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Use of Phytogenic Feed Additives for the Management of Avian Coccidiosis: A Promising Alternative Strategy

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1. Publications

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- Radhia Aitfella Lahlou. Enquête ethno-pharmaco-épidémiologique dans les élevages avicoles Efficacité des plantes médicinales dans la prévention de la coccidiose dans les élevages de poulets de chair.

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INTRODUCTION

INTRODUCTION

Coccidiosis is one of the most common and economically devastating diseases in poultry farming, particularly in broiler production systems (Attree et al., 2021; Blake & Tomley, 2014; Mesa-Pineda et al., 2021a; Parker et al., 2011). It is caused by several species of protozoan parasites from the genus Eimeria, which invade the intestinal epithelium of chickens and induce diarrhoea, dehydration, weight loss, poor feed conversion, and increased susceptibility to secondary infections (Chapman, 2017; Latha & Srikanth, 2022; Mesa-Pineda et al., 2021a, 2021b). According to Blake et al. (2020), the global economic cost of coccidiosis in chickens was estimated at approximately EUR 11.91 billion in 2016, considering both performance losses and the expenses of prevention and treatment (Blake et al., 2020). More recent estimates suggest that annual losses now exceed USD 13 billion, reflecting a combination of direct production impacts, treatment costs, and broader consequences along the poultry supply chain (Attree et al., 2021; Blake, 2025; Zaheer et al., 2022).

The parasite responsible for coccidiosis belongs to the phylum Apicomplexa and the family Eimeriidae. It completes its life cycle within 4–6 days in the host's intestinal epithelium, causing lesions whose severity depends on the *Eimeria* species and the infection intensity (Avi et al., 2023a; Chapman, 2017; Mesa-Pineda et al., 2021a). These lesions impair nutrient absorption, reduce weight gain, and worsen feed efficiency, and in severe cases may lead to bloody diarrhoea and death (El-Saadony et al., 2025; Kogut et al., 2020; Lee et al., 2022). The life cycle is further complicated by the production of highly resilient oocysts that persist in the environment and are resistant to many disinfectants, making their elimination challenging (Avi et al., 2023b; Bora et al., 2024).

From an economic perspective, coccidiosis disrupts feed efficiency, slows growth, and compromises flock uniformity in broilers, while in laying hens it reduces egg production and quality (Freitas et al., 2023). These changes inflate the feed-conversion ratio (FCR) and increase feed costs, which is particularly problematic in volatile global feed markets. Severe outbreaks cause additional losses through mortality, carcass condemnation, and the labour required for carcass removal and intensified sanitation (Waldenstedt et al., 2001). Farms also incur recurrent expenses related to anticoccidial drugs, vaccines, diagnostics, and hygiene programmes designed to limit environmental oocyst contamination. Long-term reliance on ionophores and other coccidiostats has contributed to antimicrobial resistance, thereby encouraging the adoption of newer, often more expensive control tools (Frederiksen et al., 2024). Furthermore, compliance with stringent residue regulations, such as those in the European Union, imposes additional costs and may restrict access to export markets if drug residues in poultry products lead to shipment rejections or financial penalties (European Commission, 2023). This burden is especially challenging in developing countries such as Algeria, where limited access to vaccines, diagnostics, and advanced treatments, combined with suboptimal biosecurity, leads to frequent and severe outbreaks (Amina et al., 2025). Coccidiosis remains one of the most frequently encountered parasitic diseases on poultry farms, with significant repercussions for flock health and production yields (Rahmani et al., 2024). Several surveys have identified *Eimeria tenella*, *Eimeria acervulina* and *Eimeria maxima* as the predominant species affecting broiler flocks in different regions of the country (Amina et al., 2025; Ayadi et al., 2024; Benabdelhak et al., 2024; Debbou-Iouknane et al., 2018; Djemai et al., 2022; Rahmani et al., 2024). Reported prevalence rates in Algerian broiler farms range from approximately 56% to over 80%, depending on the region and management practices (Benabdelhak et al., 2024; Debbou-Iouknane et al., 2018; Djemai et al., 2016; Peek & Landman, 2006). Poor hygiene, overcrowding, inadequate ventilation, and improper litter management are frequently cited as major risk factors that favour oocyst sporulation and persistence. In addition, field data indicate increasing resistance to commonly used anticoccidial drugs, particularly monensin and diclazuril (Djemai et al., 2016; Rahmani et al., 2024), further complicating disease control in Algerian production systems.

Traditionally, the poultry industry has relied on various anticoccidial drugs, such as monensin and amprolium, which interfere with parasite metabolism or reproduction. However, extensive and prolonged use of these drugs has driven the development of resistance, thereby reducing their efficacy (Xie et al., 2020; H. Zhang et al., 2022, 2023a; H. Zhao et al., 2024). Resistance can develop through several mechanisms, including altered membrane permeability, active drug efflux, modifications to the target site, or activation of alternative biochemical pathways (Schwarz et al., 2014, 2018; Xie et al., 2020; H. Zhang et al., 2022, 2023b). Reports from multiple regions describe total resistance to drugs such as monensin and robenidine, and partial resistance to salinomycin and toltrazuril (H. Zhang et al., 2022, 2023b; H. Zhao et al., 2024). Beyond *Eimeria*, the use of coccidiostats has also been associated with increased antimicrobial resistance in *Campylobacter* spp., raising additional public health concerns (Q. Zhang & Plummer, 2014). These findings call for integrated and sustainable approaches to managing coccidiosis and associated infections in poultry (Bukari et al., 2025; Lopez-Chavarrias et al., 2021).

In the face of rising drug resistance and the high economic cost of coccidiosis, robust biosecurity has become a central component of disease control programmes (Kadykalo et al., 2018; Ngom et al., 2024). Biosecurity is defined as a set of preventive measures aimed at limiting the introduction, persistence, and spread of pathogens within and between flocks (Huber et al., 2022). For coccidiosis, where environmentally resistant oocysts drive infection shed in faeces, environmental contamination is a key determinant of disease recurrence (Mesa-Pineda et al., 2021a). Effective biosecurity relies on controlling the movement of people, animals, equipment, and feed into and out of poultry houses (Dewulf et al., 2019; Islam et al., 2023). The absence of entry disinfection, the sharing of tools between flocks, and unregulated visitor access markedly increase the dissemination of *Eimeria* spp. (Ngom et al., 2024). Proper sanitation of facilities, including regular cleaning and disinfection of housing, feeders, drinkers, and water lines, can reduce the environmental oocyst burden and limit infection pressure (Jacobs et al., 2020; Li et al., 2024; Vaillancourt & Racicot, 2022). Litter management is equally important: damp, soiled bedding promotes oocyst sporulation and survival, whereas frequent litter renewal or treatment, combined with adequate ventilation to maintain dry conditions, suppresses the infection cycle (Beninca et al., 2021; Pajić et al., 2023; Soliman et al., 2018; Waldenstedt et al., 2001). The use of physical barriers, dedicated clothing, footbaths, and the separation of different age groups further reduces the risk of introducing oocysts from external sources (Beninca et al., 2021; Gelaude et al., 2014; Islam et al., 2023; D. Zhao et al., 2024).

In low- and middle-income settings such as Algeria, structural and economic constraints can limit the implementation of capital-intensive measures. Nonetheless, targeted low-cost interventions—such as improved litter management, basic disinfection routines, and better control of farm access—may yield substantial returns by reducing morbidity, mortality, and treatment costs (Alloui & Ayachi, 2012; Delpont et al., 2023; Rahmani et al., 2024). In such contexts, biosecurity is not a luxury but an economic necessity. It does not replace other control measures, but instead supports and enhances vaccination, nutrition, and genetic resistance, forming the basis of an integrated disease-management strategy (Mathis et al., 2025). Population growth, urbanisation, and increasing incomes are expected to drive global poultry meat consumption to around 160 million metric tonnes by 2033, accounting for nearly half of the additional meat consumed worldwide (OECD-FAO, 2023). Meeting this demand will rely heavily on intensive production systems, which, without strong biosecurity and integrated control, are particularly vulnerable to coccidiosis outbreaks.

Where practical constraints leave gaps in existing control programmes, phytogenic additives offer an additional layer of protection. Botanicals and their secondary metabolites—such as essential oils, polyphenols, tannins, and alkaloids—have attracted considerable interest due to their antimicrobial, antiparasitic, antioxidant, and immunomodulatory effects (Ahmad et al., 2024; Aitfella Lahlou et al., 2021; Al-Shaebi et al., 2023). Unlike conventional anticoccidials, which typically target specific stages of the parasite life cycle, medicinal plant products often act through multiple mechanisms due to their complex phytochemical composition (Aljohani, 2024; Ismaeil et al., 2025). Their efficacy has been demonstrated under experimental conditions using both whole dried and ground plants and plant extracts (Aljohani, 2024). These products can modulate oxidative stress, enhance immune responses, and stabilise intestinal function, all of which are particularly relevant in intestinal parasitic infections such as coccidiosis (Al-Shaebi et al., 2023; Blomstrand et al., 2022; Elshershaby et al., 2024; Felici et al., 2024; Ghaniei et al., 2022; Jamil et al., 2017; Yang et al., 2019).

In poultry, a growing body of evidence indicates that dietary phytogenic supplements can improve body weight gain, feed intake, and feed-conversion ratio (Sadek et al., 2014; Saleh et al., 2018; Y. R. Zhao et al., 2019). These improvements appear to result not only from direct antiparasitic or antimicrobial activity but also from better intestinal barrier integrity, enhanced nutrient absorption, and beneficial shifts in gut microbiota composition (Al-Garadi et al., 2025; Amad et al., 2011; Hashemipour et al., 2013; Murugesan et al., 2015; Pliego et al., 2022; Saleem et al., 2024). Medicinal plants also reduce oxidative stress and increase antioxidant capacity in tissues such as the intestinal epithelium, liver, and immune organs, which is particularly valuable in coccidiosis, where parasite metabolism and the host inflammatory response generate high levels of oxidative damage (Aitfella Lahlou et al., 2021; Pliego et al., 2022).

Plants from the Lamiaceae family, including *Mentha aquatica*, *Rosmarinus officinalis* and *Thymus algeriensis*, are rich in diverse bioactive compounds such as phenolic acids, flavonoids, terpenoids (monoterpenes and diterpenes), alkaloids, saponins, tannins, and essential oils. These phytochemicals have been widely reported to possess antioxidant, anti-inflammatory, antimicrobial, immunomodulatory, and antiparasitic properties. Despite their broad traditional use, the potential of these agents as anticoccidial agents in poultry remains insufficiently explored in controlled experimental settings.

To address these gaps, this thesis brings together three interconnected investigations.

Part 1 assesses the biosecurity status of commercial broiler farms in Boumerdès and quantifies the relationships among management practices, biosecurity scores, and *Eimeria* pressure.

Part 2 characterises the antioxidant, phytochemical, and pharmacological properties of Algerian *Mentha aquatica* extracts and identifies the fractions with the most significant potential for radical scavenging and health promotion in broiler production.

Part 3 evaluates the anticoccidial efficacy of selected phytogenic additives through parallel *in vitro* sporulation assays and controlled *in vivo* broiler challenges, using parasitological, biochemical, and zootechnical endpoints. Together, these studies aim to provide a science-based roadmap that combines rigorous biosecurity with multifunctional botanical supplements, thereby reducing drug dependence, slowing the development of resistance, mitigating oxidative stress, improving economic outcomes, and enhancing the resilience and sustainability of broiler production systems.

THEORICAL PART

CHAPTER I

The Economic and Health Impact of Parasitic Diseases in Broiler Chickens: Focus on Protozoan Coccidiosis

1. The genus *Eimeria*

Eimeria, a group of intracellular protozoan parasites belonging to the phylum Apicomplexa, is the causative agent of coccidiosis in poultry and other animals. (Chaves Hernández, 2014; Kogut et al., 2020). These parasites have a complex lifecycle and exhibit a high degree of host specificity, with each *Eimeria* species infecting a particular host and specific regions within the host's intestine (Weng et al., 2024a). *Eimeria* is particularly significant in poultry, especially chickens, where nine species are recognised as causing infection: *E. tenella*, *E. maxima*, *E. acervulina*, *E. necatrix*, *E. brunetti*, *E. mitis*, *E. praecox*, and *E. mivati*. Each species exhibits distinct pathogenicity, intestinal site tropism, and associated clinical manifestations. Here is the classification of *Eimeria* species:

Domain: Eukaryota
└─── **Supergroup**: Sar
 └─── **Clade**: Alveolata
  └─── **Phylum**: Apicomplexa
   └─── **Class**: Conoidasida
    └─── **Subclass**: Coccidia
     └─── **Order**: Eucoccidiorida
      └─── **Suborde**r: Eimeriorina
       └─── **Family**: Eimeriidae
        └─── **Genus**: *Eimeria*
         ├─── **Species**: *Eimeria acervulina*
         ├─── **Species**: *Eimeria maxima*
         ├─── **Species**: *Eimeria tenella*
         ├─── **Species**: *Eimeria necatrix*
         ├─── **Species**: *Eimeria brunetti*
         ├─── **Species**: *Eimeria mitis*
         └─── **Species**: *Eimeria praecox*

3.1. Biology and Life Cycle

The lifecycle of *Eimeria* is a highly intricate and adaptive process that ensures its survival and propagation across both host and environmental phases (Figure 1). The cycle begins when unsporulated oocysts are excreted in the faeces of infected chickens. These oocysts, protected by a durable, thick outer wall, transition to the exogenous phase, where they undergo sporulation under optimal environmental conditions such as sufficient oxygen, moisture, and temperatures around 23 °C (Belli et al., 2003; Chapman, 2014; Walker et al., 2013). Sporulation is a strictly aerobic process that typically requires 1–2 days, during which oocysts transform into infective units containing four sporocysts. Each sporocyst houses two banana-shaped sporozoites (Figure 1), producing eight sporozoites per oocyst (Shirley et al., 2005). These sporulated oocysts (Figure 1) are remarkably resilient, capable of surviving harsh conditions, including resistance to many disinfectants and environmental stressors, although they remain vulnerable to extreme heat or freezing temperatures (Cha et al., 2018; J. Liu et al., 2023; Venkateswara Rao et al., 2015). Under favourable host conditions, such as those found in the chicken's gastrointestinal tract, these sporozoites are released through a process known as excystation (Figure 1). The triggers for this process are thought to include body temperature, pH, proteases, bile salts, and other unidentified host environmental factors (Burrell et al., 2020; J. Liu et al., 2023; Walker et al., 2013; Wiedmer et al., 2020).

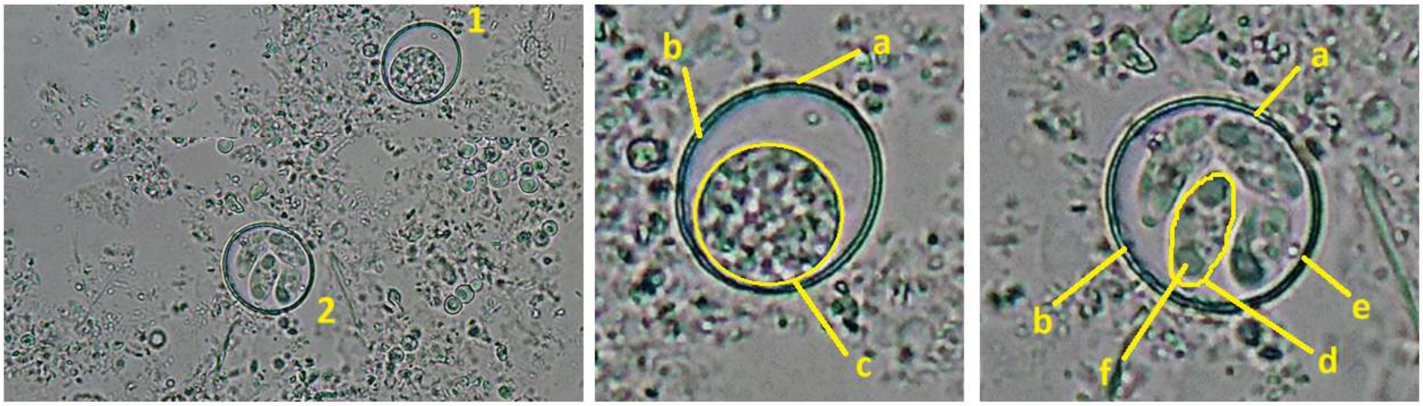


Figure 1. Oocysts of *Eimeria*. (1) Unsporulated and (2) sporulated oocysts of *Eimeria* spp, (a) Outer layer, (b) Inner layer, (c) Zygote, (d) Sporocyst, (e) refractile body, (f) sporozoites.

The endogenous phase begins when chickens ingest sporulated oocysts via contaminated feed, water, or litter (Figure 1). Within the gastrointestinal tract, digestive enzymes, bile, and carbon dioxide trigger sporozoite release (Rieux et al., 2012; Zhang et al., 2023). These motile sporozoites invade specific regions of the intestinal epithelium, where the species-specific tropism of *Eimeria* dictates their infection sites (Silva et al., 2022). The parasites initiate schizogony, developing into trophozoites within parasitophorous vacuoles within epithelial cells (Figure 2). As trophozoites grow, they undergo multiple rounds of nuclear and cytoplasmic division, resulting in the formation of merozoites (Dubey and Jenkins, 2018; Walker et al., 2013). These merozoites eventually rupture host cells, causing extensive intestinal damage and spreading to infect neighbouring cells. This cycle is repeated several times, amplifying the infection and exacerbating its pathological effects (Conway and McKenzie, 2007).

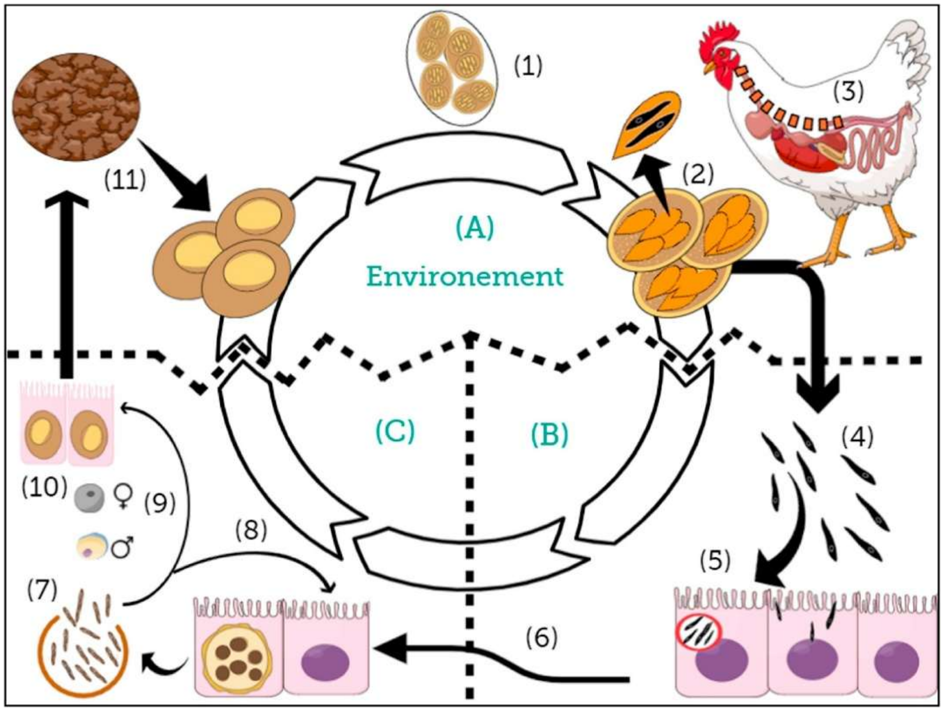


Figure 2. The life cycle of *Eimeria* in poultry (Aitfella Lahlou et al., 2021). *Eimeria* spp. is an obligate intracellular parasite with a complex life cycle that occurs both in the environment and within a single host, as illustrated. The life cycle is considered monoxenous. It takes approximately 4–7 days to complete. Following ingestion (1), the oocysts excyst within the intestinal lumen and release sporozoites (2), which penetrate the villous epithelial cells and multiply in damaged tissues (3). These multiplications constitute the asexual reproduction phase (Phase I: Schizogony or merogony - A), in which nuclear divisions and cytoplasmic differentiation lead to the formation of merozoites that detach themselves and parasitise other sites, releasing, in turn, additional generations of merozoites (4). This asexual cycle is repeated 2–4 times, depending on the *Eimeria* species. It damages the intestinal tissues, causing digestive disturbances, fluid and blood loss, and susceptibility to other diseases (5). At the end of the last merogonic cycle (6), merozoites are transformed into a female (macrogamonts) and male gametes (microgamonts) (7–8). The latter produces many microgametes that fertilise the macrogamonts (Phase II Gametogony- B). After several rounds of

multiplication, many zygotes are encased in a thick wall impervious to the harshest environmental conditions and are subsequently excreted in the droppings (9–10). The third phase of the life cycle, sporogony (C), occurs in the external environment and produces a new generation of infective oocysts (11) that can be ingested, thereby restarting the life cycle.

Each *Eimeria* species exhibits a distinct preference for specific intestinal regions, which strongly influences the clinical manifestations of the disease (Figure 2). For example, *E. tenella* primarily targets the ceca, leading to haemorrhagic lesions, bloody diarrhoea, and severe anaemia, with high mortality rates in severe cases. (CERVANTES et al., 2020). *E. maxima* infects the mid-intestine, causing inflammation, nutrient malabsorption, and stunted growth (Conway and McKenzie, 2007), while *E. acervulina* colonises the upper small intestine (Figure 2), resulting in growth retardation, reduced feed efficiency, and production losses. Other species, such as *E. brunetti* and *E. necatrix*, are associated with severe haemorrhagic enteritis. (Shirley et al., 2005), while *E. mitis* and *E. praecox* typically induce more subtle malabsorptive symptoms that nonetheless impair flock performance, particularly in subclinical infections (Allen and Jenkins, 2010; Wang et al., 2024). Despite their varying pathogenicity, even the so-called "lesser species" can contribute to production losses and impaired flock performance, particularly in subclinical infections (Figure 2).

After completing a fixed number of schizogony cycles (Figure 2), the lifecycle transitions to gametogony. During this phase, merozoites differentiate into sexual forms within epithelial cells; male microgametes fertilise female macrogametes, forming zygotes (Dubey and Jenkins, 2018; Shirley et al., 2005; Walker et al., 2013). These zygotes mature into unsporulated oocysts that are shed with the faeces, thereby re-entering the environment and perpetuating transmission (Burrell et al., 2020; Cha et al., 2018). The pathogenicity of *Eimeria* is driven by the extensive destruction of intestinal epithelial cells throughout schizogony and gametogony, which compromises the mucosal barrier, impairs nutrient absorption and triggers local inflammation (Chapman, 2014). Haemorrhagic species such as *E. tenella* cause severe blood loss and can be fatal (Table 1), whereas malabsorptive species such as E. maxima lead to chronic weight loss and reduced feed efficiency;

both conditions predispose birds to secondary infections by *Clostridium perfringens*, which can escalate to necrotic enteritis (Williams, 2005).

The remarkable environmental resilience of *Eimeria* oocysts, particularly their ability to sporulate rapidly under warm, humid conditions, explains their persistence in intensive systems with high stocking densities and inadequate litter management (Waldenstedt et al., 2001). Therefore, effective biosecurity and litter-drying protocols are critical to breaking reinfection cycles (Chapman, 2014). Successful infection hinges on multiple host–parasite interfaces: (i) parasite recognition of species-specific intestinal receptors, (ii) the structural integrity of the intestinal barrier, (iii) modulation of the gut microbiota, and (iv) an appropriately balanced immune response (Lillehoj and Lillehoj, 2000; Ritzi et al., 2014). The outcome of these interactions dictates clinical severity and long-term performance losses. A deep understanding of these dynamics underpins integrated control strategies that combine targeted vaccination, innovative therapeutics (e.g., probiotics, phytogenics), and stringent environmental management. Continued research into *Eimeria*'s molecular and cellular biology promises to reduce further the parasite's impact on poultry health and productivity, safeguarding the sector as global demand for poultry products rises.

3.2. Molecular insights into *Eimeria*–host interactions and the systemic impact of avian coccidiosis

Eimeria sporozoites do not invade indiscriminately; they recognise a precise patchwork of epithelial receptors that guides each species to its preferred intestinal niche. The MAR domain of EtMIC3, for example, binds α2-3-sialylated glycans that are abundant in the caecum. (Lai et al., 2011), while the newly described EtMIC8 latches onto EPCAM, two interactions that help explain the strict caecal tropism of *Eimeria tenella* and *E. necatrix* (Sun et al., 2024). After docking, rhoptry-neck proteins (RON2/4/5/8) insert into the host membrane and pair with apical membrane antigen 1 (AMA1) to build the moving junction, yet *Eimeria* can fall back on alternative AMA–RON pairs when AMA1 is neutralised, a redundancy that undermines single-antigen vaccines (Li et al., 2024; Ma et al., 2019).

Entry triggers an EGFR–AKT–MLCK cascade that phosphorylates myosin light chain, unzips tight-junction proteins such as ZO-1 and occludin, and widens paracellular gaps; in vivo, this translates into a two-fold rise in FITC-dextran permeability and a marked drop in nutrient transporters SGLT1, GLUT2 and GLUT5 (Q. Liu et al., 2024; Weng et al., 2024a; Zhang et al., 2022).

As the barrier function deteriorates, the parasite reprograms the host's metabolism by redirecting glycolysis, β-oxidation, and sphingolipid synthesis toward its biomass and by inhibiting antioxidant enzymes, thereby allowing reactive oxygen species to accumulate. Early-stage parasites skew intestinal macrophages towards an M2-like, STAT6-dependent profile and repress NF-κB/TLR-2/4 signalling; concentrations of IL-1β, TNF-α, and IL-6 decrease, whereas IL-10 and TGF-β increase, creating a cytokine pattern that prolongs epithelial survival and favours repeated merogonies. Microneme proteins, such as EtMIC3, EtMIC4, and EtMIC7, further delay apoptosis, thereby extending the replicative window within host cells (Jin et al., 2020; Periz et al., 2007; Tomal et al., 2023; Wang et al., 2021; Weng et al., 2024b; Yu et al., 2021).

Barrier leakage contributes to significant dysbiosis, as beneficial genera (Lactobacillus, Faecalibacterium, Roseburia) decline, while *Clostridium*, *Streptococcus*, *Salmonella*, and *Campylobacter* proliferate. The collagen-rich mucus secreted during *E. maxima* or *E. tenella* infections form a nutrient-dense scaffold that promotes the overgrowth of *Clostridium perfringens* and the emergence of necrotic enteritis. Indole metabolites from surviving commensals can partially restore tight-junction integrity through aryl-hydrocarbon-receptor signalling, highlighting the complex nature of microbial partnerships (Choi and Kim, 2022; J. Liu et al., 2024; Nicholds et al., 2021a; Schreiber et al., 2024; Tierney et al., 2007).

Beyond the intestine, the pathological cascade extends to multiple physiological systems. Extensive epithelial destruction leads to the malabsorption of amino acids, fatty acids, vitamins, and electrolytes, resulting in growth retardation and altered plasma chemistry. Significant sodium and water loss through the damaged mucosa makes birds more susceptible to dehydration and acid–base imbalances (Campos et al., 2023; Chen et al., 2025; Choi and Kim, 2022; Lee et al., 2022; Zhou et al., 2020). Ongoing haemorrhage during infections with *E. tenella* or *E. necatrix* depletes circulating erythrocytes and iron stores, causing anaemia and hypoproteinaemia that hinder oxygen delivery and tissue repair. Indicators of oxidative stress rise sharply, while superoxide dismutase and glutathione peroxidase activities decline, and by-products of lipid peroxidation accumulate, thereby worsening tissue injury and reducing mitochondrial ATP production (Cha et al., 2020; Fortuoso et al., 2019; Georgieva et al., 2006). Systemic inflammation and diversion of nutrients to the immune response further diminish feed-conversion efficiency, while persistent cytokine skewing reduces vaccine responsiveness and leaves flocks at risk from secondary pathogens (Benabdelhak et al., 2024; Campos et al., 2024; Sultan et al., 2022).

Intervention strategies now concentrate on multiple choke points. CRISPR/Cas9-engineered "precocious" lines truncate the pathogenic phase yet elicit robust immunity, and nanoparticle vaccines co-displaying EtAMA3 with RON2-like epitopes provide stronger protection than single-component formulations (Gul et al., 2022; Haseeb et al., 2022a; Jang et al., 2011; Q. Liu et al., 2023). Targeting the environmental stage is equally promising: anti-sporulation measures that block oocyst maturation in litter can reduce reinfection pressure and complement genetic or immunological tools. By targeting *Eimeria* at the attachment, invasion, replication, and environmental phases, and by mitigating the systemic consequences of infection, such integrated programmes aim to reduce both the economic burden of coccidiosis and the selective pressure for drug resistance in modern poultry production.

3.3. Pathogenicity and clinical manifestations

Coccidiosis is a complex parasitic disease caused by protozoa of the genus *Eimeria*, which target and replicate within the intestinal epithelial cells of poultry (López-Osorio et al., 2020) . The infection begins when chickens ingest sporulated oocysts from contaminated feed, water, litter, or equipment. Inside the gastrointestinal tract, sporozoites are released from the oocysts, invading epithelial cells and initiating the parasite's lifecycle (López-Osorio et al., 2020) .

Once inside the epithelium, *Eimeria* causes damage through several mechanisms, often synergistic. Mechanical rupture of parasitised cells during merogony and gamogony strips large areas of mucosa, creating a breach in the intestinal barrier that leads to plasma-protein leakage and early hypoproteinaemia (Weng et al., 2024b). Local toxic factors, well-documented for *E. tenella*, exacerbate necrosis and inhibit key digestive enzymes, such as amylase and maltase. Severe species (*E. tenella*, *E. necatrix*) also trigger pronounced vascular effects: transient prolongation of prothrombin time and massive haemorrhage that can account for most of the mortality (Nicholds et al., 2021b). Metabolic sequelae include hypoglycaemia, systemic acidosis and hypothermia in moribund birds, while sodium loss and catarrhal inflammation drive the profuse diarrhoea typical of clinical outbreaks.

Highly pathogenic species such as *E. tenella* and *E. necatrix* may provoke acute haemorrhagic caecal or mid-gut disease with case-fatality rates approaching 80 % in immunologically naïve broilers (Wang et al., 2023). By contrast, “lesser” species (*E. acervulina*, *E. mitis*, *E. praecox*) often produce subclinical infections that still impair weight gain, feed conversion, and carotenoid absorption, leading to a 3–5% weight penalty and marked depigmentation, even at low oocyst doses.

Table 1. Species tropism and clinical outcome. *Eimeria* species display strict intestinal niches that define their pathology.

Intestinal segment affected	**Predominant species**	**Typical clinical form**	**Major signs**	**Lethality / Impact on flock health***
Caeca	*E. tenella*	Caecal coccidiosis (20–28 d)	Depression, drooping wings, bloody diarrhoea from day 4	**Often fatal:** mortality can reach ≈ 80 % in naïve chicks; survivors suffer sharp weight loss and poor feed conversion
Mid-intestine	*E. necatrix*	Acute haemorrhagic enteritis (> 4 weeks)	Ballooning, muco-haemorrhagic exudate, “salt-and-pepper” serosa	**Highly fatal**: heavy bleeding, rapid losses; severe growth check in survivors
Mid-intestine & rectum	*E. brunetti*	Necro-haemorrhagic enteritis	Wall oedema, red streaks, white pseudomembranes	**Moderate to high mortality** rates occur in heavy outbreaks; chronic cases also depress growth and carcass yield.
Mid-intestine	*E. maxima*	Mucoid enteritis	Dilation, thickening, orange mucus ± blood	**Seldom fatal**, but causes a marked feed-efficiency drop and egg-production losses in layers.
Duodenum / proximal gut	*E. acervulina*	Mild mucoid enteritis	Linear white plaques, reduced appetite, depigmentation	**Low mortality**; chiefly subclinical—3–5 % weight penalty, paler skin/shanks
Duodenum	*E. praecox*	Subclinical infection	Watery contents, excess mucus; minimal gross lesions	**Non-fatal**; decreases feed efficiency and flock uniformity if oocyst pressure is high
Distal ileum – rectum	*E. mitis*	Mild mucoid enteritis	Discrete lesions, creamy contents with undigested feed	**Non-fatal**; slight weight suppression and wet litter problems

*Impact assumes typical commercial broiler or pullet conditions; actual severity depends on infective dose, bird age, immune status and management practices.

3.3.1. Lesions

The four-grade macroscopic scale proposed by Johnson and Reid (1970) remains the standard reference for assessing the severity of coccidial lesions in chickens. On this scale, birds are scored from 0 (no visible lesions) to 4 (very severe lesions or death). The score assigned depends on both the extent of tissue damage and the specific intestinal segment targeted by each *Eimeria* species (Johnson and Reid, 1970).

Eimeria acervulina: This parasite first produces fine, transverse white streaks in the duodenal loop (score 1). As the infection intensifies, streaks become more numerous and may spread up to 20 cm beyond the duodenum without yet thickening the gut wall (score 2). Coalescing plaques give the mucosa a "painted" appearance, the wall thickens, and the intestinal contents become watery (score 3). At the most advanced stage (score 4), the entire proximal intestine is grey-white, markedly thickened, and packed with a creamy exudate containing oocysts; moribund or dead birds are assigned this score (Figure 3).

Eimeria maxima: Usually confined to the mid-gut, *E. maxima* begins with scattered serosal petechiae and a trace of orange mucus (score 1). At score 2, petechiae become dense, orange mucus fills the lumen, and the wall thickens slightly. Severe infection (score 3) causes obvious ballooning, marked wall thickening, a roughened mucosa and orange mucus flecked with pinpoint blood clots. Score 4 birds show gross distension, dark digested blood, a fetid odour and extensive haemorrhage; lesions may be mistaken for necrotic enteritis unless carefully differentiated (Figure 3).

Eimeria tenella: Lesions are restricted mainly to the caeca. A light challenge reveals only a few petechiae (score 1). Moderate infection produces visible blood in the lumen and some wall thickening (score 2). Heavy cases pack the caeca with clots or caseous cores, greatly thickening the wall (score 3). In the gravest presentations (score 4), the caeca are massively distended with blood or cores and lack standard faecal material; fatalities fall into this category (Figure 3).

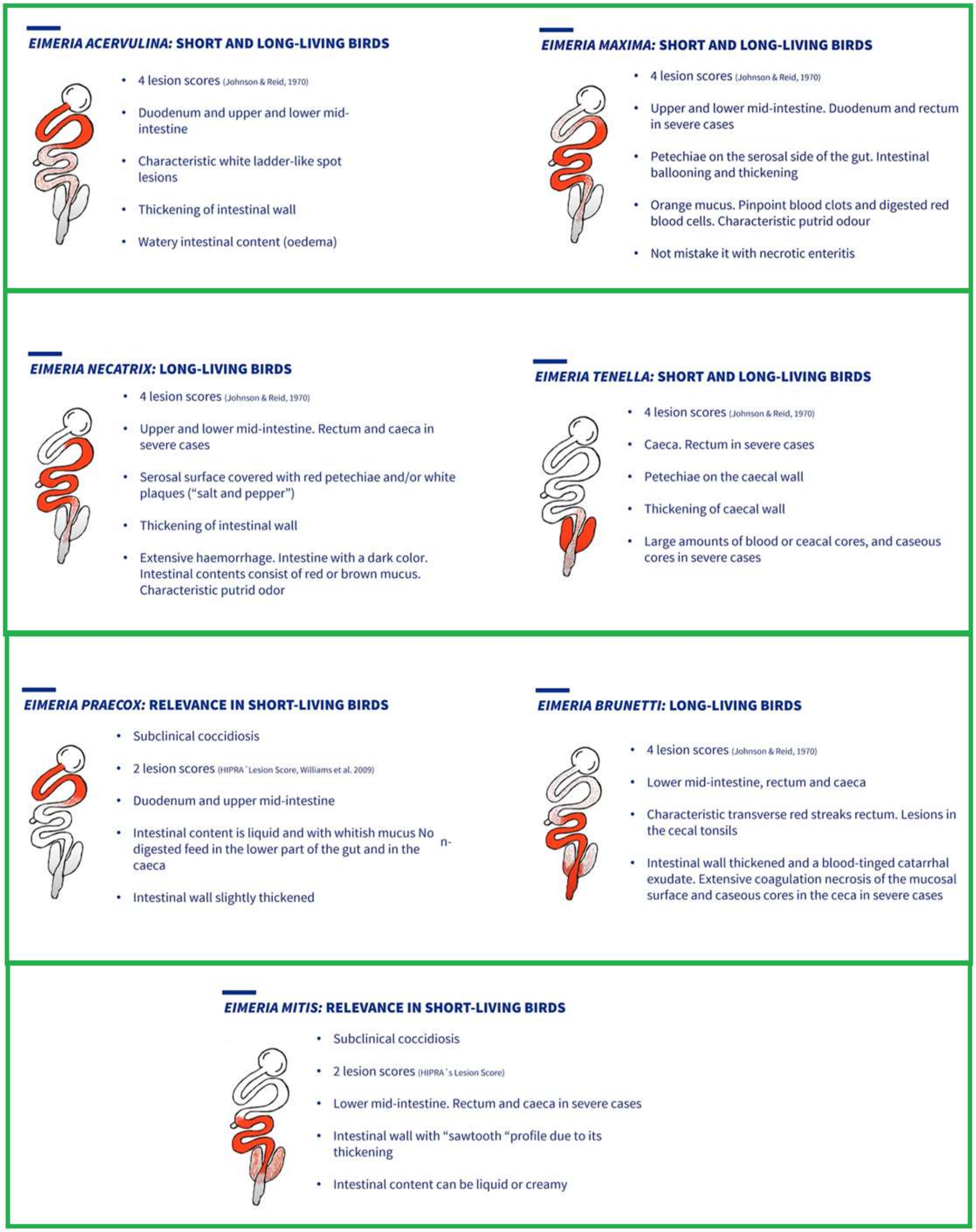
Eimeria mitis: This short-cycle species, often subclinical, colonises the lower mid-gut and rectum. At score 1, the wall appears normal, but the mid-gut contents are slightly watery. Score 2 shows mild wall thickening with a saw-tooth profile, creamy contents and undigested feed in the distal gut and caeca (Figure 3).

