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## **Aims & Scope**

Plant and animal health is closely related to human health. In this century, where the human population is rapidly increasing and technology is developing rapidly, the problem of food supply to the increasing population brings plant and animal health to the fore. Nowadays, when concepts such as artificial meat and capsule feeding are discussed, the process of growing plants and animals has begun to be discussed. For this reason, this conference focused on the changes and innovations in the field of Veterinary, Agriculture and Life Sciences.

The aim of the conference is to bring together researchers and administrators from different countries, and to discuss theoretical and practical issues of Veterinary, Agriculture and Life Sciences. At the same time, it is aimed to enable the conference participants to share the changes and developments in the field of Veterinary, Agriculture and Life Sciences with their colleagues.

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**ICVALS 2025: International Conference on Veterinary, Agriculture and Life Sciences**

## **The Influence of Food Variety and Quality on the Anatomical and Physiological Status of the Digestive System Organs**

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**Abstract:** This study focuses on the influence of food variety and quality on the anatomical and physiological status of the human gastrointestinal system (GIS). Emphasizing the interconnection of nutrition and medical research, the study shows how different food types influence the digestive process, particularly concerning the secretion of gastrointestinal juices and digestive enzymes, the functional and mechanical response of the gastrointestinal organs such as the oral cavity, stomach, small intestine, and colon, and entirety of the gut. This research contributes to the understanding of the relationship between food composition and digestive organs structure and physiology. An “Gut on a chip” model is designed to recreate the gastrointestinal environment under controlled laboratory conditions, allowing for the analysis of mechanical and biochemical breakdown of food ingested, without the need for *in vivo* trials (Valei et al., 2023). This model also allows for the observation of food substrate disintegration and quantification of secretions from various gastrointestinal organs. Through comparative evaluation of dietary components—including dietary fiber, roughage, macronutrients, and bioactive compounds—the study sheds light on their role in mastication, enzymatic hydrolysis, nutrient absorption, and microbiome balance (Conlon & Birth, 2014; Lattimer & Haub, 2010). Additionally, also examines how food quality influences the functional integrity and efficiency of digestive organs, including the role of dentition in the initial phase of digestion. The objective is to identify which food varieties best support gastrointestinal function and mucosal health, thereby informing evidence-based dietary recommendations.

**Keywords:** Food quality, Gastrointestinal system, Physiology of digestion, “Gut on chip” model.

### **Introduction**

In recent years, modern medical understanding of nutrition has largely emphasized not just the quantity of food intake, but the importance of food variety and quality in maintaining digestive system health. The gastrointestinal (GI) tract is a highly complex organ system, primarily responsible for food's mechanical and chemical breakdown into its absorbable components. These components are important not only for providing nutrition to the body but also for supporting the immune system, maintaining metabolic balance, and keeping the gut microbiome healthy. A balanced gut environment is important for overall health, with disruptions often linked with the onset of various systemic diseases.

Processing methods such as juicing, cooking, and food preservation significantly alter the physical and chemical properties of foods (Palermo et al., 2014). These modifications can influence nutrient bioavailability, digestive mechanisms, and even the physiological state of digestive organs over time. Therefore, to appreciate how different food forms affect the GI system, it is first crucial to understand the fundamental methodology by which digestion occurs. The digestive process involves two main interrelated mechanisms: mechanical and enzymatic digestion.

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Mechanical digestion begins in the oral cavity with mastication and continues through the stomach's churning process, by breaking down large food particles into smaller components. Simultaneously, enzymatic digestion plays a pivotal role, wherein specific digestive enzymes chemically dissociate macronutrients into their fundamental absorbable units—monosaccharides, amino acids, and fatty acids—which can then enter the bloodstream to support physiological functions (Particia & Dhamoon, 2025). Additionally, hormonal regulation and the involvement of accessory digestive organs such as the pancreas, liver, and gallbladder further contribute to the complexity of this system.

The form in which food is consumed significantly impacts these digestive processes. For instance, whole fruits are rich in dietary fiber, which promotes slow glucose absorption, regulates insulin response, and nurtures a diverse and healthy gut microbiome. On the other hand, fruit juices, although often thought of as nutritious, lack fiber and contain high concentrations of free sugars, leading to rapid glucose spikes just after ingestion.

Similarly, the preparation of vegetables plays a vital role in nutritional value. Raw vegetables are abundant in heat-sensitive vitamins and bioactive phytochemicals; however, cooking methods such as boiling or frying can degrade these micronutrients, lessening their bioavailability and potentially risking gut health by altering intestinal function and microbiome composition. In the case of protein sources, fresh meat and fish provide high-quality proteins and essential micronutrients that support digestive efficiency and metabolic homeostasis. However, processed meats, such as sausages, often contain additives such as sodium and nitrates, which have been associated with increased risk of gastrointestinal diseases and disruptions in enzymatic activity and microbial homeostasis.

To explore how different food varieties influence GI physiology at a cellular and molecular level, advancements in technology have provided specific laboratory tools, among which the "Gut-on-a-chip" model stands out. This innovative model replicates the three-dimensional architecture of the human intestine, simulates peristaltic motion, and maintains an oxygen gradient, thereby providing an environment closely resembling in vivo conditions. The model is constructed using live human intestinal cells, often integrated with endothelial cells within microfluidic channels, and uniquely supports the co-culture of human cells with gut microbiota. This co-culture capability allows for the investigation of host-microbe interactions, a crucial aspect of digestive health research (Particia & Dhamoon, 2025).

Understanding the significance of host-microbe interactions is essential, as recent research highlights that each individual's gut microbiome constitutes a highly personalized ecosystem of trillions of microorganisms. These microbes can metabolize the same dietary components differently across individuals, leading to variable health outcomes. Such findings underscore the need for personalized nutrition strategies to optimize digestive and systemic health.

## **Method**

This paper aims to integrate knowledge from nutritional biochemistry, gastrointestinal physiology, and technological models to provide a comprehensive understanding of how dietary patterns influence anatomical structure, physiological processes, and long-term digestive health outcomes.

## **Main Body**

### **Fiber-Power vs Sugar Shock: Gastrointestinal Processing of Whole Fruit Versus Fruit Juice**

#### **Oral Phase: Mechanical and Chemical Initiation**

Upon ingestion, both fruit juice and whole fruits undergo initial mechanical and chemical processing in the oral cavity, though via different mechanisms:

#### **Fruit Juice**

The ingestion of fruit juice requires minimal mechanical processing. The liquid immediately contacts the oral mucosa, stimulating salivary secretion (pH 6.7–7.0).

Saliva lubricates the bolus for swallowing and contains  **$\alpha$ -amylase**, which initiates minor carbohydrate digestion. Since fruit juice predominantly consists of free monosaccharides (glucose, fructose) and disaccharides (sucrose), enzymatic action at this stage is limited.

### **Whole Fruit**

Consumption of whole fruits necessitates **mastication**, a coordinated process involving:

- The **maxilla** (upper jaw), which serves as a stationary platform (BD Chaurasia, 2025).
- The **mandible** (lower jaw), which performs dynamic movements essential for grinding and tearing food (BD Chaurasia, 2025).

These movements are driven by the masticatory muscles, operating under both voluntary and reflexive control. Mechanical breakdown during mastication reduces fruit particle size, increases the surface area for enzymatic action, and mixes the food thoroughly with saliva. This chewing action not only facilitates safe swallowing but also enhances enzymatic access to the nutrients (Guyton & Hall, 2020). Additionally, **taste receptors** on the tongue detect chemical stimuli (sweet, sour, bitter, salty), initiating the **cephalic phase** of digestion via vagal nerve activation, leading to gastric acid and enzyme secretion in preparation for downstream digestive processes (Guyton & Hall, 2020).

### **Esophageal Phase: Bolus Transit**

Following oral processing, both the chewed fruit bolus and the swallowed juice are transported through the esophagus via coordinated **peristaltic contractions**. No significant digestion occurs during esophageal transit; the primary function of this phase is to propel the ingested material into the stomach (Guyton & Hall, 2020).

### **Gastric Phase: Chemical Processing and Gastric Emptying**

Upon entry into the stomach, distinct physiological differences arise between the digestion of fruit juice and whole fruit:

- **Acid Secretion:** Gastric glands secrete hydrochloric acid, reducing the intragastric pH to between 1.5 and 3.0, aiding in food sterilization and protein denaturation.
- **Pepsin Activity:**
  - In the case of fruit juice, the minimal protein content results in negligible activation of pepsinogen to pepsin.
  - In whole fruit ingestion, although fruits are not rich in proteins, stomach wall distension and neurohormonal signals promote modest pepsinogen activation, contributing slightly to proteolysis (Conlon & Bird, 2014).
- **Gastric Lipase:** This enzyme is released in response to lipid presence; however, given the low-fat content of fruits, gastric lipase plays a minimal role in both scenarios (Kuzma et al., 2017).
- **Fiber Influence:**
  - Whole fruits contain soluble fibers such as **pectin**, which hydrate and form a viscous gel, significantly slowing gastric emptying, moderating nutrient release, and promoting prolonged satiety (Dreher, 2018), (Slavin & Lloyd, 2012).
  - Fruit juice, lacking fiber, leads to rapid gastric emptying and a swift release of sugars into the small intestine.

### **Small Intestine: Enzymatic Digestion and Absorption Dynamics**

As the chyme (processed bolus) enters the **duodenum**, several digestive processes are triggered:

- **Pancreatic Secretions:** Bicarbonate is secreted to neutralize gastric acid, adjusting the pH to between 7.0 and 8.0, thereby optimizing conditions for enzymatic activity. Pancreatic enzymes, including amylase, proteases, and lipase, continue macronutrient digestion (Guyton & Hall, 2020).

- *Brush Border Enzymes:* Sucrase, maltase, and lactase at the intestinal brush border complete the breakdown of carbohydrates into monosaccharides.
- *Sugar Absorption:*
  - In fruit juice ingestion, free glucose and fructose are rapidly available for absorption.
  - Glucose is absorbed via the **SGLT-1** (sodium-glucose co-transporter) and **SGLT-2**, while fructose is absorbed through **GLUT5** by facilitated diffusion.
  - In whole fruit ingestion, the presence of intact fiber slows the enzymatic accessibility to sugars, prolonging digestion time and promoting a more gradual absorption curve (Kuzma et al., 2017).
- *Micronutrients Absorption:*
  - Vitamins such as **vitamin C**, **folate**, **potassium**, and various bioactive phytochemicals are absorbed in both cases.
  - However, the slower digestion of whole fruits enhances micronutrient bioavailability and attenuates the post-ingestion glycemic burden.
- *Fiber's Role:*
  - Soluble fibers continue to slow carbohydrate absorption, stabilize blood glucose levels, and prolong nutrient contact with the absorptive surfaces of the intestinal lining.

### **Hepatic Processing: Metabolic Consequences**

Following absorption in the small intestine:

- **Glucose** enters the hepatic portal circulation, where it may be immediately used for energy, stored as **glycogen**, or converted into lipids via **de novo lipogenesis** when in excess (Dreher, 2018).
- **Fructose** is primarily processed in the liver. A rapid influx of fructose from fruit juice can overwhelm hepatic metabolic capacity, promoting **increased triglyceride synthesis**, **hepatic steatosis**, and **insulin resistance** over time.
- *Glycemic Impact:*
  - Fruit juice consumption leads to a more rapid systemic appearance of sugars, resulting in a greater glycemic spike.
  - In contrast, whole fruits, through fiber-mediated delayed absorption, promote a moderated glycemic response (Dreher, 2018).

