU/g)**			**Bacterial isolates (%)**	
	TABC	TCC	*E. coli* count	*E. coli*	*Salmonella*
Hatch status					
Hatched	6.58 ± 1.15	6.24 ± 0.60	2.11 ± 2.34	10/18(55.5%)	0/18(0.00)
Unhatched	7.00 ± 1.25	6.22 ±0.67	4.24 ± 2.33	15/18(83.3%)	5/18(27.7)
P-value	**0.17**	**0.94**	**0.01**	**0.15**	**0.04**
Chicken Breed					
Layer	6.66 ± 1.19	6.16 ± 0.58	3.05 ± 2.74	62.5	12.5
Broiler	7.10 ± 1.23	6.36 ± 0.72	3.43 ± 2.20	83.3	16.6
P-value	**0.20**	**0.30**	**0.54**	**0.26**	**1.0**
Processing stages					
Unprocessed	6.81 ± 1.21	6.23 ± 0.63	3.20 ± 2.54	69.4	13.8
Processed	5.32 ± 1.18	4.53 ± 1.54	1.36 ± 1.84	36.1	0.00
P value	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.07**

4.2. Mineral Analysis of Hatchery Eggshell Waste Powder

4.2.1. Overall mineral contents of eggshell powder

The study revealed that hatchery eggshell waste is the best natural sources of both major and minor minerals (Figure 8). Of the major mineral, Mg, P and S were very prominent in the hatchery eggshell waste but Na and K were relatively less abundant. Hatchery eggshell waste was an excellent of trace minerals such as Fe, Cu, B, Mo and Si. However, among the trace minerals iron was the most abundant in the hatchery eggshell waste.

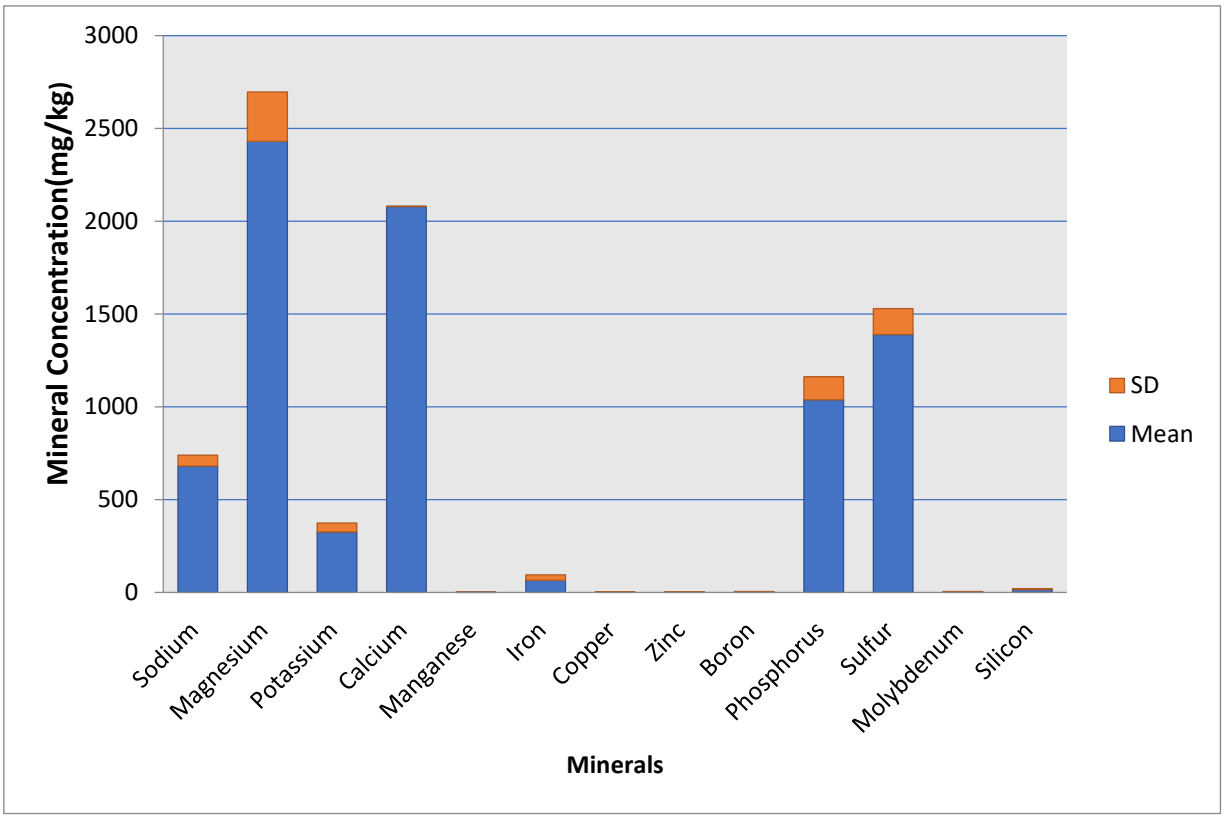

Figure 8: Overall mean and standard deviation of each analyzed mineral contents of eggshell powder.

4.2.2. Effect of incubation (hatching) on the eggshell mineral contents

Figure 9 demonstrates the impact of incubation on eggshell mineral ion content, revealing significant variations in mineral content between eggshells of hatched and unhatched egg

sources. Minerals like sodium, iron, zinc and phosphorus varied significantly ($P < 0.05$), with unhatched eggshell having the highest concentration (Table 4). The mean values of magnesium, calcium, iron, boron, sulfur, manganese, and molybdenum from eggshell of unhatched egg source had also slightly higher values compare to eggshell from hatch. The study found that unhatched eggshells have a higher mean concentration of Na, Fe, Zn, and P than hatched eggshells, while there is no significant difference in mineral content of Mg, K, Ca, Mn, Cu, B, S, and Mo. The study reveals that embryonic mobilization of certain minerals from eggshells reduces certain mineral ion contents in hatched eggshells. Depending on the mineral ion content and concentration, the proportion of mineral that embryos mobilize from the eggshell might vary significantly. For example, the percentage of Na, Mg, Ca, Mn, Fe, Zn, B, P, S, and Mo were reduced by approximately 9.83, 3.85, 0.006, 6.90, 39.72, 27.45, 13.18, 13.37, 1.56 and 3.7% in hatched eggshells, respectively as compared to unhatched eggshell. Among the major and minor minerals, Na and Fe were the most mineral that embryos mobilize from the eggshell.