Eimeria praecox: Another subclinical, short-cycle species, *E. praecox,* affects the duodenum and upper mid-gut. A score 1 intestine appears macroscopically normal, although the contents may be watery. At score 2, the duodenal wall thickens slightly, liquid contents and excess whitish mucus accumulate, and undigested feed appears distally (Figure 3).

Eimeria necatrix: This species, important in older birds, targets the mid-intestine and may extend to the rectum and caeca. Score 1 shows sparse petechiae and white spots on the serosa: numerous petechiae and slight ballooning mark score 2. At score 3, the classic "salt-and-pepper" serosa appears—red petechiae mixed with white plaques—accompanied by heavy haemorrhage and ballooning over the lower half of the mid-gut. Score 4 presents as a dark, blood-filled intestine with a putrid odour and widespread ballooning; birds found dead are scored 4 (Figure 3).

Eimeria brunetti: Typically seen in older birds, *E. brunetti* lesions begin with no noticeable macroscopic change (score 1). A grey, mildly thickened lower gut with salmon-coloured flecks signals score 2. Score 3 features marked thickening, blood-tinged catarrhal exudate, red transverse streaks in the rectum and mucus plugs in the caecal tonsils. Extensive coagulative necrosis, with dry necrotic membranes lining the gut and caseous cores plugging the caeca, defines score 4; lesions may extend forward into the mid-intestine (Figure 3).

Macroscopic lesion scoring thus offers a rapid field estimate of infection severity; however, for subclinical species such as *E. mitis* and *E. praecox*, or mixed infections, additional tools, such as oocyst counts and histology, are essential to refine the diagnosis and guide control strategies that may include vaccination, chemoprophylaxis, or phytogenic alternatives.



***EIMERIA ACERVULINA:* SHORT AND LONG-LIVING BIRDS**

- 4 lesion scores (Johnson & Reid, 1970)
- Duodenum and upper and lower mid-intestine
- Characteristic white ladder-like spot lesions
- Thickening of intestinal wall
- Watery intestinal content (oedema)

***EIMERIA MAXIMA:* SHORT AND LONG-LIVING BIRDS**

- 4 lesion scores (Johnson & Reid, 1970)
- Upper and lower mid-intestine. Duodenum and rectum in severe cases
- Petechiae on the serosal side of the gut. Intestinal ballooning and thickening
- Orange mucus. Pinpoint blood clots and digested red blood cells. Characteristic putrid odour
- Not mistake it with necrotic enteritis

***EIMERIA NECATRIX:* LONG-LIVING BIRDS**

- 4 lesion scores (Johnson & Reid, 1970)
- Upper and lower mid-intestine. Rectum and caeca in severe cases
- Serosal surface covered with red petechiae and/or white plaques ("salt and pepper")
- Thickening of intestinal wall
- Extensive haemorrhage. Intestine with a dark color. Intestinal contents consist of red or brown mucus. Characteristic putrid odor

***EIMERIA TENELLA:* SHORT AND LONG-LIVING BIRDS**

- 4 lesion scores (Johnson & Reid, 1970)
- Caeca. Rectum in severe cases
- Petechiae on the caecal wall
- Thickening of caecal wall
- Large amounts of blood or ceacal cores, and caseous cores in severe cases

***EIMERIA PRAECOX:* RELEVANCE IN SHORT-LIVING BIRDS**

- Subclinical coccidiosis
- 2 lesion scores (HIPRA´Lesion Score, Williams et al. 2009)
- Duodenum and upper mid-intestine
- Intestinal content is liquid and with whitish mucus Non-digested feed in the lower part of the gut and in the caeca
- Intestinal wall slightly thickened

***EIMERIA BRUNETTI:* LONG-LIVING BIRDS**

- 4 lesion scores (Johnson & Reid, 1970)
- Lower mid-intestine, rectum and caeca
- Characteristic transverse red streaks rectum. Lesions in the cecal tonsils
- Intestinal wall thickened and a blood-tinged catarrhal exudate. Extensive coagulation necrosis of the mucosal surface and caseous cores in the ceca in severe cases

***EIMERIA MITIS:* RELEVANCE IN SHORT-LIVING BIRDS**

- Subclinical coccidiosis
- 2 lesion scores (HIPRA´s Lesion Score)
- Lower mid-intestine. Rectum and caeca in severe cases
- Intestinal wall with "sawtooth "profile due to its thickening
- Intestinal content can be liquid or creamy

Figure 3. Poultry Intestinal localisation of the lesions of *Eimeria* spp (Conway and McKenzie, 2007).

3.4. Diagnosis and species identification

Accurate identification of the *Eimeria* species causing poultry coccidiosis is crucial for effective vaccination, drug selection, and monitoring of resistance. Classical coproscopy, based on oocyst morphometrics and lesion site, remains useful for heavy monospecific infections but is limited by subjectivity and frequent misclassification when several morphologically similar species are present in a single bird or flock (comparisons with PCR consistently reveal these limitations) (Barkway et al., 2011; Haug et al., 2008).

Multiplex PCRs targeting the ITS-1 ribosomal spacer can identify four major pathogenic species, *E. tenella, E. acervulina*, *E. maxima*, and *E. necatrix*, in a single tube with high analytical sensitivity (approximately 1–5 pg of template DNA, equivalent to a few oocysts) (You, 2014). Nested and real-time PCR formats enhance sensitivity further and enable quantification of parasite DNA; widely used TaqMan panels now encompass all seven recognised chicken *Eimeria* species and have detection limits approaching a single sporulated oocyst (Morgan et al., 2009; Vrba et al., 2010). Several field surveys using ITS-1 PCR (often combined with sequencing) identify *E. tenella* as the predominant species in broiler operations, with *E. mitis* commonly detected and notable regional variations in species composition, for example, in Iran, India, and Egypt (Gadelhaq et al., 2015; Hamidinejat et al., 2010; Kumar et al., 2015). Capillary-electrophoresis PCR has also uncovered cryptic operational taxonomic units in Australian flocks that would be overlooked by morphology alone (Cantacessi et al., 2008; Gasser et al., 2005). A comparative study in Malaysia evaluating SCAR-based PCR, LAMP, and qPCR found that although all methods detected reference strains, their performance varied on field samples, with qPCR and optimised SCAR assays more reliably detecting mixed-species infections than conventional endpoint PCR. Isothermal chemistries now enable high-specificity testing in basic farm laboratories. Species-specific LAMP assays detect approximately 1–10 genome copies in under an hour without the need for a thermocycler, and they are used for on-site monitoring of vaccine “take” or anticoccidial efficacy (Barkway et al., 2011).

The latest generation combines recombinase-polymerase amplification with CRISPR/Cas12a collateral cleavage; a fluorescence-readout assay distinguishes all seven chicken species and aligns well with qPCR results on field faecal samples, with a workflow suitable for 20–30-minute runs (Cheng et al., 2022).

These molecular platforms surpass morphology in sensitivity, specificity, and speed. A practical two-tier approach is emerging: LAMP (or RPA-CRISPR) for rapid flock-level screening, followed by multiplex or real-time PCR for definitive species identification and measurement of parasite load. Incorporating such diagnostics into routine surveillance enhances programme auditing, directs targeted treatment, and helps monitor the geographic spread of drug-resistant or genetically novel *Eimeria*, ultimately protecting poultry health and production efficiency (Barkway et al., 2011; Vrba et al., 2010).

3.5. Prevention and control of coccidiosis

3.5.1. Anticoccidial drugs

Since 1940, the primary method of controlling coccidiosis has been the use of preventive anticoccidials, and/or vaccines, along with hygienic measures and improved farm management (Chapman, 2009). Poultry production primarily used synthetic anticoccidials until the 1970s, when significant advancements in coccidiosis control were made through the discovery of ionophore anticoccidials. The growing success of the poultry industry is reportedly due to the consistent and intensive use of anticoccidials for the prevention and control of coccidiosis in poultry (Chapman and Rathinam, 2022).

Anticoccidial drugs, also known as coccidiostatics (ACDs), used in the poultry industry possess antimicrobial properties that reduce parasitic diseases (Hansen et al. 2009c). In the European Union (EU), anticoccidial compounds are classified as feed additives. In contrast, in the United States (USA), they are classified as drugs for use in animal feeds (EU, 2003). Additionally, they are also used as growth promoters in poultry production (Dibner and Richards, 2005).

The ACDs are anti-parasitic compounds (Figure 3) with the primary aim of enhancing livestock growth rates by inhibiting and killing the parasite (Elliott et al., 1998; Hansen et al., 2009; Samanidou and Evaggelopoulou, 2008). Their mechanism of action involves destroying intracellular stages of the parasite at specific times during the life cycle or exerting their effects at several phases once the parasite has invaded host cells in the intestine (Chapman, 2007). Moreover, they can act on extracellular stages (sporozoites and merozoites) to prevent cell penetration, or on intracellular stages to stop or inhibit development; a few anticoccidials also affect the sporulation of oocysts after they are excreted (Anadón and Martínez-Larrañaga, 2014).

The ACDs are widely used in the poultry industry worldwide (Kadykalo et al., 2018). In 2015, the U.S. Food and Drug Administration reported that about 81% of drug distribution is represented by anticoccidial ionophores (FDA, 2015; VICH, 2013).

As a result, the industrial production of broilers worldwide depends heavily on the utilisation of anti-anticoccidials, as it is necessary to maintain health, animal welfare, and suitable feed conversion, thereby avoiding poor growth and mortality in broilers (Bacila et al., 2017).

According to their effectiveness, there are two classes of ACDs commercially available (Figure 4): coccidiostat compounds that inhibit the growth of intracellular coccidian parasites, and coccidiocidal compounds that eradicate parasites during their development (Chapman, 2007; ERP, 2006). Some anticoccidials may be coccidiostatic on a short-term basis but coccidiocidal on a long-term basis, depending on factors as length of time on medication, dosage, and species of coccidia (Anadón and Martínez-Larrañaga, 2014; M Kahn et al., 2006). Nevertheless, they can be employed at different stages of the poultry life cycle, either alone or combined with other anticoccidial drugs generally alternated in a shuttle, continuous, and rotation programs (Clarke et al., 2014; Spínosa et al., 2014). These strategies have been developed to extend the useful life of coccidiostats by blocking the development of drug resistance while maintaining control of coccidiosis.

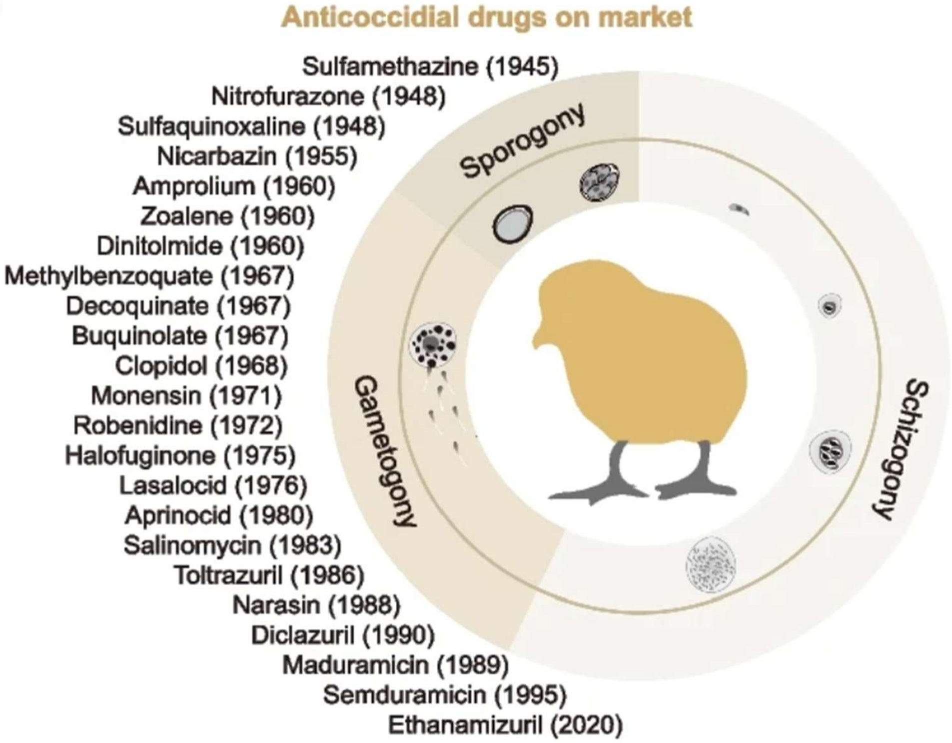


Figure 4. List of anticoccidial drugs introduced into and used in the poultry industry (Gao et al., 2024).

Synthetic coccidiostats (no polyether ionophores - NPIs) were the first ADCs to be manufactured through chemical reactions (Figure 3). There is a wide range of molecules that can be subdivided into different chemical classes: quinolones, pyridones, alkaloids, guanidines, thiamine analogues, and triazine derivatives (Kart and Bilgili 2008). Otherwise, the NPIs authorised in the EU are: Decoquinate (DEC), Robenidine hydrochloride (ROB), Halofuginone hydrobromide (HAL), Diclazuril hydrochloride (DIC), and Nicarbazin (NIC) (Anadón and Martínez-Larrañaga 2014).

The NPIs function in different ways and often act at specific stages of parasite development. They are absorbed into the host's bloodstream and kill developing parasites in the epithelial cells of the villi in the intestines (Quiroz-Castañeda, 2018; Quiroz-Castañeda and Dantán-González, 2015). Thus, this high efficiency led to their administration at lower concentrations (Hansen et al., 2009).

One of the most commonly used ACDs in the poultry industry is NIC (Anadón and Martínez-Larrañaga, 2014; EFSA, 2008). It is a chemical belonging to the carbanilide group and has been used for more than 50 years to treat broad-spectrum coccidiosis in the poultry industry (Cuckler et al., 1955). Moreover, it is authorised as a coccidiostat feed additive for individual use in chickens for fattening or in combination with the ionophore Narasin (NAR) (EFSA, 2008). Its protective action occurs on day 5 of the developing coccidia's life cycle, resulting in minimal tissue damage (Clarke et al., 2014). Furthermore, no resistance incidents that could compromise coccidiosis control have been reported to date (Chapman, 2014; Spínosa et al., 2014).

The NIC crystals complex is composed of an equimolar amount of the active ingredient 4,4'-dinitrocarbanililide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP), which participates in the absorption efficiency of DNC in the poultry GI tract, allowing the NIC complex to exhibit practical anticoccidial activities. Generally, the latter consists of the inhibition and/or destruction of first and second-generation schizonts (Aslian et al., 2014).

The mode of action appears to be via the inhibition of succinate-linked nicotinamide adenine dinucleotide reduction and the energy-dependent transhydrogenase, as well as the accumulation of calcium in the presence of adenosine-5′-triphosphate (Anadón and Martínez-Larrañaga, 2014; Clarke et al., 2014).

Before 1970, prophylactic medication with NPIs in the feed was a significant way to prevent coccidiosis. The biggest problem with this control is that when medication is withdrawn, arrested parasites may resume their life cycles and contaminate the environment with infective oocysts. Moreover, this is accompanied by the development of tolerance or a reduction in the NPIs susceptibility of the target population (Anadón and Martínez-Larrañaga, 2014). On the other hand, the intensive use of most NPIs or repeated exposure to the same drugs can result in the selection of drug-resistant strains of *Eimeria*, which may lead to a reduced efficacy of coccidiostats (McDougald and Fitz-Coy, 2009; Quiroz-Castañeda and Dantán-González, 2015).

In general, the polyether ionophore drugs (PIs) are more effective and do not induce the same degree of selective pressure on the parasite as synthetic anticoccidial drugs (Anadón and Martínez-Larrañaga, 2014; Chapman, 2007). In contrast, the IPs are characterised as potent antibiotics belonging to the broader family of naturally occurring ionophores produced by bacteria of the *Streptomycetaceae* family (Clarke et al., 2014; Riddell, 2002; Westley, 1982). Over 120 naturally occurring ionophores have been identified to date (Dutton et al., 1995). Additionally, the term ionophore refers to the molecule's ability to bind a metal ion and facilitate its transport across cellular membranes (Pressman, 1976; Pressman and deGuzman, 1977).

The PIs contain multiple tetrahydrofuran rings connected in the form of spiroketal moieties (Antoszczak et al., 2015; Riddell, 2002). Other important features, also contributing to their mode of action, include a free carboxyl group, numerous lower alkyl groups, and a variety of functional oxygen groups that form an internal cavity capable of binding metal ions (Antoszczak et al., 2015).

Thus, according to this chemical structure and the mechanism of action, the PIs can be divided into two groups (Westley, 1975): Monovalent compounds [Monensin (MON), Narasin (NAR), Salinomycin (SAL), Maduramycin (MAD), and Semduramycin (SEM)] are those that are unable to transport divalent cations. In contrast, Divalent polyether [Laslocid (LAS)] can transport both monovalent and divalent cations.

To control coccidiosis, the PIs are added to the poultry feed and are rapidly absorbed from the gut. The products function in various ways and often act on specific developmental stages of both asexual and sexual cycles of the parasite (Anadón and Martínez-Larrañaga, 2014). Furthermore, the PIs are coccidiocidal because they can move ions, usually Na+, preferentially into various stages of the parasite, thereby altering the ionic balance within the coccidian. Because coccidia intracellular parasites cannot make adenosine triphosphate (ATP) to drive their Na–K ATPase pumps, the coccidial organism loses the ability to osmoregulate and increasing intracellular sodium is transferred to other organelles, thereby causing mitochondrial lesions, cell swelling, vacuolization, and, finally, programmed cell death (apoptosis) (Anadón and Martínez-Larrañaga, 2014)

Studies have reported that PIs exhibit a broad spectrum of bioactivity and are therefore used as growth factors in animals. The compounds exhibit antibacterial, antifungal, antiparasitic, and antiviral activities. Additionally, it has been proven that PIs possess anti-inflammatory and cytotoxic properties against tumour cells. On the other hand, tests have demonstrated that derivatives formed by chemical modifications of PLs are often more antimicrobial and anticancerous compared to their parent compounds (Antoszczak et al., 2019, 2015; Kevin et al., 2009).

3.5.2. Vaccination

Immunity to *Eimeria* is complex, multifactorial and influenced by host and parasite, with different elements playing greater or lesser roles in three different types or stages of immunity: innate resistance to primary infection, acquired immunity to reinfection and maternal immunity (Allen and Fetterer, 2002; Chapman et al., 2013; Innes and Vermeulen, 2006). It is from these immunological notions that the basis of modern coccidiosis vaccinology (Shivaramaiah et al., 2014). As chickens are the most widely used animal feed in the world, the development of vaccines to control poultry diseases is crucial to global food security (Damer P Blake et al., 2015).

Vaccination would provide chickens with some parasite resistance by reducing the pathogenic effects of coccidiosis, resulting in fewer macroscopically visible lesions and/or a decrease in oocyst production, as well as increased bird performance (Peek and Landman, 2003). In 1952, the first commercial live coccidiosis vaccine, CocciVac, was registered in the United States (Edgar et al., 1952), comprising a mixture of E*imeria tenella* wild-type strains (Chapman et al., 2002; Price et al., 2014). Nevertheless, the vaccine was widely criticised for containing a single species of *Eimeria* and thus not providing sufficient protection for the flocks against other parasite species (Sharman et al., 2010). However, after several reformulation attempts and thorough reviews, different vaccines have been developed and are still used today. They are either live (first-generation) or attenuated (second-generation) vaccines, which vary depending on the number of *Eimeria* species and the number of oocysts present.

Vaccines are presented in liquid form because sporulated oocysts require a liquid suspension to remain viable and immunogenic. They contain some or all species and are typically administered in the hatchery via direct spray application to chicks, with eye drops, or in ovo, using a controlled number of oocysts within enclosed cabinets (Price et al., 2014).

Early immunity to *Eimeria* is stimulated by the vaccine and subsequently enhanced and maintained through multiple re-exposures to vaccine oocysts or wild-type oocysts present in the litter (faecal–oral transmission - "cycling"). Protective immunity is induced after two to three consecutive infections (Long et al., 1976). Strong immunological protection against a mixed *Eimeria* species infection relies on the environmental cycling of the vaccine organisms (Price et al., 2014; Williams, 2002, 1999).

Nevertheless, some instances of vaccine failure have been reported during the administration of live vaccines (Coccivac, Advent, Immucox, and Inovocox). Indeed, when placed on litter, susceptible birds can be excessively exposed to a potentially high number of virulent wild-type oocysts before they have adequate time to develop an immune response (Chapman et al., 2013). There may be a direct adverse effect on food conversion or even clinical symptoms of haemorrhagic or malabsorptive coccidiosis. Conversely, the ingestion of an inadequate dose leaves birds vulnerable to large quantities of recycled vaccine oocysts excreted by other flock members (Blake and Tomley, 2014; Chapman et al., 2002).

To achieve control over the inherent pathogenicity of the parasites, various solutions have been proposed, including alternating vaccination with the use of drugs (Chapman, 2000; Chapman et al., 2010). A partial restoration of drug sensitivity was accomplished through the use of vaccines containing drug-sensitive strains of *Eimeria* (Allen and Jenkins, 2010; Peek and Landman, 2006). However, a study by Mathis et al. (2014) found that better outcomes in terms of broiler productivity and bird immune status occurred when vaccination was combined with in-feed ionophores rather than vaccination alone (Mathis et al., 2014).

Additionally, the development of second-generation live attenuated vaccines represents another approach to mitigate the risk posed by vaccinal pathogenicity (Damer P Blake et al., 2015). They are parasites derived from a selection of their trait of precociousness. They are characterised by a faster life cycle (they lose one or two cycles of schizogony), a reduced prepatent period compared to the wild-type line (from 13 hours to 33 hours), and lower reproductive capacity and reduced pathogenicity, while maintaining their immunogenicity.

Consequently, there is a lower risk of the disease occurring, as well as a significant reduction in damage to the intestine (Mcdougald and Jeffers, 1976).

The introduction of coccidiosis vaccines in the poultry industry has significantly reduced the incidence of the disease in some parts of the world, in contrast to other adversely affected regions such as Asia, Africa, and South America, mainly because of their cost and availability, but also because of a failure to educate producers on good administration, which is essential for effective results (Bould et al., 2009). At the same time, their joint use with that of anticoccidials remains the most widely available method despite the increasingly unsatisfactory results (Damer P Blake et al., 2015).

In recent years, numerous anticoccidial vaccines have been tested (recombinant protein, DNA, dendritic cell-derived exosome, and vectored subunit vaccines). As a result of these developments, a substantial amount of new data has emerged regarding various aspects of parasite biology and the identification of potential targets for intervention (Blake et al., 2011; Ding et al., 2012; Huang et al., 2018; Sathish et al., 2011; Song et al., 2010, 2015; Vaezirad et al., 2018; Xu et al., 2013). Unfortunately, the lack of standardisation among experiments, research groups, government agencies, and industry has inhibited meaningful comparisons (Damer P. Blake et al., 2015; Bumstead and Millard, 1992; Smith et al., 2002). However, genomic, proteomic, and phylogenetic analyses of all *Eimeria* species that infect fowl promise significant improvements for coccidiosis control in the future (Blake and Tomley, 2014; Chapman, 2014).

3.5.3. Innovative coccidiosis control

High-throughput "omics" are transforming our understanding and management of *Eimeria* in poultry. Chromosome-level genome assemblies are now available for key species, including *Eimeria tenella* and *E. acervulina*, generated with long-read sequencing and Hi-C scaffolding; these resources improve gene models and facilitate identification of loci associated with virulence and drug resistance (Srivastava et al., 2025; Zhang et al., 2025). Multi-stage transcriptomic maps have also advanced: RNA-seq across seven defined life-cycle stages of *E. tenella* reveals stage-specific expression clusters and highlights gene families likely crucial for invasion, development, and sexual differentiation; full-length (PacBio Iso-Seq) transcriptomes for *E. necatrix* capture isoforms missed by short-read methods, uncovering invasion-related candidates (e.g., MICs, ROPs, CDPKs) (Chen et al., 2023; Gao et al., 2021). Beyond parasite-only datasets, dual RNA-seq has mapped host–parasite interactions *in vivo*, showing early induction of chicken interferon-γ, chemokines, and metalloproteinases alongside parasite upregulation of translation and metabolic pathways during *E. tenella* infection; in *E. falciformis*, dual RNA-seq indicates a largely "canalised" parasite programme despite differing host immune responses (Ehret et al., 2017; Sandholt et al., 2021).

Proteomics complements these layers. Comparative proteomes across *Eimeria* developmental stages have identified invasion-associated proteins and stage-enriched candidates, while a lysine-acetylome covering five *E. tenella* life stages reveals widespread, dynamic post-translational regulation that could be used for vaccine antigen selection or drug targeting (Gong et al., 2023; Ma et al., 2024).

Gene editing has transitioned from a vision to a routine practice in *Eimeria*. CRISPR/Cas9 allows for precise endogenous tagging and gene disruption in *E. tenella* (such as microneme protein EtMIC2), and nuclease variants like FnCas12a expand the options for knock-outs and fluorescent tagging, collectively establishing practical reverse genetics for an organism long considered difficult to manipulate (Cheng et al., 2021; Hu et al., 2020; Tang et al., 2020). These tools complement transgenesis to unlock next-generation vaccines: transgenic *Eimeria* can express heterologous protective antigens, and proof-of-concept studies demonstrate that *E. tenella* engineered to express *E. maxima* antigens can provide partial cross-species protection (Pastor-Fernández et al., 2020). More broadly, a recent review suggests that live attenuated/precocious lines, already successful in the field, can be further improved through genome editing and transgenesis to enhance safety and efficacy (Q. Liu et al., 2023).

Parallel advances are emerging in delivery platforms that enhance robust mucosal immunity. Oral, heat-killed yeast (*Saccharomyces cerevisiae*) expressing *E. tenella* antigens reduced parasite replication after challenge, illustrating a scalable, low-cost vector concept (Soutter et al., 2022). Rationally targeted probiotics can also serve as live vectors: a *Lactobacillus* plantarum system designed to home to intestinal tight-junction claudin-3 carried *E. tenella* vaccine cargo and elicited protective responses (L. Sun et al., 2023). Nanoparticle vaccinology is another promising area. Poly(lactic-co-glycolic acid) (PLGA) and chitosan particles provide improved protection when delivering Eimeria antigens (e.g., Em14-3-3) and enhance multiepitope vaccine performance in chickens, supporting continued exploration of tunable nano-adjuvants for durable, T-cell-skewed responses (Haseeb et al., 2022b; Yu et al., 2022). In parallel, lessons from "structural vaccinology", the structure-guided design of immunogens that induce the right specificities, are beginning to inform apicomplexan vaccine discovery, drawing on successes and strategies developed in influenza and HIV (Anasir and Poh, 2019; Ward and Wilson, 2020; Wei et al., 2020).

Since *Eimeria* control is fundamentally an ecosystem issue centred in the gut, relying solely on technology is unlikely to be sufficient. Recent integrative reviews and comparative trials endorse combining innovations (such as edited/transgenic or vectored vaccines) with gut-health modulators, probiotics, prebiotics/synbiotics, and vetted phytochemicals, and with strategic use of anticoccidials to maintain drug sensitivity and reduce performance losses (Ahmad et al., 2023; Gao et al., 2024; Javanmiri et al., 2024). In short, multi-omics provides the roadmap; CRISPR and advanced delivery methods are the tools; and holistic programmes that integrate vaccines, microbiome support, and intelligent chemotherapy represent the most practical approach to achieving resilient, sustainable control of coccidiosis (Ahmad et al., 2023; Q. Liu et al., 2023).

CHAPTER II

Poultry farm characteristics, husbandry practices, and biosecurity in the prevention of avian coccidiosis

1. Overview of Poultry Farming

Poultry farming is one of the most dynamic and rapidly expanding sectors of animal production worldwide. It plays a crucial role in global food security by providing an essential source of high-quality animal protein in the form of meat and eggs (Mottet and Tempio, 2017). Thanks to technological advancements, genetic improvements, and progress in animal nutrition, poultry farms have significantly increased their productivity in recent decades (Kleyn and Ciacciariello, 2021). However, this transformation has been accompanied by a shift towards increasingly intensive production systems, where farming conditions are optimised to maximise yields. While these changes have improved economic efficiency, they have also introduced new challenges, particularly regarding disease management and animal welfare (Attia et al., 2024; Birhanu et al., 2023; Singh et al., 2022a).

In modern poultry farming systems, the concentration of large numbers of birds in confined spaces, the reduction of production cycles, and the standardisation of husbandry practices create favourable conditions for the emergence and rapid spread of infectious and parasitic diseases (Astill et al., 2018; Elakov et al., 2025; Kovács et al., 2025a). The high sanitary pressure exerted by these high-density environments necessitates rigorous monitoring of farm conditions to mitigate health risks. Housing hygiene, litter quality, ventilation, waste management, feeding protocols, and access to veterinary care are all critical factors that directly influence poultry health and pathogen transmission (Franzo et al., 2020; Y. Li et al., 2024; Marincheva, 2023; Tsiouris et al., 2015a). Poor management of these elements can lead to significant economic losses due to decreased production performance, increased mortality rates, and rising costs associated with treatments and disease control measures (Abdisa Serbessa et al., 2023; Govindaraj et al., 2018; Jones et al., 2019).

Among the most concerning diseases affecting the poultry industry, avian coccidiosis presents a significant health challenge, particularly in intensive farming systems (Ahmad et al., 2023; Mathis et al., 2024). This parasitic disease is caused by protozoa of the genus *Eimeria*, which primarily infects the intestinal tract of poultry, resulting in severe lesions and digestive disorders (Kogut et al., 2020).

Eimeria spp. Infections compromise nutrient absorption, leading to reduced weight gain, poor feed conversion efficiency, and, in severe cases, increased mortality (Blake and Tomley, 2014). Given its substantial economic impact and global prevalence, coccidiosis is a primary concern for poultry farmers and veterinarians.

The transmission of *Eimeria* spp. follows a faecal-oral cycle, whereby oocysts excreted in faeces contaminate the environment and are subsequently ingested by other birds. Farm characteristics and husbandry practices directly influence this cycle (Bharti et al., 2024; Khursheed et al., 2022; Mesa et al., 2021). Several factors contribute to the persistence and dissemination of oocysts in poultry houses, including excessive stocking density, which increases direct and indirect contact with contaminated faeces; inadequate litter management, which allows the buildup of infectious oocysts when not properly dried or replaced; poor ventilation, which creates high humidity levels that favour oocyst sporulation; and an imbalanced diet, which weakens the birds' immune defences, making them more susceptible to infection (Alcala-Canto et al., 2020a, 2020b; Badri et al., 2024; Li et al., 2021; Liao et al., 2024).

To effectively control avian coccidiosis, an integrated approach that combines biosecurity measures, infrastructure management, health monitoring, and pharmacological control is essential (El-Saadony et al., 2025a; El-Shall et al., 2022; Lee et al., 2022). Implementing strict biosecurity protocols is crucial for preventing the introduction and spread of pathogens. Regular disinfection of housing and equipment, along with stringent control of animal and personnel movements, as well as proper waste and wastewater management, helps reduce the parasite load in the environment (Conan et al., 2012; Delpont et al., 2023; Meirhaeghe et al., 2019; Morishita and Derksen, 2021; Robertson, 2020; Vaillancourt and Racicot, 2022). Additionally, improving farming conditions, particularly through optimal litter management and adequate ventilation, helps limit oocyst survival and transmission.

This chapter offers an in-depth analysis of the characteristics of poultry farms and the husbandry practices that influence the transmission and control of avian coccidiosis. It highlights essential biosecurity measures to mitigate the impact of this disease and suggests strategies for improving farming conditions to optimise poultry health and productivity.

A better understanding of the interactions between the farming environment and Eimeria spp. dynamics is necessary to develop solutions tailored to modern poultry production systems. By integrating a multidisciplinary approach that combines prevention, biosecurity, and health management, it is possible to effectively reduce coccidiosis incidence while ensuring the sustainability of poultry farming.

2. Poultry farm characteristics

2.2. Poultry farm typology

2.2.1. Industrial vs. Traditional Poultry Farms

Poultry farms can be generally classified into industrial and traditional systems (Figure 5), each with distinct characteristics, management methods, and implications for poultry health and disease control (Gržinić et al., 2023a).

Industrial poultry farms operate on a large scale, focusing on maximising productivity, efficiency, and cost-effectiveness (Figure 5). These farms employ intensive management practices, including controlled housing, automated feeding and watering systems, optimised nutrition, and advanced veterinary care (Gržinić et al., 2023a). The use of selective breeding programmes enhances genetic traits that promote faster growth rates, higher egg production, and feed conversion efficiency. Strict biosecurity protocols, vaccination programmes, and disease surveillance systems help minimise the risks of infectious diseases (Dawkins and Layton, 2012; Jones and Wilson, 2022). However, high stocking densities, continuous flock turnover, and confined environments create ideal conditions for disease transmission, particularly for *Eimeria spp*., the causative agents of avian coccidiosis. Litter management, hygiene protocols, and prophylactic treatments such as coccidiostats are essential for controlling disease outbreaks in industrial poultry farms (Conan et al., 2012; Delpont et al., 2023; Meirhaeghe et al., 2019; Morishita and Derksen, 2021; Robertson, 2020; Vaillancourt and Racicot, 2022).

In contrast, traditional poultry farms are typically smaller-scale operations, often family-run or village-based, and rely on semi-intensive or extensive management systems (Alders et al., 2018; Wilson et al., 2022). These farms usually house birds in open or semi-confined areas, allowing them to forage naturally and benefit from a more diverse diet (Figure 5).

Unlike industrial systems, which prioritise maximum output, traditional farms emphasise low-input management, reduced reliance on synthetic feed, and limited veterinary intervention (Bist et al., 2024). While conventional farms generally maintain lower stocking densities, they face challenges related to disease exposure, restricted access to veterinary care, and variable nutritional quality. Birds in these systems are often more resilient due to natural selection and exposure to diverse microorganisms; however, they may still suffer from parasitic infections, including coccidiosis, resulting from exposure to contaminated soil, wild birds, and environmental fluctuations (Y. L. Campbell et al., 2025; Gržinić et al., 2023b; Hu et al., 2017; Karkach et al., 2023; Wu et al., 2022).

Figure 5. Contrasting industrial and traditional poultry farming systems. Top row: Three views of large-scale, high-density operations: a stockman inspecting automated drinkers in an enclosed broiler house; a long battery-cage corridor for layers; and a tunnel-ventilated, fully mechanised broiler shed. Bottom row: Examples of low-density, free-range or village setups: pasture "chicken tractors" that are moved daily; a semi-open rural flock foraging under natural shade; and hens roaming outside a small fixed coop with a ramp. Together, the images highlight the contrasting space allowance, housing style, and management practices that characterise industrial and traditional poultry production.

2.2.2. Production systems

Poultry production systems can be categorised into intensive, extensive, free-range, and organic systems (Table 2), each with specific implications for disease management, including the control of *Eimeria* spp. Infections.