### **Hormonal Responses and Appetite Regulation**

- *Insulin Response:*
  - Rapid glucose absorption following fruit juice ingestion stimulates a stronger and faster insulin response compared to whole fruit consumption.
  - Chronically elevated insulin responses are associated with an increased risk of developing **insulin resistance** and **metabolic syndrome** (Niu et al., 2025).
- *Leptin and Ghrelin Regulation:*
  - Whole fruits, rich in fiber, enhance **leptin** signaling (promoting satiety) and suppress **ghrelin** (stimulating hunger).
  - Fruit juices, lacking significant fiber content, fail to adequately stimulate satiety responses, thereby increasing the risk of overconsumption and subsequent metabolic dysregulation (Guyton & Hall, 2020).

### **Large Intestine: Microbiota and Fermentation**

- *Whole Fruits:*
  - Undigested dietary fibers reach the colon and are fermented by the gut microbiota, producing **short-chain fatty acids** (SCFAs) such as **butyrate**, **acetate**, and **propionate** (Facchin et al., 2024)

- SCFAs support **gut barrier integrity**, **reduce inflammation**, and serve as an energy source for colonocytes (Facchin et al., 2024).
- Regular intake of whole fruits promotes the proliferation of beneficial bacterial genera such as *Bacteroides* and *Parabacteroides*, thereby enhancing **microbial diversity** and overall **gut health** (Facchin et al., 2024).
- *Fruit Juices:*
  - The minimal fiber content of fruit juice limits the availability of fermentable substrates, thereby reducing SCFA production and diminishing the positive modulation of the gut microbiome (Dreher, 2018; Facchin et al., 2024).

## **Gastrointestinal Processing of Raw Versus Cooked (Boiled/Fried) Vegetables**

### **Oral Phase**

- *Raw Vegetables:*

Due to rigid cell walls and high insoluble fiber, raw vegetables require extensive mastication. This increases salivary secretion and alpha-amylase activity, initiating carbohydrate digestion. More chewing promotes satiety and slows the eating process (Guiton & Hall, 2020)

- *Boiled Vegetables:*

Softened cell walls reduce mastication needs, resulting in lower salivary stimulation and decreased alpha-amylase activation.

- *Fried Vegetables:*

Crispy texture demands moderate mastication, enhancing salivary production. However, the higher fat content delays lipid digestion initiation, as salivary glands lack lipase activity.

### **Esophageal Phase**

- In both raw and cooked forms, bolus transit through the esophagus is mediated by coordinated peristaltic contractions, without significant digestion occurring.

### **Gastric Phase**

- *Raw Vegetables:*

Bulky fiber content slows gastric emptying and prolongs gastric digestion. Increased fiber volume triggers stomach stretch receptors, enhancing satiety. Higher hydrochloric acid secretion denatures vegetable proteins, activating pepsin for proteolysis. Fiber delays gastric emptying, stabilizing postprandial blood glucose levels. Heat-sensitive nutrients like vitamin C and folate are preserved (Lattimer & Haub, 2010).

- *Boiled Vegetables:*

Soft texture leads to faster gastric emptying. Moderate gastric acid and pepsin activity facilitate digestion of softened proteins and starches.

- *Fried Vegetables:*

High fat content significantly slows gastric emptying (up to 3–4 hours), triggering prolonged hydrochloric acid secretion for fat emulsification. Higher energy density may lead to bloating and delayed satiety signals.

### **Small Intestinal Phase**

- *Raw Vegetables:*

High fiber content physically binds digestive enzymes, reducing their access to macronutrients and lowering nutrient absorption efficiency. Pancreatic enzyme activity (amylase, lipase, protease) is less effective. Minimal bile is required unless raw vegetables are consumed with additional fats. Water-soluble vitamins (C and B-complex) remain intact due to absence of heat exposure (Lattimer & Haub, 2010).

- *Boiled Vegetables:*

Gelatinized starch enhances amylase efficiency, leading to rapid glucose release and absorption. Minimal bile secretion is sufficient due to low fat content (Lee et al., 2017).

- *Fried Vegetables:*

Added fats require substantial bile secretion to emulsify lipids, activating pancreatic lipase. Presence of fat improves absorption of fat-soluble vitamins (A, D, E, K) (Hundt, 2025).

### **Nutrient Bioavailability**

- *Raw Vegetables:*

Retain heat-sensitive vitamins (C and B group) and phytochemicals. However, intact cell walls limit immediate bioavailability of some antioxidants and micronutrients (Kowalska et al., 2021).

- *Boiled Vegetables:*

Cell wall softening releases bound antioxidants, improving their absorption despite loss of some water-soluble vitamins during cooking (Toydemir et al., 2022).

- *Fried Vegetables:*

Fat-rich medium enhances bioavailability of fat-soluble vitamins results in partial degradation of water-soluble vitamins (National Research Council, 1989).

### **Large Intestine Phase**

- *Raw Vegetables:*

Insoluble fibers reach the colon, where gut microbiota ferment them to produce short-chain fatty acids (SCFAs) like butyrate and propionate. SCFAs support gut barrier integrity, modulate inflammation, and promote immune function (Facchin et al., 2024).

- *Boiled Vegetables:*

Retain some fermentable fibers, but depending on cooking method, fiber content may be reduced, moderately impacting SCFA production (Facchin et al., 2024).

- *Fried Vegetables:*

Reduced fiber content after frying limits microbial fermentation and SCFA production, weakening microbiome diversity support (Colon & Birth, 2014; Facchin et al., 2024).

### **Conclusion and Recommendations**

This study highlights that the functioning of the gastrointestinal system is closely linked to the types of food we consume. Advances in nutritional science and technology—such as the gut-on-a-chip model (National Research Council (US) on Diet and Health, 1989) have significantly deepened our understanding of digestion and nutrient bioavailability. However, emerging research underscores a crucial factor: the gut microbiota. Each individual hosts a unique ecosystem of trillions of microbes that can metabolize the same food components in remarkably different ways. As a result, metabolic and immune responses to dietary interventions—such as consuming whole fruits versus fruit juice, or raw versus cooked vegetables—can vary greatly between individuals. To the best of our knowledge, most existing research using the gut-on-a-chip model has focused on understanding the physiology of specific organs and their functions under controlled conditions. However, this paper presents a broader perspective, suggesting that the model holds significant potential for application in the field of nutrition. By simulating the dynamic environment of the human gut, the gut-on-a-chip can offer deeper insights into how nutrients are digested, absorbed, and metabolized. It also allows researchers to observe how different dietary components interact with gut microbiota in real time. This could pave the way for more precise, personalized nutritional strategies and a better understanding of diet-related diseases. This reinforces the need for personalized nutrition approaches, where dietary recommendations are tailored to the specific composition and function of an individual's gut microbiome (Conlon & Birth, 2014).

## Scientific Ethics Declaration

\* The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

## Conflict of Interest

\* The authors declare that they have no conflicts of interest.

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**ICVALS 2025: International Conference on Veterinary, Agriculture and Life Sciences**

## Breast Cancer: From Molecular Subtypes to Metastasis

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**Abstract:** Breast cancer is the most common cancer among women and is the leading cause of female mortality worldwide. According to the American Cancer Society, 13% of women (1 in 8 women) will have been diagnosed with breast cancer at least once in their lifetime. Although breast cancer is more common in women, men are also susceptible to breast cancer, but to a much lesser extent. Only 0.12% of men (one in 833 men) are diagnosed with breast cancer. Breast cancer is caused by the interplay between genes and specific environmental factors. The classification of breast cancer into different molecular subtypes that are different from each other affects the choice of the type of treatments. A number of recent studies have shown a significant relationship between breast cancer molecular subtype and metastatic status. The metastasis is a process of biological actions that must be completed by cancer cells to exit the primary tumor and develop a new tumor at a distant site. Certain breast cancer molecular subtypes are more likely to cause metastases in certain organs of the body than others. Primary and secondary breast cancer of the triple-negative molecular subtype is often the worst, followed by the HER+ molecular subtype.

**Keywords:** Breast cancer, Breast cancer molecular subtypes, Metastasis

### Introduction

When the body grows or some of its cells die, new cells are produced to replace the old cells. During this process, the number of new cells is always equal to the number of dead cells. If this biological process is disrupted and the new cells multiply more than the normal level, the body will fight against them through the mechanisms that control cell growth. When these cells are able to evade the body's mechanisms, cancer will occur. If cancer is not detected and treated, these abnormal cells will continue to grow and divide. The reason they are able to form a cancerous tumor is because these cancer cells have acquired characteristics such as their ability to evade growth inhibition or cell death mechanisms, their constant release of growth signals, their genetic instability, and their ability to induce angiogenesis and metastasis (Eroles et al., 2012).

The incidence of breast cancer is increasing every year and the incidence of breast cancer is the highest among all malignant tumors in women. In 2021, the mortality rate from breast cancer was second only to lung cancer (Li et al., 2023) and the mortality rate caused by breast cancer is expected to be the highest in 2040 (Sheva et al., 2024). This is due to the difficulty in identifying the molecular mechanisms responsible for cancer initiation and progression as well as the difficulty in determining the exact time of disease onset (Russo et al., 2000). Nevertheless, identifying the molecular subtypes of breast cancer plays an important role in determining the treatment and management of the tumor (Sheva et al., 2024).

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While primary breast cancer is not a cause of death, distant metastases of breast cancer are the leading cause of treatment failure and death. Common sites for distant metastases of breast cancer are bone, lung, liver, and brain (Liu et al., 2022). The transformation of a primary cancer into a metastatic cancer goes through several stages and the most commonly used system for breast cancer staging is the TNM system (Pourriahi et al., 2023), which describes the anatomical extent of the cancer and determines its stage (Park et al., 2011). The TNM system refers to: (T) primary tumor features (size and relationship to the surrounding environment), (N) regional lymph node features (number and location of nodes, size of nodal involvement and presence of external extension), and (M) presence of metastasis (Cserni et al., 2018). The TNM stage classification combines these parameters and categorizes the disease into one of 5 stages (0, I, II, III, and IV). Breast cancers of different stages exhibit different genomic characteristics and molecular profiles (Tsang & Tse, 2020).

## **Molecular Classification of Breast Cancer**

The breast cancer prognosis and treatment pathway are determined by its molecular classification. Research studies applying molecular techniques have determined several circulating tumor biomarkers useful for diagnosis, prognosis, therapeutic response, and monitoring minimal residual disease (Pankotai-Bodó et al., 2024). Molecular subtypes of breast cancer are identified by measuring the expression of hormone receptors including human epidermal receptor 2 (HER2), Progesterone receptor (PR) and Estrogen receptor (ER) (Guo et al., 2024). The first prognostic marker in breast cancer recognized in the 1980s was the estrogen receptor. In the late 1990s, the HER2 receptor was discovered. In addition, a cell proliferation marker such as the Ki-67 gene was incorporated to give a greater chance of differentiating molecular subtypes (Lim & Hortobagyi, 2016).

Breast growth and development is influenced by a large number of growth factors and hormones as the breast is a hormone-responsive organ. The response of the breast to hormones is selective and consequently breast cells proliferate or differentiate, leading to irreversible changes in the structure and biological characteristics of the breast (Russo et al., 2000). In principle, the most common hormones that affect the mammary gland are hormones secreted by or modulated by the ovary (Estrogens and Progesterone) (Wang et al., 2024). The role of the ovarian sex hormones Estrogen and Progesterone (PR) in the growth and development of breast cancer was discovered more than 100 years ago when George Bateson demonstrated that oophorectomy cured a woman of breast cancer (Trabert et al., 2020). Today, more than 70% of breast cancer patients have Estrogen receptor and Progesterone receptor positive tumors (Miladinova, 2019).