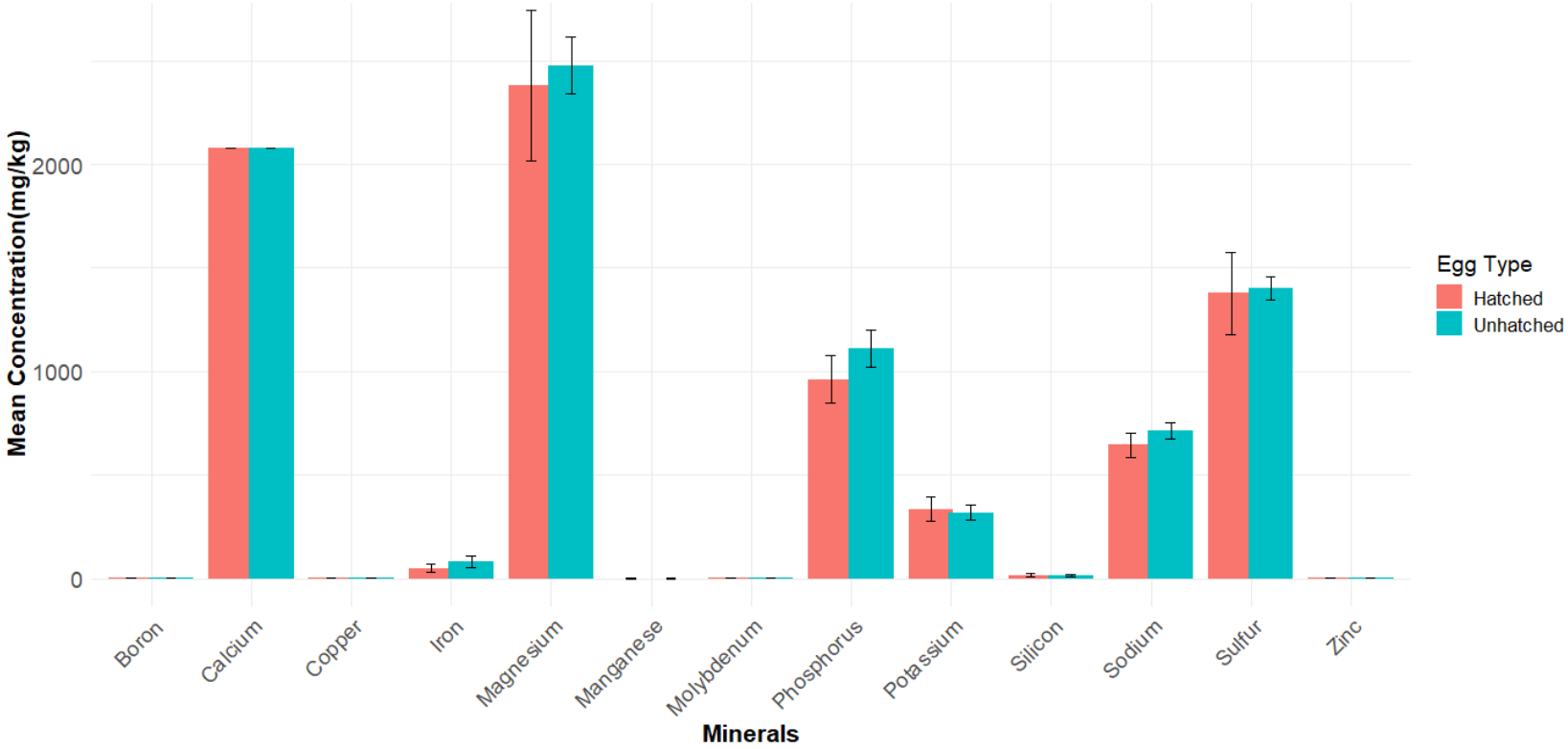

Figure 9: Comparison of Mineral Contents of Eggshells Sourced from Hatched and Unhatched Eggs

4.2.3. Comparative analysis of mineral contents in eggshell powder based on breed and hatchery plants

Regarding breeds from which eggshell are sourced the mineral content of eggshell differed between layer and broiler breeds. Of all minerals only potassium and manganese content showed a statistically significant difference ($P < 0.05$), with broiler eggshells containing higher concentration than layer eggshells (Table 4). Sodium, zinc, boron, copper and iron were also higher in broiler breeds compared to layer while calcium nearly the same in both. molybdenum and silicon levels remained almost identical between breeds.

The mineral content in eggshell powder showed variations among four hatchery plants of the study area. Most of the mineral except calcium, zink, boron and silicon content varied significantly ($P < 0.005$) among hatchery plants (Table 4). Eggshells from hatchery A contain the greatest mean concentration of sodium, magnesium, iron, and silicon while eggshells from hatchery B shows greatest concentration of potassium, copper, phosphorus, and sulfur. Hatchery C contains the greatest amount of molybdenum while hatchery D has the least mineral contents in all of the elements as shown in table 4. Generally, Hatchery A and B had higher total mineral content, while hatchery D has lower values with greater variation in some mineral.

Table 4: Comparison of mineral contents of eggshell based on hatch status, chicken breeds and hatcheries

Factors		**Mineral contents of eggshell in mg/kg**												
	Parameter	**Na**	**Mg**	**K**	**Ca**	**Mn**	**Fe**	**Cu**	**Zn**	**B**	**P**	**S**	**Mo**	**Si**
Egg Hatch Status														
Hatched	Mean	645.07	2382.09	333.99	2078.03	0.54	49.48	2.34	1.85	2.57	962.59	1378.3	2.86	16.5
	SEM	23.623	148.58	24.19	0.45	0.06	7.90	0.20	0.21	0.18	46.21	81.13	0.07	2.75
Unhatched	Mean	715.36	2477.44	317.50	2078.16	0.58	82.09	2.07	2.55	2.96	1111.13	1400.0	2.97	15.4
	SEM	15.92	55.42	14.57	0.18	0.03	11.95	0.08	0.18	0.19	37.23	22.82	0.04	1.54
	p value	**0.03**	**0.87**	**0.57**	**0.79**	**0.53**	**0.046**	**0.24**	**0.03**	**0.17**	**0.03**	**0.80**	**0.25**	**0.73**
Reduction in mg/ Kg		70.29	95.35		0.13	0.04	32.61		0.7	0.39	148.54	21.78	0.11	
% of Reduction		9.83	3.85		0.006	6.90	39.72		27.45	13.18	13.37	1.56	3.70	
Chicken Breeds														
Layer	Mean	673.4	2336.87	297.62	2078.01	0.50	57.67	2.08	2.16	2.72	1039.60	1351.8	2.91	15.9
	SEM	24.00	100.51	8.63	0.32	0.034	9.33	0.11	0.24	0.20	54.10	54.39	0.06	2.29
Broiler	Mean	693.7	2615.55	382	2078.26	0.68	82.02	2.47	2.27	2.86	1031.37	1463.9	2.93	15.9
	SEM	22.10	27.11	11.46	0.30	0.02	15.53	0.22	0.17	0.14	25.45	35.80	0.05	0.87
	P value	**0.60**	**0.08**	**<0.005**	**0.64**	**0.007**	**0.18**	**0.09**	**1.0**	**0.65**	**0.92**	**1.8**	**0.87**	**0.99**

Table 4. “Continued”: Comparison of mineral contents of eggshell based on hatcheries

Factors		**Mineral contents of eggshell in mg/kg**												
	Parameter	**Na**	**Mg**	**K**	**Ca**	**Mn**	**Fe**	**Cu**	**Zn**	**B**	**P**	**S**	**Mo**	**Si**
Hatchery plants														
Hatchery A	Mean	730.18	2641.56	362.68	2078.06	0.64	108.90	2.14	2.07	2.99	1010.21	1446.1	2.93	16.9
	SEM	14.16	46.25	6.39	0.30	0.02	0.81	0.07	0.21	0.17	17.74	25.35	0.04	1.43
Hatchery B	Mean	657.16	2589.55	401.33	2078.46	0.71	55.14	2.8	2.48	2.74	1052.53	1481.7	2.94	15.0
	SEM	8.02	30.30	0.14	0.62	0.02	1.07	0.23	0.24	0.26	51.73	80.08	0.11	0.92
Hatchery C	Mean	724.7	2536.39	279.81	2078.46	0.57	79.69	2.35	2.27	2.66	1071.03	1446.4	2.96	21.6
	SEM	14.32	62.6	6.95	0.41	0.03	6.97	0.06	0.35	0.15	35.39	45.74	0.07	1.77
Hatchery D	Mean	622.3	2137.36	315.42	2077.57	0.44	35.64	1.80	2.05	2.78	1008.17	1257.3	2.86	10.3
	SEM	27.01	129.20	9.37	0.44	0.05	5.87	0.0341	0.38	0.40	108.38	75.85	0.11	0.31
	P value	**0.02**	**0.02**	**< 0.005**	**0.46**	**< 0.005**	**<0.005**	**<0.005**	**0.86**	**0.91**	**0.02**	**0.02**	**< 0.005**	**0.46**

5. DISCUSSION

An egg is a hard-shelled reproductive body produced by birds, particularly the common domestic chicken, and its contents are consumed as food worldwide. While most freshly laid eggs are sterile, their shells can quickly become contaminated with litter, droppings, dust, and environmental factors (Smith *et al.,* 2000). The current study found that the level of bacterial contamination in eggshell powder samples from hatchery plants shown by TABC, TCC and *E.coli* indicates a high contamination level (Table 1).