- *Intensive Production Systems*

Intensive poultry farming is the predominant production model in commercial broiler and layer operations worldwide (Table 2). Birds are raised in high-density, climate-controlled environments, facilitating precise management of nutrition, lighting, temperature, and ventilation to optimise growth and productivity (Gržinić et al., 2023a). This system enables short production cycles and increased efficiency, making it ideal for large-scale meat and egg production. However, high bird densities, limited space, and continuous exposure to faeces increase the risk of coccidiosis outbreaks. *Eimeria* spp. Thrive in the warm, humid conditions of poultry houses, and if litter is not managed correctly, oocyst accumulation can reach critical levels (Bist et al., 2024). Regular litter replacement, controlled ventilation, and the use of coccidiostats or vaccines are essential for minimising disease risks in intensive poultry farms.

- *Extensive Production Systems*

Extensive poultry farming enables birds to roam in larger outdoor spaces and forage for part of their diet (Table 2). This system is commonly practised in rural areas and developing regions, where poultry production is integrated with traditional agricultural activities (E.B. Sonaiya and S.E.J. Swan, 2004; Singh et al., 2022b). Lower stocking densities in extensive systems reduce direct contact between birds, thereby decreasing disease transmission rates. However, birds are more susceptible to environmental pathogens, predators, and weather fluctuations, which can increase the risk of parasitic infections, including soil-borne *Eimeria* spp. Unlike intensive farms, which rely on medicated feed and strict biosecurity measures, extensive farms often utilise natural feed additives, probiotics, and herbal treatments to bolster disease resistance (Dal Bosco et al., 2021).

- *Free-Range Poultry Farming*

Free-range farming provides birds with daily outdoor access, enabling them to engage in natural behaviours such as foraging, dust bathing, and social interaction (Table 2). This system is regulated in many countries, necessitating that birds spend a specific amount of time outside while still having access to shelters for protection (Papatsiros et al., 2012; Pumtang-on et al., 2021; Rajkumar et al., 2019; Scott et al., 2017). While free-range conditions promote natural immunity, they also expose birds to pathogens present in soil, water sources, and wild bird populations. Coccidiosis is a significant concern, as *Eimeria* oocysts can persist in outdoor environments for extended periods (Kaboudi et al., 2016; McMullin, 2022; Silva et al., 2022a). Rotational grazing manages outdoor exposure, and the use of vaccines or natural anti-coccidial compounds is often necessary to mitigate disease risks in free-range systems.

- *Organic Poultry Farming*

Organic poultry farming adheres to strict regulations concerning feed composition, antibiotic usage, and welfare standards (Table 2). Birds must be raised without synthetic coccidiostats, antibiotics, or genetically modified feed, which makes disease control more challenging (Abd El-Hack et al., 2022; Diaz-Sanchez et al., 2015; Farooq et al., 2023; Muwal et al., 2023). Organic systems emphasise natural disease prevention by utilising probiotics, essential oils, herbal extracts, and improved gut microbiota management to boost immunity (Biradar et al., 2011; Chalova et al., 2016; Farooq et al., 2023; Muwal et al., 2023). Although low stocking densities and outdoor access enhance bird welfare, the risk of *Eimeria* spp. Infections remain high, necessitating meticulous sanitation, vaccination programmes, and rotational pasture management to mitigate outbreaks (Abd El-Hack et al., 2022; "Animal health and welfare in organic agriculture," 2004; Berg, 2002).

Table 2. Comparison of poultry production systems and coccidiosis management strategies.

Criterion	Intensive Production	Extensive Production	Free-Range Production	Organic Production
Stocking Density	Very high (20–30 kg live weight/m²), birds kept indoors continuously; optimised for short cycles and maximum yield.	Low (≥ 10 m² per bird), with birds spread over large outdoor areas and no strict climate control.	Moderate (≥ 4 m² per bird outdoors), daily outdoor access combined with sheltered housing at night.	Very low (< 6 m² per bird), densities set by EU organic regulation; outdoor runs must be free of chemical treatments
Environmental Control	Fully climate-controlled (temperature, ventilation, lighting) to ensure uniform growth	No climate control; birds face ambient temperature, humidity swings, and basic shelters	Partial control (e.g. netting, simple shelters); rotational grazing helps limit oocyst buildup.	Spacious, naturally-littered housing, rotational pastures; no chemical disinfectants or synthetic antibiotics
Feed & Supplements	Formulated feeds with synthetic coccidiostats or ionophores, applied continuously or in rotational programs.	Mixed feeding: grains supplemented by local forages (seeds, insects); empirical use of probiotics and herbal extracts	Base feed plus herbs, seeds, natural probiotics; moderate use of anticoccidial vaccines in feed.	Certified organic feeds (no GMOs or synthetic additives); phytogenics (essential oils, yeast) to support gut health.
Coccidiosis Risk	Very high: continuous exposure to faeces, humid litter, rapid oocyst accumulation (sporulation in 24–48 h at 25–30 °C).	Moderate: low bird density limits direct spread, but exposure to contaminated soil and wild birds causes sporadic outbreaks.	High: oocysts persist in outdoor runs, wild-bird vectors, favourable external conditions for sporocyst survival.	High: no chemical coccidiostats or antibiotics, untreated soil/runs; demands rigorous biosecurity and frequent pasture rotations.
Prevention Strategies	- Frequent litter replacement	-	-	-

2.2.3. Stocking density and its impact on poultry health

Stocking density is one of the most critical factors influencing poultry health, welfare, and disease prevalence (Gholami et al., 2020; Shynkaruk et al., 2023a; T. Yanai et al., 2018). High stocking densities, characteristic of industrial and intensive systems, result in increased competition for feed and water, heightened stress levels, and a greater risk of disease transmission (Gržinić et al., 2023a). The ongoing recycling of litter and accumulation of faeces create an ideal environment for Eimeria oocysts to sporulate, thereby increasing the incidence of coccidiosis outbreaks (Andrews, 1933; Bailey et al., 2022; Beninca et al., 2021a; Gholami et al., 2020; Pope and Cherry, 2000). Furthermore, elevated ammonia levels from decomposing waste can impair respiratory function, suppress immune responses, and exacerbate secondary infections (da Rosa et al., 2024; Guo et al., 2022; D. Li et al., 2023; Liu et al., 2020; Shah et al., 2020; Shi et al., 2019; Y. Zhou et al., 2021). To mitigate these risks, intensive farms must implement proper ventilation, schedule regular litter replacement, and adhere to stringent cleaning protocols.

Conversely, low stocking densities, often observed in extensive, free-range, and organic systems, offer birds better air quality, more room for movement, and reduced pathogen exposure (Erensoy et al., 2024). Birds raised in spacious environments demonstrate stronger immune responses and lower stress levels, enhancing their overall resilience to infections (Feddes et al., 2002; Gao et al., 2017; C. H. Kim et al., 2024a; Tarakdjian et al., 2020). However, low-density environments do not eliminate disease risks, as birds remain susceptible to soil-borne pathogens, predators, and climatic variations (Goo et al., 2019; Tsiouris et al., 2015b). *Eimeria spp*. Oocysts can survive for months in outdoor settings, necessitating rotational grazing, strategic housing placement, and periodic decontamination of outdoor areas to mitigate disease transmission (Andreopoulou et al., 2022; Gadelhaq et al., 2018; Jenkins et al., 2019; Kruth et al., 2024; Reperant et al., 2021; Silva et al., 2022b; Soliman et al., 2018).

The ideal stocking density varies based on several factors, including bird species, breed, production stage, housing design, and climatic conditions (Campbell et al., 2017; Downing, 2022; El Sabry et al., 2022; Khaziev et al., 2023). Broilers and layers have distinct space requirements, with guidelines differing among regulatory bodies. For instance, the European Union's organic poultry standards stipulate a maximum stocking density of 6 birds per square metre, whereas intensive farms may accommodate up to 20 birds per square metre (C. H. Kim et al., 2024b; NAHWOA, n.d.; Nasr et al., 2021). Effective density management, hygiene practices, and health monitoring are essential for ensuring optimal bird welfare, preventing disease, and promoting sustainable poultry production.

2.3.Infrastructure and Housing Management

The design and management of poultry housing play a crucial role in maintaining flock health, optimising productivity, and minimising disease risks, particularly in preventing the spread of *Eimeria spp*., the causative agents of avian coccidiosis (Blake and Tomley, 2014). A well-structured poultry house provides adequate space, ventilation, temperature control, and hygiene, all of which contribute to disease prevention and improved bird welfare, key factors influencing the occurrence and transmission of *Eimeria* spp (Beninca et al., 2021b; Collett, 2009; Elson, 2015). Include housing type, flooring materials, and perch and nest systems. Effective infrastructure design not only enhances productivity but also reduces reliance on medications by creating an environment that naturally inhibits the proliferation of pathogens (Bist et al., 2023; D. L. M. Campbell et al., 2025; Elson, 2015; Remonato Franco et al., 2022; Rodenburg et al., 2005; Wang et al., 2018).

2.3.1. Types of poultry houses

Poultry housing varies significantly depending on climate, production scale, management style, and disease control strategies. The three main housing types include conventional enclosed housing, open-sided housing, and free-range housing, each with unique implications for disease transmission and flock welfare.

- *Conventional Enclosed Housing*

Conventional poultry houses (Figure 6) are completely enclosed structures in which temperature, humidity, ventilation, and lighting are mechanically regulated to sustain optimal growing conditions (Liang et al., 2013). This type of housing is extensively used in intensive commercial poultry farming, particularly for broilers and layers. The primary features of enclosed housing include (Akter et al., 2022; Collett, 2009; Demmers et al., 2019; Elson, 2015; Remonato Franco et al., 2022):

- Automated climate control systems maintain stable environmental conditions.
- Mechanical ventilation systems (including fans, exhaust systems, and air inlets) are used to regulate air exchange and prevent ammonia accumulation.
- Controlled lighting programmes to influence feeding behaviour and growth rates.
- Biosecurity measures to prevent the entry of external pathogens.

The primary advantage of enclosed housing is its protection against external disease threats, such as infections carried by wild birds and environmental fluctuations (Kovács et al., 2025b). However, these systems heighten the risk of rapid pathogen spread, particularly in high-density settings where *Eimeria* oocysts accumulate in faeces-laden litter. Without adequate ventilation and litter management, enclosed systems can create a humid microclimate that accelerates oocyst sporulation and increases the chances of coccidiosis outbreaks (Conraths et al., 2005; Price et al., 2013; Velkers et al., 2012).

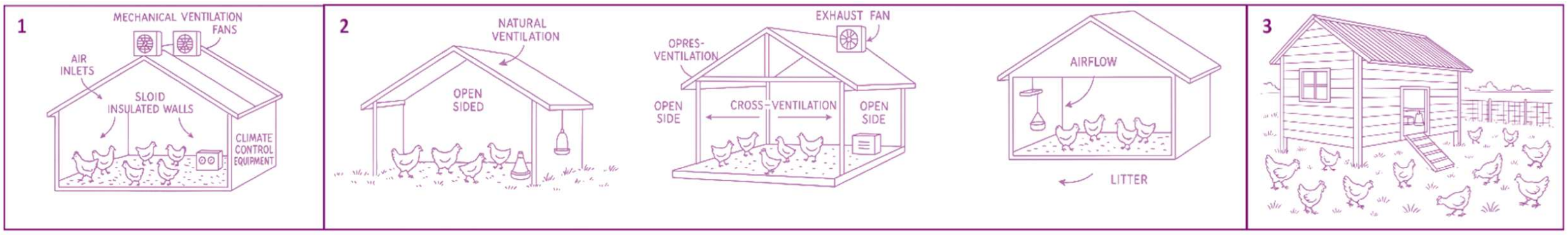


Figure 6. Three poultry-house blueprints: from high-control indoors to open pasture. The strip compares the main housing strategies used in modern broiler production. (1) A fully enclosed house, with insulated walls and powered fans, maintains a tight comfort zone, keeping temperature, humidity, and ammonia levels within a controlled range. (2) Open-sided houses, featuring mesh or curtain walls, allow natural breezes to flow; booster fans step in when the air is still. (3) Free-range shelter, a simple coop offers shade and night-time security, while pop-holes and ramps give birds daytime access to grassy pasture.

- *Open-Sided Housing*

Open-sided poultry houses (Figure 6) are partially enclosed, allowing natural airflow and daylight. These houses are commonly used in tropical and subtropical climates to minimise heat stress and improve ventilation (Saner and Shekhawat, 2023; Saraz et al., 2010). Characteristics of open-sided housing include (Kadim et al., 2008; Perera and Dematawewa, 2017; Sans et al., 2021; Saraz et al., 2013):

- Curtains, shutters, or mesh walls that can be adjusted to control airflow.
- Natural light exposure, which promotes bird activity and welfare.
- Lower energy costs compared to fully enclosed houses.

While improved ventilation reduces ammonia levels and respiratory stress, open-sided systems expose birds to external environmental contaminants, fluctuating temperatures, and potential pathogen carriers such as wild birds (Al-Aqil and Zulkifli, 2009; Du et al., 2019; Jongbo, 2024; Kovács et al., 2025c; Le Bouquin et al., 2013; Lichtner et al., 2024; Nimmermark and Gustafsson, 2004). *Eimeria* oocysts persist in soil and litter, making effective waste disposal, litter drying, and biosecurity measures essential in open-sided housing (Albeshr et al., 2025; Assis et al., 2013; Garcés-Gudiño et al., 2018; Lassen et al., 2014, 2013a; Lassen and Seppä-Lassila, 2014a).

- *Free-Range Housing*

Free-range housing (Figure 6) enables birds to access outdoor environments while providing them with indoor shelter during nighttime or adverse weather conditions. This system promotes (Ahmad et al., 2021; Arbona et al., 2011; Donaldson et al., 2012; Ferreira et al., 2021; Hofmann et al., 2020; Wang et al., 2025):

- Natural foraging behaviour, reducing reliance on commercial feed.
- Lower stress levels, improving immune function and overall health.
- Better skeletal development and muscle tone due to increased movement.

However, free-range housing also presents challenges related to disease, particularly in managing coccidiosis. Eimeria oocysts persist in outdoor environments, especially in damp soil and contaminated water sources (Lassen et al., 2014, 2013a). Infections occur when a bird ingests sporulated oocysts from contaminated grass, feed, or drinking water.

To mitigate the risk of infection, it is advisable to employ rotational grazing, facilitate pasture drying, and use natural anti-coccidial supplements (Bora et al., 2024; Chapman, 2017; Chowdhury et al., 2024; Miao et al., 2005; Yayeh, 2025).

3. Environmental conditions and their role in disease control

Maintaining optimal environmental conditions is crucial for preventing disease, promoting flock welfare, and enhancing growth performance. Three critical parameters in poultry houses are ventilation, temperature control, and humidity management.

3.1. Ventilation

Adequate airflow helps remove ammonia, excess moisture, and airborne pathogens, thereby reducing respiratory stress and improving litter quality (Ahmadi Babadi et al., 2022; Akter et al., 2022; Bhawa et al., 2023; Prodanov et al., 2016; Sun et al., 2018; Wang et al., 2018). Contemporary ventilation systems encompass:

– Negative pressure ventilation (fans create airflow by drawing air out).
– Tunnel ventilation (air moves in a straight path to cool birds efficiently).
– Natural cross-ventilation (openings on opposite sides for passive air movement).

3.2. Temperature Control

Temperature fluctuations directly affect immune response and metabolism (Ghareeb et al., 2022). Birds subjected to heat stress exhibit reduced feed intake, dehydration, and immune suppression, thereby increasing their susceptibility to infections (Sharma et al., 2025a; Sharma and Kim, 2024). Conversely, cold stress raises energy demands, making birds more vulnerable to intestinal diseases such as coccidiosis (Borsoi et al., 2015; El-Saadony et al., 2025b; Hu and Cheng, 2021; Tsiouris et al., 2015c). Automated heating and cooling systems ensure stable temperatures, thereby minimising environmental stressors.

3.3. Humidity Regulation

Humidity levels exceeding 70% result in damp litter conditions, which encourage the sporulation of Eimeria oocysts. Conversely, dry litter inhibits oocyst survival and mitigates the risk of coccidiosis (Cha et al., 2018; Lassen and Seppä-Lassila, 2014a; Soliman et al., 2018; Venkateswara Rao et al., 2015; L Waldenstedt et al., 2001). Optimal strategies for humidity control include:

– Proper ventilation to remove excess moisture.
– Litter drying techniques (absorbent bedding, heat treatment).
– Periodic litter replacement to maintain dry conditions.

Inside a modern poultry house, the birds and the building are in constant, wordless negotiation: they release heat, moisture and gases, and we must whisk those by-products away while keeping the air calm and comfortable. Exhaust fans that cycle fresh air through the house not only dilute ammonia and carbon-dioxide spikes but also stop them from rebuilding between fan runs; when minimum-ventilation fans stay off too long, ammonia rebounds within minutes, so steady air exchange is essential for air quality and litter health (Bhawa et al., 2023; Sun et al., 2018). Tunnel systems add the necessary muscle on hot days; six-inch evaporative pads can trim the incoming air by 20°F (≈11 °C), and, when combined with air speeds of around 600 ft/min, they keep birds eating and growing even under extreme heat spells (Ahmadi Babadi et al., 2022).

Suppose heat nevertheless creeps above the birds' comfort zone. In that case, physiology takes a toll: feed intake drops, growth or egg output slows, and the hypothalamic-pituitary-adrenal axis fires, sending corticosterone and other stress mediators surging through the bloodstream and tipping metabolism toward oxidative stress and immunosuppression (Akbarian et al., 2016; Liu et al., 2014; Saeed et al., 2017). Producers, therefore, blend several defences. Genomic selection programs are already isolating SNP markers linked to thermotolerance, so that future broiler and layer lines can maintain performance when temperatures spike (Nawaz et al., 2024).

Diets are also adjusted: balancing dietary-electrolyte levels around 370 mEq kg^{-1} supports water intake and gut morphology under natural heat stress, while adding organic selenium, vitamins C, and E measurably lowers cloacal temperature and enhances weight gain (Mohamed et al., 2024; Moura et al., 2024). Even before a chick hatches, short pulses of 38–39 °C during late incubation—the so-called embryonic thermal manipulation- can prime its tissues for better heat dissipation later in life without hurting hatchability (Al Amaz and Mishra, 2024).

Cutting-edge field studies over the past two years all point in the same direction: the birds thrive when the building breathes, cools and "dries out" at just the right pace. A January 2025 survey of a 15,000-bird house in southern Turkey showed that a well-sealed tunnel system with 15-cm cellulose pads held the inlet-to-exhaust temperature spread within 2 °C even in 38 °C outdoor heat, confirming that fast, linear air speeds and evaporative cooling still set the gold standard for heat removal in summer (Çayli, 2025). Inside the house, newer support technologies, such as ceiling-mounted mixing fans, have proven equally valuable in winter; North Carolina data published in early 2025 recorded a 40 % cut in ceiling-to-floor temperature stratification once the fans were switched on, saving fuel and preventing damp litter pockets that can seed ammonia spikes (Sanjay et al., 2025).

Why does all this matter? Because temperature–humidity index (THI) levels above the birds' comfort zone hit the bottom line fast. A Taiwanese trial found that broilers raised at a daily mean THI of 86 produced tougher breast meat, lost 6% in final body weight, and yielded 12% less profit than birds kept at a THI of 74 (Kuo et al., 2025). Physiologically, this performance dip corresponds to the same cascade highlighted in a study of Aryal et al (2025): heat triggers the hypothalamic-pituitary-adrenal axis to overdrive, resulting in increased corticosterone levels and reactive oxygen species accumulation, which can erode immunity and muscle deposition if left unaddressed (Aryal et al., 2025). Fortunately, the toolbox keeps expanding. A mid-2025 synthesis of "next-generation" countermeasures reports promising gains from marker-assisted selection for thermotolerance, high-dose antioxidant/vitamin blends, and smarter water-spray or pad-cooling algorithms that react to bird behaviour in real-time (Çayli, 2025). Humidity rounds out the triad.

Recent controlled-environment work from China maintained house relative humidity (RH) at 50–70 % throughout a 42-day growth cycle and used that band explicitly to stabilise litter moisture while protecting antioxidant status and weight gain, reinforcing long-held extension targets for broilers (Zhang et al., 2024). At the other extreme, a Croatian study documented that winter periods with RH well below 50 % coincided with the highest airborne dust and bacterial counts, underscoring how overly dry air trades wet-litter problems for respiratory ones (Ravić et al., 2024).

Heat stress is one of the most significant environmental challenges in modern poultry production. Broiler chickens, sheathed in feathers and almost devoid of sweat glands, already generate considerable metabolic heat; once the air temperature climbs above their thermo-neutral window of roughly 16–26 °C, that inborn furnace turns against them (Lucas J Lara and Rostagno, 2013; Wasti et al., 2020a). To cool down, the birds pant. Empirical measurements indicate that for every 1 °C rise in body temperature, the respiratory rate increases by approximately 0.56 breaths per minute. At temperatures of 35–40 °C, it can exceed 250 cycles per minute. The sustained loss of CO_2 drives blood pH toward respiratory alkalosis, a shift that, if prolonged, threatens cardiopulmonary stability (Goel et al., 2025; H.-R. Kim et al., 2024; Malila et al., 2024; Wasti et al., 2020a).

Aryal et al. (2025) show that when house temperatures climb, the mitochondrial electron-transport chain in chicken cells starts “leaking” electrons; NADPH- and xanthine-oxidases then pile on, so reactive oxygen/nitrogen species quickly outnumber the birds’ defences (Figure 7). The oxidative surge, signalled by markers such as malondialdehyde, protein carbonyls, 8-OH-dG and a falling GSH/GSSG ratio, overwhelms both enzymatic shields (SOD, CAT, GPx, Prdx) and non-enzymatic pools (GSH, vitamins E/C, carotenoids) commonly up-regulated by the Nrf2 pathway. The review highlights two practical lifelines: fortifying diets with potent antioxidants like curcumin, organic selenium, or lycopene, and breeding lines that carry markers for heat tolerance. It also recommends a simple on-farm red-flag panel, including MDA + GPx activity and the GSH/GSSG ratio, to spot oxidative damage before performance slips (Aryal et al., 2025).

Heat stress and coccidiosis share a biochemical “weak spot”: both tip the bird’s intestine into reactive-oxygen overdrive. Under high ambient temperatures, the mitochondrial electron-transport chain begins to leak electrons, sparking a ripple of ROS and RNS that overwhelms first-line enzymes such as SOD and GPx.(Aryal et al., 2025) When *Eimeria* sporozoites penetrate those already stressed enterocytes, the host mounts a respiratory burst that releases even more free radicals; malondialdehyde rises, the GSH: GSSG ratio falls, and tight junctions loosen, amplifying the parasite’s ability to damage and inflame the gut wall (Sharma et al., 2025a; Sharma and Kim, 2024).

Field work confirms that the two stressors interact. In a University of Georgia trial, broilers exposed simultaneously to 35 °C heat and *Eimeria maxima* showed altered amino-acid transporter expression and digestion patterns compared with birds facing the parasite alone, illustrating that thermal stress can rewire nutrient handling during infection (Ghareeb et al., 2022). Conversely, a study by Biabani et al. (2024) demonstrated that boosting the antioxidant armour, here with fully chelated trace mineral blends, reined in NF-κB-driven inflammation and restored Nrf2 signalling in mixed *Eimeria* challenges, thereby reducing lipid peroxidation despite infection (Biabani et al., 2024). Similar protection was observed when 0.75% grape pomace was added to heat-exposed, coccidia-infected broilers: the polyphenols quenched ROS, lowered lesion scores, and normalised Nrf2/ARE pathway activity (Sharma et al., 2025b).

Recent evidence deepens the understanding of how heat and Eimeria interact in the gut, and, just as significantly, how we can break that interaction apart. Field-scale transcriptomics from 2024 shows that combining 35 °C heat with an *E. maxima* challenge rewires more than 2,300 ileal genes. Lipid-metabolic and protein-synthesis pathways partly rebound under the double hit. At the same time, innate-immune circuits remain muted, indicating that the host is diverting energy toward maintaining redox balance rather than fueling inflammation (Ghareeb et al., 2024). Live microbes add another layer of defence. In a 240-bird Turkish trial, lactic acid bacteria probiotics effectively controlled corticosterone levels, maintained T_3 and citrulline circulation, and rebalanced the cecal microbiome, resulting in weight gain and feed efficiency under 34–36 °C heat that nearly matched those of thermoneutral controls (Aydin and Hatipoglu, 2024).

Plants can also contribute significantly: a thymol-rich *Lippia origanoides* essential oil blend (with herbal betaine) improved intestinal morphology, tibia strength, and live weight in birds cycled through 35 °C for 12 hours daily, underscoring the value of membrane-stabilising phytochemicals when oxidative and coccidial pressures coincide (Señas-Cuesta et al., 2023). One nuance: chronic heat can sometimes curtail *E. maxima* itself, as shown in a controlled study with a 35 °C reduction in oocyst shedding and sexual-stage gene expression. However, this “benefit” is offset by epithelial damage and nutrient loss, leaving overall performance in the red (Schneiders et al., 2020).

3.4.Flooring materials and their impact on *Eimeria* spp. transmission

The type of flooring impacts disease transmission, litter quality, and ease of cleaning. *Eimeria* oocysts, which are excreted in faeces, survive in damp, organic matter-rich environments, making the selection of flooring essential for disease control (Çavuşoglu and Petek, 2019; Chuppava et al., 2019, 2018a). The floor under a chicken flock is more than a comfort feature; it regulates moisture, determines how much birds come into contact with their droppings, and ultimately shapes the pressure from *Eimeria* parasites that cause coccidiosis (Dunlop et al., 2016a; Kachanova and Safiullin, 2020; Karcher et al., 2013; Mou et al., 2024; Wondimu et al., 2019). Poorly managed litter becomes an incubation chamber for oocysts, whereas raised or easily washed surfaces deny the parasite the damp environment it needs to sporulate (Abd El-Wahab et al., 2013; Jenkins et al., 2019, 2017, 2013). Picking and, above all, managing the right flooring are therefore cornerstones of on-farm biosecurity, alongside vaccination and whole-house disinfection.

3.4.1. Deep-litter beds (wood shavings, rice hulls, straw)

At 7–10 cm depth, these organics wick up drinker spills and cushion the keel, but once litter moisture drifts past ±35 % the risk curve bends sharply (Figure 7): foot-pad dermatitis scores triple by day 42, and oocyst sporulation accelerates as wet pockets create the warm (21–32 °C), oxygen-rich micro-climate *Eimeria* needs (Dunlop et al., 2016a; Jenkins et al., 2017; Taira et al., 2013).

Recent Ukrainian work shows that adding a dry disinfectant (peracetic-acid carrier) cut total *Eimeria* recovery from straw bedding by 6.7-fold after six weeks and kept pH one complete unit lower, which slows both ammonia release and oocyst survival (Fotina et al., 2024). If producers can raise litter temperature to ≥ 40 °C for 3–5 days during downtime, achievable by windrowing or in-house composting, virtually all oocysts are rendered non-infective (Zhao et al., 2024).

Maintaining healthy broiler litter comes down to steering three interconnected levers, pH, moisture and heat, so that ammonia stays in the ammonium form, *Eimeria* oocysts fail to sporulate, and foot-pad skin never spends long on a wet, caustic mat. Field and laboratory work show that sprinkling 50–75 g m^{-2} of sodium bisulfate can pull surface pH below 5.5, protonating nearly all free NH_3; in dose–response trials this cut house ammonia by as much as 91–95 % during the first day and kept levels 30–50 % lower for the rest of the first week, outperforming biochar, zeolite and flue-gas-desulfurisation (FGD) gypsum in head-to-head comparisons (Mohammadi-Aragh et al., 2025). Because the granules also hydrate slightly, growers often re-dust a lighter 20–30 g m^{-2} "booster" around day 21, when birds' protein intake, and thus uric-acid output, spikes.

Where the immediate problem is water rather than pH, alkaline powders such as quicklime provide a mirror-image tool: mixing ≈ 5 % CaO into rice-husk or similar bedding drives pH past 10, ties up moisture (litter water activity < 0.80) and has halved foot-pad-dermatitis scores in small-scale studies, while also knocking down total culturable bacteria; the trade-off is a transient burst of ammonia in the first 24–48 h, so best practice is to lime immediately after bird removal and ventilate hard before pre-heat (Lopes et al., 2013; Sujiwo and Ariyadi, 2023).

FGD-gypsum works more gently. At 10–20 % of litter mass, the calcium sulfate complexes soluble phosphorus and sorbs some ammonium, trimming ammonia by roughly 9–21 % yet, more importantly for watershed compliance, cutting dissolved-P runoff from land-applied litter by up to 40 % over multiple seasons without introducing heavy metals (Chakraborty et al., 2023; POWELL et al., 2022; Sujiwo and Ariyadi, 2023).

Engineered miscanthus biochar and clinoptilolite-rich zeolite add a purely adsorptive lever. Miscanthus biochar captures around 4–5 mg NH_3 g^{-1} and has flattened in-house ammonia peaks by 20–30 %; zeolite at 8–11 % of litter weight performs similarly, and both amendments exit the chicken house as slow-release soil conditioners, an agronomic bonus in circular-nutrient systems (Graves et al., 2024; Linhoss et al., 2023; Poudel et al., 2024).

Finally, when downtime stretches to a week or more, windrowing the bedding inside the empty barn allows microbial heat pasteurisation of the material. Core temperatures of 55–65 °C sustained for 9–10 days have repeatedly been logged; at those temperatures, *Eimeria* oocysts lose infectivity, and bacterial loads fall precipitously, giving the next flock a cleaner start without hauling litter off-site (D. K. Dittoe et al., 2018; Macklin et al., 2006).

By layering these tactics, acidify at placement, dry or hydrate-bind as the flock grows, and heat-pasteurise between flocks, producers turn litter from a liability into a controllable asset: air that smells like fresh shavings instead of ammonia, drier foot pads, a slower coccidial cycle and a fertiliser that holds on to its nutrients until it reaches the field.

3.4.2. Slatted or mesh floors (full or partial)

Elevating birds breaks the faecal–oral cycle and halves litter moisture beneath waterlines, yet welfare trade-offs are real (Figure 8): an Egyptian trial in turkeys found plastic slats pushed severe foot-pad scores (grade 2–4) from 6 % to 38 % and raised heterophil: lymphocyte ratios, an acute-stress proxy (Kamphues et al., 2012; Mousa-Balabel et al., 2024). Broiler studies comparing 100 % slats, 50 % slats and 100 % litter echo that pattern: growth improves slightly on high-slat designs, but contact dermatitis and loss of dust-bathing behaviour both rise (Aslan et al., 2024). Manure belts or scraper systems under the slats must therefore be run daily to prevent ammonia and flies from accumulating. Newer options such as nanotech-coated plastic grids are being field-tested to curb bacterial load, but performance data remain preliminary (Przybulinski et al., 2025).

Strategic refinements are reshaping how producers use slatted or mesh floors. First, targeted slats under the drinker lines are proving a "best-of-both-worlds" compromise. A German field study with 240,000 broilers demonstrated that a 40-cm strip of perforated plastic beneath each nipple line reduced litter moisture by 15 percentage points in the first two weeks and halved camera-scored foot-pad lesions at slaughter, while the rest of the house remained bedded for dust bathing and foraging (Sonnabend et al., 2022a). Second, daily manure-belt or scraper runs are non-negotiable. Meta-analyses of belt houses report a 40-70% lower ammonia release when droppings are removed every 24 hours instead of weekly, protecting both bird airways and worker health (Yang et al., 2021). Commercial broiler systems advertise similar numbers, but only when operators keep the timer set to "daily" year-round (VDL Jansen, 2022). Third, welfare add-ons are starting to close the behaviour gap that full-slat designs create:

- Hybrid layouts (≤50 % slat) leave a central litter "play zone" so birds can still dust-bathe, an enrichment repeatedly shown to lower stress-indicators like tonic immobility and boost leg strength (Baxter et al., 2018)
- Rubber-coated or nanotech-impregnated grids soften the surface and inhibit the growth of bacterial biofilms. Early trials report a 1-log reduction in *E. coli* counts and no penalty in weight gain; however, long-term performance and clean-out durability are still under review (Przybulinski et al., 2025).

Finally, regulators are watching. EU risk assessors now flag foot-pad dermatitis as a frontline "iceberg indicator" of broiler welfare and link high FPD scores directly to flooring design and litter management (Nielsen et al., 2023a). In practical terms, that means routine pad scoring, and, where slats are used, real-time adjustments to belt frequency, drinker height and ventilation rather than waiting for the next flock.

3.4.3. Concrete pads with removable bedding

Smooth, sealed concrete provides the best pest- and pathogen-barrier available, but only if three management pillars remain rock-solid (Figure 7). Concrete's high thermal conductivity can wick heat out of day-old chicks, drive condensation, and turn even good-quality shavings into a wet compress that scalds the footpad. The Aviagen foot-pad guide lists two easy safeguards: hold the litter at 0.5–1 cm for wood shavings or 1–1.5 cm for chopped straw, and pre-warm the pad to≥ 30 °C before spreading the litter. In trials, skipping either step doubled moderate-to-severe dermatitis when straw depth crept above 2 kg m^{-2} (de Jong and Jan van Harn, 2012). Where budgets allow, producers are embedding hydronic tubing inside the pad. Once the thermal mass is up to temperature, in-floor heating cuts relative humidity and CO_2, keeps litter drier, and shaves fuel bills compared with forced-air heaters, all without the open-flame fire risk (Cui et al., 2019; Olesen, 2008). In-floor hydronic heating systems can be a valuable long-term investment for poultry barns, offering significant benefits in terms of energy efficiency, thermal comfort, and moisture control. However, the high initial costs and maintenance requirements, along with climate adaptability, must be carefully considered. For regions with colder climates, these systems can provide substantial advantages, potentially leading to improved poultry health and productivity (Cui et al., 2019; Olesen, 2008; Zheng et al., 2020)

Every hairline in a pad acts like a capillary for damp litter and microbes. Breeder manuals now recommend inspecting between flocks and filling defects immediately with food-grade epoxy or urethane compounds (Joseph et al., 2021; Szycher, 2012). Both compounds offer significant benefits for use in poultry housing. Urethane compounds are particularly advantageous for their insulating properties and pathogen protection, which directly improve poultry welfare and housing conditions. On the other hand, epoxy compounds, especially when combined with eco-friendly fillers like chicken feather fibres, provide excellent corrosion protection for metal parts used in poultry housing. The choice between these compounds would depend on the specific needs of the poultry housing environment, such as the requirement for pathogen insulation versus corrosion protection (Adamska and Pankiewicz, 2024; Joseph et al., 2021; Szycher, 2012; Vijayan et al., 2020).

Addressing hairline cracks in poultry house pads by filling them with appropriate sealants is crucial for maintaining a dry, sanitary environment. This practice, combined with effective moisture management, can significantly reduce microbial risks and improve overall poultry health and safety (Dunlop et al., 2016b; Lovanh et al., 2007; Rico-Contreras et al., 2014; Winkler et al., 2017a, 2017b).

After the house is empty, pressure-wash, let the surface dry, then apply a disinfectant that can reach micro-roughness in the slab using a foam or gel spray. Chlorine-dioxide foams are gaining ground because they knock down *Salmonella* and *E. coli* biofilms yet leave no chlorinated residues (Chlibek et al., 2006; Gray, 2013; Isomoto et al., 2006; Jiang et al., 2007). German monitoring on layer farms still found a 25 % *Salmonella Enteritidis* prevalence after ordinary wash-downs, underscoring that contact time and coverage matter as much as the molecule used (Münster et al., 2023).

3.4.4. Earthen floors

Packed-soil floors are the cheapest way to house a flock, yet they behave like a long-term "savings account" for *Eimeria* oocysts (Figure 7). Laboratory and field work show that sporulated *E. maxima* can remain infective in soil for up to 602 days under warm, humid conditions, and many oocysts withstand repeated freeze–thaw cycles without losing their capacity to sporulate (Alcala-Canto et al., 2020c; Boyko et al., 2021; Cevallos-Gordon et al., 2024; Kato et al., 2004; Khashiboun et al., 2007; Langkjær and Roepstorff, 2008; Lassen et al., 2013b; Lassen and Seppä-Lassila, 2014b; Lélu et al., 2012). Because every new batch of birds arrives on a substrate seeded by countless previous flocks, the parasite pressure ratchets upward season after season.

That persistence directly translates into flock health risk. A recent South American survey of commercial broiler farms reported that houses with dirt floors carried a 4.28-fold higher odds of detecting *E. acervulina* and 2.31-fold higher odds for *E. praecox* when compared with concrete bases, figures that held even after adjusting for stocking density and anticoccidial programme (Cevallos-Gordon et al., 2024).