Estrogen is one of the most important steroidal sex hormones in women. While estrogen is important, it also plays a role in the development of a number of cancers in females. Exposure to the estrogen hormone is a risk factor for breast cancer because the majority of breast cancers have receptors for this hormone (Russo et al., 2000). Estrogen, a form of estradiol (E2), stimulates the activation of a number of genes that increase the growth of cancer cells. Both  $\alpha$  and  $\beta$  forms of the ER receptor have been discovered (Lim & Hortobagyi, 2016). In other words, Estrogen stimulates breast cancer by contributing to increased proliferation of cancer cells and reduced apoptosis. Estrogen also affects the gene expression of GATA3 and FOXA1 genes that lead to breast cancer initiation, progression and metastasis. In addition, estrogen receptors bind to G proteins on the cell membrane and this activates PI3K/AKT and Ras/MAPK signaling, which in turn stimulate the transcription of genes involved in breast cancer initiation (Wang et al., 2024). To summarize, estrogen induces breast cancer through three mechanisms: Hormonal activity mediated by the estrogen receptor which stimulates cell proliferation and increases the chances of accumulating the genetic damage that leads to breast cancer, metabolic activation through the stimulation of membrane-bound hemoproteins called cytochrome P450 which leads to toxic effects that increase mutation rates in cells and weaken the DNA repair system (Russo et al., 2000).

Progesterone is a sex steroid hormone that participates in the female menstrual cycle and pregnancy by binding to the Progesterone receptor.9 Progesterone also participates in the hormone's regulation of estrogen (Li et al., 2020). As with Estrogen, overexposure to Progesterone is a risk factor for breast cancer (Guo et al., 2024). Studies have found that women with higher levels of Progesterone are 16% more likely to develop breast cancer than other women.67 Progesterone contributes to the development of breast cancer through the following mechanisms: Progesterone receptors on the surface of mammary cells bind to the hormone Progesterone which contributes to the nourishment and proliferation of breast cancer cells. Progesterone can stimulate the proliferation of PR-positive neighboring cells, Progesterone also stimulates the proliferation of PR-negative luminal epithelial cells, Progesterone can stimulate mammary gland development by expanding the mammary stem cell population, and Progesterone can also regulate this pathway (Li et al., 2020).

HER2 (ErbB2, human epidermal growth factor receptor 2) is the second member of a family of Epidermal growth factor receptor (EGFR) trans-membrane glycoprotein receptor (Lim & Hortobagyi, 2016). The HER family consists of four receptors (HER1, HER2, HER3 and HER4). These members of the HER family of receptors participate in the regulation of many cell processes including apoptosis, migration, growth, adhesion and differentiation. This is carried out through activation of the Akt, Ras-Raf mitogen- activated protein (MAP) kinase and phosphatidylinositol 3-kinase (PI3K) pathways which lead to tumor cell proliferation, survival, adhesion and metastasis (Guo et al., 2024). HER signaling is vital for normal cell growth but dysregulated HER signaling is strongly connected to malignant transformation. Dysregulation of HER signaling can occur via a number of mechanisms: over-expression of the normal HER receptor, overexpression of the ligand or expression of the mutant HER receptor (Yarden et al., 2004). A nearly threefold increase in mortality associated with breast cancer and distant metastasis has been reported in patients with HER2 overexpression. The importance of HER2 testing has increased dramatically as it has become a strong predictive marker of response to HER2-targeted treatment (Lim & Hortobagyi, 2016).

Deregulated proliferation is a typical feature of a malignant tumor and can be evaluated by various methods, the most important of which is the expression of the Ki-67 gene. While Ki-67 is expressed in all proliferating cells, its role as a proliferation marker has attracted considerable attention (Soliman & Yussif, 2016). Ki-67 is located on the long arm of human chromosome 10 (10q25) (Ma et al., 2024). The expression rate of this gene varies according to the phases of the cell cycle, with a peak in M phase, low in G1 and early S phase, and no expression in G0 phase (Soliman & Yussif, 2016). Ki-67 has an important role in distinguishing between different molecular subtypes in breast cancers (Lim & Hortobagyi, 2016) so it is often used to distinguish between luminal A and luminal B. It is also an important marker for determining the prognosis of patients with HR+/HER2- early breast cancer. Ki-67 expression in breast cancer is associated with a higher risk of breast cancer recurrence (Ma et al., 2024). Through prognostic biomarkers such as the prevalence of estrogen receptor, progesterone receptor, HER2, and Ki-67, breast cancer can be classified into the following molecular subtypes (Li et al., 2023):

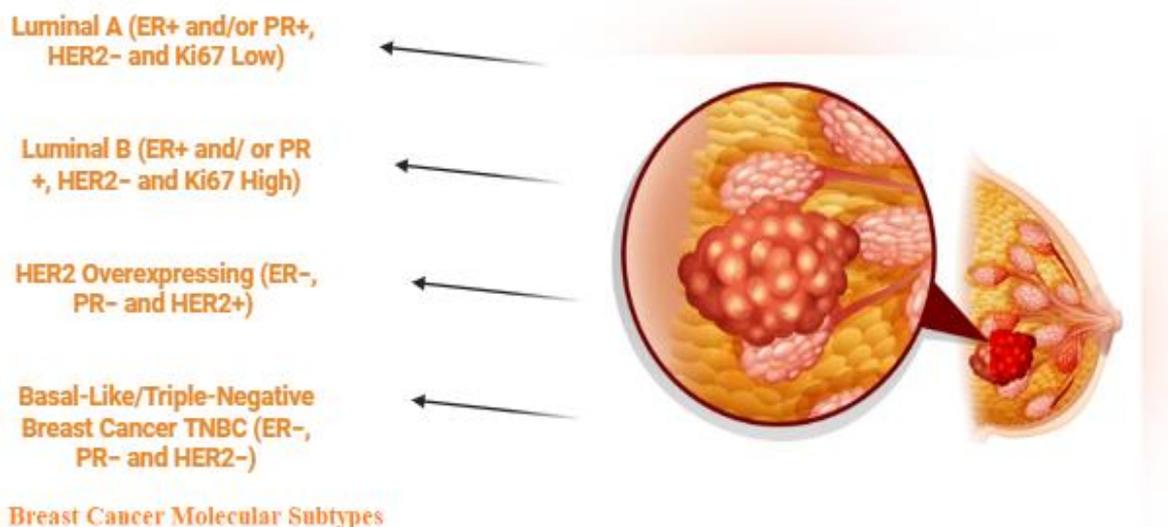


Figure 1. Breast cancer molecular subtypes

### **Luminal A (ER+ and/or PR+, HER2- and Ki67 Low < 15%)**

Luminal A breast cancer is the most common molecular subtype, with 50-60% of all breast cancer cases falling under this type (Eroles et al., 2012). This type is typically featured by the expression of genes that are transactivated by the transcription factor ER and/or PR and that are expressed in the luminal epithelium lining the ductal mammary glands. At the same time this subtype is characterized by the absence of HER2 and low gene expression of Ki-67 which is associated with cell proliferation and division (Pankotai-Bod' o et al., 2024). Tumors classified within this subtype have a better prognosis than other subtypes because they grow slowly and have a relatively low incidence rate (27.8%) and therefore a higher survival rate (median 2.2 years) (Sheva et al., 2024). Tumors of this subtype are more likely to develop bone metastases (18.7%) and may also develop metastases to other organs such as the central nervous system, liver, and lung (less than 10%) (Eroles et al., 2012).

### **Luminal B (ER+ and/ or PR+, HER2– and Ki67 High $\geq$ 15%)**

Luminal B subtype tumors account for 10%-20% of all breast cancers. This molecular subtype includes ER expression and/or PR with high Ki-67 expression and possible HER expression (Sheva et al., 2024). When comparing this subtype with luminal A subtype, tumors of this subtype are more invasive, have a higher proliferative index, a worse prognosis, and a lower survival rate (1.6 years). This is because these tumors are associated with high proliferation rates as well as potential expression of HER2. Bone is the most common site of metastasis (30%), followed by the liver (13.8%) (Pankotai-Bod' o et al., 2024).

### **HER2 Overexpressing (ER–, PR– and HER2+)**

HER2 subtype is characterized by high levels of HER2 expression as well as being ER and PR negative. Additionally, two subgroups of HER2 can be differentiated: luminal HER2 (ER-, PR-, and HER2+, Ki-67 15%-30%) and enriched HER2 (ER-, PR-, and HER2+, Ki-67>30%) (Krishnamurti & Silverman, 2014). Tumors belonging to this subtype constitute 20-25% of all breast cancer tumors (Miladinova, 2019). Tumors of the HER2+ molecular subtype are tumors with a poor prognosis. This is because HER2 is encoded by the proto-oncogene HER2/neu or c-erbB-2, which is involved in tumor cell survival, proliferation, metastasis, angiogenesis, increased cell adhesion, and impaired apoptosis (Tsang & Tse, 2020).

### **Basal-Like/Triple-Negative Breast Cancer (ER–, PR– and HER2–)**

The triple negative molecular subtype is characterized by the absence of estrogen, progesterone, and HER2 receptors (Sheva et al., 2024). Tumors of this subtype are characterized by the expression of genes in normal mammary basal/epithelial cells, especially basal cytokeratins (CK5 and CK17, P-cadherin, caveolin 1 and 2, nestin, CD44 and EGFR), so they are also termed basal-like tumors (Tsang & Tse, 2020). The basal-like subtype represents 10–20% of all breast carcinomas. This subtype is the most popular subtype of breast cancer among women under the age of 40 (Anothaisintawee et al., 2013). Triple-negative tumors have a high rate of p53 mutations; thus, these tumors are aggressive and have a poor prognosis (Chen et al., 2018). In addition, tumors with mutations in the BRCA1 gene fall into the same subtype. The pattern of metastatic relapse is characterized by aggressiveness with a predominance of lymph nodes and visceral organs (especially lungs and central nervous system) (Anothaisintawee et al., 2013). Relapses may occur within 5 years after diagnosis (Tsang & Tse, 2020).

Table 1. Features of molecular breast cancer subtypes

Subtype	ER/PR/HER2	Frequency	Prognosis	Significant Genes
Luminal A	ER+ and/or PR+, HER2– and Ki67 Low	50-60%	Good	PI3KCA-MAPK3K1- GATA3- CCDN1
Luminal B	ER+ and/ or PR+, HER2– and Ki67 High	10%-20%	Poor/Bad	PI3KCA-MAPK3K1- GATA3- CCDN1- TP53- RB- FOXM1
HER2 Overexpressing	ER–, PR– and HER2+	20-25%	Poor	HER2-GRB7
Basal-Like/Triple- Negative	ER–, PR– and HER2–	10%-20%	Poor	TP53- BRCA1- MYC- RB1- CK5/6

## **Breast Cancer Subtypes and Locations of Metastasis**

Metastasis is a sequence of biological processes completed by cancer cells to exit the original tumor location to other locations where the primary tumor is transformed into a distant secondary tumor. First, the tumor cells must be able to perform invasion, which is the first step in the metastatic process. For the process of invasion, changes occur in the adhesion of cells to their neighboring cell as well as the adhesion of the cell to the extracellular matrix. Tumor cells invade the basement membrane and surrounding cells. Ultimately, cells that are able to survive leak into blood or lymphatic circulation and some cells migrate to form a tumor in a new location. The 5-year survival rate in primary breast cancer is 99%. Nevertheless, once distant metastases appear, this rate decreases to 23% (Mego et al., 2010).