These are more than the standard of Food and Drug Administration (FDA), which indicated 5×10^3 CFU/g for total count and 1 CFU/g for coliform count (Gilbert *et al.,* 2000). According to De Reu *et al*. (2008), bacterial count of eggshells below 5 $\log_{10}$ CFU/g is considered as acceptable for hygienic quality which was not the case in this study. According to Ferasyi *et al.,* (2018) the presence of coliforms on the eggshell indicates poor egg handling. As a result, eggshell meal or powder sourced from various hatchery facilities cannot be directly incorporated into animal feed as an ingredient; it necessitates prior treatment through a range of methods.

The present results are consistent with Zeweil *et al*., (2015), who shows total aerobic mesophilic bacteria, varying between 4.0 and 7.0 log10 CFU per clean egg. Similarly, Eman and Saad (2015) reported high TABC (log 6.7±1.07 CFU/ml) and TCC (log 7.41±5.22 CFU/ml) respectively from leaking chicken table eggs. Chaemsanit *et al.* (2015) also reported higher total aerobic count on an eggshell, which ranges from 2.9 to 6.2 log10 CFU/ml in the market layer whereas 7.2 to 8.0 log CFU/mL in farm layers samples.

On the contrary, Mebkhout *et al.* (2022) reported low total bacteria count by the rapid method (2.95 ± 1.06 Log CFU/cm²) and by the classical method (2.85 ± 0.99 Log CFU/cm. Furthermore, the findings of Buhr *et al.,* (2009) reported 1.1 log10CFU/mL coliforms of rinsate of eggs, which is much lower than the present finding. In the same way, the current *E.coli* count result is slightly lower than the result of previous studies by Karlsson, (2010) who reported highest *E. coli* colony count (mean $\log_{10}$ 3.13 CFU/cm²) on eggshells from floor systems. These variations might be due to differences in management practices, environmental

conditions, and sanitation protocols in the egg collection area and the overall hygiene of the farm, all of which affect bacterial contamination of the eggs (Hafez *et al.*, 2007).

According to available standards referenced by FAO and relevant standards, the preferred *E. coli* count in animal feeds is less than 10 colony-forming units per gram (CFU/g), and counts above 50 CFU/g are not recommended and are considered high. Generally, the excessive coliform counts in hatched and unhatched eggshell powders originated from different hatchery plants found in current study indicated the unhygienic conditions on the hatchery plants.

In terms of bacterial isolates, 25 out of 36 samples (69%) were positive for *E. coli*, the most prevalent organism in this research. Unlike *E.coli, Salmonella* was found in only five of the 36 samples (13.8%), indicating less presence in the examined eggshell powder samples relatively. However, Kapena *et al.* (2020) reported a lower prevalence of *Salmonella* (2.31%) than the present study, while Chousalkar *et al.* (2010) and Mahdavi *et al.* (2012) did not find any *Salmonella* on the eggshells. The higher incidence of Salmonella detected in previous study compared to current research could be attributed to environmental contamination and insufficient cleaning and disinfection of equipment for egg handling and storage (Davies and Breslin, 2003).

Comparison between the mean of TABC, TCC and *Escherichia coli* counts in eggshell samples of four hatchery plants indicated variations among hatcheries (Table 2). However, none of them were significantly associated with hatchery plants. The non-significance shows bacterial contamination is common in all hatchery plants. However, the study by Oviasogie *et al*. (2016) reported significant variation in the number of bacterial colonies from eggshells collected from various sites. The researchers indicated that the bacterial count of free-range chicken eggshells ranged from 9.7 ± 0.7 x 104 to 1.27 ± 0.2 x 105, whereas battery cage eggshells had a bacterial count ranging from 3.3 ± 0.8 x 104 to 7.4 ± 0.5 x 104. The research suggested that bacterial surface loads on eggshells are established based on environmental conditions in a hatchery, sanitation protocol, and egg-handling practices. Generally, the contamination level of *E. coli* depends strongly on factors including the diet of the laying hens, the environmental conditions of the egg during and after laying, and the bacterial charge of the air which is consistent to study reported by De Reu *et al*. (2005).

The results of this study on *E. coli* frequency among hatcheries indicated that, despite the apparent differences in contamination rates, there is insufficient evidence to conclude that the hatchery of origin has a measurable impact on *E. coli* presence in eggshell meal. In accordance with the present results, Yousef *et al.* (2023) reported highest rate of *E. coli* in the manual hatcheries (57.1%), then semiautomatic types (43.8%), and automatic types (33.3%); however, the differences were not significant. In the present finding, the overall frequency of *Salmonella* in four hatcheries was ranging from 0.0% to 33.3%. However, the variations was found no considerable difference among the hatcheries which suggests comparable sanitation practice among hatcheries ($p = 0.39$). Conversely, extensive research work by Oastler *et al.* (2022) on broiler hatcheries found *Salmonella* prevalence ranged significantly from 0% to 33.5% among hatcheries.

Overall, 78% of the hatcheries had at least one *Salmonella* serovar, and some had multiple serovars. The study highlighted some hatcheries being more affected than the others are. Another study by Xu, *et al.* (2020) who investigated *Salmonella* occurrence in hatcheries of different sizes indicated statistically significant differences: medium-scale hatcheries had a Salmonella occurrence of 27.52%, large-scale hatcheries had 11.11%, while small-scale hatcheries had no detection of *Salmonella*. It revealed difference in frequency among hatchery scales was highly significant. Differences in significance of Salmonella prevalence among hatcheries may be attributed to difference in size, application of biosecurity control or degree of contamination in hatchery environment.

By comparison, the overall low frequency of *Salmonella* (13.8%) in this study than *E. coli* (69%) primarily due to its high shedding rate, environmental persistence. However, *Salmonella* must possess specific conditions (e.g., temperature, humidity) to exist and develop on eggshells, which may be less frequent (Messens *et al.*, 2005). This finding is consistent with the study of Assefa *et al.* (2011) who reported a general prevalence of 11.5% in Kombolcha, Ethiopia and with Tadesse and Ali (2024) who reported 19.1% overall prevalence of *Salmonella*. However, Tessema *et al.* (2017) had studied at Haramaya University Poultry Farm and reported a combined *Salmonella* prevalence of 2.9% in eggs and 2.3% on eggshells, which is far less compared to the present study. The differences in Salmonella prevalence

attributed to the differences in the management systems used, the housing types, and the biosecurity measures employed, despite contamination being possible even under environmental and handling conditions (Abayneh *et al*., 2023).

The current study analyzed bacterial load of eggshell powder in terms of egg status (unhatched or hatched) and breed of origin (layer or broiler) which presented in Table 3. The unhatched eggs had a higher TABC and TCC compared to eggs that had hatched, though the difference was not significant. The *E. coli* was greater in unhatched samples but was not significantly different ($p = 0.15$). For Salmonella, a difference was significant: it was not present in hatched eggshells but found in the unhatched samples ($p = 0.04$).

This demonstrated that compared to hatched eggs, eggshells taken from unhatched eggs are more vulnerable to bacterial contamination. The high moisture and nutrient content of unhatched eggs, which are crack-free and have not lost their native substance, may be the cause of this. According to Lipdo *et al.* (2024), dead-in-shell embryos should be handled and processed carefully to prevent deteriorating because of their high moisture content, particularly if they are used in organic fertilizers or animal feed. Likewise, previous research has highlighted how crucial moisture control is, to preserve hatchery waste's quality and prevent deterioration (Kim and Park, 2021).