Small-flock extension guides echo the concern, noting that oocysts can lie dormant for close to a year in backyard soil, ready to sporulate when warmth and moisture return (Boyko et al., 2021; Khashiboun et al., 2007; Langkjær and Roepstorff, 2008; Lassen et al., 2013b; Lassen and Seppä-Lassila, 2014b; Lélu et al., 2012).

Control therefore hinges on breaking the environmental stage of the parasite's cycle rather than trying to disinfect the impossible. Portable coops or paddock systems that let ground lie fallow for six to twelve weeks deprive oocysts of hosts long enough for natural die-off. Where rotation is impractical, producers turn the top 10-15 cm of soil and incorporate hydrated-lime or quick-lime "hot-mixes": the surge in pH (≥ 12) and the ammonia released during curing can knock viable oocyst counts down by several logs, provided birds are removed, and ventilation is maximised (Cockerill et al., 2020). Still, whole-house lime blankets are discouraged; alkaline litter can accelerate ammonia release and cause footpad burns. Spot treatments on wet patches are safer and equally effective (De Jong et al., 2014; Fotina et al., 2024; Kamphues et al., 2012; Mousa-Balabel et al., 2024) . Finally, keeping the run dry by fixing dripper leaks, improving drainage, and adding fresh carbon (straw, chips) slows sporulation. It buys time for vaccines or low-level ionophores to keep the infection pressure below the disease threshold.

3.5.Perch and nest systems: impact on stress and parasite spread

Well-designed perch and nest systems can reduce stress, limit pathogen exposure, and improve flock welfare: providing raised structures encourages birds to roost off the floor, decreasing contact with contaminated litter, and experimental work shows broilers readily use low perches/platforms and that elevated structures support better welfare indicators (Malchow et al., 2019; Malchow and Schrader, 2021; Norring et al., 2016). Conversely, poorly designed or placed perches increase the risk of injury, especially keel-bone damage in layers; therefore, dimensions, stability, and positioning must be evidence-based (Riber et al., 2018; Rufener and Makagon, 2020; Toscano et al., 2020). Nest hygiene is equally critical: clean, dry nests and timely replacement of nest material lower eggshell bacterial loads (including Salmonella) and reduce overall contamination along the egg chain (Bosland and Sartini, 2023; De Reu et al., 2006; Hannah et al., 2011).

Overcrowding around perches and nests exacerbates stress and disease spread; reducing stocking density improves welfare, performance, and litter conditions (Estevez, 2007a; Shynkaruk et al., 2023b). Practically, placing perches over slatted/cleanable areas and keeping the zones beneath easy to sanitise prevents waste build-up and helps keep litter drier, findings consistent with studies showing slatted elements can improve litter quality, footpad health, and bacterial profiles (Chuppava et al., 2018b; Heitmann et al., 2020; Sonnabend et al., 2022b). Finally, because *Eimeria* oocysts sporulate as a function of temperature, humidity, and oxygen, maintaining dry, well-ventilated litter and nest areas is a direct lever to curb coccidiosis pressure (Mesa-Pineda et al., 2021; Vertommen et al., 1994; L. Waldenstedt et al., 2001).

Figure 7. Four poultry-house flooring systems, left to right: (1) Deep-litter bed of wood shavings with hens scratching; (2) Plastic slatted/mesh floor equipped with automated feeders; (3) Bare concrete alley ready for a removable bedding layer; (4) Earthen dirt floor inside a rustic backyard coop.

4. Husbandry practices and poultry health management

Husbandry practices sit at the heart of poultry health and productivity, and they are pivotal for reducing the risk of avian coccidiosis, an *Eimeria*-driven enteric disease that damages the intestinal mucosa and depresses performance, because day-to-day management directly shapes gut integrity and exposure pressure (Mesa-Pineda et al., 2021; Wickramasuriya et al., 2022). In practical terms, a resilient flock starts with nutrition that supports the microbiota–immune axis and epithelial barrier, now a central paradigm in poultry production, so feed formulation and functional ingredients should be planned with gut health in mind (Ducatelle et al., 2023; Kogut, 2022). Intensive systems add multiple stressors (e.g., heat, crowding, handling), and these stressors are well-documented to suppress immunity and heighten disease susceptibility, underscoring the value of environmental control and low-stress routines in routine husbandry (Lucas J. Lara and Rostagno, 2013; Wasti et al., 2020b). Genetic tools complement management: coccidiosis responses show meaningful between-line variation and heritable biomarkers, and recent work links breed differences to intestinal microbial signatures, evidence that selective breeding can incrementally harden flocks against *Eimeria* (Boulton et al., 2018a; Broadwater et al., 2025a). Pulling this together, sustainable production relies on an integrated approach that couples nutrition, stress reduction, and targeted genetics with core control measures for coccidiosis (biosecurity, vaccination/drug programs, and gut-health support) (Gao et al., 2024a; Madlala et al., 2021a; Mesa-Pineda et al., 2021).

4.1.Daily Management Practices

Daily management is where coccidiosis prevention really takes hold: keep the litter dry and the air fresh, keep the water lines clean, maintain a comfortable light and space, and keep a close eye on the birds. Practically, controlling humidity and ventilating to avoid ammonia protects feet and mucosa and reduces the environmental pressure for *Eimeria,* high relative humidity drives litter caking and ammonia, and >25 ppm ammonia harms performance and welfare, so removing wet patches, adjusting minimum ventilation, and checking in-house NH_3 are core daily tasks (Kristensen and Wathes, 2000; Weaver and Meijerhof, 1991; Zhou et al., 2020).

Dry, friable litter also matters because moisture levels influence oocyst sporulation dynamics, so daily spot-repairs around drinkers and prompt removal of wet litter are warranted (L. Waldenstedt et al., 2001; Weaver and Meijerhof, 1991). Water systems deserve equal attention: biofilms readily form in drinking lines and can seed opportunists into the gut, so routine flushing and sanitation are evidence-based hygiene steps (Maes et al., 2019a; Mustedanagic et al., 2023a). Light and rest cycles are another lever you adjust every day. Modern data show that thoughtfully managed photoperiod/intensity improves behaviour and efficiency while easing stress, so aim for programs that provide true dark periods and allow birds to choose brighter areas for activity and dimmer zones for rest (E. Linhoss et al., 2025; Kang et al., 2023). Stocking density interacts with all of the above; staying within science-based allowances improves litter quality, welfare, and performance, reducing enteric disease pressure (Buijs et al., 2009; Estevez, 2007b). Finally, routine health monitoring closes the loop: regular (e.g., weekly) faecal oocyst counts and targeted lesion scoring during challenges, plus tracking expected oocyst cycling after live vaccination, let you catch problems early and verify that control programs are working (Johnson and Reid, 1970a; Snyder et al., 2021a).

4.2. Monitoring and Health Surveillance

Monitoring and health surveillance in modern poultry production work best as an integrated set of measures based on animal-, environment-, and pathogen-related factors. On the animal side, structured flock walks that include gait scoring can catch lameness early. Recent reviews and automated pose-estimation studies support the reliability and practicality of these assessments, demonstrating how computer vision can flag subclinical problems before they escalate (Fodor et al., 2023; Kwon et al., 2024). Precision-livestock approaches can then track behaviour and environment continuously, so anomalies trigger timely checks rather than late, post-hoc responses (Schillings et al., 2021). Facility-level trend tracking of mortality, feed, and especially water consumption provides sensitive “lead indicators”; the EFSA on-farm welfare opinion explicitly notes that deviations in barn water usage are an early warning for bird or equipment problems (Nielsen et al., 2023b).

For coccidiosis specifically, weekly faecal oocyst counts using modified McMaster remain a field standard for quantifying pressure, while recent image-analysis methods enable faster, more objective counts at scale (Adams et al., 2022; Haug et al., 2006). When birds are culled or at checkpoints, standardised lesion scoring provides a consistent clinical anchor for interpretation across houses and flocks (Johnson and Reid, 1970b). Molecular tools now complement these routines: species-specific qPCR from cloacal swabs or tissues improves sensitivity and speciation, and even dust qPCR can surveil house-level loads of *Eimeria* (and co-pathogens such as *Clostridium perfringens*) with minimal disturbance (Ahaduzzaman et al., 2021; Nolan et al., 2015; Velkers et al., 2010). After live vaccination, oocyst-shedding curves follow predictable cycles; tracking these against published baselines helps verify vaccine "take" and detect drift in anticoccidial programs (Snyder et al., 2021b). Pulling these streams into a simple dashboard, counts, scores, and trends, helps teams spot trouble sooner and act faster, sustaining welfare and performance.

4.2.1. Poultry nutrition and feeding

Nutrition is one of the most effective tools for managing coccidiosis because it influences gut integrity, microbiota balance, and immune readiness precisely where *Eimeria* invades (Table 3). Energy and macronutrients support immune functions and tissue repair. At the same time, long-chain n-3 fatty acids can reduce inflammatory responses and bolster barrier health, benefits well documented in poultry facing infectious and production-related stressors (Alagawany et al., 2019a; Bedford and Apajalahti, 2022; Snyder et al., 2021b). Micronutrients are equally important: vitamin A deficiency weakens local intestinal immunity and increases oocyst shedding during *Eimeria* challenges, whereas vitamin E and selenium help counteract the oxidative stress linked with coccidiosis (Table 3); organic selenium (yeast) has been shown to improve growth and tissue selenium status under challenge conditions (Dalloul et al., 2002; Khalifa et al., 2021; Wickramasuriya et al., 2023). Trace minerals such as zinc (and, depending on the context, copper) also support epithelial integrity and regulate inflammation during *Eimeria* infection (Bortoluzzi et al., 2020; Santos et al., 2020) (Table 3). Beyond basic nutrition, several feed additives provide additional, evidence-based benefits.

Probiotics (e.g., Bacillus and *Lactobacillus* spp.) have been shown to decrease lesion scores and oocyst output, especially when combined with live coccidia vaccination, and can positively influence the microbiome during challenge experiences (Memon et al., 2022; Ritzi et al., 2016, 2014) (Table 3). Prebiotics like mannan-oligosaccharides (MOS) and fructo-oligosaccharides (FOS) selectively promote beneficial microbes, boost mucosal immunity, and decrease parasite excretion in chicks exposed naturally (Gmez-Verduzco et al., 2009; Youssef et al., 2023). Phytogenic compounds, including oregano, thyme, and garlic extracts rich in carvacrol, thymol, or allicin, demonstrate anticoccidial and gut-protective effects *in vitro* and *in vivo* (Table 3), alleviating lesion severity and performance declines in challenged broilers(Aitfella Lahlou et al., 2021; Mohiti-Asli and Ghanaatparast-Rashti, 2015a; Sidiropoulou et al., 2020; Zhang et al., 2023a). Organic acids improve the gut environment and barrier function (Table 3). Butyrate supports epithelial regeneration and has reduced coccidial severity in experimental systems, while encapsulated organic-acid blends (including citric acid) have shown anticoccidial and immune-stimulating activity (Table 3). Results with individual acids like citric can vary, so formulation and delivery are crucial (Balta et al., 2021; Melaku et al., 2021; Nouri, 2022; Proszkowiec-Weglarz et al., 2020). Finally, yeast β-glucans act as immunomodulators that enhance both innate and adaptive immune responses, leading to better outcomes during *Eimeria* challenges (Omara et al., 2021; Schwartz and Vetvicka, 2021) (Table 3). A balanced diet supplemented with targeted additives can strengthen host defences, stabilise the microbiota, and reduce reliance on synthetic anticoccidials as part of an integrated approach to coccidiosis control (Madlala et al., 2021b) (Table 3).

Table 3. Nutrition-based strategies for coccidiosis mitigation: macronutrients, micronutrients, phytogenics, and evidence.

Category	Nutrient / Additive	Central mechanisms of action (succinct)	Evidence (peer-reviewed)
Macronutrients	Threonine (AA)	Fuels mucin (MUC2) synthesis; supports epithelial barrier and local immunity, especially under *Eimeria* challenge.	(Saadatmand et al., 2019; Q. Zhang et al., 2016)
	Arginine (AA)	Substrate for nitric-oxide synthase (iNOS); modulates cytokines and improves outcomes during *Eimeria* infection.	(Castro et al., 2020; Liu et al., 2023)
	NSP (carbohydrates) & xylanase	NSP raise digesta viscosity and impairs digestion; xylanase/NSP-ases reduce viscosity, improve nutrient access, and stabilise gut environment.	(Chen et al., 2023; Wang et al., 2024)
	Fermentable fibre → SCFA (butyrate)	SCFA (especially butyrate) reinforce tight junctions, stimulate mucus production, and temper inflammation; they support recovery during enteric challenges.	(Alagawany et al., 2019b; Allen et al., 1996)
	n-3 fatty acids (lipids)	Shift eicosanoids toward pro-resolution mediators; *in vivo*, n-3 diets reduce *E. tenella* lesion scores.	(Liu et al., 2021; Ma et al., 2022))
Micronutrients	Vitamin A	Maintains mucosal integrity and IgA responses; deficiency increases oocyst shedding and alters cytokines after *Eimeria* challenge.	(Dalloul et al., 2002)
	Vitamin D_3	Via VDR, it induces host-defence peptides (e.g., avian β-defensins); supports innate immunity at the mucosa.	(White, 2022; L. Zhang et al., 2016)
	Vitamin E + Selenium	Antioxidant network (membrane VE + Se-GPx); mitigates oxidative stress during coccidiosis and improves performance under stress.	(Khalifa et al., 2021; Pecoraro et al., 2022)
	Zinc (± Copper)	Supports tight junction proteins and the barrier; modulates inflammatory signalling; improves integrity in *Eimeria*/*C. perfringens* co-challenge.	(Bortoluzzi et al., 2019; Santos et al., 2020)
Feed additives	Probiotics (e.g., *Bacillus*, *Lactobacillus*)	Competitive exclusion, bacteriocins & SCFA; TLR/cytokine tuning; ↑ tight-junction expression; lower coccidial lesions/OPG (esp. with live vaccine).	(Park et al., 2019; Ritzi et al., 2016)
	Prebiotics - MOS	Mannose moieties bind type-1 fimbriae → reduce pathogen adhesion; modulate mucosa & microbiota; reports of lower oocyst output.	(Ahmad et al., 2023; Chacher et al., 2017)
	Prebiotics - FOS/inulin	Selectively fuel beneficial microbes → more SCFA, better barrier & immune tone; supportive data in *Eimeria* settings.	(Adhikari et al., 2020; El-Shall et al., 2024)
	Phytogenics - oregano oil (carvacrol, thymol)	Antimicrobial & anti-inflammatory; direct anti-*Eimeria* effects (reduced lesions/performance loss) in challenged broilers.	(Mohiti-Asli and Ghanaatparast-Rashti, 2015a; Zhang et al., 2023a)
	Phytogenics - garlic (allicin)	Antiparasitic activity against *Eimeria* oocysts; lowered oocyst shedding/lesions in vivo.	(Abd-ELrahman et al., 2022; Kim et al., 2013b)

	Organic acids - butyrate	Lowers luminal pH (general OA effect), but specifically: fuels enterocytes, tight-junction upregulation, anti-inflammatory; improved outcomes in coccidiosis trials.	(Hansen et al., 2021; Melaku et al., 2021)
	Organic acids - encapsulated blends (e.g., citric + others)	Undissociated OA diffuse into bacteria → intracellular acidification & metabolic disruption; encapsulation targets distal gut; anticoccidial and immunogenic benefits shown.	(Dana K. Dittoe et al., 2018; Nouri, 2022)
	β-Glucans (yeast cell wall)	Dectin-1–mediated innate "training"; macrophage activation; modulate cytokines and enhance responses during *Eimeria* challenge.	(Omara et al., 2021; Schwartz and Vetvicka, 2021)
	Enzymes (NSP-ases)	Hydrolyse NSP → lower viscosity, improve digestibility, reduce substrate for dysbiosis; supports post-coccidial gut stability.	(Chen et al., 2023)

AA: amino acids, MUC2: mucin-2 (major intestinal gel-forming mucin), iNOS : inducible nitric oxide synthase, NO: nitric oxide, NSP: non-starch polysaccharides, NSP-ases: NSP-degrading enzymes (e.g., xylanase), SCFA: short-chain fatty acids, OPG: oocysts per gram, FCR: feed conversion ratio, ADG: average daily gain, VDR: vitamin D receptor, IgA: immunoglobulin A, GPx: glutathione peroxidase, ZO-1: zonula occludens-1 (tight-junction scaffold), PUFA: polyunsaturated fatty acids, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, AMEn: apparent metabolizable energy (nitrogen-corrected), TLR. Toll-like receptor, HDAC-i: histone deacetylase inhibitor, TJ: tight junction(s), MOS: mannan-oligosaccharides, FOS: fructo-oligosaccharides, Dectin-1: β-glucan receptor (C-type lectin).

- *Phytogenic strategies for controlling avian coccidiosis*

Across broiler trials and mechanistic studies, phytogenic extracts target different *Eimeria* stages, from excystation and invasion (MIC/RON–dependent) to schizogony, while also supporting host defences (antioxidant enzymes, barrier integrity, and immune modulation) (Table 4, Figure 8). For example, *Artemisia annua* (qinghao) consistently reduced oocyst output and lesion scores in broilers, both as dried powder and as standardised "qinghao" products, even in floor-pen experiments when combined with fennel (*Foeniculum vulgare*) (Coroian et al., 2022; Drăgan et al., 2014a; Wang et al., 2021). Thyme/oregano essential oils and their phenolics (thymol, carvacrol) inhibit sporozoite invasion and decrease early schizonts, with *in-vitro* DF-1/MDBK models and *in-vivo* validations; recent work compares these oils directly with anticoccidials (Felici et al., 2024, 2023, 2020). Garlic (allicin) exhibits direct anti-*Eimeria* activity (reducing sporulation/infectivity) and causes *in vivo* decreases in oocyst shedding and lesions (Abd El-Ghany, 2024; Abd-ELrahman et al., 2022). Neem (*Azadirachta indica*) improves outcomes in challenged broilers; both conventional extracts and nano-formulations lower oocyst counts and modulate immunity (Algammal et al., 2024; R. Ishaq et al., 2022; Onyiche et al., 2021).

Polyphenol-rich botanicals are also promising (Figure 8). Pomegranate peel (tannins/punicalagins) reduced faecal oocysts and repaired caecal histology in *E. tenella* models, including controlled broiler infections (Ahad et al., 2018; Hafeez et al., 2023; Khorrami et al., 2022). Turmeric (curcumin) improves lesion scores and anticoccidial index while supporting antioxidant defences, and ginger contributes similar benefits in co-supplementation trials (Algammal et al., 2024; Ali et al., 2019; R. Ishaq et al., 2022). Green tea (*Camellia sinensis*) catechins lowered OPG/lesions and improved FCR in broilers, echoing earlier proof-of-concept work (Jang et al., 2007; Jelveh et al., 2022).

Compounds that act on membranes or parasite development introduce another layer. *Quillaja saponaria* (saponins) disrupt parasite membranes and work synergistically with vaccination, with multiple trials in vaccinated broilers and new pen studies showing approximately 40–50% OPG reductions and lower lesion scores; recent research also reports improved performance under coccidial challenge (Bafundo et al., 2021, 2020; Saddoris-Clemons et al., 2024).

Cinnamon/cinnamaldehyde has achieved moderate anticoccidial indices and performance improvements in infected birds, along with reports of enhanced digestion and gut health (Ali et al., 2021; Orengo et al., 2012; Qaid et al., 2020). Clove/eugenol provides supportive benefits for performance and gut function in poultry and is being tested in EO panels that include anti-*Eimeria* outcomes (Ibrahim et al., 2022; Zoroaster et al., 2025). Piper betle essential oil is a relatively new entry with *in vitro* and *in vivo* activity against *E. tenella* (Ristanti et al., 2024).

Several botanicals primarily modulate host immunity and oxidative stress while still reducing disease markers (Figure 8). *Aloe vera* polysaccharides (acemannan) improved growth, lowered OPG and lesions, and enhanced immune responses in broilers, including as vaccine or drug adjuncts (Akhtar et al., 2012; Hassan et al., 2024; Khaliq et al., 2017). Eclipta (coumestans) and *Acacia nilotica* (condensed tannins) interfered with oocyst sporulation and lowered field and pathology indices; Acacia's dose-dependent sporulation block has been demonstrated *in vitro* (Ahmed et al., 2022; Michels et al., 2011). Onion (quercetin and sulfur compounds) has early poultry data suggesting reduced coccidial impact, though results are more variable than with garlic (Abo-Aziza et al., 2022). *Phyllanthus emblica* (amla) inhibited sporulation and faecal oocysts and boosted immunity to coccidial challenge in broilers (Kaleem et al., 2014; Sharma et al., 2021). *Nigella sativa* shows protective effects in broiler Eimeria challenges, including trials with seed oil and even NS-mediated ZnO nanoparticles, but outcomes vary by dose and formulation (Lail et al., 2023; Manjunatha et al., 2023). Berberine (from Coptis and Berberis) reduces oocyst shedding, protects the caecal barrier, and can work synergistically with amprolium (Fang et al., 2016; Malik et al., 2016; Nguyen et al., 2021). *Citrus aurantium* leaf extract has also demonstrated anticoccidial activity in experimental broiler infections (A. N. Ishaq et al., 2022).

Recent syntheses recommend using standardised phytochemicals within integrated programmes, alongside vaccination, litter and stress management, microbiome-supporting feed additives, and judicious anticoccidial rotation, to build more resilient, drug-sparing control of coccidiosis in modern broiler production (El-Shall et al., 2022).

Table 4. Anticoccidial activities of selected medicinal plants in chickens, preparations, principal bioactives, and proposed mechanisms of action.

Plant name	Botanical family	Preparation type	Main bioactives	Mechanism of action	Key evidence	Ref
Artemisia annua	Asteraceae	Aqueous/ethanolic extract; artemisinin	Artemisinin, sesquiterpene lactones	Inhibits parasite development (incl. calcium homeostasis/ATPase pathways); ↓ oocyst output; improves lesion scores	Field/pen trials show anticoccidial efficacy; *A. annua* and artemisinin mitigate *E. tenella* infection.	(Coroian et al., 2022; Drăgan et al., 2014b; Jiao et al., 2018)
Allium sativum (garlic)	Amaryllidaceae	Crude/ethanolic extract; allicin	Allicin, organosulfur compounds	Direct anti-*Eimeria* activity; modulation of phagocytes & cytokines; ↓ OPG/lesions; ↑ performance	*In vivo* broiler studies (garlic, allicin) reduce oocysts and lesions; reviews corroborate.	(Abd El-Ghany, 2024; Abd-ELrahman et al., 2022; Adjei-Mensah and Atuahene, 2023)
Azadirachta indica (neem)	Meliaceae	Ethanolic/aqueous extract	Azadirachtin, limonoids	Antioxidant/anti-inflammatory; impairs sporulation & parasite fitness; ↓ OPG	Broiler and rodent models show protection against *Eimeria* spp.; recent nano-formulations are active against *E. tenella*.	(Algammal et al., 2024; Dkhil et al., 2013; R. Ishaq et al., 2022; Onyiche et al., 2021)
Thymus vulgaris (thyme)	Lamiaceae	Essential oil	Thymol, carvacrol	Blocks sporozoite invasion/excystation; ↓ intracellular replication; gut barrier support	*In vitro* inhibition of sporozoite invasion and in vivo benefits of thymol/carvacrol blends.	(Felici et al., 2024, 2020)
Curcuma longa (turmeric)	Zingiberaceae	Methanolic extract; powder	Curcumin	Anti-inflammatory/antioxidant; preserves mucosa; ↓ OPG & lesions; ↑ anticoccidial index	Multiple broiler trials under *Eimeria* challenge show improved AI, lesions, and oxidative markers.	(Chen et al., 2024; Khalafalla et

						al., 2011; Kim et al., 2013a)
Punica granatum (pomegranate)	Lythraceae	Aqueous/peel extract	Tannins, punicalagin	Inhibits oocyst sporulation & wall formation; ↓ invasion; anti-inflammatory.	Peel extracts reduce oocysts/lesions; punicalagin improves cecal integrity in *E. tenella*.	(Ahad et al., 2018; Hafeez et al., 2023; Khorrami et al., 2022; Yang et al., 2022)
Origanum vulgare (oregano)	Lamiaceae	Essential oil/extract	Carvacrol, thymol	↓ epithelial desquamation; inhibits invasion; microbiota/AMEn benefits	The studies show lower oocyst counts and lesions, and performance benefits.	(Giannenas et al., 2003; Mohiti-Asli and Ghanaatparast-Rashti, 2015b)
Quillaja saponaria	Quillajaceae	Saponin-rich extract	Triterpenoid saponins	Disrupts parasite membranes; synergises with vaccines; ↓ OPG & lesions	Meta-analysis and trials in vaccinated broilers; the study shows dose-responsive OPG/lesion reductions.	(Bafundo et al., 2021, 2020; Saddoris-Clemons et al., 2024)
Zingiber officinale (ginger)	Zingiberaceae	Aqueous/ethanolic extract	Gingerols, shogaols	Antioxidant; ↓ lesion scores & oxidative stress; immune modulation	Broiler trials (ginger ± garlic) show anticoccidial benefits vs. amprolium; supportive mouse data.	(Ali et al., 2019; Mubaraki et al., 2022)
Eclipta alba (*E. prostrata*)	Asteraceae	Aqueous extract	Wedelolactone; coumestans	Inhibits parasite development; immunomodulation	The trial shows that coumestans control *E. tenella* in broilers.	(Michels et al., 2011)
Acacia nilotica	Fabaceae	Aqueous/leaf extract	Condensed tannins	Disrupts oocyst wall; anti-inflammatory; ↓ sporulation	*In vitro* inhibition of *E. tenella*; tanniferous leaves screened for anticoccidial effects.	(Ahmed et al., 2022)
Aloe vera	Asphodelaceae	Crude/polysaccharide extract	Acemannan, polysaccharides	Immune stimulation; ↓ sporulation; supports mucosa	The studies show immunoprotective and anticoccidial effects of Aloe polysaccharides/extracts.	(Akhtar et al., 2012; Hassan et al., 2024b; Khaliq et al., 2017)

Allium cepa (onion)	Amaryllidaceae	Aqueous extract	Quercetin, sulfur compounds	Antioxidant/cytokine modulation; adjunct anti-*Eimeria* effect	A comparative broiler study (onion vs diclazuril/garlic/moringa) reports benefits against *E. tenella.*	(Abo-Aziza et al., 2022)
Cinnamomum cassia (cinnamon)	Lauraceae	Ethanolic extract; cinnamaldehyde	Cinnamaldehyde	Immunomodulation (↑ protective cytokines); anti-inflammatory; ↓ lesions	Cinnamaldehyde protects *against avian coccidiosis in vivo*; broader poultry data reviewed.	(Ali et al., 2021; Lee et al., 2011; Orengo et al., 2012; Qaid et al., 2020)
Phyllanthus emblica (amla)	Phyllanthaceae	Aqueous/ethanolic extract; tannins	Gallic acid, tannins	Inhibits oocyst sporulation; enhances antioxidant enzymes; boosts immunity	The study shows reduced OPG/lesions; *P. emblica* tannins immunostimulate.	(Kaleem et al., 2014; Sharma et al., 2021)
Withania somnifera (ashwagandha)	Solanaceae	Aqueous/ethanolic extract	Withanolides	Antioxidant/immune support; histology improvements reported in poultry health	Evidence in poultry is mainly immunomodulatory (not strictly anticoccidial). Use as a supportive adjunct.	(Salem et al., 2022)
Terminalia chebula (haritaki)*	Combretaceae	Methanolic extract	Chebulinic/chebulagic acids (hydrolysable tannins)	Tannins may impair oocyst wall/enzymes; gut integrity support	Poultry-specific anticoccidial data are limited; cited in reviews/patents—treat as emerging.	(Gokila et al., 2013)
Nigella sativa (black cumin)	Ranunculaceae	Seed/oil	Thymoquinone	Anti-inflammatory & antimicrobial; ↓ lesions/OPG; supports performance, esp. in mixed *Eimeria*/*C. perfringens* challenges	Controlled broiler trials with NS oil; adjunct data in rabbits/poultry.	(Lail et al., 2023; Manjunatha et al., 2023; Mohammed Kuraa et al., 2021)
Camellia sinensis (green tea)	Theaceae	Leaf extract/powder	Catechins (EGCG)	Antioxidant; immunomodulatory; ↓ OPG/lesions; performance benefits	Studies show anticoccidial effects comparable to toltrazuril at some doses.	(Jang et al., 2007; Jelveh et al., 2022)
Coptis chinensis / *Berberis* spp. (berberine)	Ranunculaceae / Berberidaceae	Alkaloid extract	Berberine	Direct anti-*Eimeria*; immune modulation; synergy with amprolium	Broiler studies across 5 *Eimeria* spp.; mechanistic and synergy data available.	(Fang et al., 2016; Malik et al., 2016;

						Nguyen et al., 2021)
Sophora flavescens (RSF)	Fabaceae	Root extract	Matrine, oxymatrine	Anti-inflammatory; promotes mucosal immunity; adjunct with toltrazuril	The chicken model shows that RSF + toltrazuril reduces inflammation and improves mucosal immunity.	(B. H. Zhou et al., 2021)
Citrus aurantium	Rutaceae	Ethanolic leaf extract	Flavonoids (naringin, hesperidin)	Antioxidant & anti-inflammatory; ↓ lesions/OPG	Broiler trial demonstrates anticoccidial activity vs *E. tenella*.	(A. N. Ishaq et al., 2022)
Syzygium aromaticum (clove/eugenol)	Myrtaceae	Essential oil; eugenol nano-emulsion	Eugenol	Damages parasite membranes; ↓ inflammation; improves barrier	Eugenol-based formulations reduce lesions/OPG; clove EO improves performance under coccidial stress.	(Geng et al., 2024; Zhang et al., 2023b)
Foeniculum vulgare (fennel)	Apiaceae	Aqueous/seed extract	Anethole	Adjunct with *A. annua*; anti-inflammatory; supports recovery	A floor-pen study shows that A. annua and fennel mitigate *E. tenella* infection.	(Drăgan et al., 2014a)
Piper betle (betel leaf)	Piperaceae	Essential oil	Chavibetol, eugenol	Anti-*Eimeria* activity; ↓ lesions/OPG; immunomodulation	2024 *Scientific Reports* shows the efficacy of PBEO against *E. tenella* in broilers.	(Ristanti et al., 2024)

MIC: Microneme proteins (parasite adhesins released from the apical organelles to start host-cell invasion); RON: Rhoptry neck proteins (partners of MIC that help form the "moving junction" during invasion); Schizogony: Asexual replication phase of *Eimeria* inside the host (multiple fission/merogony); Excystation: Release of sporozoites from sporocysts/oocysts to initiate infection; SOD: Superoxide dismutase, a core antioxidant enzyme (with CAT and GPx); TNF-α: Tumor necrosis factor-alpha, a pro-inflammatory cytokine; IL-1β: Interleukin-1 beta, a rapidly induced pro-inflammatory cytokine in birds and mammals.

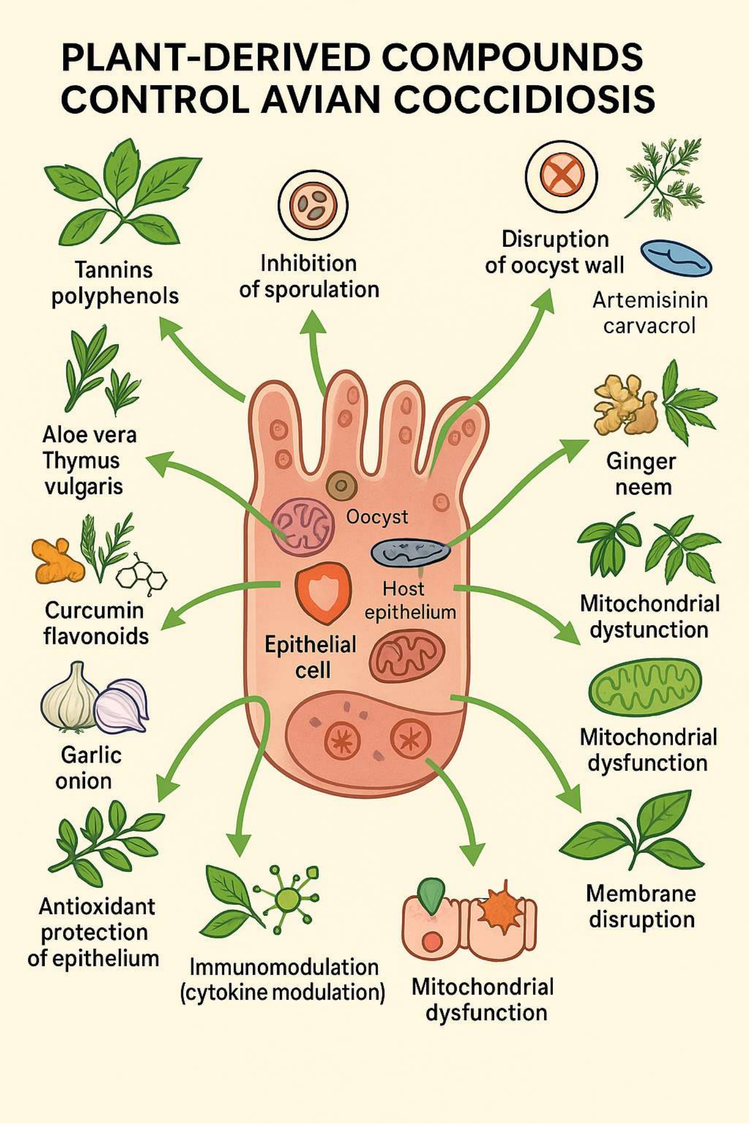


Figure 8. Illustrative infographic summarising the central plant-derived mechanisms against avian coccidiosis.

4.2.2. Breeding systems and genetic selection

Selective breeding provides a robust, sustainable approach to improve poultry resistance to *Eimeria*-induced coccidiosis while sustaining productivity. Genetic variation in resistance to *Eimeria tenella* has been established, such as in *Fayoumi* × *Leghorn* crosses, where quantitative trait loci (QTLs) associated with traits like lesion score and weight gain were identified, demonstrating the potential for targeted selection for disease resistance (Bacciu et al., 2014; Boulton et al., 2018b; Lillehoj et al., 1989; Qiao et al., 2024). The role of the major histocompatibility complex (MHC) in immune response against *Eimeria* has long been acknowledged; for instance, Lillehoj and colleagues showed that interactions between MHC genes and other background genes influence susceptibility to *E. tenella*-induced coccidiosis (Lillehoj et al., 1989). More recently, Boulton et al. confirmed that inbred lines such as 15I (MHC type B15) and C.B12 (MHC type B12) show significant differences in oocyst shedding following *E. maxima* infection, highlighting the influence of the host's genetic background on immune outcomes (Boulton et al., 2018b). Additionally, selecting for broader immune responsiveness, not just pathogen-specific resistance, has been proposed as a promising breeding target, which could enhance vaccine response and overall resilience (Broadwater et al., 2025b; Qiao et al., 2024; Hailiang Yu et al., 2023). Collectively, these studies suggest that integrating genomic tools and marker-assisted selection to incorporate favourable alleles, including those at the MHC locus, can produce chicken lines that are both resilient to *Eimeria* infection and capable of maintaining high production performance.

4.3.Stress management and animal welfare

Stress physiology is closely linked to coccidiosis outcomes in chickens. Activation of the hypothalamic–pituitary–adrenal axis increases corticosterone, which is strongly immunosuppressive in birds. Experimental corticosterone administration leads to rapid lymphoid depletion in the thymus, bursa, and spleen, reducing the cellular and humoral responses necessary to control *Eimeria* infections (Schat and Skinner, 2013). In broilers infected with *Eimeria acervulina*, dietary corticosterone does not affect the immediate peak of oocyst shedding. However, it extends the shedding period and worsens production losses, indicating a delayed development of protective immunity under stress (Graat et al., 1997).

Beyond endocrine effects, common farm stressors weaken the intestinal barrier, the main frontline against *Eimeria*. Heat stress repeatedly damages tight junction integrity, increases intestinal permeability, and activates systemic inflammation, thereby mechanistically linking environmental stress to enteric disease risk. Controlled trials and reviews show heat-stressed broilers develop leaky guts, higher endotoxemia, increased corticosterone and cytokines, and changes in epithelial tight-junction gene expression (Rostagno, 2020; Tabler et al., 2020; Varasteh et al., 2015). As Eimeria invades and multiplies within gut epithelia, a stressed, permeable mucosa worsens lesion severity and performance decline; recent reviews of coccidiosis pathogenesis and control in chickens highlight this host–environment–parasite interaction (Ahmad et al., 2023; Mesa-Pineda et al., 2021).