Breast cancer cells metastasize preferentially to selected organs, known as “organ metastasis,” which is highly regulated by breast cancer subtypes, the microenvironment of host organs, and interactions between cancer cells and organs. (Chen et al., 2018). Breast cancer metastases majorly to the lymph nodes, skeleton, lungs, liver, and brain by way of the blood circulation (Ma et al., 2015). 70% of metastatic breast cancer patients have metastases to the bone, 30% to the liver and 10-30% to the brain. Molecular subtypes of breast cancer play a critical role in the process of identifying the target organ for metastasis (Chen et al., 2018).

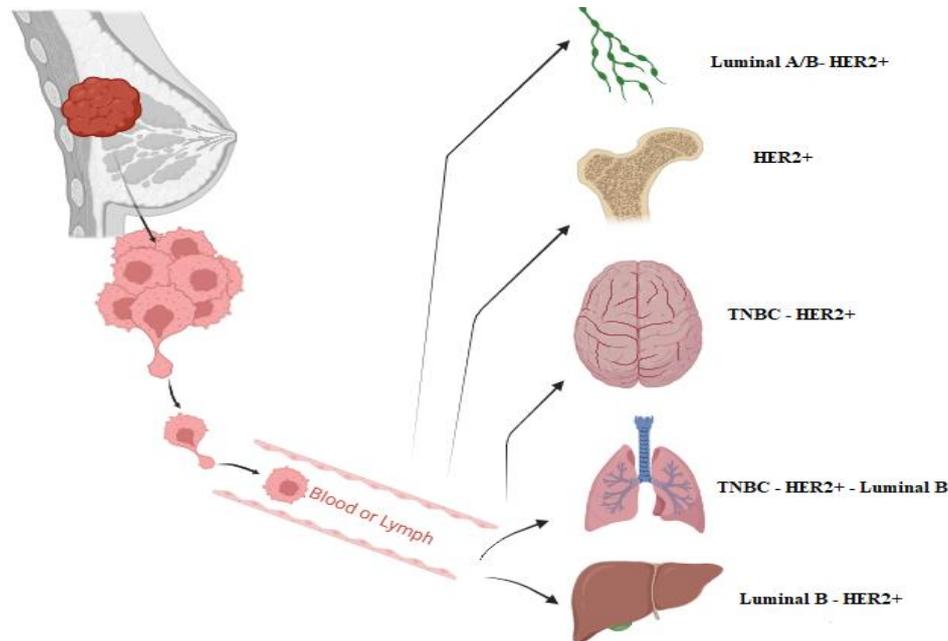


Figure 2. Breast cancer subtypes and locations of metastasis

### Bone Metastases

**Luminal** The majority of women with advanced breast cancer have clinical evidence of bone metastases by the time of death. The most common site of metastatic breast cancer is bone and represents about 70% of metastases. Bone metastases remain asymptomatic for a long time but then pain will be common and hypercalcemia and fractures will occur. Metastatic breast cancer cells infiltrate from the capillaries into the bone matrix and mimic bone, leading to adhesion, proliferation, and survival in the bone microenvironment.<sup>76</sup> Thus, bone loss occurs by enhancing bone degradation (Weilbaecher et al., 2011). While all subtypes are susceptible to bone metastasis, luminal A/B tumors develop bone metastases at a much higher rate (80.5%) (Jin et al., 2018) than TN tumors (41.7%) and HER2+ tumors (55.6%) (Savci-Heijink et al., 2016).

### Lung Metastases

Lung metastases often begin to form within 5 years after the initial diagnosis of breast cancer. Lung metastases have a major influence on patient morbidity and mortality because it interrupts normal lung function (Medeiros & Allan, 2019). Specifically, 60-70% of metastatic breast cancer patients who die are diagnosed with lung metastasis. Life expectancy is reduced when lung metastases are diagnosed, with a median of only 22 months. Lung metastases are often developed from triple-negative primary subtype breast cancer; it occurs in about 40% of patients (Jin et al., 2018). Research has found that genes such as epidermal growth factor receptor ligand epiregulin are implicated in lung metastases by promoting tumor angiogenesis, releasing cancer cells into the circulation and penetrating lung capillaries (Chen et al., 2018). HER2+ breast cancer tends to metastasize to the lungs at about 25% while this rate is 21% for Luminal B breast cancer (Medeiros & Allan, 2019).

### Liver Metastases

The third most common site of spread of metastatic tumors from breast cancer is the liver (Liu et al., 2022). Patients with metastatic breast cancer have a 15-32% incidence of liver metastases (Medeiros & Allan, 2019).

The liver is a densely vascularized organ and its endothelium has no basement membrane, which allows the transport of large molecules into the liver and also affects the interactions between tumor cells and the liver microenvironment. Liver metastases can then develop a suitable environment for their survival, replacing liver cells and internalizing blood vessels (Liu et al., 2022). Liver metastases are poorly diagnosed and if left untreated, the survival rate is only 4-8 months (Ma et al., 2015). Liver metastasis is strongly associated with ER expression and luminal subtype B is the most likely to develop liver metastases. In addition, the HER2+ subtype also has more liver metastases compared to other breast cancer subtypes (Chen et al., 2018). Triple-negative breast cancers also exhibit a high risk of liver metastasis (Liu et al., 2022).

### **Brain Metastases**

The second most popular reason for metastatic brain disease is breast cancer (Rostami et al., 2016). About 10-30% of metastatic breast cancer patients develop brain/central nervous system metastases. Brain metastases are often a late complication of breast cancer, appearing after lung, liver and/or bone metastases. Brain metastases are associated with poor prognosis and neurological impairment affecting cognitive and sensory functions. Many risk factors for brain metastases have been identified, including young age and poorly differentiated tumors (Lanigan et al., 2007). HER2+ and triple negative breast cancer subtypes have higher probability of brain metastasis (17 and 15 %, respectively) compared to luminal A and luminal B subtype (9 and 11 %, respectively) (Rostami et al., 2016).

### **Lymph Node Metastases**

Determining the presence of lymph node metastases is critical for prognosis, staging, and treatment guidance in breast cancer patients (Zhou et al., 2020). The presence of lymph node metastases indicates a high risk of distant metastases and the absence of lymph node metastases indicates a low risk of distant metastases. The metastases of the tumor to distant sites are well known to occur not exclusively through axillary lymph nodes but also through the circulation. Luminal A, Luminal B, and HER2+ breast cancer subtypes are associated with lymph node metastasis. High lymphovascular invasion and high Ki67 expression are independently predictive of metastasis of axillary lymph nodes (Chen et al., 2018).

### **Conclusion**

The most prevalent malignant tumor among women all over the world is breast cancer. Breast cancer is a complex disease involving very distinct morphological and molecular structures. The heterogeneity that occurs in breast cancer cannot be explained by some characteristics such as histological grade, age, tumor size, and lymph node involvement. Today, researches are heavily focused on the molecular biology of breast cancer. Thus, there is also a need to consider some biomarkers such as estrogen receptor (ER), progesterone receptor (PR), and epidermal growth factor receptor 2 (HER2). These biomarkers are important because they are routinely used in the diagnosis and treatment of breast cancer. Despite the possibility of different classifications, in general, breast cancer can be divided into four molecular subtypes: Luminal A, Luminal B, HER2 +, and triple negative breast cancer (TNBC). Molecular subtypes of breast cancer are associated with prognosis and the occurrence of distant metastases. Metastasis is a multi-stage mechanism by which cancer cells acquire new characteristics that enable them to leave their original location and migrate to other distant locations, thus transforming the original primary tumor into a distant secondary tumor.

Metastasis is the final stage in most cancers and is the leading cause of death rather than the primary tumor in breast cancers. In both primary and secondary breast cancer, luminal A subtype shows the best survival rate, followed by luminal B subtype. With the exception of the TNBC subtype, it was observed that bone was the most common location of metastasis in all breast cancer molecular subtypes. Briefly, HER2+ subtypes show a noticeably higher rate of metastases to brain, liver, and lung than luminal A/B subtype. TN subtypes have a high rate of metastases to brain, lung, and distant lymph-node.

### **Scientific Ethics Declaration**

\* The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

## Conflict of Interest

\* The authors declare that they have no conflicts of interest

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**ICVALS 2025: International Conference on Veterinary, Agriculture and Life Sciences**

## **Effect of Green Synthesized Silver Nanoparticles Supplementation on Broiler Growth, Carcass Traits, Blood Indices and Cecal Microbial Load**

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**Abstract:** This study evaluated the effects of green-synthesized silver nanoparticles (AgNPs) derived from garlic and ginger extracts on broiler nutrition at 4, 8, and 12 mg/kg concentrations. Characterization showed spherical AgNPs with an average diameter of 240 nm, O–H and C–H functional groups, and an elemental composition of 22.03% silver along with traces of Cl, C, N, Na, and S. XRD analysis revealed a crystalline structure with prominent peaks at  $2\theta$  angles of  $38.10^\circ$ ,  $46.05^\circ$ ,  $64.04^\circ$ , and  $76.36^\circ$ , corresponding to specific planes. Growth performance results indicated that increasing AgNP concentrations led to a significant rise in body weight, with a final weight of 745.30 g at 12 mg/kg compared to 510.39 g in the control and a reduction in feed conversion ratio (FCR) from 2.78 to 1.59. Carcass evaluation showed the highest dressing and organ weights at 12 mg/kg, while kidney and gizzard weights peaked at lower concentrations. Liver function remained unaffected, while renal function tests showed increased creatinine, urea, globulin, total protein, and albumin values at specific concentrations. Antimicrobial analysis demonstrated reduced fungal and *E. coli* counts at higher AgNP doses, although aerobic bacterial growth increased. In conclusion, supplementing broiler diets with 12 mg/kg of AgNPs positively impacted growth, carcass traits, liver and renal health, and microbial balance in the intestine.

**Keywords:** Silver nanoparticles, Green synthesized AgNPs, Broiler, Microflora, Growth performance

### **Introduction**

Antibiotics are extensively employed in poultry feed to promote growth and mitigate disease prevalence (El-Abd, Hamouda, Al-Shaikh, & Abdel-Hamid, 2022) particularly in broilers, where diseases significantly threaten the poultry industry's development in emerging economies. These diseases often stem from high levels of pathogenic bacteria, molds and yeasts adversely affecting flock health, meat quality and economic viability (Hamouda, Youssef, Saleh, El Sabry, & Nasr, 2023). Unfortunately, the efficacy of antibiotics is diminishing due to the global rise of antibiotic-resistant microbes. While new antibiotics are essential, their development is time-consuming and costly, meanwhile, the infections led by resistant strains keep increasing worldwide (Bruna, Maldonado-Bravo, Jara, & Caro, 2021). Eventually, the notion of nanotechnology has emerged as a promising

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solution for resistant microorganisms, involving the development, alteration and imaging of nanostructures, known as nanoparticles, with size ranges of 1–100 nm. With applications spanning various sectors including food packaging, veterinary, agriculture, electronics, medicine and healthcare offers diverse possibilities. Nanoparticles (NPs) are categorized into metallic and non-metallic forms (Alharbi, Alsubhi, & Felimban, 2022). Metal and metal oxide nanoparticles, distinctly Au, Ag and Se, being more capable and efficient than non-metal forms, have captured the attention of scientists (Abbasian & Jafarizadeh-Malmiri, 2020).