The increase in the number of *E. coli* and *Salmonella* percentage on unhatched eggshells indicates their involvement in hatch failure. *E. coli* can penetrate the eggshell; infect the embryo, and cause mortality or stunted chicks, reducing hatchability (A EL-Sawah *et al*., 2016). Although overall bacterial contamination would not be quite dissimilar, the presence of *E. coli* is a determining factor due to unhatched eggs. This means *E. coli* infection is more directly associated with embryonic infection and hatch failure than with overall bacterial load. The finding of the present study is in consistent with research of Kowshik *et al*, (2024) who reported that unhatched eggs include a uniform microstructure but are permeable by bacteria and contaminated. Similarly, Mousa-Balabel *et al*. (2017) stated that unhatched eggshells contain higher bacterial counts, which are more dangerous to hatchability and chick quality if not sanitized in due time.

Furthermore, Punom *et al.* (2020) determined that *E. coli* and *Salmonella* are responsible for embryonic death and low hatchability, which supports the findings of the current study. They reported a 56% rate of occurrence of *E. coli* in the inner contents and 53% on surfaces of Bangladesh mini-hatchery unhatched duck eggs. They also reported a prevalence of 59% *Salmonella spp.* on the surfaces and 56% in the inner contents of the unhatched duck eggs. Further, Hameed *et al*. (2014) conducted a study to assess the impact of *Salmonella* on hatchability and fertility of layer hens and found that hatchability is decreased due to the existence of *Salmonella* infection in the reproductive system of the hen. Similarly, Gast and Porter (2020) clarified that incubation of eggs infected with *Salmonella* leads to increased microbial growth.

When bacterial loads of eggshell powder compared based on breed of origins, the results did not reveal any statistically significant differences in TABC, TCC, or E. coli count (Table 3). The study implies that breed or genetic variations in eggshell powder are less prevailing or other factors like housing systems, hen age, and egg handling practices, which can indirectly reflect breed-specific impacts in bacterial loads, are more significant than breed itself. Finding of Protais *et al.* (2003) and De Reu *et al.* (2005) also found that eggshell contamination is influenced by housing system that shows higher mesophilic aerobic bacteria in alternative systems compared to conventional and furnished cages. Generally, the findings suggest that management factors (housing, hygiene) significantly influence bacterial loads on eggshells, which may indirectly relate to breed-specific practices (Huneau-Salaün, *et al.*, 2010).

The results of the present finding clearly indicate that eggshell preliminary processing such as (washing, drying, and grinding) influences the bacterial count of the resulting eggshell meal immensely (Table 3). The technique used to treat the hatchery waste eggshell reduced the microbial load to the safe or acceptable standard (5 log CFU). Notably, there was a significant decrease in TABC, TCC and *E. coli* count following preliminary processing. The frequency of *E. coli* and *Salmonella* in eggshell powder also shows considerably reduction (Table 3), although the latter reduction was not statistically significant ($p = 0.07$). These findings show that the utilized procedures considerably reduced the bacterial contamination. The results of

the current research are in agreement with those reported by Braun *et al.* (2005 who recorded a remarkable reduction of the shell germ account by washing process in about 3-log.

Furthermore, Hutchison *et al.* (2004) research reported more than 5-log reduction of Salmonella of artificially contaminated eggs by spray jet washing. Bell (2002) further discovered that washing and sanitizing eggs under optimal conditions can reduce microbial load by 2-3 log CFU/egg. Emagnaw (2018) similarly started, that drying is a standard practice to remove water, which could aid bacterial growth after washing, and grinding eggshells into meal still reduces microbial contamination, demonstrating the effectiveness of these combined steps in ensuring hygienic quality. Similar with the current study, Ezz, (2023), described eggshell washing, oven drying at about 70°C, and grinding as processes involved in eggshell powder preparation that was effective in preventing microbial contamination, including pathogens like *E. coli* and *Salmonella*. Generally, the study confirmed that preliminary processing of the eggshells significantly reduces the bacterial load of eggshell meal thereby making it more microbiologically safe for use. This aligns with a European Union report that characterizes eggshells as a low-risk material that may be properly recycled and used again in other applications that reported by Pagonis *et al*. (2024).

The present study revealed that hatchery waste eggshell powder is abundant in magnesium and calcium (Figure 8). The large amount of Ca and Mg in relation to other minerals in this research supports a previous discovery by Ajala *et al.* (2018) who found that eggshells typically have 2300.33 mg/L (equivalent to 2300.33 mg/kg) of Ca and 850.00 mg/L (equivalent to 850.00 mg/kg) of Mg. This is in line with current finding that eggshells are a natural source of magnesium and calcium.

Likewise, Halgrain *et al.* (2022) noted that eggshell is the main source of Ca, Mg, and S. Similar to this finding the results of Schaafsma *et al.* (2000) indicated that major elements in Slovakian chicken eggshell powder are Ca and Mg. However, the Ca concentration (401 ± 7.2 mg/g) reported was much higher than present results. Hassan (2015) had also reported the highest value of Ca (35080mg/100g) in eggshell powder. In the same way, this study noted much lower calcium content, which is much lower than values reported by Urjintseren *et al.* (2024), who found calcium concentrations ranges from 72.6% to 85.7%, in eggshell analyzed

without grinding. These differences in mineral content may result from variations in hen breed, layer diet, and environmental conditions, as well as differences in sample preparation and analytical techniques.

The current finding of calcium concentration was higher than the finding of Adeyeye, (2009) who reported 61.9 mg/100 g, 42.3 mg/100 g and 50.1 mg/100 g which is equal to 619 mg/kg, 423 mg/kg and 501 mg/kg respectively from egg shells of three bird *spp* (francolin, duck and turkey respectively. These differences might be due to differences in species of bird on which study conducted or differences in sample preparation and analytical techniques.

Although Ca content is lower than many reports indicated above, the magnesium content in the present samples was notably higher than the report of Ajala *et al*. (2018) and Adeyeye, (2009). Regarding Ca and Mg relationship, Skrivan *et al*. (2016) explained that calcium Ca and Mg have an antagonistic relationship towards eggshell formation. An increase in dietary Mg can inhibit Ca concentration in eggshells but improve overall shell quality. For the other minerals, for instance, potassium, sodium, phosphorus, and sulfur, the present finding results generally conform to the reports of Schaafsma *et al.,* 2000 and Urjintseren *et al*. (2024), who also reported comparable concentrations in eggshells of various bird species.

As indicated in figure 8 trace minerals like manganese, iron, copper, and zinc were found in fairly low concentrations, which correspond with those of Drabik *et al*. (2021) and Schaafsma *et al.*, 2000). However, minerals like boron, molybdenum, and silicon levels were higher than some of the previous researchs, and this may reflect regional dietary differences. The variation in the mineral content of eggshells in the aforementioned studies was largely dependent on dietary supplementation in birds (Brodacki *et al*. 2018). Overall, while the mineral makeup of eggshells within this study conforms to the literature, there are considerable discrepancies in calcium, magnesium and certain trace elements that indicate the influence of local variables and are worth pursuing further.

The eggshells mineral contents of Na, Mg, Ca, Mn, Fe, Zn, B, P, S, and Mo were decreased for hatched eggshells as compared with unhatched eggshells (Table 4). Proportional amounts of minerals mobilized by embryos from the eggshell may vary considerably among species

and among populations of the same species (Wang *et al.*, 2014). In present finding, the mineral content of eggshell powder varied noticeably between hatched and unhatched egg sources. Minerals like sodium, iron, zinc, and phosphorus varied significantly ($P < 0.05$), with unhatched eggshell having the highest concentration. The higher concentration of minerals of eggshell powder from unhatched eggshells was also observed in certain minerals like, magnesium, sulfur, and molybdenum compared to eggshells that sampled from hatched eggs. This finding is in agreement with Hincke *et al*. (2012), who reported that unhatched eggshells typically contain more minerals due to reduced mobilization of minerals in embryonic development. The research established that the development of chick mobilizes shell minerals, especially towards the later stages of incubation periods.