Animal-welfare–oriented management can lessen these vulnerabilities. Environmental enrichment that encourages species-specific behaviours (e.g., dust-bathing, perching, and foraging) decreases stress reactivity and supports mucosal defences. In laying hens, providing a dust-bathing environment upregulated tight-junction (claudin-1, claudin-4, occludin, ZO-1/2) and immune genes (e.g., IL-4, IL-6, IL-8, IFN-γ, IgA). It shifted microbiota towards beneficial taxa linked with mucosal immunity, findings that align with improved barrier function and resilience under challenge (Hanlin Yu et al., 2023). Broader syntheses in Poultry Science also conclude that well-designed enrichments enhance behaviour, affective state, and health, promoting welfare and robustness (Campbell et al., 2019; Jacobs et al., 2023).

Nutritional strategies complement environmental measures by buffering stress and enhancing anticoccidial immunity. In commercial-style *Eimeria* challenges, probiotics improved performance, reduced duodenal/jejunal lesion scores, and (in some treatments) lowered oocyst shedding, demonstrating tangible protection without anticoccidials (Ritzi et al., 2014). Combining probiotics with live coccidiosis vaccination further lowered lesion scores and improved weight gain compared to vaccine alone, indicating additive benefits for mucosal immunity (Ritzi et al., 2016). Central additional controlled studies report probiotic-fed birds resisting *E. tenella* with improved antioxidant status and tight-junction/transport gene expression, consistent with barrier preservation under stress (Mohsin et al., 2022).

Recent reviews integrate these findings, noting that microbiota-directed feed additives (probiotics, prebiotics, phytogenics) can modulate the stress–immunity–barrier triad in favour of the host (Abd El-Hack et al., 2020).

Stress management is not ancillary to coccidiosis control; it is central. By minimising environmental stressors (heat load, crowding, abrupt disturbances), enabling natural behaviours through enrichment, and supporting gut integrity and microbial balance via targeted nutrition, producers can shorten the oocyst shedding window, reduce intestinal damage, and improve both welfare and productivity. Contemporary overviews of coccidiosis control explicitly call for such integrated approaches that marry good husbandry with immunological and nutritional support (Ahmad et al., 2023).

5. Biosecurity and strategies for controlling avian coccidiosis

Biosecurity is a foundational pillar in modern poultry systems, especially given the high infectivity and environmental resilience of *Eimeria* oocysts, which can persist for months in litter, dust, or water systems (Gao et al., 2024b; Kogut et al., 2020). Because coccidial transmission follows the faecal–oral route, the rigorous separation of infected from healthy flocks, strict hygiene protocols, and environmental controls are essential to curtail the oocyst burden (Attree et al., 2021). Systematic on-farm biosecurity, including controlled access (personnel, equipment, vehicles), proper disposal of litter, and sanitation of housing, substantially reduces *Eimeria* spread and contributes to decreased reliance on anticoccidial medications (Ngom et al., 2024).

5.1. On-Farm Biosecurity Measures

Effective control of *Eimeria* on poultry farms relies on a layered biosecurity approach that prevents pathogen entry, reduces environmental oocyst levels, and halts transmission within houses. At the perimeter, restrict the movement of people, vehicles, and equipment; establish clean and dirty zones; require dedicated protective clothing and footwear to be sanitised; and isolate any potential introductions.

Quarantine and "all-in/all-out" flock management are essential principles proven to diminish transmission between groups in food-animal systems and are directly applicable to poultry houses (Barrington, 2013).

Between flocks, cleaning and disinfection should follow a sequence of dry-cleaning, detergent wash, rinse, oocyst-active disinfectant, and thorough drying, because organic matter inactivates most chemical agents, and adequate contact time on clean, dry surfaces is crucial. Multiple experimental and review papers concur that *Eimeria* oocysts are particularly resilient: unsporulated forms are more susceptible than sporulated ones, and efficacy varies significantly by active ingredient and concentration. Across comparative tests, 8% ammonia has demonstrated the most substantial oocysticidal effect; phenolics (e.g., chlorocresol/cresol), strong organic acids (e.g., acetic acid), and certain aldehydes can inhibit sporulation or damage oocyst walls, whereas peroxygen formulations and some quaternary ammonium compounds perform inconsistently against oocysts in practical conditions. These data justify selecting chemistry proven for coccidia and applying it under low-soil, high-contact-time conditions (El-Sherry et al., 2021; Gadelhaq et al., 2018; Guimarães et al., 2007; Mohammed, 2017; You, 2014; Zhao et al., 2024).

House microclimate and litter management have a direct impact on sporulation pressure. *Eimeria* sporogony requires oxygen and a suitable temperature–humidity environment. In controlled studies, sporulation occurs quickly at ~20–30 °C with good aeration; however, overly wet, compacted litter can block oxygen flow, reducing sporulation while still promoting the growth of other pathogens and raising welfare concerns. In commercial-style litter, viable oocysts can stay for weeks, highlighting the need to keep litter dry and friable, promptly clear caked areas, and ensure ventilation prevents moisture build-up around drinkers (L. Waldenstedt et al., 2001; Williams, 1995; Zhao et al., 2024)

Water and feed hygiene are equally essential to ensure health and safety. Broiler waterlines often develop resilient biofilms that seed drinkers and birds; recent research in commercial settings shows complex microbial communities within these lines and supports using validated sanitation programmes (such as regular flushing, mechanical cleaning where feasible, and continuous or pulsed disinfection with agents proven to be safe and effective in poultry). Controlled trials and evaluations suggest that chlorination methods, including sodium dichloroisocyanurate or hypochlorous acid–based treatments, can reduce bacterial loads in waterlines and faeces without affecting performance when applied at appropriate levels, thereby aiding in physical biofilm control. Feed should be stored dry, sealed, and protected from pests and wild birds to prevent contamination (J. Li et al., 2023; Ogundipe et al., 2024; Zou et al., 2023).

Finally, biosecurity is a foundational element, not a substitute, for other control tools. The most reliable programmes combine robust on-farm biosecurity measures (movement control, sanitation, litter and water management) with vaccination and/or anticoccidials, tailored to local risk and resistance conditions. Recent comprehensive reviews in poultry science emphasise this integrated approach to reduce environmental *Eimeria* burden, protect flock health, and maintain performance (Gao et al., 2024b; Mesa-Pineda et al., 2021).

5.1.1. Access Control: Personnel, Equipment, and Animal Movements

Access control is a crucial, evidence-based component of biosecurity that prevents the entry of *Eimeria* oocysts and other pathogens into poultry farms. Peer-reviewed field studies indicate that farms enforcing strict visitor restrictions, dedicated work clothing, and controlled movement between poultry houses experience significantly lower rates of coccidiosis and other enteric diseases (Tilli et al., 2022). Segregating farm areas into "clean" and "dirty" zones, and using dedicated equipment in each zone, reduces the risk of cross-contamination by more than half compared to farms sharing equipment (Gelaude et al., 2014). Vehicles and machinery are identified as major indirect vectors; even after superficial cleaning, *Eimeria* oocysts and other pathogens can remain in tyres, wheel wells, and enclosed compartments. Modelling studies on livestock movements show that poorly sanitised vehicles can facilitate the long-distance spread of pathogens, sustaining regional outbreaks (Galvis and Machado, 2022). Therefore, comprehensive disinfection of wheels, undercarriages, and load compartments with proven sporocidal agents, along with maintaining vehicle and personnel movement logs, is recommended as a key preventive measure. Access control also applies to other animals, notably rodents, wild birds, and domestic pets, which can mechanically carry oocysts and must be kept out of production areas through structural barriers and pest control measures.

5.1.2. Cleaning and disinfection of housing and equipment

Thorough cleaning and disinfection of poultry housing and associated equipment between production cycles are consistently associated with lower environmental loads of *Eimeria* oocysts and fewer coccidiosis outbreaks. Laboratory and field trials have identified specific chemical agents with high oocysticidal efficacy. For instance, El-Sherry et al. (2021) demonstrated that quaternary ammonium compounds, chlorocresol, glutaraldehyde, and Kilcox achieved up to 100% destruction or inhibition of sporulation in *Eimeria tenella* oocysts under optimal contact times (El-Sherry et al., 2021). Babashani et al. (2019) further reported that cresol- and formaldehyde-based disinfectants at elevated concentrations significantly improved sporocidal activity against fully sporulated oocysts (Mohammed, 2017). However, *Eimeria* oocysts are highly resistant to many typical disinfectants due to their thick, multilayered wall, meaning that mechanical cleaning to remove organic matter is essential before chemical disinfection (Gao et al., 2024b). Moisture control is also vital; wet, compacted litter not only encourages oocyst sporulation but can also shield them from disinfectant penetration.

Effective sanitation protocols therefore include: (1) complete litter removal, (2) thorough drying of the facility, (3) application of high-efficacy disinfectants at the concentration and contact times recommended by the manufacturer, and (4) targeted treatment of high-risk zones such as drinker lines, feeders, and ventilation ducts where moisture and biofilms support persistence. Incorporating heat treatment, such as flaming floors or using steam, can further increase oocyst kill rates, providing an additional layer of protection before restocking.

- *Litter and Waste Management*

Maintaining low *Eimeria* pressure begins with litter physics and microclimate management (Table 5): oocyst sporulation depends on oxygen and moisture, and classic research showed *E. maxima* sporulates best in drier, friable litter (around 16% moisture) and least in very wet litter (around 62%), likely due to oxygen limitations in moist substrates (L. Waldenstedt et al., 2001). Temperature and ambient humidity mainly influence the onset and rate of sporulation (faster at 33 °C than at 21 °C, with earlier development at 80% RH compared to 40% RH), highlighting the importance of avoiding warm, humid pockets (Vertommen et al., 1994).

At the same time, overly dry, dusty litter is undesirable because airborne particles hinder respiratory defences and are linked to poorer health and performance; maintaining ventilation that dries litter without producing dust is essential (Al Homidan et al., 2003; Wang et al., 2023). Litter structure also matters (Table 5): compaction and caking restrict airflow and trap moisture, so regular turning and maintaining a porous surface are effective management practices (D. K. Dittoe et al., 2018). The choice of substrate can influence outcomes, wood shavings are a common, absorbent baseline, but fine sawdust can increase dust levels and footpad problems if not properly managed; straw tends to retain moisture and is repeatedly associated with more severe footpad dermatitis; while sand often remains drier with lower bacterial counts in side-by-side trials (Boussaada et al., 2022; Durmuş et al., 2023; Macklin et al., 2005). Maintenance options form a ladder of intensity: total clean-out and disinfection after severe disease or at long intervals; partial or top-layer replacement during cycles to control moisture and microbes while managing costs; and built-up litter with in-house windrow composting, which achieves pathogen-lethal temperatures and improves litter quality when done correctly (sustained around 50–55 °C in the pile) (D. K. Dittoe et al., 2018; Macklin et al., 2008). To further condition the litter, chemical amendments are well-supported: aluminium sulfate (alum) lowers pH and reduces ammonia in the housing and in the litter; sodium bisulfate decreases NH_3 and suppresses *Salmonella* when applied at effective rates; and hydrated lime can reduce *Salmonella* survival (with care to prevent irritation at high levels) (Bennett et al., 2005; Joerger et al., 2020; Li et al., 2013; Madrid et al., 2012; Moore et al., 2000). Several formulations of acidifiers and organic acids have also been shown in controlled studies to reduce litter pH, ammonia, and pathogenic microbiota (de Toledo et al., 2020). Biological tools complement chemical approaches: *Bacillus*-based probiotics can shift the litter microbiota towards less harmful profiles and interact with the gut–litter microbial loop, while essential oils (notably oregano, thyme, garlic) demonstrate anti-coccidial activity both *in vitro* and *in vivo* (lower lesion scores and oocyst output) when used as feed additives within an integrated programme (Bindari et al., 2021; Horyanto et al., 2024; Mohiti-Asli and Ghanaatparast-Rashti, 2015a; Sidiropoulou et al., 2020).

Table 5. Common types of poultry litter and their characteristics.

Litter Type	Absorption	Dust Level	Insulation	Pathogen Risk	Maintenance Needs
Wood Shavings (Pine, Cedar, Hardwood)	High	Low	High	Low (if managed correctly)	Moderate
Sawdust	Very High	High (causes respiratory issues)	Moderate	High (if wet)	Frequent stirring required
Rice Husks	Moderate	Low	Good	Moderate	Requires drying in humid conditions
Straw/Hay	Low	Low	Good	High (prone to mould & compaction)	Frequent turning
Sand	Low	Very Low	Poor	Very Low	Regular cleaning required
Peanut Shells/Corn Cobs	Moderate	Moderate	Poor	High (prone to mould)	Needs frequent replacement

5.1.3. Feed and Water Hygiene Management

Feed and drinking water are continuous inputs that can either dilute or amplify enteric pathogen pressure; therefore, good hygiene in both streams underpins any coccidiosis control programme. In water systems, biofilms establish readily inside drinker lines and nipples, harbouring *Pseudomonas*, *Stenotrophomonas*, and other opportunists that seed the gut; routine line sanitation and periodic shock treatments are warranted because standard farm water treatments often fail to clear established biofilms and bacterial loads (Maes et al., 2019b; Mustedanagic et al., 2023b). Acidification of drinking water (using organic acids) has been shown to improve intestinal barrier features and favourable microbiota shifts in broilers, and in challenge settings can reduce Salmonella recovery from the crop, benefits that complement coccidiosis control by stabilising the gut environment (Avila et al., 2003; Zhang et al., 2022). Because wet litter accelerates oocyst cycling, while very wet substrates can paradoxically hinder sporulation through oxygen limitation, careful water management (leak prevention, timely flushing, and ventilation to keep litter dry-but-friable) is essential to moderate the timing and intensity of *Eimeria* pressure (Vertommen et al., 1994; L. Waldenstedt et al., 2001) . In parallel, farms should monitor water quality at the house (aerobic counts, Enterobacteriaceae, and indicator genera) since high bacterial loads are standard in practice and correlate with performance penalties (J. Li et al., 2024; Mustedanagic et al., 2023b).

On the feed side, contamination can occur in mills, transport, and bins; pelleting provides a crucial thermal kill step that significantly reduces *Salmonella* (and validated surrogates), although re-contamination is still possible after pelleting and must be managed with good mill and on-farm hygiene (Boltz et al., 2021, 2019; Coe et al., 2022; Jones, 2011). Acidifying the feed (e.g., formic/propionic acids) is an evidence-based adjunct that lowers *Salmonella* levels in feed and along the intestinal tract when fed from hatch, and has consistently improved microbiological outcomes in broilers (Izat et al., 1990; Ricke et al., 2020; Van Immerseel et al., 2006). Vigilance for mycotoxins is equally essential: even low, field-relevant levels of aflatoxin, DON, or fumonisin can impair immunity and gut integrity, and aflatoxin specifically increases mortality and worsens outcomes during *Eimeria tenella* infection, highlighting the importance of thorough ingredient screening, storage, and detoxification strategies (Gómez-Osorio et al., 2024). Taken together, clean waterlines (biofilm control + optional acidification), dry and well-managed litter via sound drinker management, thermally hygienised and (where appropriate) acidified feed, alongside routine microbial/mycotoxin monitoring, form a comprehensive feed–water hygiene package that stabilises the intestinal ecosystem and reduces the biological “noise” against which coccidiosis control programs must operate (Jones, 2011; Maes et al., 2019b; L. Waldenstedt et al., 2001).

CHAPTER III

Impact of Medicinal Plants on Avian Coccidiosis: focus on the Lamiaceae family

1. Lamiaceae family

1.1. Morphological characteristics

The mint family, Lamiaceae, is a sizable and aromatic branch of flowering plants, encompassing roughly 3,350 to 4,000 species that thrive mainly in warm-temperate regions of the Northern Hemisphere but extend to nearly every continent (Haas et al., 2024). Most members exist as annual or perennial herbs, although a few mature into woody shrubs. Members of the mint family (Lamiaceae) can be identified by a range of microscopic and macroscopic traits that form a reliable framework for classification (Figure 9). Epidermal cells often exhibit irregular, hexagonal, or polygonal outlines on the leaf surface. Meanwhile, stomata are primarily anomocytic but may also be diacytic, paracytic, or tetracytic, a diversity that varies among species (Zaman et al., 2022). Equally distinctive is the extensive variety of trichomes: glandular types such as capitate, peltate, and clavate hairs are frequently involved in essential oil secretion, while non-glandular hairs range from simple unicellular forms to multicellular conical, falcate, cylindrical, or stellate structures (Figure 10), which together influence herbivore interactions and environmental stress responses (Jabeen et al., 2024). Above ground, stems are characteristically square in cross section and bear oppositely arranged leaves that vary from broadly to narrowly ovate, occasionally appearing cordate, ovate, or suborbicular in outline, imparting a familiar aroma of basil, sage, or thyme when crushed (Gul et al., 2019). The dry fruits, or nutlets, further enhance identification: their shapes vary from sub- globose to obovoid or elliptic, their surfaces from smooth to intricately sculptured with reticulations or tubercles (Figure 10), and their colours from pale yellow- orange to dark brown or black, features that are often species- specific under scanning electron microscopy (Bano et al., 2020). Finally, pollen morphology offers additional taxonomic resolution, with pollen grains exhibiting three apertures (tricolporate or tricolpate) or multiple colpi (trizonocolpate or hexazonocolpate) and displaying exine sculpturing patterns that are reticulate, reticulate scabrate, or perforate, differing subtly between genera and species (Baran & Özdemir, 2009). Botanists achieve a nuanced and reliable classification of Lamiaceae members by integrating these epidermal, trichome, fruit, and pollen characteristics (Figure 10).

Figure 9. Morphological details of some Lamiaceae species: inflorescence of (1) *Nepeta racemosa*, (2) *Teucrium capitatum*, (3) *Melittis melissophyllum*, (4) *Salvia nemorosa, Monarda fistulosa*, (5) *Leonurus cardiaca*, (6) *Prunella vulgaris*, (7) *Glechoma hederacea* (INaturalist, 2025; POWO, 2024).

1.2. Geographical Distribution

The mint family (Lamiaceae) comprises about 245 genera and 7886 species globally and is found on every continent except Antarctica. Still, its epicentre of diversity lies in the temperate and subtropical belt mosaics of the Northern Hemisphere, nowhere more vividly than in the Mediterranean Basin and western Asia (Figure 10) (Bedair et al., 2024; GBIF, 2025; Rundel, 2019; Rundel et al., 2018). In this seasonally extreme cradle, hot, water-stressed summers and cool, rain-soaked winters have favoured aromatic subshrubs such as Thymus, Origanum, and Salvia rosmarinus, whose small, pilose or wax-coated leaves and terpene-rich essential oils minimise transpiration while discouraging herbivores (J. J. Chen & Sun, 2025; Gavalas et al., 2011; Jené et al., 2024a; Lai et al., 2024; Lianopoulou & Bosabalidis, 2014; Naeem et al., 2013; Ouahzizi et al., 2023; Zahedi et al., 2021; Zhou et al., 2015).

Comparable drought-ready lineages, Nepeta and Ziziphora, for instance, colonise the steppes and high plateaus of western Asia, whereas in the New World, the family's richness coalesces around Hyptis and Lepechinia from humid Amazonian lowlands to the cloud-cooled Andes (Babar et al., 2021; Deng et al., 2016; Lyu et al., 2022; Mahmood et al., 2015; Mu et al., 2022; Sivalingam et al., 2021) (Figure 11). South of the Sahara, lamiaceous shrubs track rainfall gradients from Sahel scrub to equatorial rain forest, underscoring an ecological flexibility unmatched by most temperate herb families (Bayala et al., 2022; Diedhiou-Sall et al., 2021; Guilbert et al., 2024; Nyong et al., 2012).

North Africa reflects this adaptability, merging widespread Mediterranean elements with pockets of narrow endemism (Figure 10, Table 6). In Tunisia, botanists have recently documented Vitex trifolia along the coast, discovered Algerian-centred *Thymus dreatensis* in the Kroumirian mountains, and noted the naturalisation of *Volkameria inermis* near Hammamet-Sud (Figure 11, Table 6). The Kabylia–Numidia–Kroumiria corridor straddling Algeria and Tunisia shelters the regional endemic *Thymus numidicus*, while the windswept Aurès host *T. saturejoides*, a species that reappears across Morocco's High, Middle, and Anti-Atlas as well as the Saharan Atlas. Libya completes the Maghreb arc with *Thymus hirtus* subsp. *algeriensi*s stretches from sub-humid wadis to lower-arid slopes across all four North African countries. The North African flora also includes cosmopolitan genera Te*ucrium*, widespread from Atlantic capes to inland massifs, and the ruderal *Lamium purpureum*, common along field margins and ancient ruins (Bukhari et al., 2015; Zhao et al., 2024).

Elsewhere around the Mediterranean (Figure 10, Table 6), Italy records 272 infrageneric taxa dominated by Mediterranean elements but interlaced with peninsular and insular endemics (Pezzetta, 2019); Greece tallies 111 endemic taxa, concentrated on Kriti and the southern Peloponnese (Cheminal et al., 2020a); and Turkey, with 46 genera and 782 taxa (~44 % endemic), ranks among the world's richest and most economically important lamiaceous floras (Çallś & Baśer, 2021) (Figure 10). Egypt likewise lists Lamiaceae among its most species-rich *Tubiflorae* families, with endemic diversity peaking in the granitic massifs and hidden wadis of Sinai (Abd El-Ghani et al., 2024; El-Husseini et al., 2007b, 2007a) (Figure 10).

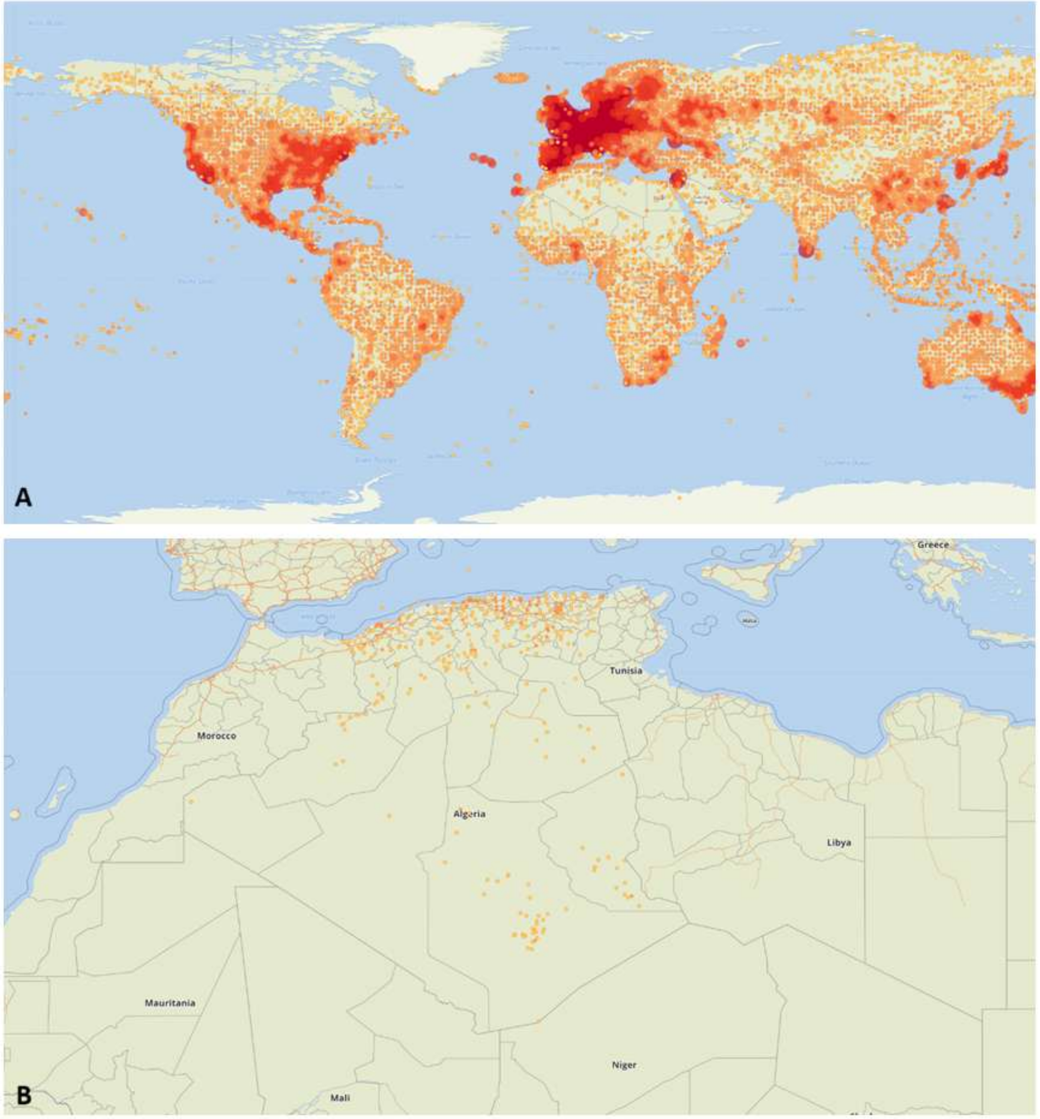


Figure 10. Localisation of the Lamiaceae family members worldwide and in Algeria (GBIF, 2025)

Across oceans, Mexico claims 32 genera and 591 species, 65.8 % endemic, most thriving in temperate highlands, yet some pushing into hot, semi-arid basins (Martínez-Gordillo et al., 2013) (Figure 11, Table 6). Brazil follows with 46 genera and 525 species in four subfamilies, *Ajugoideae* and *Viticoideae* now rehomed from *Verbenaceae*, emphasising South America's deep lamiaceous lineage (Monteiro et al., 2018) (Figure 10). Further north, Ukraine's riverside broadleaf forests nurse linear populations of the rare woodland skullcap *Scutellaria altissima* (Melnyk et al., 2022), and in Afghanistan's Jaji Aryob district, the family ranks among the most significant and most medicinally valuable components of the local montane flora (Adil et al., 2023) (Figure 10, Table 6).

From the thyme-scented maquis of ancient Numidia and the sage-covered slopes of the Peloponnese to the peppermints thriving in Mexican pine–oak forests and the lavender expanses of Brazil's cerrado, the Lamiaceae family embodies both morphological ingenuity and phytochemical virtuosity (Frezza et al., 2019). Their remarkable ability to colonise blazing deserts, mist-laden cloud forests, granite ravines, and windswept islands has driven extraordinary patterns of endemism (Stankovic, 2020), underpinned by adaptations that span Mediterranean, temperate, and tropical ecosystems (Figure 10, Table 6).

This botanical versatility is matched by their chemical richness, with species producing an exceptional diversity of essential oils, flavonoids, terpenoids, and phenolic compounds, traits rooted in evolutionary innovations in specialised metabolism (Boachon et al., 2018; Rattray & Van Wyk, 2021). These attributes have forged a deep and enduring relationship with human cultures, where Lamiaceae species continue to be cultivated and harvested for their flavours, fragrances, and remedies, contributing to both traditional medicine and modern pharmacopoeias (Mamadalieva et al., 2021; Uritu et al., 2018)

Table 6. Listing of the total and endemic taxa of the Lamiaceae family in some countries.

Region	Total Species	Endemic genera	Notable Genera/Species	Ref
Italy	191	4	*Sideritis italica; Thymus richardii* subsp. *Nitidus; Thymus serpyllum* var. *picentinus* Lacaita; *Phlomis ferruginea* Ten; *Lavandula austroapennina.*	(Pezzetta, 2019; Royal Botanic Gardens, 2024; Venditti et al., 2013)
Mexico	591	32	*Hyptis obtusiflora; Hedeoma piperita; Hypenia violacea.*	(Martínez-Gordillo et al., 2013, 2017; Royal Botanic Gardens, 2024)
Brazil	525	46	*Lepechinia annae; Salvia lachnostachys; Marsypianthes dunensis; Eplingiella sanoi; Hypenia kalunga.*	(Antar et al., 2019, 2022, 2024; de Souza Soares et al., 2023; Erbano et al., 2012; Monteiro et al., 2018; Royal Botanic Gardens, 2024; Soares et al., 2025)
Greece	238	111	*Sideritis scardica; Origanum Dictamnus; Stachys iva; Sideritis sipylea.*	(Aneva & Zhelev, 2018a; Cheminal et al., 2020a, 2020b; Di Giacomo et al., 2022; Royal Botanic Gardens, 2024; Solomou et al., 2024; Vantsioti et al., 2025)
Turkey	642	45	*Stachys milasensis; Stachys siirtensis; Scutellaria yildirimlii; Marrubium sivasense; Clinopodium troodi* subsp. *Vardaranum; Clinopodium grandiflorum.*	(Büyükkartal et al., 2016; Çallś & Baśer, 2021; Güner, 2022; Kaya, 2017; Royal Botanic Gardens, 2024; Yildirim et al., 2023)
Egypt	38	24	*Teucrium spp.; Phlomis aurea; Clinopodium serpyllifolium* subsp. *barbatum; Origanum syriacum* subsp. *Sinaicum.*	(Abdelaal et al., 2018; El-Gazzar et al., 2019; El-Husseini et al., 2007c; El-Sayed Ghareb et al., 2020; Rabei et al., 2019; Royal Botanic Gardens, 2024; Zaghloul et al., 2014)
Algeria	157	8	*Teucrium spp.; Thymus saturejoides; Thymus dreatensis; Calamintha candidissima; Salvia balansae* de Noé.	(Chaachouay et al., 2023; El Mokni & Domina, 2024; Meddour et al., 2023; Royal Botanic Gardens, 2024)
Spain	471	7	*Thymus loscosii; Thymus moroderi; Teucrium roselloi; Micromeria rodriguezii; Teucrium thymifolium; Teucrium pumilum* Aggregate*; Sideritis leucantha* subsp. *Leucantha.*	(Crespo et al., 2018; Delgado et al., 2006; Ferrer-Gallego et al., 2015, 2022; Ferriol et al., 2006; López-Pujol et al., 2004; Marco-Medina & Casas, 2015; Royal Botanic Gardens, 2024; Senar et al., 2020)
Paraguay	81	3	*Cyanocephalus poliodes; Hyptis pachyarthra; Salvia aridicola.*	(Royal Botanic Gardens, 2024)

Portugal	113	3	*Thymus camphoratus; Teucrium salviastrum; Lamium coutinhoi; Thymus lotocephalus.*	(Coelho et al., 2012; Delgado et al., 2006; Royal Botanic Gardens, 2024)
Angola	192	15	*Fuerstia rara; Haumaniastrum cubanquense; Leucas bakeri; Orthosiphon cinereus; Plectranthus mechowianus; Stachys gossweileri.*	(Royal Botanic Gardens, 2024)
South Africa	297	42	*Thorncroftia lotterii; Tetradenia tuberosa; Killickia grandiflora; Plectranthus brevimentum; Plectranthus stylesii; Teucrium africanum; Teucrium kraussii; Teucrium trifidum; Salvia runcinata; Salvia chamelaegnea; Salvia radula; Salvia verbenaca.*	(Edwards, 2005, 2006; Kamatou et al., 2008; Kgaboesele et al., 2021; Rattray & Van Wyk, 2021; Royal Botanic Gardens, 2024; Ruiters et al., 2016)
Peru	190	21	*Salvia hunzikeri; Salvia vargas llosae; Salvia speciosa;*	(Alva & Rodríguez, 2012; Fragoso-Martínez et al., 2021; Paucar, 2010; Rodriguez, 2006; Royal Botanic Gardens, 2024)
China	274	12	*Wenchengia alternifolia; Pogostemon dielsianus; Isodon attenuatus; Isodon gongshanensis; Scutellaria tsinyunensis; Elsholtzia zhongyangi; Paraphlomis yingdeensis; Eriophyton sunhangii; Lamiophlomis rotata.*	(Chen et al., 2017, 2024; G. X. Guo et al., 2023; Hu et al., 2021; X. J. Jin et al., 2022; Li et al., 2014; J. Liu et al., 2006; X. Liu et al., 2020; Royal Botanic Gardens, 2024; Wu et al., 2007; Xu et al., 2009)
Czech Republic	144	112	*Salvia pratensis; Salvia verticillata; Betonica officinalis.*	(DuŠeka et al., 2010; Kaplan, 2012; Royal Botanic Gardens, 2024)
Bulgaria	169	6	*Sideritis scardica; Satureja pilosa; Satureja kitaibelii; Thymus aznavourii; Thymus perinicus; Thymus jalasaianus.*	(Aneva & Zhelev, 2018b; Ivanova et al., 2022; Kancheva et al., 2025; Royal Botanic Gardens, 2024; Stoyanov & Marinov, 2021; Zheljazkov et al., 2022)
Global	7,886	245	*Salvia, Scutellaria, Lamium, Dracocephalum*	(Abdelhalim & Hanrahan, 2021)

The Lamiaceae family, renowned for its aromatic and medicinal plants, displays remarkable taxonomic diversity in Algeria, as illustrated in the provided image (Figure 11). A total of 257 species are distributed across 34 genera, reflecting both the ecological heterogeneity of the region and the evolutionary adaptability of this botanical family. The chart highlights a notable imbalance in the distribution of species per genus, with a small number of genera comprising a disproportionately large number of species (APD, 2022; GBIF, 2025; WFO, 2025).

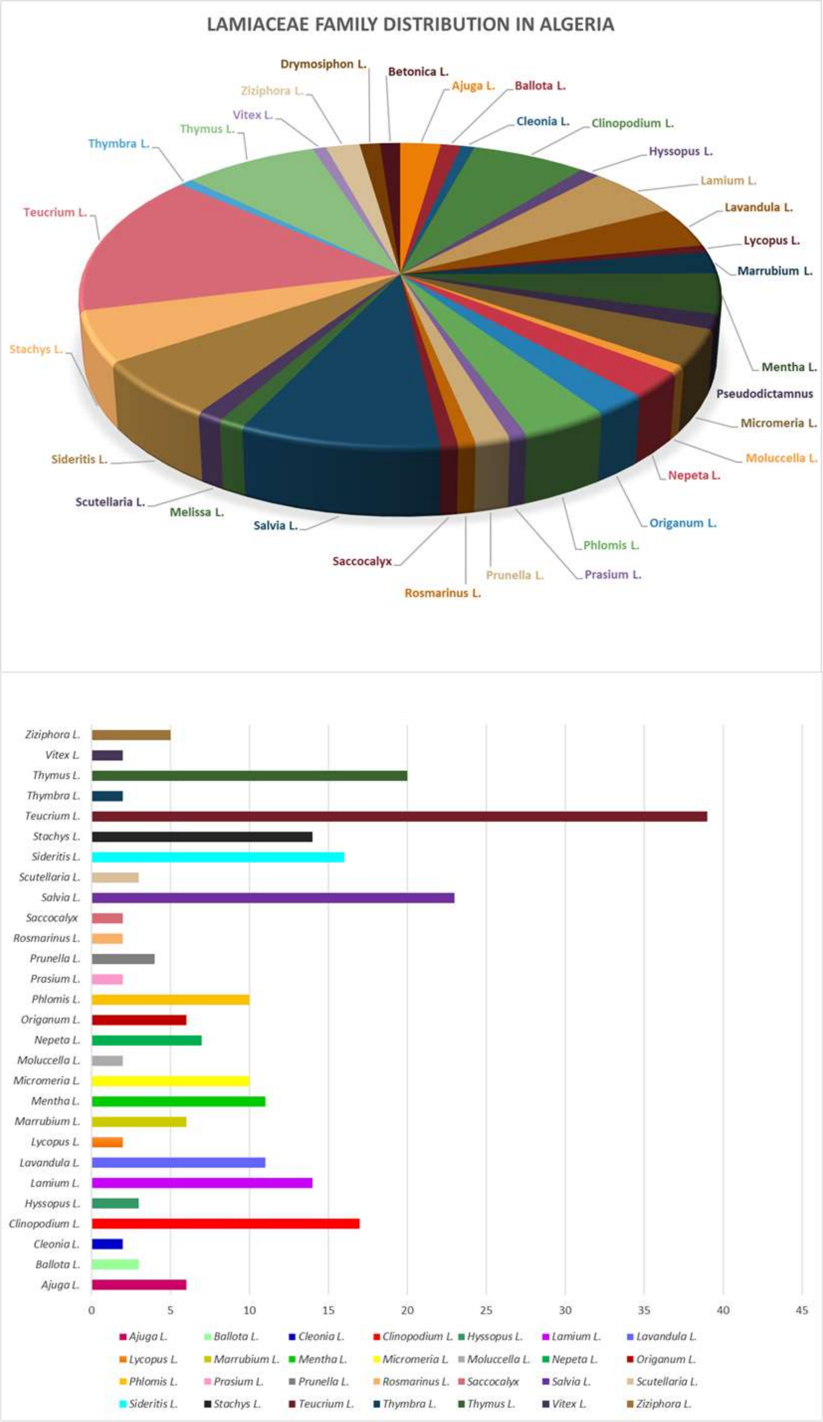
At the forefront is the genus *Teucrium*, which dominates the Algerian Lamiaceae flora with 39 recorded species, underscoring its ecological success and taxonomic complexity (Figure 11). This is followed by *Salvia* (23 species) and *Thymus* (20 species), two genera widely recognised for their ethnobotanical significance and rich essential oil profiles (Figure 9). Together, these three genera account for nearly one-third of the Lamiaceae species diversity in the country (Figure 11).