Silver (Ag) has a long history of use as an antimicrobial agent (Khan et al., 2022). Likewise, silver nanoparticles (AgNPs) have also been shown to possess significant antimicrobial properties. These are utilized in the poultry industry to prevent or abolish antibiotic-resistant strains, hence reducing mortality risks (Awaad et al., 2021). Astonishingly, green synthesized silver NPs are either less toxic or harmless to human beings (Abu-Dief et al., 2020), yet exhibit strong lethality against various prokaryotic cells like bacteria, fungi and viruses (Samuggam et al., 2021). The antimicrobial mechanism of silver ions involves the generation of reactive oxygen species (ROS) within microbial cells, leading to cascade of disruptive effects such as DNA and mRNA inhibition, cell membrane and cell wall destruction, protein synthesis inhibition, mitochondrial dysfunction and ETP (electron transport chain) inhibition (Jain, Pawar, Sarkar, Junnuthula, & Dyawanapelly, 2021). AgNPs possess unique chemical, electrical, optical magnetic and mechanical properties, that make them valuable in nanomedicine for antimicrobial, anti-plasmodial, antifungal and anti-cancerous, as well as for purposes like sensing, imaging, targeted drug delivery and wound healing (Alharbi et al., 2022). Taking into account their antimicrobial nature, the AgNPs have successfully been utilized in biolabeling, food packaging, electoral devices, wastewater treatment, paint and ceramics (Abbasian & Jafarizadeh-Malmiri, 2020). Additionally, these are deemed excellent alternatives to antibiotics because of their prevalent antimicrobial activities (Bruna et al., 2021).

The novelties in the synthesis of NPs have revolutionized various research domains. Traditionally, NPs are developed through three approaches encompassing physical, chemical and biological. However, physical and chemical methods are less favored because of their low yield and toxicity, respectively. Green synthesis, which involves the utilization of medicinal plants or microbes to develop NPs, is gaining prominence as a preferred mechanism due to its energy efficiency, low toxicity, high yield, availability and cost- and time-effectiveness (Alharbi et al., 2022). Plant-derived phytochemicals, renowned for their numerous antioxidant and antimicrobial properties, enhance the capabilities of nanoparticles (Shahzad Shirazi et al., 2022). Several plant parts including stems, leaves, fruits, flowers, bark and roots are commonly employed in the development of AgNPs (Alharbi et al., 2022). Garlic (*Allium sativum L.*) is globally recognized as a food, spice and traditional medicine, possessing anti-cardiovascular, anticarcinogenic and anti-aging attributes. Its extract, being an abundant source of bioactive compounds, plays an essential role in the reduction process of metal NP synthesis (Shafea & Mahmoud, 2021). Similarly, ginger extract, also employed in NP development, is valued for its antioxidant, anti-inflammatory and anticancer properties attributed to its phenolic compounds, gingerols and shogaols (Bakr, Abdelgayed, El-Tawil, & Bakeer, 2020).

The study seeks to elucidate the effects of green synthesized AgNPs on broiler chickens, specifically focusing on growth rate, carcass characteristics and microbial load. Due to the problems associated with antibiotic resistance and achieving sustainable poultry farming, this study explores the potential of AgNPs as growth promoters and antimicrobial agents. Utilizing green synthesis techniques, the present study aims to evaluate both the yield response and the health benefits of AgNPs derived from garlic and ginger in broiler chicken. A key objective is to determine the optimal concentration of AgNPs for maximizing growth performance while avoiding the negative impacts. Overall, this research contributes to the existing literature reviewing the application of nanotechnology in poultry farming and society particularly in the growth and feed of broiler chickens hence informing the development of effective strategies for the improvement of the poultry farming ecosystem.

## **Materials and Methods**

### **Procurement of Ingredients, Chemicals and Reagents**

All the chemicals including AgNO<sub>3</sub> (99.9% w/w) were analytical grades and utilized without further purification after being acquired from Future Chemicals, Faisalabad, Pakistan. Bulbs of garlic and ginger were collected from a local farm in Faisalabad, peeled and rinsed with deionized water to remove contaminants. A total of 32 chicks (with an average weight of 26g) were purchased from the bird market in Faisalabad, Pakistan.

## Preparation of Garlic and Ginger Extracts

The extracts were prepared following the method of (Keshari, Srivastava, Singh, Yadav, & Nath, 2020). The ginger and garlic extracts were prepared by making 19 grams of dried plant powder dissolving it in 100mL of deionized (DI) water. The white powder was combined with distilled water in a 250 mL beaker and heated at 55°C on a hotplate for 15 min with constant stirring and afterward left to cool. For filtration, Whatman grade 1 filter paper was used, and the resultant filtration was kept in a sealed container and was also being kept in a refrigerator at 4 °C for further use.

## Development of Garlic and Ginger Based AgNPs

Garlic and ginger extracts-based silver nanoparticles were prepared according to the guidelines of (Shafea & Mahmoud, 2021). The green silver NPs were developed by dropwise addition of mix extracts (20 mL) to 50 mL of 0.05 M silver nitrate (AgNO<sub>3</sub>) while continuously stirring at 65 °C for 2 h. Formation of nanoparticles was indicated by the change in color from pale yellow to dark brown (Alzubaidi et al., 2023); (Baran et al., 2023); (Singh et al., 2023). The attained precipitate was washed out with methanol and deionized water to take out appropriate precipitation. In the end, calcination was done by placing the mixture in a furnace (350 °C) for 2 hours to remove extract residues. The received AgNPs were added in different proportions (T<sub>1</sub>–T<sub>3</sub>) to the chicks feed as shown in Table 1 and Table 2.

Table 1. Research designs showing treatment groups with their specific garlic and ginger extracts based AgNPs concentrations to be administered to chicks orally

Groups	Treatments
Control group (T <sub>0</sub> )	No supplementation
Treatment group (T <sub>1</sub> )	Green AgNPs supplementation (4 mg/kg)
Treatment group (T <sub>2</sub> )	Green AgNPs supplementation (8 mg/kg)
Treatment group (T <sub>3</sub> )	Green AgNPs supplementation (12 mg/kg)

Table 2. Percentages of feed ingredients fed to chicks at pre-starter (1–10 days), grower (11–21 days) and finisher (22–28 days) age stages

Ingredients	Pre-starter (1–10 days)	Grower (11–21 days)	Finisher 22–28 days
Corn	50.0	59.7	66.1
Wheat	5.3	5.2	5.2
Gluten	9.8	10.2	12.2
Soybean meal	27.2	17.3	10.14
Soybean oil	3.53	3.43	3.05
Lime stone	1.65	1.32	1.2
Dicalcium phosphate	1.92	1.81	1.85
Salt (NaCl)	0.38	0.38	0.38
Mineral premix	0.27	0.27	0.27
Vitamin premix	0.27	0.27	0.27
L-Lysine	0.54	0.59	0.56
DL-Methionine	0.27	0.05	0.05

## Characterization Techniques

### UV-Visible Spectroscopy

UV-vis spectroscopy analysis was done as described by Yassin et al. (2022). The green-synthesized silver NPs were diluted in distilled water (blank) and absorption of the blank solution was recorded in the 200–800 nm range using a UV-vis spectrophotometer.

### FTIR Analysis

FTIR analysis was performed according to the description of Yassin et al. (2022) in order to evaluate the surface chemistry of green-silver nanoparticles. The silver nanoparticles were dispersed in a KBr matrix, later

compacted to a transparent disc, pallet and used as a standard. The functional groups (stretches) were detected at resolution of  $4 \text{ cm}^{-1}$ .

### **XRD Analysis**

A diffractometer mounted with a graphite monochromator was used to perform XRD patterns employing Cu-K radiation to analyze the crystalline form of biosynthesized silver nanoparticles as done by Yassin et al. (2022). To note XRD measurement on a film of the silver nanoparticles, step-scanning having 0. In the present study, the dose rate per step was 0.02 and the acquisition time per step was set at 5 sec. at 2-theta.

### **Scanning Electron Microscopy (SEM)**

The technique was implemented to characterize samples. The electron beams scanned samples to generate magnified images in order to calculate nanoparticle size. The images were recorded at an applied potential of 5 kV via Nova NanoSEM.

### **EDX Analysis**

The elemental composition analysis of biosynthesized silver nanoparticles was carried out through SEM coupled with an EDX analyzer following the method prescribed by Yassin et al. (2022).

### **Antimicrobial Activity of Silver NPs**

Antimicrobial efficiency of green synthesized AgNPs was assessed against selected microbial strains (*Listeria monocytogens*, *Staphylococcus aureus*, *Bacillus* and *Salmonella*) using disk diffusion method as suggested by M1. The microbial cultures were uniformly plated on Muller Hinton Agar (MHA) media. Then, sterilized discs were aseptically transferred onto the MHA plates that have been inoculated beforehand. To the discs, 150 ppm strength of green silver nanoparticles in the solid state were used. The solvent and standard drugs like gentamycin and penicillin (20-30  $\mu\text{g}$ ), were utilized as negative and positive controls, respectively. Afterwards, MHA plates were incubated at  $37^\circ\text{C}$  for the duration of 24 hours and inhibition zones were determined in mm.

### **Experimental Design**

The research was conducted at the PARS campus, University of Agriculture, Faisalabad. A total of 32 chicks (with an average weight of 26g) were divided into 4 treatment groups with successive supplementation of AgNPs as shown in Table 1. Before the examination, all the chicks were adapted to the lab environment for one week. All the chicks were reared under the same conditions at an ambient temperature ( $25^\circ\text{C}$ ) and humidity range of 50-70% with an appropriate supply of feed (Table 2) and water. Uninterrupted provision of light was made sure and the chicks were vaccinated on day 1 and 21.

### **Growth Performance**

The growth performance variables were calculated at the end of every week from days 7 to 28. Weight gain and FCR were computed using the following formulas:

Weight gain = Final wt. – Initial wt.

FCR = Feed intake/Wt. Gain

Feed intake = Food offered – Food reused

### **Carcass Yield**

At the end of the research period, 3 chickens from each treatment group were randomly chosen and starved for 12 hours. The birds were then weighed individually and slaughtered. The percentage live body weight was

calculated on the relative weights of liver, heart, pancreas, gizzard, kidney, spleen, stomach, intestine, breast, thigh and abdominal fat. The dressing percentage was estimated using the weight of the dressed carcass against the weight of the live birds and further presented in percentage following (Reda et al., 2020).

### Microbial Analysis

The caeca and ileum contents collected from intestines of broilers were analyzed for microbial profile analysis involving coli form bacteria, enterobacteria, anaerobic bacteria, lactic acid bacteria and *C. perfringens* were recorded according to Sheiha et al. (2020).

### Blood Chemistry

Blood specimens with anticoagulant were collected to determine the serum bilirubin, alkaline phosphatase, AST, ALT, serum protein, albumin, globulin, A/G ratio, blood urea, serum creatinine, cholesterol, triglyceride, HDL, LDL and VLDL of the experimental animals using Randox kit of USA. The hematological variables were estimated through the help of the biochemical analyzer machine (Bio-Rad) and those include; hemoglobin, total leukocyte count, total red blood cells, packed cell volume, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelet count, lymphocytes, neutrophils, monocytes and eosinophils.

### Statistical Analysis

Measurement of the differences of the values was done using basic parametric statistical method; one way ANOVA via Statistix 8.1 software. Dunnet comparison test was employed to compare means and the results were depicted as mean  $\pm$  SE (n=8).

## Results and Discussion

### Development of Silver Nanoparticles

The extracts were interfused with  $\text{AgNO}_3$  solution, turning color from pale yellow to dark brown as an indication of the formation of silver NPs. Similar change in color was observed by (Keshari et al., 2020) during the production of *Cestrum nocturnum*-based AgNPs where color changed from light yellow to dark brown. Such a change in color is attributed to SPR (Hamouda et al., 2023).