In present finding is consistent with the study reported by Ji *et al*. (1997) who noted the mobilization of calcium by embryo nearly by 14% for hatchling eggshells. Dennis *et al*. (1996) and Chien *et al.* (2008) also strengthen this in their study report of mobilization of calcium for skeletal mineralization during the embryonic development. According to the study of Halgrain *et al.* (2022), the transfer of calcium from the eggshell to the embryo is mediated by a 3-layered structure, namely the chorioallantoic membrane (CAM). The chorionic epithelium of the CAM lines the eggshell membranes and is involved both in the solubilization of calcium from the inner eggshell, and in the transfer of solubilized ions to the embryo via its capillary network (Halgrain *et al.,* 2022). Besides, Wang *et al.* (2014) noted that the mean percentage of ash was significantly higher in unhatched eggshells (24.6%) than in hatched eggshells (22.3%). Thus, this study evidenced the embryonic mobilization of minerals from the eggshell.

With the current finding, the mineral composition between layer and broiler breeds varied with broiler eggshells showing a tendency towards elevated mineral content. This significant difference in mineral content of broiler and layer chicken breed eggshell might be due to the difference in the rearing system of hens and feed types which is consistent with result reported by Abrha and Asefa (2022). The study by Tumova *et al.* (2014) found that broiler breeder egg shells contained significantly more P ($P = 0.004$) and Mg ($P = 0.001$) than those from laying hens which is in line with the current finding. This may be due to differences in the duration of egg formation, the rate of mineral transfer from blood to shell gland, and the efficiency of

mineral utilization. Broiler breeders may deposit more minerals in the shell because of their larger body size and different metabolic priorities.

Broiler breeders and layers are often fed diets formulated for their specific production needs, which can influence the availability and deposition of minerals in the eggshell. Broiler breeders may receive diets with higher mineral content to support both reproductive and growth demands, contributing to higher mineral deposition in the shell. Beside, Grower (2024) found that nutrition and management play a significant than genetics on eggshell mineral contents, which indirectly contributes to increased mineral concentration in eggshells of broilers over layers. Furthermore, Abrha and Asefa (2022) observed that the local breed's egg powder had significantly different levels of iron, zinc, and manganese compared to the exotic breed, with the local breed's egg powder having significantly higher levels of these elements than the exotic breed, while the local and exotic egg types did not significantly differ in copper content.

The present study found variations in mineral concentration among four hatchery plants, with Hatchery A having high levels of sodium, magnesium, and iron, and Hatchery D having the lowest levels. These differences likely explain variations in management regimes, feed composition, chicken breeds that different hatchery plants maintain and other environmental parameters. Variations in the mineral content of eggshells, such as potassium, iron, and copper, are often reflective of the hens dietary intake. The research conducted by Shao *et al.* (2025) has also supported this idea by revealing that feeding patterns and phosphorus levels significantly interacted with egg production ratios, soft shell and broken egg ratios. Similarly, in current study, higher potassium levels in Hatchery B compared to Hatchery C could be due to differences in feed formulations. Notably, Stefanello *et al.* (2014) has shown that supplementing hens' diets with trace minerals such as manganese, zinc, and copper from inorganic or organic sources affects eggshell quality and mineral deposition. Additionally, housing systems influence eggshell characteristics that could contribute to the variations in mineral content across different hatcheries. Sekeroglu *et al.* (2007) has supported this idea by revealing that the eggs of the deep litter system were the higher quality, when the egg shell colour and the mineral elements consider together.

Generally, the high concentration of both major and minor mineral contents enable it to use as an alternative source of mineral supplement for animals and humans if pathogenic microorganisms present in eggshell can be eliminated by proper processing. Quail eggshells have higher calcium contents, which implies that they could be a better source of this necessary mineral for agricultural and nutritional purposes. Olawale *et al*. (2021) highlighted the significance of calcium concentration in assessing the commercial viability of eggshell products, especially for human and animal use.

6. CONCLUSION AND RECOMMENDATIONS

The present study revealed that eggshell powder recovered from hatchery by-products, particularly when produced from unhatched eggs, was highly contaminated with bacteria including *E. coli* and *Salmonella spp,* which could cause environmental and health issues. However, preliminary treatment of eggshell with washing, drying and grinding was capable of significantly minimizing bacterial contamination, particularly in eliminating major pathogen risks such as Salmonella, which is a major safety concern in animal feed. Although *E. coli* and coliform were not totally eliminated, their diminution to very low levels suggest that by further thermal treatment or other sanitary steps, microbial safety can be improved further. Mineral analysis confirmed that eggshell powder of the study area rich in several minerals particularly when derived from unhatched eggs. The analyses indicated relatively high magnesium and calcium contents compared to other minerals and trace elements. However, the calcium content observed was somewhat lower than values reported in previous literature, which suggests that, it can be extracted further by different processing methods. Therefore, eggshell from hatchery waste can offer a valuable mineral supplement that can be recycled safely and effectively as alternative source of mineral-enriched green feed ingredients of animals.

Based on the results of this study, the following recommendations are made:

- Additional thermal treatment or chemical sanitization steps should be used to eliminate pathogenic microorganisms in eggshells before incorporating them into animal feed.
- Hatcheries and feed producers should integrate eggshell recycling into their waste management and production systems to promote sustainability and resource efficiency.
- Government and regulatory agencies should establish microbiological and chemical standards for eggshells and other recycled hatchery by-products.
- Future research should focus on optimizing treatment methods and assessing the long-term effect and benefits of this resource in animal feed applications.

7. REFERENCES

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8. APPENDICES

Appendix 1: Summary of Biochemical Test Results of *Escherichia coli* and *Salmonella*

Test	**E. coli (Result & Color)**	**Salmonella (Result & Color)**	**Remarks**
Indole	Positive (+), Red ring	Negative (−), No color change	Indole production
Methyl Red (MR)	Positive (+), Red	Positive (+), Red	Mixed acid fermentation
Voges-Proskauer (VP)	Negative (−), No red color	Negative (−), No red color	Acetoin production test
Citrate Utilization	Negative (−), Green	Positive (+), Blue	Utilization of citrate as sole carbon source
TSI	A/A, Gas (+), No H_2S – Yellow slant/butt	K/A, H_2S (+), Gas (±) – Red slant, yellow butt, black precipitate	Fermentation and H_2S production
Urease	Negative (−), Yellow/No change	Negative (−), Yellow/No change	Urease enzyme production
Motility	Positive (+), Diffuse growth	Positive (+), Diffuse growth	Flagellar movement

Legend:

- A/A = Acid slant / Acid butt (glucose, lactose/sucrose fermentation)
- K/A = Alkaline slant / Acid butt (only glucose fermentation)
- H_2S = Hydrogen sulfide production
- Gas = Gas production
- (+) = Positive result
- (−) = Negative result

Appendix 2: PCR Protocol for Detection of *E. coli* and *Salmonella spp.*

I. DNA extraction

DNA extraction was performed using a Qiagen extraction kit following the manufacturer's guidelines. Bacterial isolates were grown overnight in nutrient agar (HIMEDIA, India) at 37 °C. A loop full of the colonies was added to 100 µl of sterile water. A 200 µL bacterial

suspension was mixed with 20 µL of proteinase K and 200 µL of lysis buffer, vortexed for 15 seconds, and incubated at 56 °C for 10 minutes. After incubation, 200 µL of 96% ethanol was added, and the mixture was homogenized. The 620 µL suspension was transferred to a mini spin column and centrifuged at 8,000 rpm for 1 minute. The flow-through was discarded and 500 µL of AW1 (Aqueous Wash 1) buffer was added, followed by centrifugation at 8,000 rpm for 1 minute. After discarding the flow-through, 500 µL of AW2 wash buffer was added, and the column was centrifuged at 14,000 rpm for 3 minutes. The mini spin column was then placed in a clean 2 mL collection tube and centrifuged at 14,000 rpm for 1 minute with the rotor cap open to dry the column matrix. Finally, the column was transferred to a labeled Eppendorf tube, and DNA was eluted by adding 50 µL of elution buffer and centrifuging at 8,000 rpm for 1 minute. The eluted DNA was stored at −20 °C until needed.