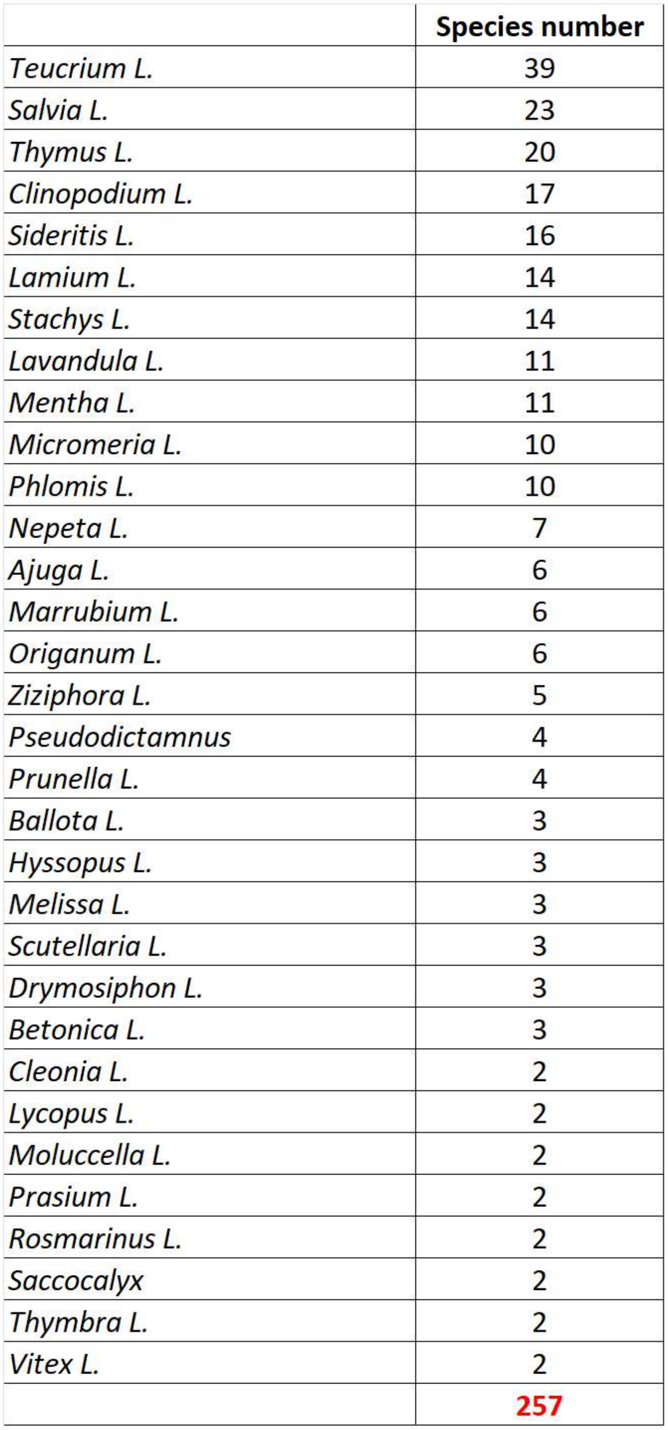
In contrast, a considerable number of genera, including *Vitex*, *Thymbra*, *Rosmarinus*, and *Saccocalyx,* are represented by only two species each, illustrating a “long-tail” distribution where numerous minor taxa contribute modestly to the overall richness (Figure 11). Despite their low species count, these genera often possess significant medicinal or ecological value, particularly in traditional medicine and habitat-specific interactions. The mint family is woven into Algeria's every bioclimatic belt, from the humid Mediterranean littoral to the wind-scoured steppes and the Saharan fringe (Mehalaine, 2023). Along the coast, mild, rainy winters and scorching, desiccating summers favour drought-hardy, aromatic shrubs such as *Salvia Rosmarinus* (rosemary), *Origanum glandulosum* and the North-African endemic *Thymus algeriensis* (Houmani et al., 2024; Jené et al., 2024; Lahlou et al., 2022; Mechergui et al., 2016; Miara et al., 2019; Ouelbani et al., 2016; Zouaoui et al., 2020). Their compact cushions cling to rocky slopes and degraded garrigue, and their small, gland-dotted leaves concentrate essential oils so markedly that coastal floristic surveys consistently rank Lamiaceae as the most abundant medicinal family: they make up 14.3 % of remedy plants in the Dahra Mountains of Chlef and about 12 % in Relizane (H. H. Ali et al., 2023; Senouci et al., 2019).

Moving inland, the Kabylie, Aurès and Djurdjura massifs provide cooler, mist-laden niches where thyme meets *Lavandula stoechas* (Spanish lavender) and wild *Mentha* species; here, slightly deeper soils and heavier dew allow denser foliage (Belhadj et al., 2023; Benkhettou et al., 2022; Boukerker et al., 2021; Meddour & Sahar, 2021). Further south, rainfall collapses across the Hauts Plateaux and the pre-Saharan steppe (Bezzih et al., 2021; Daoud & Kadik, 2024; Y. F. Guo et al., 2021). In these fiercely continental zones, hardy genera such as *Satureja* (savoury) and xerophilous lavenders deploy thick cuticles, woolly trichomes and cushion-like growth to endure aridity and brutal thermal swings (Jacobsen et al., 2008; Peguero-Pina et al., 2020).

Coastal populations add another layer of interest. *Thymus algeriensis*, prized in folk medicine for antimicrobial, antioxidant and anti-inflammatory properties, now faces mounting harvest pressure (Ben Elhadj Ali et al., 2024a). *Lavandula stoechas*, long distilled for perfume and pharmaceutical uses, also tolerates moderate salinity, an asset for revegetating degraded littoral soils (Dadach et al., 2021). Even small shrubs such as *Teucrium marum* exhibit striking chemotype shifts from shoreline sands to inland limestone, illustrating the family's capacity to fine-tune its secondary metabolites to local microclimates (Gobbo-Neto & Lopes, 2007; Kremer et al., 2015; Maccioni et al., 2023). Essential oils from Algerian thymes and lavenders are chemically polymorphic, a variability that underwrites their therapeutic breadth and the distinctive flavours of regional cuisine (Chograni et al., 2021; Thompson et al., 2003).

Across every zone, rural households weave these plants into daily life: rosemary and thyme lend depth to broths and couscous (Stefanaki & van Andel, 2021)Sweet mint tea remains a national emblem of hospitality and digestive comfort (Brahmi et al., 2023a). Wild gathering and backyard plots perpetuate a centuries-old cycle of selection that spreads particularly fragrant or potent landraces through seed exchange and local markets (Ali-Shtayeh et al., 2019). However, popularity brings peril. Over-collection of rosemary, *Thymus algeriensis* and other slow-growing shrubs already threatens some wild stands, while habitat fragmentation erodes genetic diversity in narrowly distributed coastal species (Ben El Hadj Ali et al., 2012).





	Species number
Teucrium L.	39
Salvia L.	23
Thymus L.	20
Clinopodium L.	17
Sideritis L.	16
Lamium L.	14
Stachys L.	14
Lavandula L.	11
Mentha L.	11
Micromeria L.	10
Phlomis L.	10
Nepeta L.	7
Ajuga L.	6
Marrubium L.	6
Origanum L.	6
Ziziphora L.	5
Pseudodictamnus	4
Prunella L.	4
Ballota L.	3
Hyssopus L.	3
Melissa L.	3
Scutellaria L.	3
Drymosiphon L.	3
Betonica L.	3
Cleonia L.	2
Lycopus L.	2
Moluccella L.	2
Prasium L.	2
Rosmarinus L.	2
Saccocalyx	2
Thymbra L.	2
Vitex L.	2
	257

Figure 11. Distribution of the Lamiaceae family in Algeria (APD, 2022; GBIF, 2025; WFO, 2025).

Safeguarding Algeria's lamiaceous heritage, therefore, demands more than admiration. Systematic surveys, fine-scale phylogeography and community-based harvest guidelines are needed to map genetic reserves, manage extraction and encourage small-scale cultivation (Dincheva et al., 2023; Gamoun & Louhaichi, 2024). Only by coupling traditional know-how with modern conservation can the country protect the ecological resilience, cultural identity and economic value embedded in its diverse Lamiaceae flora (Ben Elhadj Ali et al., 2024b).

2. The genus *Mentha* L.

The genus *Mentha* L. (Lamiaceae: Nepetoideae) is a widely distributed and taxonomically complex group of aromatic, perennial herbs, known for its extensive hybridisation, frequent polyploidy, and a complex evolutionary history. Taxonomic treatments currently recognise between 18 and 30 core species and over 100 cultivated hybrids and varieties. However, frequent gene flow at the population level and historical hybrid swarms complicate the definition of species boundaries (Yousefian et al., 2023). Here is the taxonomic classification:

Rank	**Taxon**
Kingdom	Plantae
Clades	Tracheophytes → Angiospermes → Eudicots → Astérids
Order	Lamiales
Family	Lamiaceae
Sub-family	*Nepetoideae*
Tribe	*Mentheae*
Genus	*Mentha* L.

Cytogenetically, the genus is characterised by a basic chromosome number of $x = 12$, with diploid ($2n = 24$), triploid ($2n = 36$), and tetraploid ($2n = 48$) cytotypes well documented. Among them, the allopolyploid peppermint (*Mentha* × *piperita*), a hybrid between *M. aquatica* and *M. spicata,* is widely studied, with reported chromosome counts of up to $2n = 96$ (Vining et al., 2017). Genome size variation has been correlated with ploidy level and essential oil yield, which are critical factors in breeding programs (Firl et al., 2024).

Molecular phylogenetic analyses based on chloroplast markers such as rbcL and trnL-F, nuclear ribosomal ITS regions, and genome sequencing have identified five major clades within the genus: *Menth*a sect. *Mentha, Pulegium*, Eriodontes, Audibertia, and Preslia; however, frequent introgressive hybridisation often results in complex patterns of shared haplotypes and non-monophyletic taxa (Yousefian et al., 2023). The genus encompasses 25 recognised species, including *M. alaica, M. aquatica, M. arvensis, M. atrolilacina, M. australis, M. canadensis, M. × carinthiaca, M. cervine, M. choujunensis, M. cunninghamii, M. dahurica, M. × dalmatica, M. darvasica, M. diemenica, M. × dumetorum, M. gattefossei, M. × gayeri, M. × gracilis, M. grandiflora, M. japonica, M. × kuemmerlei, M. laxiflora, M. × locyana, M. longifolia, M. micrantha, M. pamiroalaica, M. × piperita, M. pulegium, M. × pyramidalis, M. requienii, M. × rotundifolia, M. royleana, M. satureioides, M. spicata, M. suaveolens, M. × suavis, M. × verticillate, M. × villosa, M. × villosa-nervata, M. × wirtgeniana.* (POWO, 2024).

Several species of the genus *Mentha* (family Lamiaceae) share distinctive morphology, square stems, and zygomorphic flowers, and, although their most significant diversity lies in Western Europe, they are now distributed worldwide, often as naturalised or cultivated plants (Mogosan et al., 2017; Šarić-Kundalić et al., 2009a).

Historically, Mentha species have been cultivated and used since ancient times. Archaeological and textual records from Egypt, Greece, and Rome report the use of mint species for their aromatic, digestive, and antiseptic properties (Oroian et al., 2017). These traditional uses continue today, with the genus playing a prominent role in modern culinary, cosmetic, and pharmaceutical industries. Recent advances in plant genomics and transcriptomics have enabled the generation of chromosome-scale genome assemblies, such as those of *Mentha suaveolens* (apple mint). These genomic resources have enabled the identification of genes involved in monoterpene biosynthesis and opened the path toward marker-assisted selection of chemotypes with elevated menthol content and improved abiotic stress tolerance (Firl et al., 2024). Furthermore, environmental conditions such as light, soil, and altitude have been shown to influence essential oil profiles, thus affecting the expression of terpene synthase genes (Kokkini et al., 1995). The integration of classical taxonomy, cytogenetics, molecular biology, and genomics continues to enhance our understanding of Mentha biodiversity and its vast economic potential.

Mentha species share classic Lamiaceae characters, including square (four-angled) stems, opposite decussate leaves, and an indumentum of glandular peltate trichomes (40–60 μm diameter) and non-glandular multicellular hairs (J. Jin et al., 2014). Leaf blades range 2–8 cm, from broadly ovate in *M. suaveolens* to narrowly lanceolate in *M. longifolia*, with margins serrate to crenate. Stomatal density spans 100–300 mm^2, predominantly anomocytic but with paracytic and diacytic types in xeric-adapted taxa based on field micromorphological surveys of *M. spicata* at different altitudes (Kokkini, 2011). Inflorescences form verticillasters of 6–12 zygomorphic, bilabiate flowers (4–6 mm long) in shades of white, pink, or purple; nutlets (1–2 mm) bear species-specific surface sculpturing, which is reticulate, foveolate, or tuberculate, and is helpful in SEM-based identification (Krawczyk & Głowacka, 2015). Native to the Mediterranean Basin and temperate Eurasia, *Mentha* has naturalised globally, including *M. arvensis* in Canada and *M. spicata* in South America (Pyšek & Richardson, 2006). Ecological niche models project poleward range expansions of approximately 200–500 km for spearmint and peppermint under RCP 8.5 by 2050, driven by warming and altered precipitation, with measurable impacts on essential oil yield (Elith et al., 2011).

Commercial cultivation of spearmint (*M. spicata*), peppermint *(M. × piperita*), and corn mint (*M. arvensis*) yields over 23,000 t/year of essential oil, generating more than USD 400 million annually (Fejér et al., 2017). Agronomically, these perennials spread via stolons and are harvested just before flowering to maximise oil content (0.5–2 %) (Fejér et al., 2017; Santos et al., 2012; Upadhyay et al., 2014). Steam distillation produces oils rich in oxygenated monoterpenes (menthol, menthone, carvone, pulegone, 1,8-cineole) and terpene hydrocarbons (Al-Marzouqi et al., 2007; Tsai et al., 2013).

Species belonging to the genus *Mentha* are characterised by a remarkable biochemical richness, comprising a wide array of compounds such as cinnamic and organic acids, flavonoid aglycones and glycosides, sterols, steroidal glycosides, alkaloids, lignans, hydrocarbons, fatty acids, tocopherols, proteins, and free sugars (M. Ali et al., 2002; Al-Sabahi et al., 2016; Miguel et al., 2016; Triantaphyllou et al., 2001). Among these, flavonoids are particularly abundant, with luteolin and its derivatives, eriocitrin, naringenin-7-O-glucoside, narirutin, diosmin, isorhoifolin, eriodictyol, and apigenin identified as major constituents.

Water extracts of *Mentha* species are particularly rich in phenolic acids, including caffeic, chlorogenic, rosmarinic, and salvianolic acids. Fatty acids, including linoleic, linolenic, and palmitic acid, have also been reported.

The essential oils of *Mentha* are widely recognised as the principal bioactive fraction, with monoterpenes such as linalool, pulegone, menthone, carvone, menthol, and piperitenone constituting the dominant components (Brahmi et al., 2017). These volatile oils exhibit significant variations between species due to differences in metabolic pathways, particularly in the biosynthesis of monoterpene compounds. In peppermint (*Mentha × piperita*), for instance, this biosynthetic route is well defined, and the rate of monoterpene accumulation is governed by developmentally regulated enzymatic activity (McConkey et al., 2000; Ringer et al., 2005).

A chemotaxonomic analysis of 60 Mentha accessions from Tunisia encompassing seven species (*M. aquatica*, *M. longifolia*, *M. piperita*, *M. pulegium*, *M. rotundifolia*, *M. spicata*, and *M. spicata* var. *crispa* "moroccan") revealed substantial biochemical variability. Statistical clustering identified six major chemotypes based on terpenoid composition: (i) pulegone/isomenthone/menthone; (ii) isomenthone/pulegone; (iii) menthone/pulegone; (iv) piperitenone oxide; (v) linalool/linalyl acetate/1,8-cineol/myrcene; and (vi) carvone/limonene/1,8-cineol. Notably, *M. pulegium* formed two chemotypes, one rich in pulegone and the other in isomenthone, while *M. longifolia* and *M. spicata* also exhibited distinct chemotypic patterns. *M. rotundifolia* was rich in piperitenone oxide, whereas *M. aquatica* and *M. piperita* were dominated by linalool and linalyl acetate. These findings highlight the considerable biochemical diversity within Tunisian Mentha germplasm (Soilhi et al., 2019).

Pharmacologically, *Mentha* spp. Exhibits broad activities, antispasmodic, analgesic, antiemetic, anti-inflammatory, anticancer, antidiabetic, anti-obesity, and immunomodulatory, attributable to both essential oils and polyphenols (luteolin, rosmarinic acid, catechins) and flavonoids (Eftekhari et al., 2021). Traditionally, infusions relieve gastrointestinal (flatulence, spasm, dyspepsia) and respiratory complaints and serve as mild diuretics (Mamadalieva et al., 2020).

Industrially, their essential oils are used in food and beverage products (confectionery, drinks, baked goods), perfumery, cosmetics, oral hygiene, and as biopesticides, leveraging their antimicrobial and insect-repellent properties (Almeida et al., 2018; Anwar et al., 2019; Eftekhari et al., 2021; Mamadalieva et al., 2020). Finally, the ease of hybridisation, genus-wide polymorphism, and chemical variability driven by climate and terroir make cultivar selection both complex and promising for optimising future aromatic and medicinal profiles (Šarić-Kundalić et al., 2009a).

2.1. *Mentha aquatica*

2.1.1. Morphological description and geographic distribution of *Mentha aquatica*

Water mint (*Mentha aquatica*) (Figure 12) is a remarkably plastic species whose form and function shift in response to landscape and lineage. Field surveys in the Hyrcanian hotspot of northern Iran show that traits such as stem and collar diameter, the number and size of inflorescences, and calyx length and diameter vary widely among ecotypes; a principal-component analysis of fifteen Iranian populations revealed that those characters together account for more than 90 % of the total morphological variation (Schanzer et al., 2012). In Europe, detailed population studies in Bosnia-Herzegovina and Slovakia confirmed that finer traits, including the dentation of the leaf margin, overall hair density and, above all, the architecture of capitate glandular hairs, provide the clearest diagnostic signals where gross morphology overlaps (Šarić-Kundalić et al., 2009b) working with sixty Tunisian accessions showed that only the integration of such descriptors with molecular markers yields a robust picture of intraspecific diversity and taxonomic limits (Soilhi et al., 2020).

Figure 12. Water Mint (*Mentha aquatica*) in bloom: morphology and ecosystem (INaturalist, 2025; Lahlou et al., 2024).

The species' range is as fluid as its phenotype. Besides its Iranian stronghold, *M. aquatica* is well documented along river systems in Bosnia-Herzegovina and Slovakia (Šarić-Kundalić et al., 2009a), occupies wet habitats from the Maghreb to inland oases in Tunisia (Soilhi et al., 2020), and forms extensive riverside clones in Abkhazia, western Ukraine and southern Russia, where individual ramets may be metres apart yet belong to the same genet (Schanzer et al., 2012). Wherever it appears, the plant gravitates to the shallow margins of streams, ditches, canals, wet meadows and marshes, tolerating substrates that range from mildly acidic peat to calcareous marl (Avelar et al., 2015). That aquatic affinity makes water mint a practical ecological engineer: in horizontal-flow constructed wetlands treating domestic wastewater, stands of *M. aquatica* maintain vigorous growth under four-fold differences in organic loading, while their leaves accumulate nitrogen and potassium in proportion to the load applied—evidence of high productivity and nutrient-capture capacity under permanent water-logging (Avelar et al., 2015). Along eastern European rivers, the species often propagates clonally. Nevertheless, where flowering partners are available, it hybridises readily with *M. arvensis*, a process confirmed by ISSR marker analyses that detect first-generation hybrids and back-crosses in natural stands (Asadollah-Pour et al., 2021; Šarić-Kundalić et al., 2009b); such episodic sexual exchange injects fresh genetic variation into otherwise uniform clonal mosaics, underscoring why both morphological and molecular lenses are indispensable for tracing the whole tapestry of diversity in this adaptable mint (Schanzer et al., 2012).

2.1.2. Phytochemistry

Mentha aquatica L. stands out among aromatic and medicinal plants for its distinctive chemical profile, comprising volatile oils, phenolic compounds, terpenoids, and a wide range of secondary metabolites with therapeutic potential (Table 7).

Its essential oil, a highly volatile and complex mixture of mainly mono- and sesquiterpenes, plays a central role in its pharmacological profile. The oil composition of *M. aquatica* is known to vary considerably depending on environmental conditions, plant part, developmental stage, and geographic origin. Altitude, temperature, sunlight, soil type, and biotic interactions can significantly influence terpene biosynthesis and metabolic fluxes (Agostini et al., 2009; Deschamps et al., 2014; Ngoc Dai et al., 2015).

In Iran, Morteza-Semnani et al. (2006) identified 29 constituents accounting for 99.3% of the essential oil, dominated by 1,8-cineole (27.2%), menthofuran (23.2%), β-caryophyllene (12.8%), and limonene (5.2%) (Morteza-Semnani et al., 2006). In Ethiopia, menthofuran was the principal component (70.5%), followed by limonene (9.2%) and p-menthone (7.2%) (Getahun et al., 2008). Similarly, wild-growing *M. aquatica* in Corsica produced oil rich in menthofuran (50.7%), with smaller amounts of 1,8-cineole (5.9%), limonene (5.3%), and borneol (3.5%) (Sutour et al., 2011). However, a Brazilian ecotype showed a distinct profile where menthone was dominant (77.76%), followed by pulegone (14.39%), linalool (2.01%), and 1,8-cineole (1.49%) (Agostini et al., 2009), while in another study, carvone (62.34%), limonene (19.5%) and 1,8-cineole (3.9%) were the main constituents (de Oliveira Braga et al., 2022). Other variants, such as those reported by Chaker et al. (2014), showed linalyl acetate (26.1%) and α-pinene (22.7%) as major constituents (Chaker et al., 2014). Significant differences in chemical variability were also observed in the major components of each Indian collection across different seasons (Table 7). Linalool represents the dominating compound in every six collections from April to September comprising a total of 30.1 %, 25.2 %, 33.5 %, 48.4 %, 31.1 % and 45.7 % respectively of total oil composition while linalool acetate represents the dominating compound in following three collections from October to December comprising a total of 46.98 %, 42.1 % and 48.0 % respectively of total oil compositions (Singh et al., 2020).

Oil composition also shifts between plant parts (Table 7). In Iranian specimens, β-caryophyllene (22.4%), viridiflorol (11.3%), and 1,8-cineole (10.9%) were dominant in stem extracts, whereas leaf oil was rich in piperitenone oxide (25.7%), β-caryophyllene (12.0%), and 1,8-cineole (10.3%) (Esmaeili et al., 2006). Growth stage influences composition too: in Turkish plants, menthofuran (51.26–58.59%), limonene (5.94–12.06%), trans-β-ocimene (5.59–6.10%), ledol (3.01–4.06%), and β-caryophyllene (2.92–3.56%) were found to fluctuate across phenological stages (Anca-Raluca et al., 2013).

Beyond its volatile components, *M. aquatica* is a remarkable source of phenolic compounds. These natural antioxidants exhibit broad structural variability and exist in various forms, including monomeric, polymeric, glycosylated, and aglycone forms. They are not evenly distributed within the plant, and external conditions can influence their stability (Alara et al., 2021). In the genus *Mentha*, phenolics include around 53% flavonoids and 42% phenolic acids (Riachi & De Maria, 2015). In *M. aquatica*, the most significant compounds are rosmarinic acid, caffeic acid, ferulic acid, chlorogenic acid, and several flavonoids like eriocitrin, eriodictyol-7-O-rutinoside, luteolin-7-O-glucoside, and naringenin (Brahmi et al., 2017; Pereirao et al., 2012). Pereira et al. (2012) quantified eriodictyol-7-O-rutinoside at 50% of total phenolics, hesperetin-7-O-rutinoside at 15%, and naringenin-7-O-rutinoside at 8%. In another study, rosmarinic acid and eriodictyol-O-rutinoside were quantified at 68 ± 7 µg/mg and 145 ± 2 µg/mg, respectively, in Portuguese extracts (Pereira et al., 2019).

In addition to phenolics, *M. aquatica* synthesises several triterpenoids, including rare molecules like maquatic acid and 3-O-benzoyltormentic acid, along with pentacyclic triterpenes such as ursolic, oleanolic, maslinic, and tormentic acids (de Oliveira Braga et al., 2022). Other identified compounds include coumarins (e.g., umbelliferone), alkaloids, saponins, tannins, tocopherols, β-sitosterol, and polyunsaturated fatty acids like linoleic acid (Dhifi et al., 2011).

Table 7. Phytochemical profile of *Mentha aquatica*: compound classes, key constituents, and chemotypes.

Chemical group	Representative compounds	Notes/chemotypes/plant part	References
Monoterpene hydrocarbons	limonene; α-pinene; β-pinene; sabinene; myrcene; p-cymene; trans-β-ocimene; γ-terpinene; terpinolene; camphene	Core hydrocarbon backbone in many chemotypes; ocimene/limonene varies with phenological stage.	(Benabdallah et al., 2018; Bozin et al., 2006; Sutour et al., 2011)
Oxygenated monoterpenes	menthofuran; 1,8-cineole (eucalyptol); menthone; isomenthone; linalool; linalyl acetate; α-terpineol; terpinen-4-ol; borneol; camphor; piperitenone oxide; piperitone; piperitone oxide; carvone; geranyl acetate; neryl acetate	Major drivers of chemotypes. Examples: menthofuran-rich (Ethiopia, Corsica); menthone-rich (Brazil); rare linalool/linalyl-acetate chemotype; leaves in Iran rich in piperitenone oxide.	(Agostini et al., 2009; Esmaeili et al., 2006; Ferrati et al., 2023; Getahun et al., 2008; Sutour et al., 2011)
Sesquiterpene hydrocarbons	β-caryophyllene; germacrene D; α-humulene; δ-cadinene	β-caryophyllene is frequently abundant, especially in stems (Iran). Other sesquiterpenes vary with origin.	(Esmaeili et al., 2006; Hassanpouraghdam et al., 2022; Ngoc Dai et al., 2015)
Oxygenated sesquiterpenes	viridiflorol; caryophyllene oxide; spathulenol; ledol	Viridiflorol is notable in stems; ledol fluctuates with phenology/site.	(Esmaeili et al., 2006)
Phenolic acids	rosmarinic acid; caffeic acid; chlorogenic acid; ferulic acid; p-coumaric acid	Dominant non-volatile phenolics in extracts; rosmarinic acid is the major.	(Pereira et al., 2019; Zeljković et al., 2021)
Flavanones	eriocitrin (*eriodictyol-7-O-rutinoside*); hesperetin-7-O-rutinoside; narirutin (*naringenin-7-O-rutinoside*); naringin; eriodictyol	In MA, eriocitrin can dominate total phenolics, quantified in Portuguese extracts.	(Lahlou et al., 2024; Pereira et al., 2019)
Flavones & flavonols	luteolin-7-O-glucoside; apigenin-7-O-glucoside; rutin (quercetin-3-O-rutinoside); quercetin glycosides	Recurrent in MA aqueous/hydroethanolic extracts (HPLC-MS/MS).	(Lahlou et al., 2024; Pereira et al., 2019; Zeljković et al., 2021)
Phenylethanoid glycosides	verbascoside (acteoside) and related PhGs	Reported/quantified in MA hydroethanolic extracts; contribute to antioxidant activity.	(Lahlou et al., 2024)
Triterpenoids (non-volatile)	maquatic acid (new); 3-O-benzoyltormentic acid (new); tormentic acid; 1-O-benzoylhyptadienic acid; 3-epi-ursolic acid; hyptadienic acid; 3-epi-maslinic acid; 3-epi-tormentic acid; ursolic acid; oleanolic acid;	Isolated from aerial/underground parts; several first reports of *Mentha* are recorded in this study.	(Ferhat, Kabouchea, et al., 2017; Lahlou et al., 2024)

	pomolic acid; micromeric acid; 21α-hydroxyursolic acid		
Sterols, tocopherols & amyrins (lipophilic)	β-sitosterol; α/γ-tocopherol; α/β-amyrin; policosanols (e.g., triacontanol)	Cross-species metabolomics (9 *Mentha* spp.): Water mint exhibited the highest total policosanol content, with sterols/tocopherols/amyrins also quantified.	(Tavaszi-Sárosi et al., 2025)
Fatty acids (non-volatile)	palmitic, linoleic, linolenic (± oleic)	Reported among MA lipophilic constituents and genus-level lipid panels.	(Lahlou et al., 2024; Pires et al., 2024)
Other classes (reported in MA extracts)	tannins; saponins; alkaloids; coumarins (e.g., umbelliferone)	Qualitative phytochemical screenings of MA leaf extracts report these classes (method-dependent; follow-up isolation recommended).	(Lahlou et al., 2024; Ngoc Dai et al., 2015; Pires et al., 2024)

2.1.3. Uses in traditional medicine and reported activities of *Mentha aquatica*

Mentha aquatica L., commonly known as water mint, is widely recognised in traditional medicine across various cultures due to its diverse therapeutic properties. Historically, it has been used to address gastrointestinal ailments, including constipation, diarrhoea, stomach aches, indigestion, and biliousness (Pires et al., 2024). It has also been employed in the treatment of respiratory conditions such as the common cold, bronchitis, and coughs, often administered in the form of herbal infusions or inhalations (Asadollah-Pour et al., 2021; Dhifi et al., 2011). Moreover, in ethnomedical practices, particularly in regions of North and Southern Africa, the plant has been recommended for managing liver dysfunctions and regulating blood pressure (Belhacini et al., 2024; Brahmi et al., 2023b; Cock et al., 2024; El Hassani, 2020a, 2020b; Lahyaoui et al., 2025). Beyond these applications, *M. aquatica* has traditionally served as a tonic, a mild sedative for anxiety and nervous exhaustion, and a vermifuge for expelling intestinal parasites in children. In the Zulu cultural context, it holds spiritual significance, used in rituals for purification and ancestral communication (Asadollah-Pour et al., 2021; de Oliveira Braga et al., 2022).

Pharmacological studies support these traditional claims by demonstrating a broad spectrum of bioactivities. *Mentha aquatica* extracts have shown potent antimicrobial activity against several Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*, suggesting its potential as a natural preservative or complementary antimicrobial agent (Alizadeh Amoli et al., 2019; Behbahani et al., 2022; Dhifi et al., 2011; Fancello et al., 2017; Ferhat, Erol, et al., 2017; Golestan et al., 2016; Hoai, Quoc, Raes, et al., 2023; Mimica-Dukić et al., 2003). The phytochemicals explain the plant's wide-ranging pharmacological activities. The essential oil has demonstrated potent antibacterial activity, particularly against *Staphylococcus aureus* and *Bacillus pumilus*, with MIC values below 5 µg/mL (Getahun et al., 2008). It also displays strong antioxidant properties, as shown in DPPH and deoxyribose degradation assays (IC_{50}: 11.2 and 3.74 µL/mL, respectively) (Getahun et al., 2008). *Mentha aquatica* food systems enhanced sensory qualities and microbial safety, preserving *Lactobacillus reuteri* and *Bifidobacterium animalis* while controlling pathogens like *Clostridium perfringens* (Golestan et al., 2016).

Additionally, its aqueous and ethanol extracts exhibit marked antioxidant activity, which is primarily attributed to high levels of phenolic compounds such as rosmarinic acid, eriocitrin, and luteolin derivatives (Conforti et al., 2008; Hoai, Quoc, Hoang, et al., 2023; Thi et al., 2020). The anti-inflammatory potential of the plant has been linked to its essential oils and phenolic acids, which inhibit key pro-inflammatory mediators, such as nitric oxide and COX enzymes (Dhifi et al., 2011). The oil suppressed skin carcinogenesis in experimental models by reducing keratin 14 and COX-2 expression (Chang et al., 2019). In a mouse model, *Mentha aquatica* has chemopreventive effects against PLX4032-induced cutaneous side effects. The EO and its major constituents, limonene and carvone, inhibit keratinocyte activity, induce cell-cycle arrest, and promote apoptosis. They also modulate the macrophage population, shifting from M2 to M1 phenotype, which is associated with anti-tumour activity.

The plant exhibits neurochemical effects by modulating the GABAergic system, binding to GABA-benzodiazepine receptors, which may underlie its traditional use as a calming agent or sedative (Jäger et al., 2007; López et al., 2010). Cytotoxicity assays have demonstrated that *M. aquatica* extracts possess anticancer properties, inducing apoptosis in various cancer cell lines, although further investigation is necessary to validate these findings *in vivo*. The ethanolic extract and its fractions from *M. aquatica* showed promising anti-proliferative effects against human tumour cell lines, including NCI-H460 and MCF-7 cells. Fraction F2 demonstrated potent and irreversible anti-proliferative effects without inducing cell cycle arrest (de Souza et al., 2022). Another study confirmed that the ethanolic extract of *M. aquatica* significantly reduced cell viability in HeLa and MCF-7 cells after 48 hours, indicating strong cytotoxic effects (Eftekhari et al., 2022). The extracts of *M. aquatica* were found to elevate reactive oxygen species (ROS) levels and decrease intracellular glutathione levels, indicating oxidative stress that can lead to apoptosis. The anti-proliferative effects were associated with apoptosis, as evidenced by the increase in ROS and the induction of apoptosis-related pathways (Eftekhari et al., 2022). Additionally, other studies have shown that *Mentha aquatica* extracts exhibit significant cytoprotective effects against oxidative stress-induced damage in human hepatocarcinoma cells. This suggests that the plant's extracts can protect liver cells from damage caused by ROS (Pereira et al., 2019).

The plant has also demonstrated significant larvicidal activity, especially against mosquito larvae (*Culex pipiens*), suggesting its utility in vector control (Asadollah-Pour et al., 2021). Environmentally, its capacity for phytoremediation, removing heavy metals like cadmium and lead, as well as faecal pathogens from wastewater, adds ecological value to its therapeutic role (Dahija et al., 2019, 2022).

The chemical profile of *Mentha aquatica* is dominated by terpenoids, including menthofuran, 1,8-cineole, trans-caryophyllene, and viridiflorol, which contribute to its aromatic and pharmacological properties. These compounds are relevant to the plant's medicinal properties and applications in the cosmetic industry, where they are increasingly incorporated into formulations for their soothing, refreshing, and deodorising effects. *Mentha aquatica*'s wide range of traditional uses and scientifically supported bioactivities affirms its importance as a multifunctional plant with therapeutic, environmental, and commercial potential (Pires et al., 2024).

2.2. The (former) genus *Rosmarinus* - now *Salvia* subg. *Rosmarinus*

2.2.1. Taxonomy and current nomenclature

Molecular phylogenies place the former genus *Rosmarinus* firmly inside *Salvia*. In 2017, Drew et al. proposed uniting *Rosmarinus* with *Salvia*; this is now widely adopted in authoritative databases and floras. Accordingly, culinary rosemary is *Salvia rosmarinus* (≡ *Rosmarinus officinalis*), while the other two Mediterranean "rosemaries" are *Salvia jordanii* (≡ *R. eriocalyx*) and *Salvia granatensis* (≡ *R. tomentosus*). Hybrid and infraspecific names have been recombined under *Salvia*, e.g., *S.* × *lavandulacea* (= *S. jordanii* × *S. rosmarinus*) and *S.* × *mendizabalii* (= *S. granatensis* × *S. rosmarinus*); *S. rosmarinus* subsp. *valentina* (≡ *R. officinalis* subsp. *valentinus*) is maintained for white-flowered, prostrate Valencian populations (Drew et al., 2017; IPNI, 2025).

2.2.2. Species-level diversity and natural hybrids

S. rosmarinus is widespread around the western Mediterranean; *S. jordanii* is native to Northwest Africa and southern Spain and differs in having smaller, narrower leaves and often a lower, creeping habit; *S. granatensis* is a south-eastern Iberian endemic (Drew et al., 2017; IPNI, 2025). Natural hybrid zones occur where ranges meet, producing *S.* × *lavandulacea* and *S.* × *mendizabalii*, both of which are formally recognised under *Salvia*. Subspecies *S. rosmarinus* subsp. *valentina* bears distinctive white corollas and prostrate architecture, with an HPLC-profiled phenolic signature (Figure 13).