### Fourier Transform Infra-red Spectroscopy Analysis

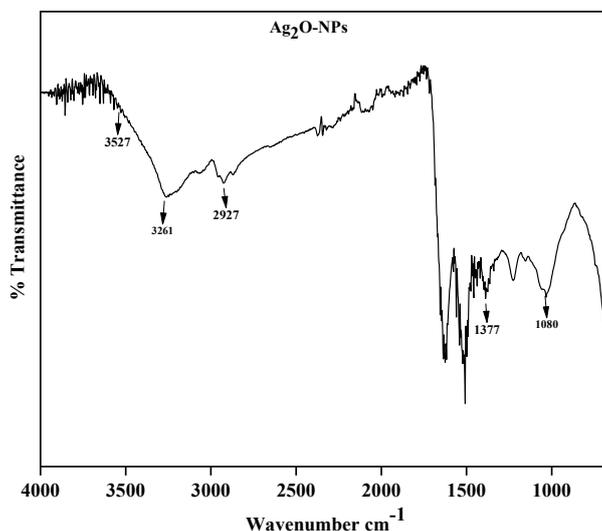


Figure 1. FTIR spectra of green synthesized AgNPs

FTIR analysis of garlic extract based green synthesized AgNPs exhibited distinct bands at 1153, 1377, 2927, 3261 and 3527/cm. The bands represent molecular vibrations: 1153  $\text{cm}^{-1}$  is linked to O–H bonds in tertiary C–O–H groups, while 1377  $\text{cm}^{-1}$  denotes C–H bond stretching. The stretching vibrations observed at 2927 and 3261  $\text{cm}^{-1}$  are related to C–H and O–H bonds, respectively, with respect to garlic extract sugars. The bending vibration of C–N bond (3527  $\text{cm}^{-1}$ ) represents the presence of amines. (Keshari et al., 2020) analyzed peaks at 3477  $\text{cm}^{-1}$  (O–H stretch), 3348  $\text{cm}^{-1}$  (N–H stretch of amines and amides) and 2917  $\text{cm}^{-1}$  (C–H stretch in methyl groups) in *Cestrum nocturnum* based green silver nanoparticles. The FTIR analysis (Fig.1) portrays the successful manufacture of silver nanoparticles. (Hassanen, Hussien, Mehanna, & Morsy, 2023) observed peaks in a chemo-/bio-synthesized silver granules as 3453, 2922 and 2864  $\text{cm}^{-1}$  indicating O–H stretching, C–H stretching of alkanes and N–H bend of primary amines, respectively (Fig. 1).

### Scanning Electron Microscopy Analysis

The images of AgNPs were taken at different magnifications i.e., 10,000, 25,000, 50,000 and 70,000X (Fig.2). The images taken below 50,000X lacked clarity and distinguishable identification of shapes, whereas images captured at 50,000X magnification demonstrated spherical shapes of particles. The SEM images at 70,000X depicted randomly arranged nanoparticles. The sizes of AgNPs ranged 100–350 nm with an average size of 240 nm (Fig.3). The findings are in line with the results of (Keshari et al., 2020) who observed spherical shape and random arrangement of silver nanoparticles at 80,000X in green synthesized silver nanoparticles developed using *Cestrum nocturnum*. Hamouda et al. (2023) observed a size range of biosynthesized AgNPs as 38.3–103 nm, while an average size of coffee extract based AgNPs was noted as 153 nm by (Abbasian & Jafarizadeh-Malmiri, 2020).

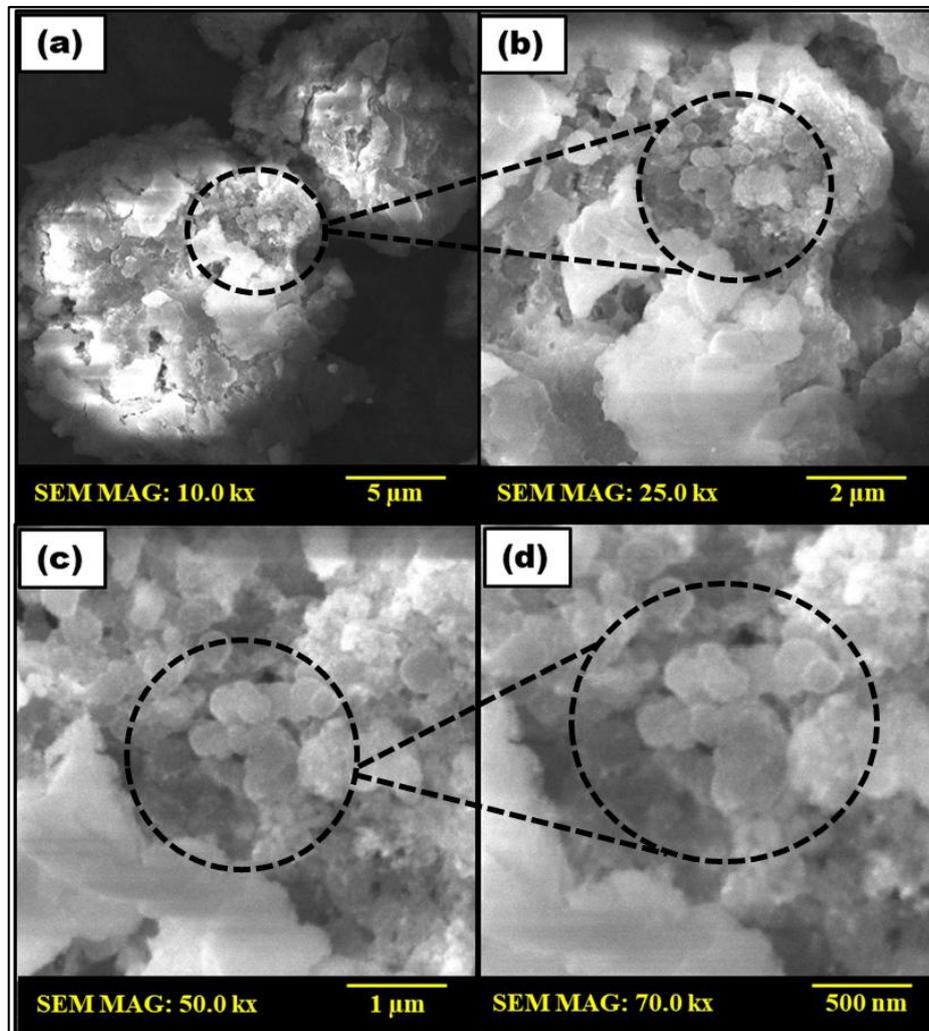


Figure 2. SEM images of AgNPs (a) at 10,000X magnification, (b) magnified at 25000X, (c) spherical shape Ag nanoparticles (50,000X) and (d) uneven surface and randomly arranged AgNPs (70,000X).

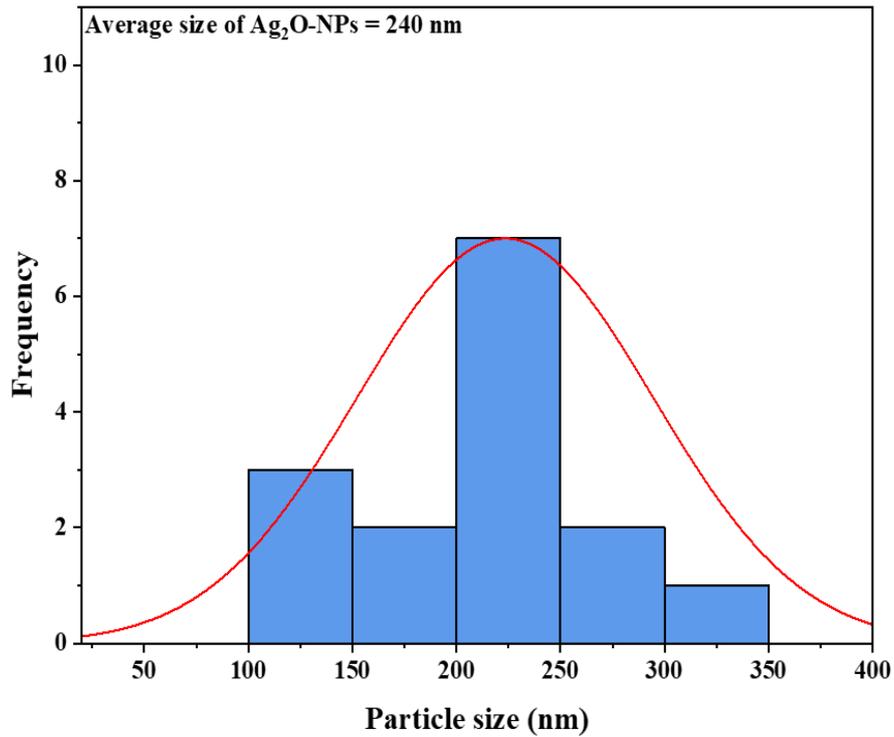


Figure 3. Distribution curve of frequency and particles size of spherical green AgNPs

#### EDX Analysis of AgNPs

EDX analysis depicts peaks stemming from characteristics X-ray emissions and elemental composition, exhibiting primary constituents of silver and oxygen, as well as certain impurities (Fig.4). The metallic silver showed 3.57 and 22.03% atomic and weight percentages, respectively. In a study by (Amin, 2020), *Ulva lactuca* based green Ag nanoparticles illustrated the absorption peak with 51.72%. The significant peak detected at 2.98 keV indicates L<sub>α</sub> transition (M–L shell, n=2 to n=3) of Ag, while a minor peak observed at 0.525 keV portrays K<sub>α</sub> transition (L–K shell, n=2 to n=1) of oxygen.

Mohamed et al. (2021) observed strong absorption peaks at 2.4-4 keV for AgNPs prepared using garlic extract. The rest of the peaks signify the presence of impurities like Cl, C, N, Na and S elements. A detailed summary of the EDX spectra for the samples is presented in Table 3. EDX analysis of *Origanum majorana* leaf extract-based AgNPs performed by Yassin et al. (2022) revealed the presence of silver, oxygen, chlorine and carbon as 71.37, 3.69, 17.89 and 2.93%, respectively.

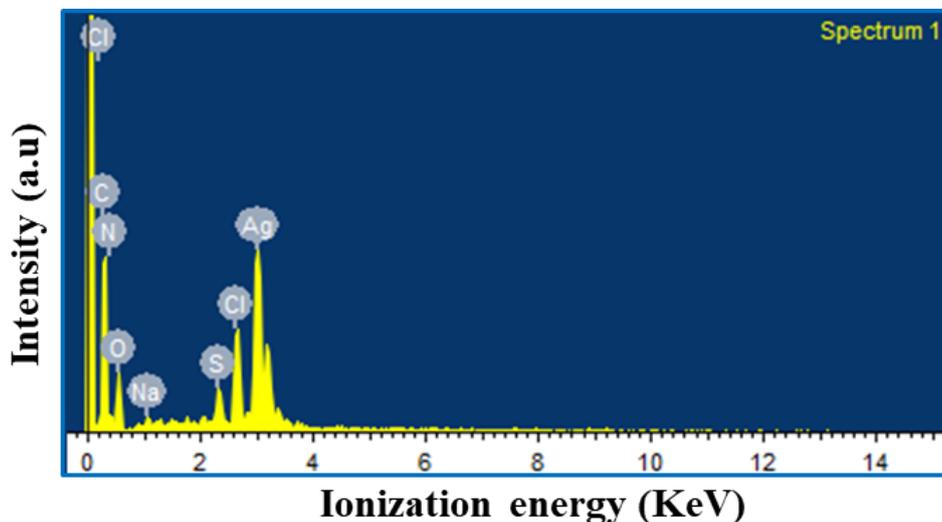


Figure 4. EDX pattern of Ag<sub>2</sub>O nanoparticles

Table 3. EDX summary of Ag<sub>2</sub>O NPs

Element	K ratio	Intensity corn.	Wt. %	Atomic%
Cl	0.02269	0.8654	3.95	1.94
C	0.07305	0.809	29.62	43.05
N	0.01737	0.1047	25.16	31.34
Na	0.00128	0.7476	0.58	0.44
S	0.00733	0.9974	1.27	0.69
O	0.01248	0.3032	17.39	18.97
Ag	0.12093	0.832	22.03	3.57
Total			100	

### XRD Analysis of AgNPs

The XRD analysis, using Bruker D8 X-ray powder diffractometer, intended to analyze structure and crystalline nature of silver NPs, presented four significant peaks in the whole spectrum of  $2\theta$ , encompassing values as  $38.10^\circ$ ,  $46.05^\circ$ ,  $64.04^\circ$  and  $76.36^\circ$ , corresponding to planes at 111, 200, 202 and 311. The values are close to the findings of (Keshari et al., 2020) in *Cestrum nocturnum* based green AgNPs at  $2\theta$ , who observed peaks values as  $38.06^\circ$ ,  $44.23^\circ$  and  $67.43^\circ$  at 111, 200 and 200 planes, respectively. The sample exhibited diffraction peaks underscoring standard silver and its face-centered cubic structure, confirming AgNPs formation. Moreover, additional peaks were also observed due to bioorganic phase of green extracts on the surface of NPs (Fig.5). The crystalline structure of Ag-NPs was noted. Sharpness of peaks is the reason behind highly crystalline structure of green AgNPs (Shaik et al., 2019). Keshari et al. (2020) also detected crystalline structure in silver nanoparticles developed using *Cestrum nocturnum*.