II. Conventional PCR Protocol for Salmonella universal identity test

1. Master Mix Preparation per sample

- 3 µl of RNase-free water was added.
- 2 µl of Forward Primer (Salmon-Fow, 5 pmol/µl) 5-ACTGGCGTTATCCCTTTCTCTGGTG-3’ was added.
- 5 µl of Reverse Primer (Salm-Rev, 5 pmol/µl) 5’ATGTTGTCCTGCCCCTGGTAAGAGA-3’ was added.
- 70 µl of iQ™ Supermix was added.
- 5 µl of DNA Template was added.

The total reaction volume was 22 µl.

2. PCR Reaction

- The initial denaturation was carried out at 94°C for 5 minutes 1 cycle.
- Denaturation was performed at 94°C for 30 seconds 30 cycles.
- Annealing was conducted at 56°C for 30 seconds for 30 cycles.
- Elongation was done at 72°C for 45 seconds 30 cycles .
- Final elongation was completed at 72°C for 5 minutes 1 cycle.
- The gel was stored at 4°C until the machine was turned off.

3. Agarose Gel Preparation and Electrophoresis

- A 2% agarose gel was prepared.
- with loading dye, 10 µl of PCR product, and 10 µl of DNA ladder 100bp (Himedia; India) were added.
- Electrophoresis was run for 1 hour at 120V.
- The result was read using UV light and documented using a gel documentation system (UVtec 08 100554).

III. PCR Protocol for Detection of *E. coli spp*.

1. Master Mix Preparation per sample:

- 6 µl of RNase-free water was added to the reaction mixture.
- 2 µl of Primer EVT1-Fow- 5pm/ µl 5CAAC ACTGGATGATC TCAG-3 was added to the reaction.
- 2 µl of Primer EVT2 - 5pm/ µl 5CCCCCTCAAC TGCTAATA -3 was added to the reaction.
- 10 µl of 1Q Super Mix was added to the reaction.
- 5 µl of DNA template was added to the reaction.
- The total reaction volume was adjusted to 25 µl.

2. Multiplex PCR Reaction:

- The PCR reaction was run under the following conditions:
    - Initial denaturation was performed at 95°C for 5 minutes for 1 cycle.
    - Denaturation was carried out at 95°C for 1 minute for 35 cycles.
    - Annealing was done at 55°C for 1 minute for 35 cycles.
    - Elongation was conducted at 72°C for 1 minute for 35 cycles.
    - Final elongation was performed at 72°C for 7 minutes 1 cycle.
    - The PCR tube was stored at 4°C until the machine was turned off.

3. **Agarose Gel Preparation and Electrophoresis:**

- A 2% agarose gel was prepared.
- 1 µl of GelRed with loading dye, 10 µl of PCR product, and 10 µl of DNA marker (ladder) 100bp (Himedia; India) were added to the respective wells.
- Electrophoresis was run for 1 hour at 120V.
- The result was read using UV light.
- A visible band around 349 bp indicated a positive result.

Appendix 3: Mineral composition analysis procedures

Dry Ash Procedure

Reagents

- 1 N hydrochloric acid - Dilute 83.3 ml conc. Hcl to 1 L Deionized H20.
- 6 N hydrochloric acid - Dilute 50 ml conc. Hcl to 100 ml deionizer H20.

Apparatus

1. Muffle furnace (german)
2. high form" porcelain crucibles
3. 100 ml volumetric flasks
4. 13 x 100 mm flint glass test tubes

Dry Ashing Principle

The decomposition of eggshell using high temperature to destroy the organic components in order to obtain a solution of inorganic ions contents. In this procedure, the eggshell material was calcinated in a muffle furnace, dissolved in 1 normal hydrochloric acid and filtered for the determination of heavy metals.

Procedure

1. 1.25 g of the sample Weighed into a "high form" porcelain crucible.

2. The crucible placed in a furnace and increased the temperature gradually until it reached 540°C.
3. The sample Ashed for 6 hours after reaching the target temperature.
4. The ash wetted with a small amount of distilled water, then 5–10 mL of 6 N HCl was added and the mixture brought to near dryness on a hot plate.
5. The ash dssolved by adding 10 mL of 1 N HCl to the crucible.
6. The dissolved ash transferred into a 100 mL volumetric flask.
7. The remaining sample was washed down in the crucible with distilled water.
8. The volume diluted to with distilled water and shook well to mix.
9. An aliquot of the solution placed into an ICP test tube.

Analysis of Aqueous Solutions by ICP-OES with radial plasma Observation

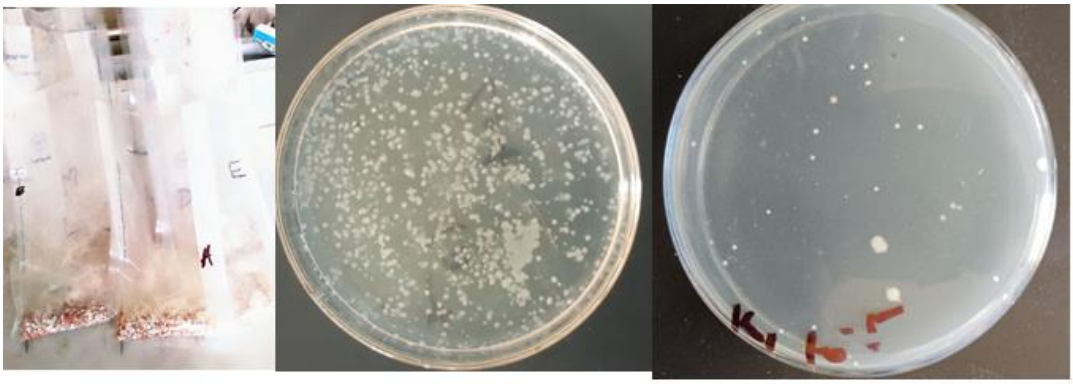
All measurement were performed using sector arcos optical emission spectrometer optimized with small volume and 32 linear CCD detectors the wave length range between 130-770 nm. Simultaneously the analyzed sample was nebulizer in to the argon gas field plasma and the energy excitement was proceed in the single spectra and the energy is expressed in intensity which is directly proportion to concentration ,the concentration is calculated on the linear graph of the standard Concentration and the Corresponding Intensities.

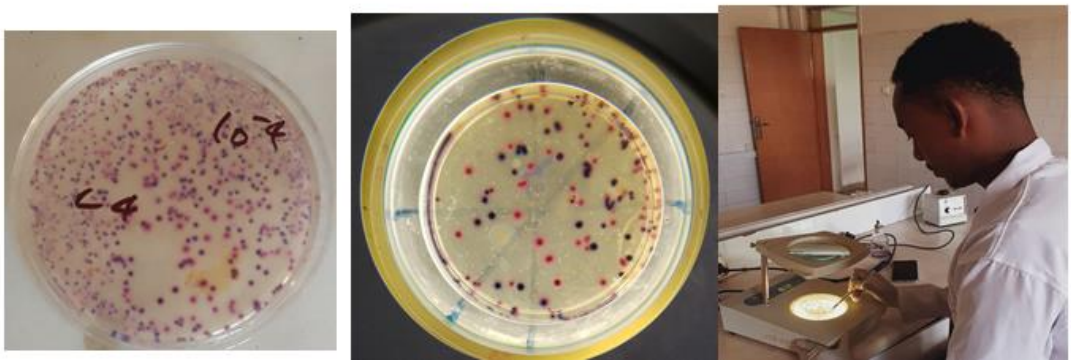
All the measuring conditions, like plasma power ,gas flows torch positions and measuring time are configure. According to the standard the calibration and standardization of the spectra method was performed but standardization is daily and it was a quick procedure for correct measuring intensities so that the correct concentration are obtained using the original calibration