2.2.3. Morphology, micromorphology, and phenology

Members of *Salvia* subg. *Rosmarinus* are evergreen, highly branched shrubs (often over 1 metre) with narrow, revolute leaves that bear abundant peltate and capitate glandular trichomes storing essential oils in a subcuticular space. X-ray CT and GC–MS imaging of *S. rosmarinus* trichomes reveal distinct reservoirs of volatiles (e.g., 1,8-cineole, camphor, α-pinene) at the gland apex (Figure 14). In the Mediterranean, flowering typically occurs from late winter to spring; cultivated hybrids can display a clear sequence of vegetative (Dec–Feb), pre-flowering (Aug–early Sep), full bloom (late Sep–Nov), and post-flowering phases, each associated with shifts in essential-oil (EO) yield and profile (Fiume et al., 2020; Gaspar et al., 2001).

The lineage is well-suited to dry, nutrient-poor Mediterranean environments, enduring seasonal drought and high light exposure; populations range from sea level to approximately 1600 m. This stress resistance is linked to high inherent and inducible production of polyphenols and volatile compounds, traits associated with photoprotection and defence against herbivores and pathogens-features that support successful low-input cultivation on marginal lands (Fiume et al., 2020).

Figure 13. Morphological features and flowering habit of *Rosmarinus officinalis* L. in its natural habitat

2.2.4. Phytochemistry

Rosemary leaves (*Salvia rosmarinus*) characteristically accumulate high levels of the phenolic diterpenes carnosic acid (CA) and carnosol (CAR), which contribute to the antioxidant capacity of lipophilic fractions. Across different genotypes, environments, and extraction methods, CA content in dried leaves generally ranges from approximately 0.1–7% w/w, with CAR typically present at lower concentrations; these broad ranges align with surveys of Lamiaceae diterpenes and process-optimised extractions (Pavić et al., 2019). For routine quality control, a validated HPLC-PDA assay measures CA and CAR directly in fresh herbs, achieving recoveries of approximately 85–105% and %RSD ≤4–5% (ICH-compliant), facilitating reliable standardisation and genotype screening. Matrix-matched techniques have also been developed for food products (oils, meats, dressings), supporting the traceability of these markers during processing and storage (Zhang et al., 2012). For example, a Tunisian study demonstrated that, with tuned ethanolic extraction, titers could reach approximately 101.0 mg CA/g DW and 10.1 mg rosmarinic acid (RA)·g/DW, consistent with a response-surface model predicting roughly 86.3 mg·g^{-1} CA and 8.37 mg/g RA at the optimal temperature–time conditions (Yeddes et al., 2022).

In the polar phenolic pool, RA (with caffeic acid derivatives) predominates and exhibits pronounced seasonality: a 12-month UHPLC series on wild rosemary measured RA levels from approximately 12.6 to 32.2 mg/g DW (winter maxima). In a broader comparison within the Lamiaceae, RA content in culinary herbs ranged from trace amounts to over 20–50 mg/g DW, depending on species and method (Bejenaru et al., 2024; Shekarchi et al., 2012).

Rosemary also contains flavonoids (luteolin/apigenin-type glycosides) and triterpenes (notably ursolic and oleanolic acids), which contribute additional antioxidant and anti-inflammatory properties, as documented in pharmacological and food models (Andrade et al., 2018; Borrás-Linares et al., 2014) .

The essential oil (EO) fraction consists of reproducible chemotypes, typically 1,8-cineole-dominant, camphor-dominant, or verbenone-dominant, often with α-pinene/borneol as co-majors, whose composition varies with genetics, location, and phenology. A global synthesis describes these patterns, and a two-year field study in Tunisia demonstrated that season, growth stage, and genetic pool collectively influence both yield and composition, with implications for antimicrobial and antioxidant activity. Typical EO yields by hydrodistillation or steam distillation are around 1–2.5% (DW) in well-grown material, although process optimisation can modify these values (Arfa et al., 2022; Conde-Hernández et al., 2017; Satyal et al., 2017).

At the tissue level, volatiles and many phenolics are synthesised and stored in glandular trichomes (peltate and capitate) on the leaf surface; stereological and histochemical studies in rosemary map these structures and their lipophilic secretions. (Boix et al., 2011; Marin et al., 2006) Online Biosynthetically, CA/CAR originate from the plastidial MEP diterpenoid pathway (abietane-type intermediates expressed in trichomes), while RA derives from phenylpropanoid and tyrosine branches, explaining the co-occurrence of potent lipophilic (CA/CAR) and hydrophilic (RA) antioxidants within the same organ. (Brückner et al., 2014; Guan et al., 2022) Environment can further influence composition and yield: for instance, a field comparison reported approximately 52.7% higher EO percentage in saline habitats compared to non-saline controls, with a shift towards camphor and a reduction in verbenone (Soliman et al., 2024).

Finally, for regulatory use in foods, rosemary extracts are authorised in the EU as E 392. EFSA’s refined exposure assessment (2018) aligns with JECFA’s ADI of 0–0.3 mg/kg bw/day, expressed as the sum of CA + CAR, and specifications cover solvent systems (e.g., ethanol, acetone, supercritical CO_2) and compositional criteria, benchmarks that enable effective use well below toxicological concern (Younes et al., 2018).

2.2.5. Biological activities and mechanisms

CA and CAR represent the primary contributors to rosemary extract's antioxidant activity in lipid systems (chain-breaking and ROS/RNS scavenging), while RA plays a role in aqueous phases. Importantly, carnosic acid acts as a Keap1/Nrf2 activator, inducing cytoprotective genes and reducing NF-κB/NLRP3-linked inflammation in both cellular and *in vivo* models. This effect is observed across neurological and inflammatory contexts (Lin et al., 2023; Satoh et al., 2007).

Rosemary EO shows bacteriostatic/bactericidal activity against both Gram-positive and Gram-negative species, but MICs vary with chemotype and test conditions. A 2018 synthesis reported broad activity across *E. coli, Bacillus, Staphylococcus, Clostridium*, and other species; targeted testing against *S. aureus* found MICs of ~0.7–11.25 mg/mL, with combinations sometimes outperforming single oils. Mechanistically, major monoterpenes (1,8-cineole, camphor, α-pinene) disrupt membranes, increase permeability, and perturb energy metabolism; at sub-MICs, they can dampen quorum sensing and enzyme targets (Nieto et al., 2018).

Additionally, purified carnosic acid acts as a specific inhibitor of the *S. aureus* Agr quorum-sensing system, decreasing agrA/rnaIII expression and reducing hemolytic and toxin production. It also degrades pre-formed MRSA biofilms (0.5–4× MIC) and inhibits early biofilm formation. These effects highlight the central role of Agr in *S. aureus* virulence and biofilm dispersal and demonstrate an adjuvant approach that limits pathogenicity without exerting strong bactericidal pressure (Boles & Horswill, 2008; Nakagawa et al., 2020).

Rosemary EO also inhibits several foodborne moulds, although its potency varies by species and formulation. Research on aflatoxigenic *Aspergillus flavus* shows antifungal and antiaflatoxigenic effects, while broader reviews highlight strong activity against *A. niger* but weaker effects on *A. flavus* and some *Penicillium* spp, emphasising the significance of chemotype and dosage. Mechanistic microscopy and transcriptomics studies with *Colletotrichum gloeosporioides* reveal EO-induced damage to cell walls and membranes, decreased ergosterol levels, leakage of nucleic acids and proteins, and inhibition of TCA/ATP metabolism (da Silva Bomfim et al., 2020; Mutlu-Ingok et al., 2020).

In meats, rosemary extracts and EOs slow lipid oxidation and microbial growth. Process-engineering studies show a synergy between about 1% rosemary EO and supercritical CO_2 on chicken meat, leading to greater *E. coli* reductions than CO_2 alone while maintaining acceptable colour. Further research expands this approach to shelf-life outcomes and other matrices, with micro- or nano-encapsulation (such as gum arabic combined with maltodextrin spray-drying) enhancing EO stability and controlled release in foods (Santi et al., 2023; Younes et al., 2018).

Multiple studies report AChE/BChE inhibition by rosemary EO/actives (e.g., 1,8-cineole, α-pinene, ursolic acid), with IC_{50} values typically in the tens of µg/mL for EO and in the tens of µM for purified phenolics *in vitro*, alongside small, hypothesis-generating human aromatherapy trials. These are not clinical endpoints, but they align mechanistically with the Nrf2 and POP (prolyl oligopeptidase) data and justify reporting EO composition and dose in any cognitive-adjacent claims (Sayorwan et al., 2012).

Because activity tracks chemotype, harvest stage, and delivery system, applied studies should report: (i) GC–MS top 5–10 constituents with sampling season/site; (ii) extract type and CA/CAR/RA quantitation; and (iii) formulation (emulsion, microcapsule, carrier ratios). This is already the norm in the latest analytical/QC papers and regulatory opinions (Paloukopoulou & Karioti, 2022).

2.3. The genus *Thymus*

Thymus (Lamiaceae) is an Old-World genus with its centre of diversity located in the Mediterranean Basin, extending across Europe, North Africa, and western–central Asia into steppe and montane habitats. Species richness decreases towards the Atlantic coast and the arid east. Plants are typically low, aromatic chamaephytes (evergreen subshrubs) with four-angled (quadrangular) stems, opposite, exstipulate leaves, and verticillaster inflorescences, a typical Lamiaceae architecture adapted to sunny, dry, often calcareous environments, such as garrigue, maquis, open pinewoods, and rocky slopes. Epidermal peltate and capitate glandular trichomes on leaves and bracts synthesise and store the essential oils that support culinary and medicinal uses; recent genome-scale studies link the development of these secretory trichomes to the regulation of monoterpenoid biosynthesis in thyme (Halat et al., 2022; Sun et al., 2022).

Taxonomically, modern syntheses following Jalas and Morales acknowledge approximately 200 species divided into eight sections (*Micantes*, *Mastichina*, *Piperella*, *Teucrioides*, *Pseudothymbra*, *Thymus*, *Hyphodromi*, and *Serpyllum*), with many regional endemics and frequent intraspecific polymorphism. This taxonomic complexity reflects both hybridisation and strong ecogeographic structuring across the Basin (Halat et al., 2022).

Reproductively, gynodioecy, the coexistence of female and hermaphrodite individuals within populations, is common in *Thymus* and is maintained by well-studied interactions between cytoplasmic male-sterility factors and nuclear restorers. Classic and contemporary studies in *T. vulgaris* and related species demonstrate widespread, habitat-linked variation in female frequency, showing how nucleocytoplasmic dynamics can stabilise gynodioecy in nature. Beyond its evolutionary interest, this sexual polymorphism can also be associated with chemotype structure (e.g., phenolic versus non-phenolic oil profiles) at the population level (Delph et al., 2007; DOMMÉE et al., 1978; Kosakowska et al., 2020).

Cytologically, the *Thymus* genus displays notable karyological diversity. While many cultivated and wild populations are diploid (2n ≈ 30 is common), tetraploid cytotypes are widespread, and induced or natural polyploidy can alter morphology and secondary metabolite production, traits of agronomic importance.

Flow cytometry and cytological surveys across various *Thymus* species report diploid and tetraploid levels (e.g., 2n = 30; 2n = 56–60). Experimental doubling of chromosome number in *T. vulgaris* affects trichome density, essential oil yield and composition, and plant stature (Etri & Pluhár, 2024).

2.4. *Thymus hirtus* Willd. ssp. *Algeriensis*

2.4.1. Morphological description and geographic distribution

Often treated as *Thymus hirtus* Willd. ssp. *Algeriensis* (Figure 14), this North African endemic occurs in sub-humid to lower arid zones across Tunisia, Algeria, Morocco, and Libya. Populations span ≈120–1,100 m on rocky, calcareous slopes; flowering is typically April–June. The species bears dense secretory trichomes on small, pubescent leaves, consistent with high aromaticity (Lahlou et al., 2022; Zouari et al., 2012).

Kingdom: Plantae
Clade: Tracheophyta
Clade: Euphyllophyta
Clade: Spermatophyta
Clade: Magnoliopsida
Clade: Mesangiospermae
Clade: eudicotyledons
Clade: Gunneridae
Clade: Pentapetalae
Clade: Asterids
Clade: Lamiids
Order: Lamiales
Family: *Lamiaceae*
Subfamily: *Nepetoideae*
Tribe: *Mentheae*
Genus: *Thymus* L.
Type species: *Thymus algeriensis* Boiss. & Reut.

Figure 14. Systematic classification and botanical aspects of *Thymus algeriensis* Boiss. and Reut flowers and leaves. (**a**,**b**) from Tunisia, (**c**) from Algeria, (**d**) Systematic classification of *T. algeriensis* (INaturalist, 2025; Lahlou et al., 2022).

2.4.2. Phytochemistry

Across North African populations, *T. algeriensis* exhibits a monoterpene-rich essential oil profile, characterised by notable chemotypic variation, which is primarily influenced by geography and, to a lesser extent, by phenology (Lahlou et al., 2022). Across North African populations, the essential oil (EO) is rich in monoterpenes but exhibits chemotypic diversity: surveys identify thymol-dominant and α-pinene/1,8-cineole/1,8-cineole-dominant profiles in Algeria (EO yield approximately 0.9–2.35% w/w; up to 61 volatiles identified), and in Tunisia, multiple chemotypes range from thymol, linalool, 1,8-cineole/α-pinene, bornyl/linalool to camphor clusters. Major volatiles commonly reported include α-pinene, 1,8-cineole, camphor, terpinen-4-ol, linalool, borneol, γ-terpinene, p-cymene, α-terpinyl acetate, carvacrol acetate, thymol, carvacrol, elemol, and viridiflorol. Phenology and location can influence the balance among these constituents (Etri & Pluhár, 2024; Lahlou et al., 2022; Zouari et al., 2012).

Non-volatile phenolics share a similar structure. Rosmarinic acid is the predominant phenolic across most populations, accompanied by caffeic acid, a caffeoyl-rosmarinic acid (m/z 537), salvianolic acid K (m/z 555), salvianolic acid E (m/z 717), and monomethyl-lithospermate (m/z 551). The flavonoid profile is extensive and well documented by LC-MS, including kaempferol-O-glucuronide (often the main flavonol), kaempferol-O-dihexoside, quercetin-3-O-glucoside (isoquercitrin), quercetin-3-O-glucuronide, and a quercetin-O-malonyl-glucoside. Flavones such as luteolin-7-O-glucoside, luteolin-7-O-glucuronide, luteolin-6-C-glucoside isomers, apigenin-7-

O-glucuronide, apigenin-8-C-glucoside (vitexin), and apigenin-6,8-di-C-glucoside (vicenin-2); as well as flavanones like eriodictyol-O-hexoside (isomers) and naringenin-O-hexoside are also present. A minor isoflavone signal (sissotrin = biochanin-A-7-O-glucoside) has been reported. Phenolic fingerprints at the population level correlate with antioxidant and anti-acetylcholinesterase activities; notably, extracts from upper arid sites contained carvacrol in the methanolic fraction (which was otherwise absent), indicating environment-linked phenolic–terpenoid interactions. Secretory peltate and capitate glands on leaves and bracts (confirmed by SEM) support these findings and their developmental variations (I. B. E. H. Ali et al., 2010; Benkiniouar et al., 2007; Guesmi et al., 2019; Jaouadi et al., 2019, 2023; Lahlou et al., 2022).

2.4.3. Uses in traditional medicine

Maghrebi ethnomedicine primarily utilises *T. algeriensis* in the form of infusions, decoctions, or powdered aerial parts, which are added to food and drinks. In Naâma (south-western Algeria), people use these preparations for colds, as anti-inflammatory agents, and for metabolic or gynaecological issues (e.g., hypercholesterolemia, menstrual cycle problems); during the COVID-19 pandemic, they were reportedly also consumed, reflecting local practice rather than proven effectiveness. In El-Kantara (the Sahara gate), leaves and flowers are infused "against abdominal stomach pain, wound infections, and food poisoning," and the plant is cited for antihypertensive and "heart disease" uses. In Morocco, it is traditionally used for diabetes and as a tonic or expectorant for coughs, fever, and wound infections; in Tunisia, it serves both as a culinary herb (including as a tea flavour) and as a popular remedy "protective" against digestive disorders and reproductive problems. These country-specific uses and preparations are documented (with primary sources) in a recent peer-reviewed review (Baba Aissa, 2011; Benkiniouar et al., 2007; El-Gharbaoui et al., 2017; Fakchich & Elachouri, 2021; Hachi et al., 2016; Kachmar et al., 2021; Karous et al., 2021; Lahlou et al., 2022; Mechaala et al., 2022). Quantitative ethnobotany from the Algerian highlands further shows *T. algeriensis* ranks among the most culturally important species (use value, UV = 0.82; 197 use-reports), with the strongest informant consensus for digestive (ICF ≈ 0.90) and respiratory (ICF ≈ 0.87) ailments, patterns that mirror its local indications for gastrointestinal discomfort and colds (Maghni et al., 2025).

2.4.4. Biological and pharmacological applications

Experimental pharmacology aligns with traditional claims. *In vivo*, the essential oil accelerated healing of HCl/ethanol-induced gastric ulcers in rats while improving mucosal redox status, supporting gastroprotective use. Co-formulation studies report dose-dependent anti-inflammatory, analgesic, and antioxidant effects for *T. algeriensis* oil (alone or in combination with *Artemisia herba-alba*), with toxicity screens indicating acceptable acute safety at the tested doses. Antimicrobial applications range from *in vitro* inhibitory activity against Gram-positive/Gram-negative bacteria and fungi to food-technology trials, where *T. algeriensis* oil prevented *Penicillium* contamination during storage and showed MIC/MBC values of 0.03–0.15 mg/mL against common foodborne bacteria, with low-range MICs for fungi. Additional studies have documented the ACE-inhibitory activity of the oil (IC_{50} ≈ 150 µg/mL), and population-level phenolic fingerprints correlate with anti-acetylcholinesterase activity, suggesting that the cardiometabolic and neurofunctional relevance of this compound warrants further exploration. Overall, chemotype-aware standardisation (botanical voucher + GC–MS/HPLC profiling) is essential because thymol/carvacrol-rich oils often show more potent bioactivity but also demand careful dose selection (Bukvicki et al., 2018; Guesmi et al., 2014; Jaouadi et al., 2019; Ouahdani et al., 2021; Zouari et al., 2011).

PRACTICAL PART

PART I.

Prevalence of Coccidiosis and assessment of biosecurity status in commercial chicken farms found in Boumerdes

1. Materials and methods

1.1.Overview of the study area

The study was conducted in the wilaya of Boumerdes, located in the north-central region of Algeria (Figure 15). It covers an area of 1,456.16 km² and has a coastal strip exceeding 80 km, extending from the Cape of Boudouaou Elbahri in the west to the eastern limit of the commune of Afir. This region has a population of 801,068 inhabitants, comprising nine daïras and 32 communes. The Mediterranean Sea borders Boumerdès to the north, Tizi Ouzou to the east, Bouira to the south, and Blida and Algiers to the west.

Boumerdès is one of Algeria's most active poultry hubs, with commercial registers listing more than 50 specialised poultry and egg production firms spread across the nine study communes. These semi-intensive units typically stock approximately 3,000 to 5,000 broilers per house and conduct 4 to 5 cycles per year, relying on corn- and soybean-based compound feed that constitutes about 80% of production costs (Alloui & Bennoune, 2013a). On a national scale, the sector produces approximately 340,000 tonnes of chicken meat and 4.8 billion eggs annually. Supporting roughly 500,000 jobs, the local industry highlights its economic significance. The industry underscores its economic significance (Alloui & Bennoune, 2013b).

The communes concerned in the study are Beni Amrane, Boudouaou, Cap djenat, El Kharrouba, Ouled Haddadj, Souk El Had, Thénia, Tidjelabine, and Zemouri (Figure 15).

Figure 15. Location of the nine study districts in Boumerdès Province (Wilaya), northern Algeria**.** (1) Cap djenat, (2) Zemmouri, (3) Thénia, (4) Souk El Had, (5) Beni Amrane, (6) Tidjelabine, (7) Boudouaou, (8)El Kahrrouba, (9) Ouled Haddadj.

1.2. Study design and questionnaire development

A cross-sectional population survey was conducted from December 2017 to April 2018 to assess the biosecurity measures adopted by commercial chicken farms in Boumerdes city. The survey was conducted on intensive breeding farms using the deep litter system in the Boumerdès region. The list of commercial chicken farms was obtained from the DSA. A questionnaire was sent to all registered owners of backyard flocks in the DSA-Boumerdès poultry register. There are two types of registered poultry farming: official (declared at the institutional level) and unofficial (undeclared). The farmers were informed of the purpose of this study. Verbal consent for participation was obtained from only 158 farms, while others refused to participate, and the reasons for refusal were not explored. The survey consisted of face-to-face interviews with farm owners, conducted using a structured questionnaire. The same person carried out the interview. In all the farms visited, the Chickens were stocked at day old and sold after 52 days of growth. All flocks were visited three times at 2 – 8 weeks of age. The questionnaire was designed based on findings from scientific literature and risk factors previously associated with coccidiosis in broiler chickens. The analysis of parameters related to housing, husbandry, and biosecurity practices was carried out by comparing the collected data with national standards for poultry production provided by the Technical Institute of Animal Farming (I.T.E.L.V.) and prior field studies conducted in Algeria (ITELV, 2025).

The questionnaire consisted of 75 items, including both binary (yes/no) and open-ended questions, and was administered through face-to-face interviews. Each broiler farm was visited individually by one of four trained investigators, who also collected visual observations to ensure accuracy. A sample of the questionnaire is provided in Supplementary Table A. The questions were grouped into four major thematic categories, each designed to evaluate a specific dimension of farm management and its potential impact on coccidiosis occurrence.

The first category assessed the physical and structural characteristics of the farm, including building layout, equipment, environmental conditions, and access to resources (Category A). These factors are essential for understanding the hygiene and bio-physical conditions in which birds are raised. Inadequate infrastructure or poorly maintained environments can contribute to the persistence and spread of pathogens, including *Eimeria* species.

The second category focused on the farm's level of isolation and control over external movements. This included evaluating spatial distancing, traffic control, and the potential for disease transmission from neighbouring farms, visitors, vehicles, or shared equipment (Category B). These factors are critical for preventing the introduction of infectious agents from external sources and for designing effective external biosecurity protocols.

The third category examined internal biosecurity practices and health management strategies. It explored how hygiene measures, veterinary oversight, pest control, and cleaning routines are implemented within the farm (Category C). This category also reflected the level of disease monitoring and emergency response capacity. These practices play a direct role in limiting the spread of coccidiosis within the flock and reducing its severity once an outbreak occurs.

The fourth and final category encompassed key production parameters, including feed efficiency, growth performance, and slaughter data (Category D). These indicators provide an indirect assessment of animal health and welfare. Suboptimal performance may reflect the impact of disease pressure, including subclinical or clinical coccidiosis, and help identify management weaknesses that affect productivity.

Together, these four dimensions offer a comprehensive framework for evaluating the biosecurity status of broiler farms and for identifying the most critical leverage points to reduce the risk of coccidiosis outbreaks.

To quantify biosecurity performance across farms, each variable included in the four thematic categories, Farm Characteristics (A), Farm Isolation and External Traffic Management (B), Health Status and Biosecurity Practices (C), and Production Characteristics (D), was assigned a score ranging from 1 to 10, based on its relative importance and adherence to best practices. The cumulative score for each category was then calculated, yielding the following totals: 150 points for Category A, 230 points for Category B, 270 points for Category C, and 80 points for Category D, with a maximum combined score of 730. To normalise the total, each farm's global score was converted to a percentage scale (out of 100) by dividing by 10. The prevalence of farms with negative coccidiosis status aligned with the highest scoring tier (270/270) in health-related practices, highlighting the critical role of internal biosecurity.

Farms were then classified into two categories based on their final scores: those scoring ≥70/100 were considered to have good biosecurity, while those with scores <70/100 were classified as having poor biosecurity. This classification enabled an objective comparison of farm-level sanitary practices and their relationship with disease presence.

1.3. Coccidiosis prevalence

1.3.1. Coprological and necroscopic examination

Dead or live subjects (8 birds/flocks = S1) are randomly sampled from broiler farms at 2-3 weeks of age. The live birds are humanely euthanised by cervical dislocation. The necropsy is performed according to standard veterinary practitioner protocols. The feathers are wetted down with a disinfectant solution to minimise the spread of feathers during dissection. The skin of the abdomen and chest is removed. The abdominal muscle is incised and cut through the ribs on the sides of the heel bone. The thoracic cavity and internal organs are exposed after the keel bone near the abdomen is pulled upwards. The liver and air sacs are examined. The intestines have been removed, and their outer and inner surfaces have been examined for coccidial lesions. All the guts were cut into different portions (duodenum, jejunum, ileum, large intestine and caeca), and the contents (S1) of respective portions were examined microscopically to reveal the presence of coccidial oocysts. According to the Johnson and Reid method (Johnson & Reid, 1970), the lesion scale or index corresponds to a score of 0 to 4, depending on the increasing severity of the observed lesions specific to each identified species (*Eimeria acervulina, Eimeria maxima, Eimeria brunetti, Eimeria preacox, Eimeria tenella, Eimeria Necatrix*) (Johnson & Reid, 1970). During the study, excreta samples (10 per flock =S2) were also collected by moving back and forth across the building, along feed chains and water troughs, and by selecting the most contaminated areas.

The different preparations (S1 + S2) were weighed and homogenised with distilled water in a mortar. They are then sieved through a colander to remove the troublesome debris. All faecal sample was placed in a pre-labelled bottle containing 2% potassium dichromate ($K_2Cr_3O_7$) and conserved at 4 - 5 ° C in the Laboratory Valorisation of Bioresources (VALCOR), Faculty of Sciences, University M'hamed Bougara Boumerdes.

1.3.2. *Eimeria* species oocysts collection and identification

Different species of the genus Eimeria were identified based on their predilection for the intestinal site, lesion score, sporulation time, and oocyst morphology and size. For the positive sample, the Eimeria oocysts were incubated in a 2.5% potassium dichromate solution for sporulation at 25-29°C for 48 hours, with a humidity level maintained at 60-80%. The sporulation of the oocyst was monitored by examining sporocysts using a light microscope (Leica DMLS®, objective 40×, 100×). A standard Flotation was then used to purify the oocysts in a saturated solution (NaCl) to proceed to the qualitative and quantitative examination of oocysts. Identification of Eimeria species was based on microscopic morphometric observations. The measurement of length, width, and the ratio required the use of an ocular micrometre. The identification of the *Eimeria* species present in the sample was performed based on the conformity of the shape and size of oocysts with that reported by Reid et al. (1978) (MW REID et al., 1978). All coprological analyses were carried out at the Laboratory of Parasitology and Mycology of the Hospital of Mustapha Bacha –Algiers.

1.4. Data analysis

Data collected on farming conditions and health and welfare characteristics were summarised as means (± SD) or frequencies for the flocks. Farm prevalence was determined using an exact (Clopper-Pearson) 95% confidence interval (epitools.ausvet.com.au). Data from the questionnaires were collected and analysed using IBM SPSS Statistics for Windows, Version 29.0 (IBM Corp., Armonk, N.Y., USA). Overlapping variables or small categories were merged or summarised when possible. Potential risk factors for the presence of *Eimeria sp.* were initially examined using univariable analyses, including a chi-square test for categorical variables and logistic regression for continuous variables. Variables associated ($p < 0.05$) with the outcome of interest (presence of *Eimeria sp*) were considered for inclusion in a stepwise, backwards, multiple logistic regression analysis. The selected variables for the multivariable analysis were tested for mutual correlation. A likelihood ratio test was performed to eliminate variables from the multivariable model. Variables had to be significant ($p< 0.05$) to remain in the final model. The goodness-of-fit of the final model was assessed using the Hosmer-Lemeshow test.

2. Results and discussion

2.1. The socio-demographic and geographic profile of the farmers

The socio-demographic and geographic profile of the farmers included in this study provides a valuable context for interpreting biosecurity practices and coccidiosis risk. As shown in Table 8, the surveyed population was overwhelmingly male (98.73%), reflecting the region's male-dominated nature in commercial poultry farming (Table 7). The majority of participants (84.18%) were aged 20-65 years, corresponding to the economically active segment of the rural population. Educational attainment varied widely: over half (52.53%) of the farmers had no formal education, and only a small proportion (5.7%) had reached higher education (Table 8). This low level of schooling may represent a barrier to the effective implementation of technical biosecurity recommendations, particularly those that require literacy or procedural understanding (Table 8).

Table 8. Socio-Demography of Farm Owners**.**

Characteristics	Category	Number	Percentage (%)
Gender	M	156	98.73
	F	2	1.27
Age	20–45 years old	78	49.37
	46–65 years old	55	34.81
	Over 65 years old	25	15.82
Educational level	No education	83	52.53
	Elementary	43	27.22
	Secondary education	23	14.56
	Higher education	9	5.70
Experience in rearing (Number of years of working on the farm)	<10	72	45.57
	>10	86	54.43
Aviculture training	Yes	43	27.22
	No	115	72.78

Regarding professional background, 54.43% of respondents had been working in poultry farming for more than 10 years, indicating sustained involvement in the sector. However, this experience did not necessarily translate into better biosecurity performance. Only 27.22% reported having received formal training in poultry production (Table 8). This discrepancy between extensive practical experience and limited structured training raises concerns about the effectiveness of knowledge transfer, particularly regarding emerging diseases and updated sanitary guidelines. It also underlines the need for targeted, context-adapted training programmes to strengthen farmers' biosecurity competencies.

The geographic and legal distribution of farmers further underscores the informal nature of poultry farming in the region. According to Table 9, 158 farmers were distributed across nine municipalities. The highest concentrations of farmers were in Thénia (20.9%), Tidjelabine (16.5%), and Boudouaou (15.8%), indicating that poultry production is geographically clustered in these areas. In terms of legal status, only 50 farms (31.6%) were officially registered, while 108 farms (68.4%) operated informally (Table 9). Officially registered farms were most commonly found in Thénia (30% of all officially registered farms) and Tidjelabine (22%). In contrast, informal operations dominated across all regions, particularly in Souk El Had (14.8% of unofficial farms), Boudouaou (15.7%), and Thénia (16.7%) (Table 9).

Table 9. Regional breakdown of total, official, and unofficial farmers with percentage representation in each category

Regions	Total Farmers (N/%)		Official farms (n/%)		Unofficial farms (n/%)	
Beni amrane	8	5.10%	2	4.0%	6	5.6%
Boudouaou	25	15.80%	8	16.0%	17	15.7%
Cap djenat	11	7.00%	1	2.0%	10	9.3%
El kharrouba	13	8.20%	6	12.0%	7	6.5%
Ouled haddadj	12	7.60%	1	2.0%	11	10.2%
Souk el had	19	12.00%	3	6.0%	16	14.8%
Thenia	33	20.90%	15	30.0%	18	16.7%
Tidjelabine	26	16.50%	11	22.0%	15	13.9%
Zemouri	11	7.00%	3	6.0%	8	7.4%
Total	158	100.0%	50	100.0%	108	100.0%

This imbalance between the formal and informal sectors suggests a dual production system in which regulatory standards and support services are more accessible to a minority of farms (Table 8). Officially registered farms are more likely to benefit from veterinary supervision, health certification, and eligibility for subsidies or technical assistance (Alloui & Ayachi, 2012; Alloui & Bennoune, 2013a). In contrast, the majority of farmers, who operate informally, face limitations in accessing structured guidance and government programs. This situation may contribute to inconsistent application of preventive measures, such as vaccination schedules, proper litter management, or the rational use of anticoccidial drugs (Islam et al., 2024; Umunna et al., 2022).

Moreover, the high concentration of unofficial farms in municipalities such as Thénia, Souk El Had, and Boudouaou may heighten regional vulnerability to disease outbreaks. These clusters, in the absence of coordinated oversight, create environments where pathogen transmission can be sustained through proximity, shared equipment, and informal labour exchanges between farms (Delabouglise et al., 2020). This is particularly relevant for coccidiosis, a disease whose persistence in poultry houses is closely linked to environmental hygiene and biosecurity discipline (Gentile et al., 2024).

The spatial distribution of official versus unofficial farms also reflects potential disparities in knowledge dissemination and training coverage. Municipalities with higher proportions of official farms, such as Tidjelabine and El Kharrouba, may benefit from closer proximity to veterinary services or more organised poultry networks. However, even within these areas, informal practices remain prevalent and may undermine collective efforts to improve sanitary standards (Tasie et al., 2021).

The implications of this structure are significant: efforts to control endemic diseases, such as coccidiosis, must extend beyond farm-level interventions and include systemic approaches that address training accessibility, regulatory integration, and community-level coordination. Targeted interventions should focus on municipalities with high densities of informal producers, where minor improvements in awareness and practice could yield substantial public health and economic benefits (Ellwanger et al., 2021).

Furthermore, building trust and engagement with unofficial farmers will be essential to promote voluntary participation in health programs and data reporting systems.

2.2.Coccidiosis prevalence

The data (Table 10) reveal that coccidiosis was detected in 115 farms, corresponding to an overall prevalence of 72.8%, while 43 farms (27.2%) were free of the disease. This confirms that coccidiosis remains a widespread and persistent health challenge in the region's poultry sector. Prevalence rates varied across communes, with the highest levels recorded in Zemmouri (90.9%), Tidjelabine (88.5%), and Beni Amrane (87.5%), indicating a near-endemic circulation of *Eimeria* spp. in these areas. Intermediate prevalence rates were found in Thénia (60.6%), Souk El Had (63.2%), and Ouled Haddadj (58.3%). Boudouaou, which comprised the most significant number of surveyed farms (25, 15.8% of the total), had a prevalence of 64%, comparable to the overall average. Cap Djenat and El Kharrouba also showed considerable infection levels of 81.8% and 84.6%, respectively.

Table 10. Prevalence of *Eimeria* oocyst-positive poultry samples across nine communes of Boumerdes Province (Algeria).

	Absent	**Present**	**Total**
Commune	**N/%**		
Beni Amrane	1 (2.30%)	7 (6.10%)	8 (5.10%)
Boudouaou	9 (20.90%)	16 (13.90%)	25 (15.80%)
Cap Djenat	2 (4.70%)	9 (7.80%)	11 (7.00%)
El Kharrouba	2 (4.70%)	11 (9.60%)	13 (8.20%)
Ouled Haddadj	5 (11.60%)	7 (6.10%)	12 (7.60%)
Souk El Had	7 (16.30%)	12 (10.40%)	19 (12.00%)
Thenia	13 (30.20%)	20 (17.40%)	33 (20.90%)
Tidjelabine	3 (7.00%)	23 (20.00%)	26 (16.50%)
Zemouri	1 (2.30%)	10 (8.70%)	11 (7.00%)
Total	43 (100.00%)	115 (100.00%)	158 (100.00%)

To determine whether these observed differences between municipalities were statistically significant, a Pearson Chi-Square test was conducted. The result yielded a chi-square value of 12.895 with 8 degrees of freedom and a p-value of 0.116. Since the p-value exceeds the standard significance threshold of 0.05, the variation in infection rates across communes is not statistically significant. The results suggest that the observed differences may be due to random variation rather than a true epidemiological disparity. A supporting Likelihood Ratio test produced a similar result ($\chi^2 = 13.831$, $p = 0.086$). Although 27.8% of the expected cell counts were below 5, the minimum expected value (2.18) was sufficient to retain the test's validity.

These findings indicate that coccidiosis is uniformly distributed across the region, highlighting its endemic nature. Although the observed differences in prevalence between communes were not statistically significant, they may have been influenced by various factors related to farming conditions and management practices. These results align with those of other studies conducted in various geographical contexts. For instance, a study in Benadir, Somalia, found that although prevalence varied slightly across age and sex groups, there was no statistically significant difference in infection rates, suggesting a relatively homogeneous distribution of the disease across the surveyed areas (Mio et al., 2022). Similarly, research in Ambo, Ethiopia, showed no significant differences in prevalence between sexes or age groups, reinforcing the idea of an even distribution of the disease in the region (Oljira et al., 2012). A predictive model-based study in the Horn of Africa also observed that while some countries showed differing overall prevalence, no statistically significant differences were detected between several of the regional distributions analysed, supporting the hypothesis of widespread endemicity without sharp spatial clustering (Muñoz-Gómez et al., 2024a).

In our study, the high overall infection rates suggest the presence of shared risk factors affecting poultry health across the province. However, it is important to consider that the apparent uniformity of prevalence might mask a more complex underlying pattern of species diversity and genetic variation in *Eimeria* populations (Ayadi et al., 2024; Chengat Prakashbabu et al., 2017; Clark et al., 2016). Indeed, geographic location plays a crucial role in shaping the composition of Eimeria species and their genetic structure. Although the main pathogenic species are globally distributed, numerous studies have demonstrated that significant regional differences exist in the prevalence and diversity of these parasites (Alcala-Canto et al., 2020; Kumar et al., 2015; Silva et al., 2022).