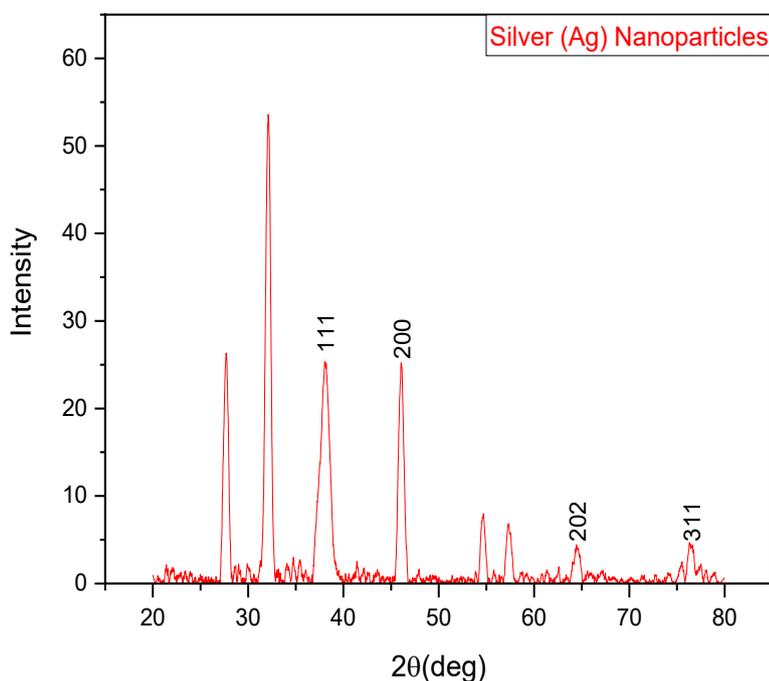


Figure 5. X-ray powder diffractometer

### Growth Performance

The data (Fig.6) underscores that weight of broilers improved with the time and increasing concentrations of AgNPs. At day 28, the lowest weight was observed in control as 510.3g, followed by the groups fed 4 mg/kg, 8 mg/kg and 12 mg/kg green synthesized AgNPs, demonstrating body weights as 610.3, 675.7 and 745.30g, respectively. At the 28th day, the values for net body weight gain for control, 4 mg/kg, 8 mg/kg and 12 mg/kg groups were recorded as 262, 299, 361 and 440 g, respectively. The comparable effect of various dose levels of AgNPs ( $T_0$ - $T_3$ ) on body weight gain of chicks is presented in Fig.7. The results on FCR of broilers (Fig.8) elucidated that FCR decreased with increasing concentrations of green silver nanoparticles. Notably, control

represented the highest FCR value as 2.78, while the littlest FCR scores were exhibited by 12mg/kg group as 1.59.

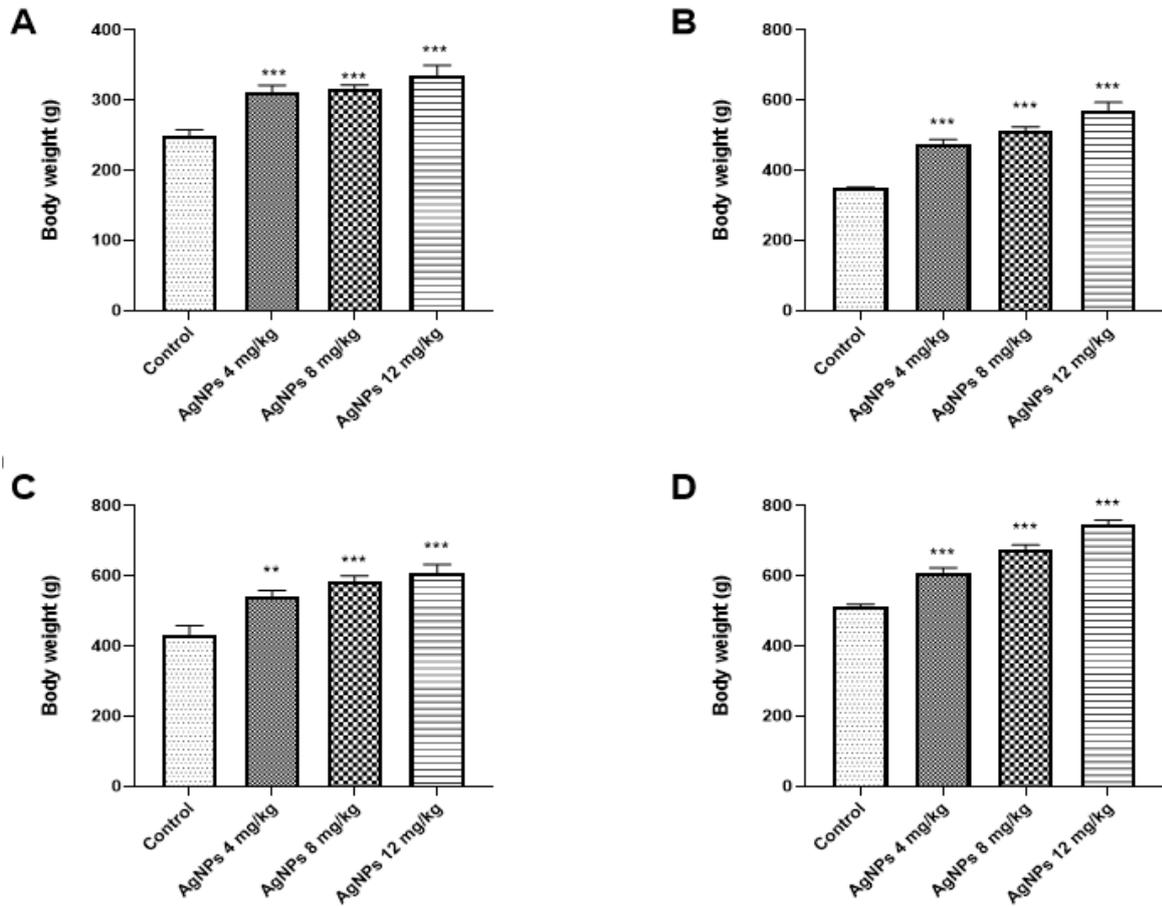


Figure 6. Graphical representation of weekly body weight of chicks (A: is referred as 1<sup>st</sup> week, B: is the weight of chicks in the 2<sup>nd</sup> week of treatment, C: is the weight of 3<sup>rd</sup> week and D: is the weight of 4<sup>th</sup> week During all this period Chicks were treated with green synthesized AgNPs at the dose of 4 mg/kg, 8 mg/kg and 12 mg/kg).

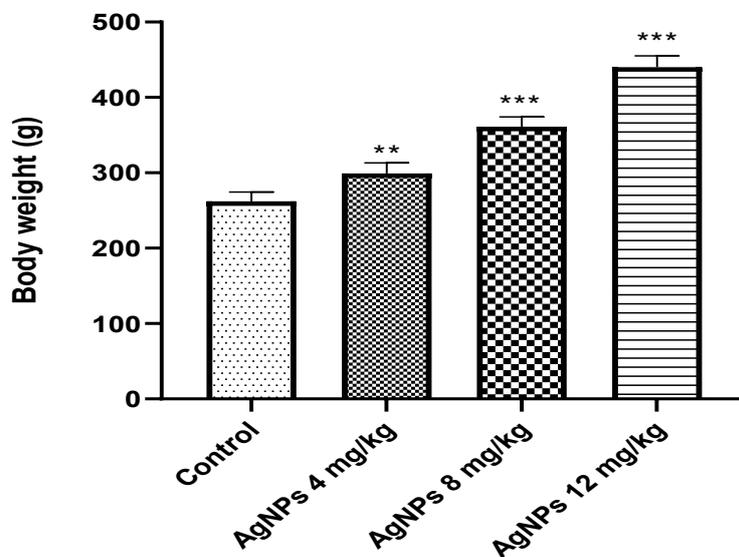


Figure 7. Graphical presentation of body weight gain of chicks with the different doses of green synthesized AgNPs

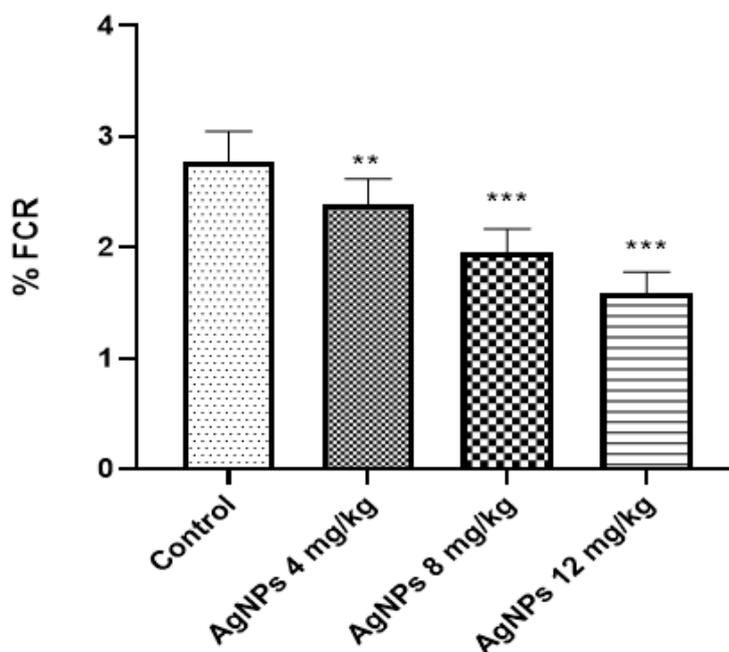


Figure 8. Graphical presentation of FCR of broiler chicks on treatment with different doses of green synthesized AgNPs

Similar trends were observed by (Keshari et al., 2020) while assessing impact of AgNPs dietary supplementation on growth and many other aspects of broilers in two phases (8–28 and 28–42 days). The findings suggested that AgNPs supplementations at 2.5 mg/kg demonstrated the highest body weight and weight gain as 2600 and 2480 g, respectively after 42 days. However, higher NP concentrations i.e., 20 mg/kg, notably suppressed these parameters. Additionally, the least FCR value (1.89) was noted at 2.5 mg/kg supplementation of silver nanoparticles. (Ahmadi et al., 2013) noted boosted growth rate in the chicks and reduced the feed conversion rate upon feeding broiler chicks AgNPs at 300, 600 and 900 ppm (Youssef, El-Banna, Elzorba, & Galal, 2019). Likewise, (Al-Sultan et al., 2022) observed that dietary incorporation of AgNPs enhances the final body weight of broiler chickens. Another study by Kumar et al. (2020) suggests that different concentrations of silver nanoparticles mixed with drinking water made growth performance of the broiler chickens much better than control group. The results of the current study are however different from those of Dosoky et al. (2021), who showed that silica-silver nanoparticles have no effect on the growth rate of the birds and the hematological, biochemical and oxidative stress parameters.