Appendix 4: Pictures taken during thesis work

i) Eggshell sample collection from hatchery wastes

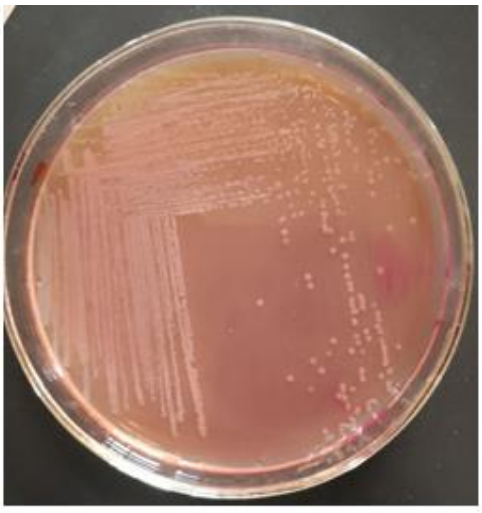
ii) Bacterial load Counts

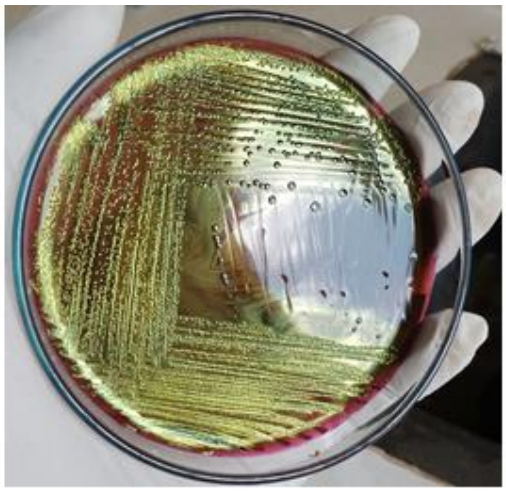




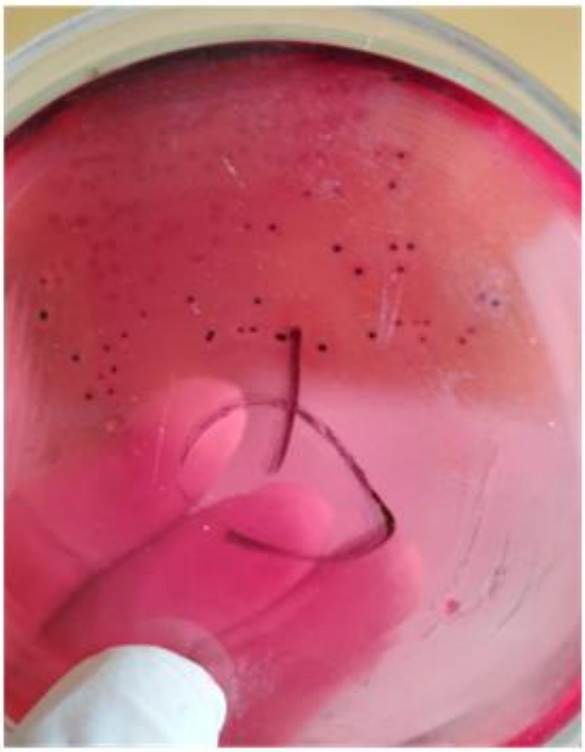
iii) Eggshell cleaning (washing), drying and grinding

iv) Isolation of E. coli and salmonella

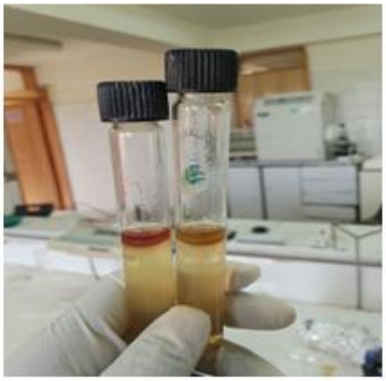
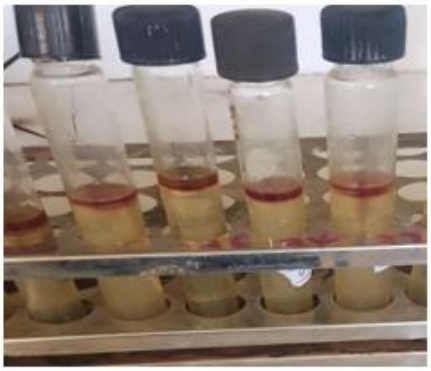




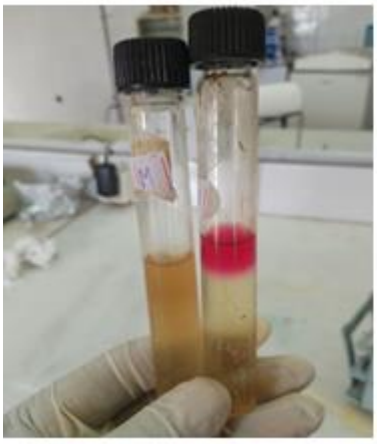
E.coli growth on MacConkey Agar *E.coli* growth on EMB Agar

Salmonella growth on XLD Agar

Indole test



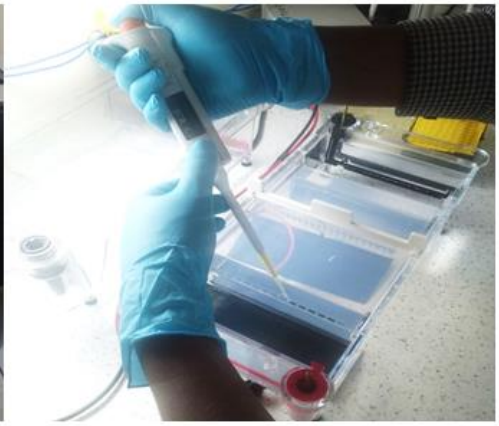
Methyl red test

Citrate Utilization Test

TSI Test

Motility test

PCR thermal cycling



Sample loading for electrophoresis

Analysis of Aqueous Solutions by ICP-OES with radial plasma Observation

Horticoop Ethiopia (Horticulture) PLC Soil,

Water and Plant Analysis Laboratory Laboratory

Test Report

Test Overview

Customer: AAU

Tel: +251 92 408 6631

Adress: Bishoftu

Country: Ethiopia

1

Information about sample			
Sampled By	Client	Order Number	25HEON-0080
Report Date	May 21, 2025	Received date	May 9, 2025
Location			

Lab Code	Description	Concentration	Na	Mg	K	Ca	Mn	Fe	Cu	Zn	B	P	S	Mo	Si
			mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
25H 1817	O	Conc.1	716.02	2595.31	356.29	2077.76	0.66	108.09	2.20	2.27	2.82	992.47	1420.72	2.89	18.28
25H 1817'	T.	Conc.2	744.34	2687.81	369.06	2078.35	0.62	109.70	2.07	1.86	3.15	1027.94	1471.41	2.96	15.43
25H 1818	D	Conc.1	579.19	1937.66	330.93	2076.92	0.39	25.74	1.84	1.31	1.99	827.11	1147.89	2.70	10.03
25H 1818'	H	Conc.2	571.97	1890.97	331.97	2076.91	0.36	25.46	1.73	1.49	2.20	822.85	1119.23	2.67	9.59
25H 1819	Q	Conc.1	732.19	2399.11	285.02	2077.97	0.61	93.25	2.26	2.84	2.45	1123.39	1356.87	2.91	18.61
25H 1819'	R	Conc.2	761.70	2461.60	295.85	2078.43	0.62	90.11	2.26	2.92	2.41	1140.22	1389.46	2.96	18.54
25H 1820	F	Conc.1	649.14	2559.25	401.19	2077.84	0.69	54.07	2.57	2.24	2.48	1000.80	1401.61	2.82	15.94
25H 1820'	G	Conc.2	665.18	2619.84	401.46	2079.08	0.73	56.21	3.03	2.71	3.00	1104.25	1561.76	3.03	14.11
25H 1821	B1	Conc.1	672.27	2370.96	302.83	2078.78	0.51	47.85	1.84	2.81	3.45	1249.30	1441.82	3.14	11.03
25H 1821'	D1	Conc.2	665.61	2349.83	295.94	2077.68	0.48	43.52	1.80	2.58	3.48	1133.45	1320.17	2.94	10.54
25H 1822	K1	Conc.1	707.35	2649.62	275.07	2079.62	0.57	68.89	2.50	1.65	3.06	1017.86	1559.11	3.14	24.40
25H 1822'	L1	Conc.2	697.59	2635.22	263.31	2077.80	0.49	66.51	2.39	1.67	2.71	1002.64	1480.19	2.82	24.98

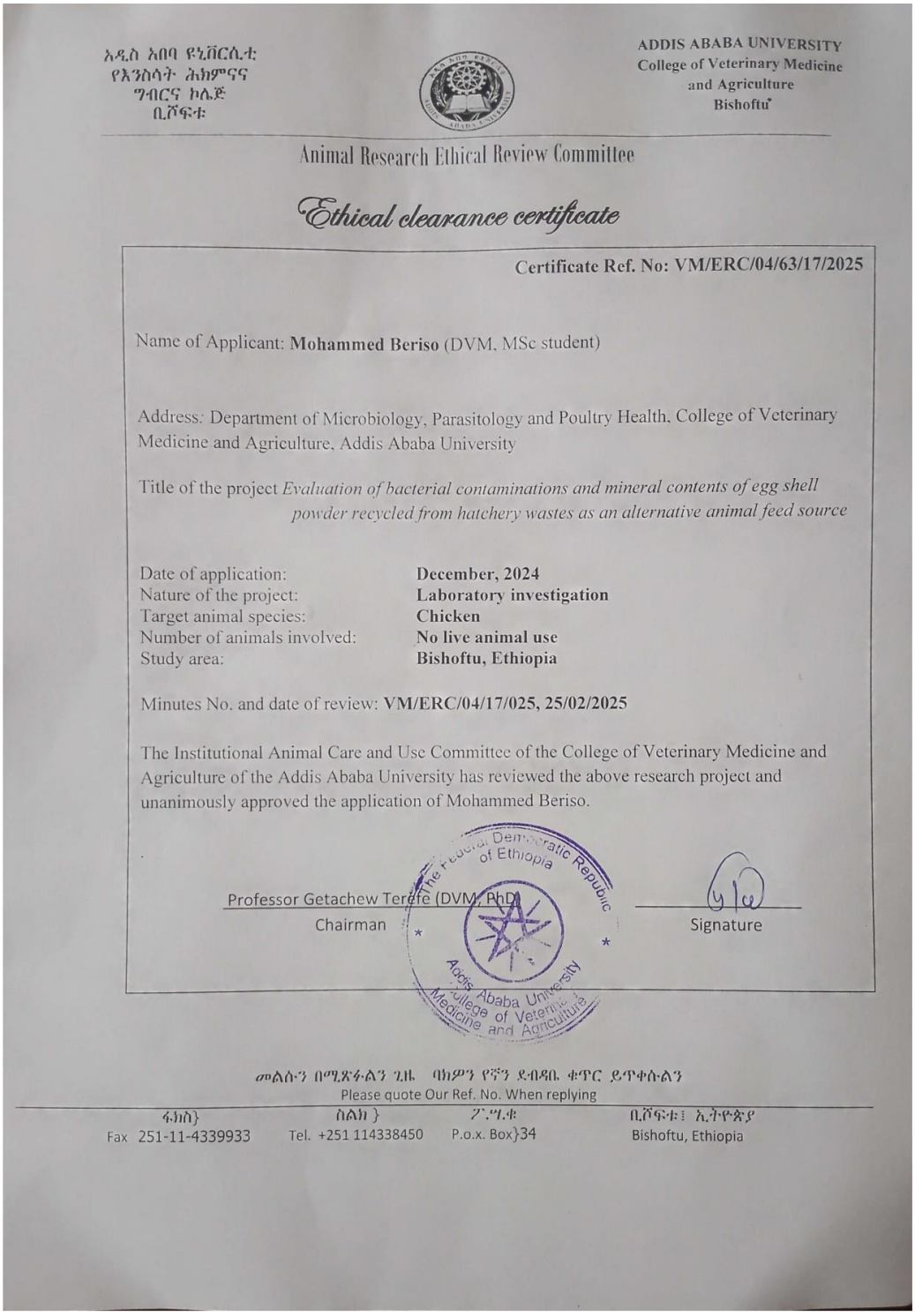
Parametrs	Applied Standard
All Metals	Dry Ash + ICP- OES Determination

T: +251 11 652 55 89 P.O.BOX: 1646 Debere Zeit, Ethiopia E-mail: Laboratory.horticoop@gmail.com

Zufan Gizidan Lab Head

Laboratory test result of eggshell mineral analysis

Appendix 5: Ethical clearance certificate



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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/04/63/17/2025

Name of Applicant: **Mohammed Beriso** (DVM, MSc student)

Address: Department of Microbiology, Parasitology and Poultry Health, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project *Evaluation of bacterial contaminations and mineral contents of egg shell powder recycled from hatchery wastes as an alternative animal feed source*

Date of application:	**December, 2024**
Nature of the project:	**Laboratory investigation**
Target animal species:	**Chicken**
Number of animals involved:	**No live animal use**
Study area:	**Bishoftu, Ethiopia**

Minutes No. and date of review: **VM/ERC/04/17/025, 25/02/2025**

The Institutional Animal Care and Use Committee of the College of Veterinary Medicine and Agriculture of the Addis Ababa University has reviewed the above research project and unanimously approved the application of Mohammed Beriso.

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

Professor Getachew Tereffe (DVM, PhD)
Chairman

Signature

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Please quote Our Ref. No. When replying

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Fax 251-11-4339933 Tel. +251 114338450 P.o.x. Box}34

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Bishoftu, Ethiopia

Appendix 6: Thesis originality report

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Digital Receipt

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The first page of your submissions is displayed below.

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File name:	Final_my_Msc_Thesis.docx
File size:	8.44M
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Character count:	121,704
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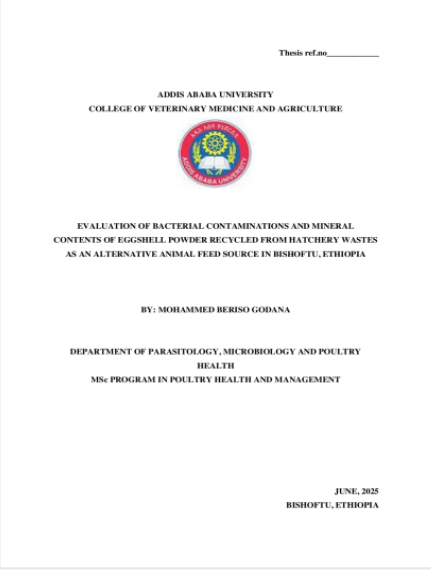

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ADDIS ABABA UNIVERSITY
COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

EVALUATION OF BACTERIAL CONTAMINATIONS AND MINERAL CONTENTS OF EGGSHELL POWDER RECYCLED FROM HATCHERY WASTES AS AN ALTERNATIVE ANIMAL FEED SOURCE IN BISHOFTU, ETHIOPIA

BY: MOHAMMED BERISO GODANA

DEPARTMENT OF PARASITOLOGY, MICROBIOLOGY AND POULTRY HEALTH
MSc PROGRAM IN POULTRY HEALTH AND MANAGEMENT

JUNE, 2025
BISHOFTU, ETHIOPIA

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