### Effect of Silver NPs on Carcass Characteristics of Broilers

Carcass characteristics encompassing dressing, liver, heart, kidney, gizzard and thigh weights of broilers were calculated from each treatment groups (control, 4mg/kg, 8mg/kg and 12 mg/kg) on the final day of experiment. The findings portrayed in Table 4 emphasize that the broilers consuming 12mg/kg treatment group supplementation of AgNPs had the highest dressing weight (325g), liver weight (10.55g), heart weight (2.86g) and thigh weight (82.66g). The highest values for kidney weight (0.91g) and gizzard weight (14.58g) were noted for broilers feeding on 4 mg/kg and 8 mg/kg AgNPs treatment groups, respectively. (Awaad et al., 2021) observed that AgNPs had a significant impact on the dressing weight and the thigh weight but failed to exert substantial changes on heart or gizzard weight in broiler chickens.

Ahmad et al. (2022) referred that the nanoparticles @ 4, 8 and 12 ppm enhanced small intestine and abdominal fat weights in broiler chickens and were minimal on liver and gizzard percent weight. Further, in a study conducted by (Saleh & El-Magd, 2018) on broiler nutrition, an elevation in small intestine and liver's relative weights was delineated, while the gizzard, proventriculus & pancreas organs didn't show any variation due to the intake of various concentrations of AgNPs and silver nitrate. The improved and excellent broiler performance rate, carcass and relative dimensions of vital organs were analyzed in the group of broiler chickens with fed 2.5 ppm AgNPs as compared to the control group (Al-Sultan et al., 2022). In contrast, Al-Saeedi et al. (2021) reported that dressing % and the relative weight of heart and spleen in broiler chickens were not significantly influenced by the different levels of AgNPs interfused with drinking water.

Table 4. Effect of varied doses of green-synthesized AgNPs on carcass characteristics of broilers

Treatment Groups	Dressing weight	Liver weight	Kidney weight	Heart weight	Gizzard weight	Thigh weight
Control	256.66 ±4.05	9.07±0.01	0.50±0.02	2.50±0.28	13.35±0.23	44.66±3.17
4 mg/kg AgNPs	288.66 ±4.05**	10.45±0.35*	0.91±0.04***	2.45±0.27 <sup>ns</sup>	13.75±0.38 <sup>ns</sup>	54±1.70*
8 mg/kg AgNPs	307.33±2.60***	10.33±0.31*	0.86±0.04***	2.61±0.24 <sup>ns</sup>	14.58±0.712 <sup>ns</sup>	67.66±2.02***
12 mg/kg AgNPs	325±2.33***	10.55±0.33*	0.85±0.02***	2.86±0.26 <sup>ns</sup>	13.67±0.33 <sup>ns</sup>	82.66±1.45***

\*Values are presented as Means ± SE (n=8). Significant differences are indicated by \*\* (p<0.01) and \*\*\* (p<0.001) when compared to the control group

### Blood Chemistry

Liver functioning analysis was carried in order to estimate the potential toxic impact of green-synthesized silver nanoparticles' different doses on broiler chickens. The results (Table 5) suggest that ALT, AST, ALP, GGT and total bilirubin were enhanced with increasing doses in the green-AgNPs treated groups as compared to control, recording the topmost values at 12 mg/kg of green-AgNPs. At a 12 mg/kg of AgNPs dose, ALT, AST, ALP, GGT and total bilirubin presented the utmost values as 49.33 U/L (control: 15), 257.33 U/L (control: 124), 1025 U/L (control: 349), 59 U/L (control: 16.9) and 0.90 mg/dL (control: 0.48), respectively. Decreased serum AST activity was noted in subjected animal treated with 8 and 12 mg/L AgNPs, however there was no alteration in serum ALT (Pourgholam, Khara, Safari, Sadati, & Aramli, 2017).

Table 5. Effect of varied doses of green-synthesized AgNPs on liver functioning parameters of broilers

Parameters	ALT (U/L)	AST (U/L)	ALP (U/L)	GGT (U/L)	Total Bilirubin (mg/dL)
Control Group	15±0.57	124±2.30	349.33±1.45	16.9±0.37	0.48±0.01
4 mg Ag NPs	18±0.57 <sup>ns</sup>	138.66±1.45**	708.66±2.33***	24±1.15**	0.37±0.01**
8 mg Ag NPs	29±1.15***	218.33±2.02***	923±2.30***	39±1.15***	0.69±0.01***
12 mg Ag NPs	49.33±2.02***	257.33±2.33***	1025±2.30***	59±1.15***	0.90±0.01**

\*Values are presented as Means ± SE (n=8). Significant differences are indicated by \*\* (p<0.01) and \*\*\* (p<0.001) when compared to the control group

### Renal Function Analysis

Renal function analysis aimed at assessing key parameters such as creatinine, albumin, globulin, total protein and urea. The calculations (Table 6) indicate that creatinine and urea demonstrated the highest values in the broilers fed 12 mg/kg green-AgNPs treatment group as 1.37 mg/dL (control: 0.91) and 23 mg/dL (control: 12), respectively, while albumin had the highest levels at AgNPs dosage of 4 mg/kg as 3.15 g/dL (control: 3.4). Globulin and total protein presented topmost values at a dosage of 8 mg/kg as 2.26 g/dL (control: 1.37) and 5.40 g/dL (control: 4.44).

The founded results of a study by Al-Sultan et al. (2022) showed that the total protein (mg/dL), total cholesterol, urea, creatinine and phosphorus of the broilers chickens that were treated with nanoparticles were significantly lower than those of the control group ( $p < 0.05$ ). In research conducted by Ahmadi and Branch (2012), it was depicted that AgNPs-supplemented feed (20–40 ppm/kg) negatively affected the broilers blood lipid profile for the higher level of cholesterol, LDL and triglycerides.

Table 6. Effect of varied doses of green-synthesized AgNPs on renal function parameters

Parameters	Creatinine (mg/dL)	Albumin (g/dL)	Globulin (g/dL)	Total Protein (g/dL)	Urea (mg/dL)
Control Group	0.91±0.03	3.4±0.15	1.37±0.008	4.44±0.02	12±0.57
4 mg AgNPs	0.63±0.01 <sup>ns</sup>	3.15±0.02 <sup>ns</sup>	2.24±0.01 ***	5.14±0.08***	13±0.57 <sup>ns</sup>
8 mg Ag NPs	0.40±0.29 <sup>ns</sup>	2.86±0.08 **	2.26±0.02 ***	5.40±0.11 ***	18±0.57**
12 mg AgNPs	1.37±0.01 **	2.2±0.05 ***	1.57±0.01 <sup>ns</sup>	4.67±0.03 <sup>ns</sup>	23±1.15***

\*Values are presented as Means ± SE (n=8). Significant differences are indicated by \*\* (p<0.01) and \*\*\* (p<0.001) when compared to the control group

## Microbial Profile Analysis

The antimicrobial activity of varied concentrations of green-AgNPs was evaluated against a number of bacterial strains, encompassing *E. coli*, *P. aeruginosa*, *C. diphtheria*, *B. subtilis*, *S. aureus*, *B. cereus*, *S. typhimurium* and *Serratia marcescens* using standard methods. The results (Table 7) disclosed significant differences ( $p<0.001$ ) in the broilers fed 12 mg/kg green silver nanoparticles compared to control group. With respect to fungal content, control group showed value as  $4.1\times 10^1$  CFU/g, while values at 4, 8 and 12 mg/kg were recorded as  $2.6\times 10^1$ ,  $2.2\times 10^1$  and  $2.0\times 10^1$  CFU/g, respectively. Total aerobic bacteria were enhanced from  $2.2\times 10^5$  (control) to  $3.1\times 10^6$  CFU/g (12 mg/kg AgNPs dose) with increasing concentrations of AgNPs. As for coli group bacteria, the colony forming units decreased significantly ( $p<0.001$ ) from  $3.6\times 10^5$  (control) to  $1.1\times 10^5$  CFU/g (12 mg/kg AgNPs dose). The outcome of the current study on antimicrobial activity revealed significant effect against different strains of bacteria. These findings were in-line with the previous studies on the antimicrobial activity (Alavi & Hamblin, 2023; Hayat et al., 2023; Wasilewska et al., 2023).

Table 7. Effect of varied doses of green-synthesized AgNPs on microbiological analysis of the contents of the jejunum and ceca of broiler (CFU/g)

Parameters	Total number of fungi	Total number of aerobic bacteria	Total number of E.coli group bacteria
Control	$4.1\times 10^1$	$2.2\times 10^5$	$3.6\times 10^5$
4mg/kgAgNPs	$2.6\times 10^1$ ***	$2.5\times 10^6$ **	$2.2\times 10^5$ **
8 mg/kgAgNPs	$2.2\times 10^1$ ***	$2.8\times 10^6$ **	$1.5\times 10^5$ ***
12 mg/kgAgNPs	$2.0\times 10^1$ ***	$3.1\times 10^6$ **	$1.1\times 10^5$ ***

\*Values are presented as Means  $\pm$  SE (n=8). Significant differences are indicated by \*\* ( $p<0.01$ ) and \*\*\* ( $p<0.001$ ) when compared to the control group

The study demonstrates that green synthesized AgNPs from garlic and ginger extract had an overall positive impact on broiler growth rate and carcass characteristics, particularly at 12 mg/kg of AgNPs, whereas higher kidney and gizzard weights were observed at low concentrations. Liver function remained stable across treatments, whereas renal function exhibited dose-dependent changes, underlining the need for dosage control. It was observed that green-synthesized AgNPs possessed the antimicrobial potential in the given context and significantly depicted the reduction in the fungal and coliform bacteria but a slight increment in the aerobic bacteria pointed towards further research. In conclusion, feeding broilers 12 mg/kg of AgNPs opens up a beneficial potential to enhance poultry production but since this field is relatively new, it becomes important to conduct more research to determine safety measures and gain the most benefits.

## Scientific Ethics Declaration

\* The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

\*All animal experiments performed in this study complied with the guidelines of the Institutional Review Board (IRB), University of Agriculture Faisalabad. Each of the abovementioned methods was done in a manner that complied with the relevant guidelines and regulations. All surgical operations were performed following the guidelines of the Institute of Laboratory Animal Resources, Commission on Life Sciences University, National Research Council (1996).

## Conflict of Interest

\* The authors declare they have no conflict of interest.

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## Authors' Contribution Statement

\* Arifa Mehreen & Adnan Afzal: Conceptualization, Supervision, Muhammad Saleem & Muhammad Rizwan Javed: Data curation, Investigation, Methodology, Hafiz Aamir Ali kharl: Writing –original draft, Data curation, Investigation, R&D.

## Data Availability

\* The data supporting the findings of this study are available upon reasonable request from the corresponding author. Access to the data will be provided contingent on compliance with any relevant ethical and legal requirements. For any inquiries regarding data availability, please contact the corresponding author directly.

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