Tables 11 and 12 summarise the identification of *Eimeria* species across 115 poultry farms, based on both necroscopic examination of chicks and coprological examination of faeces. Across both diagnostic approaches, *Eimeria tenella* (ET) emerged as the most frequently identified species, accounting for 24.89% of detections in chicks and 28.09% in faeces. This aligns with global findings that highlight *E. tenella* as one of the most prevalent and pathogenic species in poultry (Blake et al., 2020). However, important regional distinctions were observed. For instance, the commune of Tidjelabine reported the highest counts of *Eimeria tenella* (ET) in both chick necropsy (48.91%) and faecal analysis (39.13%). In contrast, regions like Souk El Had and Zemouri showed markedly lower detection rates. Interestingly, *Eimeria maxima* (EMAX), which is often associated with subclinical infections and productivity loss, was highly prevalent in Zemouri (86.25% in chicks and 69.00% in faeces), suggesting a strong regional presence that may be linked to environmental or management factors. Conversely, this species was absent in El Kharrouba necropsies and infrequent in Beni Amrane.

Some regions also showed unexpectedly high levels of species such as *Eimeria necatrix* (EN) and *Eimeria brunetti* (EB), both of which are considered moderately to highly pathogenic. Notably, Ouled Haddadj displayed high frequencies of *Eimeria necatrix* (35.71% in chicks and 28.57% in faeces), suggesting localised epidemiological dynamics that may require tailored control strategies.

The slight differences between necropsy and coprological data may reflect the stage of the parasite life cycle, lesion distribution in the gut, and the sensitivity of each method. For example, *E. acervulina* (EA) was more commonly detected in chicks (10.22%) than in faeces (9.04%), while *E. necatrix* showed a significant increase in faecal detection (12.52%) compared to necropsy (5.87%). Such discrepancies highlight the complementarity of both diagnostic methods when assessing the burden of Eimeria species on farms.

These observations are consistent with broader findings across Algeria. Several studies report alarmingly high prevalence rates of *Eimeria* in both broiler and layer farms, with values ranging from 63% to nearly 100%. In Médéa province, *E. mitis* (92.5%), *E. tenella* (81%), and *E. necatrix* (76%) were the most frequent species, while neither *E. acervulina* nor *E. praecox* were detected (Alloui & Bennoune, 2013a; Amina et al., 2025). In six northeastern provinces, *E. necatrix* (63%) and *E. maxima* (53%) predominated among future laying and breeding hens, with co-infections more common than mono-infections (Ayadi et al., 2024). Similarly, in Béjaïa province, *E. acervulina* (32%) and *E. tenella* (27%) were most prevalent, with overall infection rates in broilers reaching 73.8% and seasonal peaks in autumn (Debbou-Iouknane et al., 2018). Notably, all seven recognised *Eimeria* species have been identified in some Algerian regions, although their relative abundance varies depending on geography, production system, and diagnostic approach.

Table 11. Prevalence and regional distribution of *Eimeria* species identified in intestinal samples from chicks (n = 115 Poultry Farms). This table summarises the detection rates of seven Eimeria species, *Eimeria acervulina* (EA), *Eimeria tenella* (ET), *Eimeria brunetti* (EB), *Eimeria necatrix* (EN), *Eimeria mitis* (EM), *Eimeria praecox* (EP), and *Eimeria maxima* (EMAX), based on post-mortem intestinal examination of chicks collected from 115 poultry farms. Values are presented as counts and percentages by region, providing insight into species distribution and infection prevalence among birds.

Regions	**Total Farms**	**EA***	**ET***	**EB***	**EN***	**EM***	**EP***	**EMAX***
Beni Amrane	7	20 (35.71 %)	20 (35.71 %)	7 (12.50 %)	1 (1.79 %)	0 (0.00 %)	5 (8.93 %)	3 (5.36 %)
Boudouaou	16	10 (7.81 %)	10 (7.81 %)	5 (3.91 %)	0 (0.00 %)	8 (6.25 %)	35 (27.34 %)	60 (46.88 %)
Cap Djenat	9	6 (8.33 %)	5 (6.94 %)	5 (6.94 %)	10 (13.89 %)	20 (27.78 %)	14 (19.44 %)	12 (16.67 %)
El Kharrouba	11	20 (22.73 %)	45 (51.14 %)	4 (4.55 %)	4 (4.55 %)	10 (11.36 %)	5 (5.68 %)	0 (0.00 %)
Ouled Haddadj	7	5 (8.93 %)	2 (3.57 %)	5 (8.93 %)	20 (35.71 %)	7 (12.50 %)	2 (3.57 %)	15 (26.79 %)
Souk El had	12	0 (0.00 %)	20 (20.83 %)	12 (12.50 %)	8 (8.33 %)	8 (8.33 %)	8 (8.33 %)	40 (41.67 %)
Thenia	20	23 (14.38 %)	35 (21.88 %)	26 (16.25 %)	10 (6.25 %)	20 (12.50 %)	26 (16.25 %)	20 (12.50 %)
Tidjelabine	23	10 (5.43 %)	90 (48.91 %)	20 (10.87 %)	0 (0.00 %)	1 (0.54 %)	5 (2.72 %)	58 (31.52 %)
Zemouri	10	0 (0.00 %)	2 (2.50 %)	2 (2.50 %)	1 (1.25 %)	1 (1.25 %)	5 (6.25 %)	69 (86.25 %)
Total	115	94 (10.22 %)	229 (24.89 %)	86 (9.35 %)	54 (5.87 %)	75 (8.15 %)	105 (11.41 %)	277 (30.11 %)

*n (%)

Table 12. Prevalence and regional distribution of Eimeria species *Eimeria acervulina* (EA), *Eimeria tenella* (ET), *Eimeria brunetti* (EB), *Eimeria necatrix* (EN), *Eimeria mitis* (EM), *Eimeria praecox* (EP), *and Eimeria maxima* (EMAX) detected in faecal samples from poultry farms (n = 115 Farms).

Regions	**Total Farmers**	**EA***	**ET***	**EB***	**EN***	**EM***	**EP***	**EMAX***
Beni amrane	7	20 (28.57 %)	20 (28.57 %)	7 (10.00 %)	15 (21.43 %)	0 (0.00 %)	5 (7.14 %)	3 (4.29 %)
Boudouaou	16	10 (6.25 %)	42 (26.25 %)	5 (3.13 %)	0 (0.00 %)	8 (5.00 %)	35 (21.88 %)	60 (37.50 %)
Cap djenat	9	6 (6.67 %)	23 (25.56 %)	5 (5.56 %)	10 (11.11 %)	20 (22.22 %)	14 (15.56 %)	12 (13.33 %)
El kharrouba	11	20 (18.18 %)	45 (40.91 %)	4 (3.64 %)	4 (3.64 %)	10 (9.09 %)	5 (4.55 %)	22 (20.00 %)
Ouled haddadj	7	5 (7.14 %)	2 (2.86 %)	5 (7.14 %)	20 (28.57 %)	7 (10.00 %)	16 (22.86 %)	15 (21.43 %)
Souk el had	12	0 (0.00 %)	44 (36.67 %)	12 (10.00 %)	8 (6.67 %)	8 (6.67 %)	8 (6.67 %)	40 (33.33 %)
Thenia	20	23 (11.50 %)	55 (27.50 %)	26 (13.00 %)	30 (15.00 %)	20 (10.00 %)	26 (13.00 %)	20 (10.00 %)
Tidjelabine	23	10 (4.35 %)	90 (39.13 %)	20 (8.70 %)	46 (20.00 %)	1 (0.43 %)	5 (2.17 %)	58 (25.22 %)
Zemouri	10	10 (10.00 %)	2 (2.00 %)	2 (2.00 %)	11 (11.00 %)	1 (1.00 %)	5 (5.00 %)	69 (69.00 %)
Total	115	104 (9.04 %)	323 (28.09 %)	86 (7.48 %)	144 (12.52 %)	75 (6.52 %)	119 (10.35 %)	299 (26.00 %)

*n (%)

These high prevalence rates and diverse species profiles confirm that coccidiosis remains a major health threat to Algerian poultry. This burden poses significant challenges for producers, particularly in the absence of tailored control strategies (Alloui & Ayachi, 2012; Alloui & Bennoune, 2013a; Rahmani et al., 2024). The regional diversity in species composition underscores the importance of localised surveillance and adaptive interventions, whether through vaccine formulation, anticoccidial rotation, or biosecurity improvements, to mitigate the economic and health impacts of this endemic disease (Gao et al., 2024).

In addition to morphological identification, recent PCR-based studies have offered more profound insights into the genetic diversity and distribution of *Eimeria* species in Algeria (Mares et al., 2023). Molecular detection techniques, particularly those targeting the ITS1 region, have confirmed both the high prevalence and the complexity of species co-circulation in poultry flocks. A study conducted in six northeastern provinces using ITS1-PCR on samples from 32 farms detected six *Eimeria* species: *E. necatrix* (63%) and *E. maxima* (53%) were the most dominant, followed by *E. tenella*, *E. brunetti*, *E. acervulina*, and *E. mitis*, with *E. praecox* notably absent (Ayadi et al., 2024). Co-infections were more frequent than mono-infections, and breeding hen farms exhibited higher oocyst loads than pre-laying pullet farms, suggesting an accumulation of infection pressure with age and production stage.

Furthermore, genetic studies in Jijel province using PCR on litter samples from broiler farms identified only *E. acervulina* and *E. maxima*. Importantly, these isolates were tested for resistance to commonly used anticoccidial drugs, revealing full resistance to monensin and narasin, partial resistance to salinomycin and lasalocid, and susceptibility to diclazuril (Djemai et al., 2016). These findings not only demonstrate the power of molecular tools for precise species identification but also emphasise the urgent need to integrate drug resistance monitoring into routine coccidiosis control programs.

Altogether, the convergence of morphometric and molecular data in Algeria paints a comprehensive picture of a complex, evolving *Eimeria* landscape marked by high species diversity, widespread co-infections, and emerging drug resistance. These realities call for an evidence-based approach to disease control, tailored to regional species profiles and resistance patterns, and supported by enhanced diagnostic capacities at both farm and laboratory levels.

2.3.Management and Biosecurity Evaluation in the farms

To better understand the dynamics of coccidiosis prevalence in poultry production in the Boumerdès region, a comprehensive assessment of farm management and biosecurity measures was conducted. The evaluation aimed to capture not only routine husbandry practices but also the implementation of disease prevention protocols and the condition of farm infrastructure.

We examined the influence of various aspects of farm management on coccidiosis prevalence in broiler farms. The analysis was based on 75 survey items grouped into four thematic categories: farm characteristics (Category A, Table 13), farm isolation and external traffic management (Category B, Table 14), biosecurity and healthcare practices (Category C, Table 15), and production parameters (Category D, Table 16). Using binary logistic regression, we identified significant associations between specific practices and coccidiosis, with effect sizes expressed as odds ratios (ORs), along with their corresponding 95% confidence intervals (CIs) and p-values.

The first category (A) examined the structural and environmental features of the farm (Table 13). It included variables such as the farm's juridical status (officially registered or not), building type, flock size, number of chickens per square meter of breeding area, and the age of the chickens. Other factors considered were the presence of fans, extractors, and humidifiers, the type of building ground (concrete, soil, sand), and the type of litter used (wood shavings, chopped straw, or mixture). This category also encompassed feeding and watering practices, including the mode of food distribution (automatic or manual), the condition of the feeders, the water distribution system, the source of drinking water (public supply, well, or borehole), and the state of the water troughs.

Notably, farms with non-registered legal status had markedly higher odds of infection (OR = 4.50; 95% CI: 1.957-10.349; $p < 0.001$), suggesting that informal or poorly regulated operations may lack access to veterinary support or oversight (Table 13).

Similarly, insufficient space per bird (high stocking density) significantly increased the risk (OR = 11.83; CI: 3.88–36.09; $p < 0.001$), likely due to increased faecal contact and stress-induced immunosuppression (Table 13). Overcrowding compromises bird immunity and accelerates *Eimeria* transmission through contaminated litter and surfaces (Cheru et al., 2023; Guardia et al., 2011; Lunden et al., 2000; Tsiouris et al., 2015).

The presence of earthen flooring (OR = 8.78; CI: 3.52–21.90; $p < 0.001$) and non-standard litter materials (OR = 5.82; CI: 1.87–18.11; $p = 0.002$) also increased the risk of infection by facilitating oocyst accumulation and persistence in the environment. This is supported by Dalloul and Lillehoj (2005), who highlighted that porous and moisture-retentive floors allow oocysts to survive longer, and that poor litter quality exacerbates this effect (Dalloul & Lillehoj, 2005). Similarly, Muñoz-Gómez et al (2024) found significantly higher coccidiosis rates in broiler houses with dirt floors and reused or inadequately managed litter (Muñoz-Gómez et al., 2024b)

Interestingly, the use of public water sources emerged as a protective factor (OR = 0.089; CI: 0.01–0.80; $p = 0.031$), possibly due to more stable and monitored water quality compared to private wells or surface sources. These results support the idea that poor infrastructure and overcrowding create a physical context highly favourable to the development and spread of *Eimeria spp*. This finding aligns with the results of Wondimu et al. (2019), who reported a higher prevalence of coccidiosis in farms using untreated or surface water, with access to public water associated with better health outcomes (Wondimu et al., 2019a).

These results support the idea that poor infrastructure and overcrowding create a physical context highly favourable to the development and spread of *Eimeria* spp.

Additional risk factors included manual food distribution (OR = 22.46; CI: 6.75–71.40; $p < 0.001$) and manual water systems (OR = 22.46; CI: 6.75–71.40; $p < 0.001$), both of which may reflect outdated practices or reduced hygiene standards that facilitate pathogen transmission (Table 12). Similar conclusions were drawn by Kachanova & Safiullin (2019), who emphasised the role of automated systems in reducing contamination risk and ensuring consistency in feed and water hygiene (Kachanova & Safiullin, 2019).

Moreover, farms with poorly maintained feeders were more likely to report infection, particularly when feeder condition was classified as "very bad" (OR = 13.33; CI: 1.14–156.45; $p = 0.039$), reinforcing the importance of regular maintenance and cleanliness in limiting disease spread (Table 13). These findings align with those of Lakshmi Namratha et al. (2019), who emphasised that feeder hygiene is directly correlated with reduced oocyst ingestion (Lakshmi Namratha et al., 2019).

In terms of ventilation, the absence of fans, extractors, or humidifiers did not significantly alter the odds of infection; however, their role in overall animal welfare and environmental stability should not be overlooked. Good ventilation helps maintain dry litter and reduce humidity levels that favour oocyst sporulation (Gottardo Balestrin et al., 2021).

Finally, the cleanliness of water troughs also played a role, with poorly maintained troughs associated with increased prevalence (OR = 4.93 for “bad” vs. “good”; CI: 1.19–20.46; $p = 0.026$), highlighting water hygiene as a key component of disease prevention. This aligns with the findings of Andreopoulou et al. (2022), who emphasised that microbial contamination of drinkers significantly increases the likelihood of *Eimeria* transmission (Andreopoulou et al., 2022).

Table 13. Influence of Farm Characteristics (Category A) on Coccidiosis Prevalence in Broiler Farms.

Nº	Variables	Reference variable	Answered	Coccidiosis prevalence (N,%)		ORR	95% Confidence interval (%)	p
				Absente 43 (27.2%)	Presente 115 (72.8%)			
1	*Juridical status (Officially registered or not)*	Yes	50 (31.6%)	18 (60%)	12 (40%)	4.500	1.957-10.349	0.000
		No	108 (68.4%)	32 (25%)	96 (75%)	-	-	-
2	*Building type*	Concrete building	22 (13.9%)	11 (36.7%)	11 (8.6%)	7.062	2.634-18.934	0.000
		Greenhouse	129 (81.6%)	16 (53.3%)	113 (88.3%)	1.333	0.240-7.405	0.742
		Other	7 (4.4%)	3 (10%)	4 (3.1%)	-	-	-
3	*Flock size*	<1500	78 (49.4%)	18 (41.9%)	60 (52.2%)	1.515	0.747-3.075	0.250
		>1500	80 (50.6%)	25 (58.1%)	55 (47.8%)	-	-	-
4	*Number of the chicken* m^2 *of breeding air*	<12	46 (29.1%)	20 (66.7%)	26 (20.3%)	7.846	3.278-18.779	3.7E-06
		>12	112 (70.9%)	10 (33.3%)	102 (79.7%)	-	-	-
5	*Age of the chickens*	>2 weeks	77 (48.7%)	29 (67.4%)	48 (41.7%)	0.346	0.165-0.723	0.005
		<2 weeks	81 (51.3%)	14 (32.6%)	67 (58.3%)	-	-	-
6	*Fans*	Yes	59 (37.3%)	13 (43.3%)	46 (35.9%)	1.363	0.608-3.056	0.452
		No	99 (62.7%)	17 (56.7%)	82 (64.1%)			
7	*Extractors*	Yes	48 (30.4%)	12 (40%)	36 (28.1%)	1.704	0.746-3.891	0.206
		No	110 (69.6%)	18 (60%)	92 (71.9%)			
8	*Humidifier*	Yes	51 (32.3%)	7 (23.3%)	44 (34.4%)	0.581	0.231-1.460	0.248
		No	107 (67.7%)	23 (76.7%)	84 (65.6%)	-	-	-
9	*Building ground*	Concrete	50 (31.6%)	21 (70%)	29 (22.7%)	8.780	3.521-21.898	0.000003
		Soil	105 (66.5%)	8 (26.7%)	97 (75.8%)	1.448	0.123-17.041	0.768
		Send	3 (1.9%)	1 (3.3%)	2 (1.6%)	-	-	-
10	*Litter*	Wood shavings	98 (62%)	17 (39.5%)	81 (70.4%)	4.467	1.858-10.740	0.001
		chopped straw	29 (18.4%)	11 (25.6%)	18 (15.7%)	1.534	0.548-4.293	0.415
		Mixture	31 (19.6%)	15 (34.9%)	16 (13.9%)	-	-	-
11	*Food distribution*	Automatic	40 (25.3%)	19 (63.3%)	21 (36.7%)	0.114	0.047-0.273	0.000
		Manually	118 (74.7%)	11 (16.4%)	107 (83.6%)	-	-	-
12	*Condition of the feeders*	Good	41 (25.9%)	22 (73.3%)	19 (26.7%)	0.063	0.025-0.163	0.000
		Bad	117 (74.1%)	8 (14.8%)	109 (74.1%)	-	-	-
13	*Water distribution*	Automatic	77 (48.7%)	23 (53.5%)	54 (46.5%)	0.770	0.381-1.554	0.465
		Manually	81 (51.3%)	20 (47%)	61 (53%)	-	-	-
14	*Source of water*	Public water	48 (30.4%)	7 (16.3%)	41 (35.7%)	3.626	1.258-10.452	0.017
		Well	76 (48.1%)	23 (53.5%)	53 (46.1%)	1.427	0.611-3.329	0.411

		Borehole	34 (21.5%)	13 (30.2%)	21 (18.3%)	-	-	-
15	*State of the water troughs*	Good	86 (54.4%)	22 (51.2%)	64 (55.7%)	1.198	0.594-2.417	0.614
		Bad	72 (45.6%)	21 (48.8%)	51 (44.3%)	-	-	-

The second category B (Table 14) evaluated the farm's level of isolation and exposure to external contamination. In this category, the study examined how the physical separation of the poultry house from external sources of contamination and the degree of access control influence coccidiosis prevalence on broiler farms (Atif et al., 2023a). Farms with minimal isolation (for example, those adjacent to public roads, lacking perimeter walls or fences, or featuring multiple uncontrolled entrances) are more vulnerable to oocyst introduction via contaminated footwear, vehicles, and fomites (Atif et al., 2023a; Bauer et al., 2023; N. Begum et al., 2024). Conversely, operations that employ well-defined boundaries, single access points, and durable, easy-to-clean building materials establish practical barriers against environmental contamination and pathogen incursion, in line with international biosecurity recommendations (FAO, 2025).

Overall, perimeter security and access control were strongly associated with lower coccidiosis prevalence, while multiple entry points and human proximity increased risk (Table 14). Specifically, having a wall or fence around the farm reduced the odds by about 60% (OR = 0.38; 95% CI: 0.19–0.79; $p = 0.009$), whereas having multiple entrances approximately tripled them (OR = 3.01; 95% CI: 1.45–6.26; $p = 0.003$). These patterns are consistent with international biosecurity standards that emphasise a defined boundary, a single controlled entry, and managed flows of people and vehicles (Atif et al., 2023b; Pajić et al., 2023; Wondimu et al., 2019b).

Concerning site access, farms directly fronting a paved road showed higher, though non-significant, odds of coccidiosis compared with those accessed via an alleyway (reference) (Road: OR = 2.74; 95% CI: 0.98–7.67; $p > 0.05$), plausibly reflecting heavier traffic and visitor flow. Access via track was also not significant (Track: OR = 1.87; 95% CI: 0.72–4.86; $p > 0.05$) (Table 14). Compliance with road-distance regulations did not change risk (OR = 0.83; 95% CI: 0.41–1.69; $p = 0.61$). Adjacent human dwellings nearly tripled the odds of coccidiosis (OR = 2.83; 95% CI: 1.37–5.83; $p = 0.005$), whereas having no habitation nearby was clearly protective (OR = 0.19; 95% CI: 0.06–0.58; $p = 0.004$), and a distance of < 50 m showed a non-significant protective trend (OR = 0.34; 95% CI: 0.10–1.17; $p = 0.086$). In practice, these findings support the implementation of buffer zones, parking outside the perimeter, and pre-entry authorisation for people and vehicles, as recommended in authoritative manuals (Amina et al., 2025; Atif et al., 2023b; OIE, 2021; Pajić et al., 2023).

The building envelope and micro-environment were also major determinants. Good floor insulation reduced the odds of coccidiosis by about 94% (OR = 0.064; 95% CI: 0.023–0.175; p ≈ 0.0000001), and moderate insulation by roughly 73% (OR = 0.27; 95% CI: 0.11–0.68; p = 0.005), compared to poor insulation (Table 14). Chimney-only ventilation was associated with a higher risk (OR = 3.01; 95% CI: 1.42–6.38; p = 0.004), whereas the presence of windows (OR = 0.74; 95% CI: 0.36–1.51; p = 0.408) and natural corridor ventilation (OR = 1.09; 95% CI: 0.54–2.20; p = 0.808) was not significantly associated with disease. Biologically, this is coherent with the sporulation biology of *Eimeria*: oocysts require oxygen, suitable temperature, and especially moisture to sporulate and become infective; therefore, a dry litter and well-managed airflow directly suppress sporulation and reduce environmental oocyst load (Chapman, 2000; Hafez, 2008; López-Osorio et al., 2020; Mesa-Pineda et al., 2021)

Our moisture and temperature management practices align with this mechanism (Table 14). Actively monitoring building moisture was strongly protective (OR = 0.18; 95% CI: 0.05–0.65; p = 0.009). In contrast, routine temperature checks were associated with higher odds of coccidiosis (OR = 2.48; 95% CI: 1.16–5.29; p = 0.019), likely reflecting surveillance bias (farms perceiving greater risk monitor more). The literature emphasises that temperature control only translates into biological impact when coupled with adequate ventilation and moisture control (Ma et al., 2022; Z. Yang et al., 2022).

Regarding flooring, concrete was associated with a higher prevalence of coccidiosis (OR = 4.12; 95% CI: 1.94–8.74; p = 0.0002) compared with dirt (OR = 1.70; 95% CI: 0.15–20.02; p = 0.672) or sand (sparse data). Because hard, intact surfaces should, in principle, be easier to sanitise, we interpret this as a flag for technical audit (slope, drainage, cracks, ponding) and for possible confounding with litter and ventilation management rather than as an indictment of concrete itself (Course et al., 2021; Fotina et al., 2023). Prevailing guidance prioritises moisture management and effective cleaning/disinfection regardless of substrate (Battersby et al., 2017; Jiang et al., 2018; Wales et al., 2006).

Several movement-related variables were not significant in our univariable analysis: equipment sharing (OR = 1.47; 95% CI: 0.68–3.18; p = 0.323), vehicle sharing (OR = 1.43; 95% CI: 0.69–2.93; p = 0.336), employees working on other farms (OR = 1.11; 95% CI: 0.54–2.29; p = 0.772), the presence of other animals on site (OR = 0.68; 95% CI: 0.33–1.43; p = 0.314), and distance to the nearest poultry farm (< 50 m: OR = 0.58; 95% CI: 0.22–1.56; p = 0.28; 50–200 m: OR ≈ 0.56; 95% CI: 0.26–1.24; p = 0.155). This does not contradict current standards; effects may be muted by strong perimeter controls already in place, low exposure frequency, or limited power. Manuals still recommend avoiding inter-farm sharing or enforcing strict cleaning/disinfection when sharing is unavoidable (Amalraj et al., 2024; Souillard et al., 2024; Van Limbergen et al., 2018).

Finally, the visitor policy stood out as one of the most impactful levers: strictly limiting visitor access reduced the odds of coccidiosis by about 85% (OR = 0.151; 95% CI: 0.046–0.491; p = 0.002), whereas maintaining a visitor register was not significantly associated with prevalence (OR = 1.46; 95% CI: 0.65–3.25; p = 0.359). This supports a practical message from official guidance: blocking unvetted entry at the gate through authorisation, a clear, clean–dirty line, dedicated boots and clothes, and vehicle controls is more protective than documenting entries after the fact (Fathelrahman et al., 2020; Ornelas-Eusebio et al., 2020)

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Table 14. Associations between farm isolation, external traffic management variables (Category B), and coccidiosis prevalence in broiler farms.

Nº	**Variables**	**Reference variable**	**Answered**	**Coccidiosis prevalence N (%)**		**ORR**	**95% Confidence interval (%)**	**p**
				Absente 43 (27.2%)	Presente 115 (72.8%)			
1	*Access to the site*	Road	58 (36.7%)	12 (27.9%)	46 (40%)	2.738	0.977-7.674	2.738
		Track	76 (48.1%)	21 (48.8%)	55 (47.8%)	1.871	0..720-4.860	1.871
		Alleyway	24 (15.2%)	10 (23.3%)	14 (12.2%)	-	-	-
2	*Does the distance from roads comply with the regulations?*	Yes	61 (38.6%)	18 (41.9%)	43 (37.4%)	0.829	0.406-1.694	0.608
		No	97 (61.4%)	25 (58.1%)	72 (62.6%)	-	-	-
3	*Does the farm have a wall or fence around it?*	Yes	55 (34.8%)	22 (51.2%)	33 (28.7%)	0.384	0.187-0.791	0.009
		No	103 (65.2%)	21 (48.8%)	82 (71.3%)	-	-	-
4	*Does the farm site have multiple entrances?*	Yes	86 (54.4%)	15 (34.9%)	71 (61.7%)	3.012	1.450-6.258	0.003
		No	72 (45.6%)	28 (65.1%)	44 (38.3%)	-	-	-
5	*Are there any human habitations next to the farm?*	Yes	88 (55.7%)	16 (37.2%)	72 (62.6%)	2.826	1.369-5.832	0.005
		No	70 (44.3%)	27 (62.8%)	43 (37.4%)	-	-	-
6	*Do the habitations have direct access to the farm?*	Yes	63 (39.9%)	13 (30.2%)	50 (43.5%)	1.775	0.840-3.750	0.133
		No	95 (60.1%)	30 (68.8%)	65 (56.5%)	-	-	-
7	*Distance to the nearest habitation*	No Habitation	71 (44.9%)	27 (62.8%)	44 (38.3%)	0.186	0.060-0.582	0.004
		<50	48 (30.4%)	12 (27.9%)	36 (31.3%)	0.343	0.101-1.165	0.086
		>50	39 (24.7%)	4 (9.3%)	35 (30.4%)	-	-	-
8	*Building ground*	Concrete	50 (31.65%)	23 (46.0%)	27 (54.0%)	4.117	1.939-8.742	0.0002
		Dirt	105 (66.46%)	18 (17.1%)	87 (82.9%)	1.704	0.145-20.022	0.672
		Sand	3 (1.90%)	1 (33.3%)	2 (66.7%)	-	-	-
9	*Roof condition*	Good	22 (13.9%)	3 (7%)	19 (16.5%)	2.830	0.755-10.613	0.123
		Moderate	68 (43%)	19 (44.2%	49 (42.6%)	1.152	0.551-2.411	0.707
		Bad	68 (43%)	21 (48.8%)	47 (40.9%)	-	-	-
10	*Floor Isolation*	Good	30 (19%)	20 (46.5%)	10 (8.7%)	0.064	0.023-0.175	0.0000001
		moderate	40 (25.3%)	13 (30.2%)	27 (23.5%)	0.266	0.105-0.677	0.005
		Bad	88 (55.7%)	10 (23.3%)	78 (67.8%)	-	-	-
11	*Presence of the path (Natural ventilation)*	Yes	87 (55.1%)	23 (53.5%)	64 (55.7%)	1.091	0.540-2.204	0.808
		No	71 (44.9%)	20 (46.5%)	51 (44.3%)	-	-	-
12	*Windows*	Yes	63 (39.9%)	19 (45.2%)	44 (37.9%)	0.740	0.362-1.511	0.408
		No	95 (60.1%)	23 (54.8%)	72 (62.1%)	-	-	-
13	*Chimneys*	Yes	44 (27.8%)	19 (45.2%)	25 (21.6%)	3.007	1.418-6.377	0.004
		No	114 (75.2%)	23 (54.8%)	91 (78.4%)	-	-	-

14	*Do you check the building's temperature?*	Yes	42 (26.6%)	17 (40.5%)	25 (59.5%)	2.475	1.159-5.285	0.019
		No	116 (73.4%)	25 (21.6%)	91 (78.4%)	-	-	-
15	*Do you monitor the building's moisture content?*	Yes	11 (7.0%)	7 (16.7%)	35 (83.3%)	0.179	0.049-0.646	0.009
		No	147 (93.0%)	4 (3.4%)	112 (96.6%)	-	-	-
16	*Do you have other livestock or pets on your farm?*	Yes	95 (60.1%)	28 (66.7%)	14 (33.3%)	0.684	0.326-1.433	0.314
		No	63 (39.9%)	67 (57.8%)	49 (42.2%)	-	-	-
17	*Distance to the nearest poultry farm*	<50m	78 (49.4%)	16 (38.1%)	62 (53.4%)	0.580	0.216-1.558	0.280
		50–200m	25 (15.8%)	8 (19%)	17 (14.7%)	0-5625	0.255-1.243	0.155
		>200m	55 (34.8%)	18 (42.9%)	37 (31.9%)	-	-	-
18	*Do you exchange your equipment with other farms?*	Yes	55 (34.81%)	12 (21.8%)	30 (29.1%)	1.473	0.683-3.175	0.323
		No	103 (65.19%)	43 (78.2%)	73 (70.9%)	-	-	-
19	*Have you exchanged your vehicles with other farms?*	Yes	58 (36.71%)	18 (31.0%)	40 (69.0%)	1.425	0.693-2.931	0.336
		No	100 (63.29%)	24 (24.0%)	76 (76.0%)	-	-	-
20	*Do your employees work on other farms?*	Yes	61 (38.61%)	17 (27.9%)	44 (72.1%)	1.113	0.541-2.289	0.772
		No	97 (61.39%)	25 (25.8%)	72 (74.2%)	-	-	-
21	*Is visitor access to poultry limited?*	Yes	48 (30.38%)	16 (80.0%)	32 (37.6%)	0.151	0.046-0.491	0.002
		No	57 (36.08%)	4 (20%)	53 (62.4%)	-	-	-
22	*Do you have a visitor register?*	Yes	37 (23.42%)	12 (32,4%)	30 (24,8%)	1.456	0.653-3.249	0.359
		No	121 (76.58%)	25 (67.6%)	91 (75.2%)	-	-	-
23	*Do you buy the chicks from a reputable source?*	Yes	57 (36.08%)	13 (12.4%)	44 (41.9%)	1.731	0.629-4.763	0.288
		No	48 (30.38%)	7 (6.7%)	41 (39.0%)	-	-	-

In our dataset, Category C (Table 15) produced some of the clearest signals, confirming that day-to-day behavior and the consistency of measure implementation strongly influence coccidiosis risk. Having a cloakroom was associated with higher odds of reported coccidiosis (Yes vs No: OR = 3.11; 95% CI: 1.47–6.57; p = 0.003), a pattern that likely reflects reverse causality (installation on already-affected, often larger or more intensively managed farms) rather than a harmful effect of the measure itself. In contrast, several routine hygiene practices showed a marked protective effect when applied systematically: good litter quality ("good" vs "very bad") was associated with significantly reduced odds (OR = 0.10; 95% CI: 0.03–0.43; p = 0.002), handwashing on each occasion when accessing the poultry area (vs "never") was strongly protective (OR = 0.20; 95% CI: 0.08–0.49; p < 0.001), and always wearing specific/clean clothing when entering poultry areas (vs "never") further reduced risk (OR = 0.12; 95% CI: 0.04–0.35; p < 0.001). Similarly, systematically isolating birds showing signs of disease from healthy birds ("on each occasion" vs "never") substantially lowered the odds of coccidiosis (OR = 0.18; 95% CI: 0.08–0.44; p < 0.001) (Table 15).

By contrast, several measures that are frequently emphasised in guidelines did not show a statistically significant association with coccidiosis in this sample. Footbaths (Yes vs No: OR = 1.50; 95% CI: 0.52–4.32; p = 0.448), vehicle disinfectant mats (Yes vs No: OR = 0.74; 95% CI: 0.32–1.73; p = 0.483), drinking-water disinfection (Yes vs No: OR = 0.89; 95% CI: 0.41–1.92; p = 0.766), and water-circuit control (Yes vs No: OR = 0.56; 95% CI: 0.27–1.15; p = 0.116) were all non-significant, with ORs close to 1. This pattern is consistent with the idea that these tools offer little benefit when poorly implemented (e.g., dirty footbaths, wrong disinfectant concentration or contact time, failure to renew solutions, or inadequate cleaning of water lines), and that implementation quality is at least as important as their nominal presence. A longer downtime between flocks (>7 days vs no specific period/immediate restocking) was unexpectedly associated with higher odds (OR = 3.08; 95% CI: 1.09–8.71; p = 0.034), which probably reflects targeted extension of downtime on farms that have already experienced health problems, rather than a causal increase in risk.

Other management indicators fitted the same picture of reverse causality. Having a mortality record book that was up-to-date and regularly reviewed was associated with increased odds of reported coccidiosis (OR = 2.86; 95% CI: 1.14–7.18; p = 0.025), suggesting that more meticulous recording is implemented where disease pressure is already recognised as high. Most anticoccidial drugs showed no independent association with disease status in this model; only one product, diaveridine, demonstrated a statistically significant protective signal (OR = 0.075; 95% CI: 0.006–0.954; p = 0.046), although the low number of users means this result should be interpreted cautiously and mainly supports the importance of structured programmes and rotation rather than ad hoc treatment (J. A. Begum et al., 2023; Souillard et al., 2024; Tilli et al., 2022).

Overall, these findings reinforce that the measures most likely to reduce faecal–oral transmission, and thus to directly limit Eimeria cycling and sporulation, which depend on adequate moisture, oxygen, and temperature (Gadelhaq et al., 2018; Soliman et al., 2018), are those that keep litter and air dry and are implemented consistently rather than reactively. In practical terms, our results argue for preventive, standardised biosecurity: single controlled entry with a verified clean–dirty line, reliable routines for handwashing and changing into dedicated clothing, systematic isolation of sick birds, well-maintained footbaths and mats (pre-clean boots, correct disinfectant mix, adequate contact time, frequent renewal), and robust waterline hygiene—elements that are all emphasised in WOAH and FAO guidance (FAO, 2025; Souillard et al., 2024; Tilli et al., 2022).

Tableau 15. Associations between biosecurity and healthcare practices (Category C) and the prevalence of coccidiosis in broiler farms.

Nº	Variables	Reference variable	Answered	Coccidiosis prevalence (%)		ORR	95% Confidence interval (%)	p
				Absente 43 (27.2%)	Presente 115 (72.8%)			
1	*Cloakroom*	Yes	79 (50.00%)	13 (30.20%)	66 (57.40%)	3.108	1.471-6.570	0.003
		No	79 (50.00%)	30 (69.80%)	49 (42.60%)	Referent	Referent	Referent
2	*Foot bath*	Yes	24 (35.24%)	5 (11.60%)	19 (16.50%)	1.504	0.524-4.317	0.448
		No	134 (84.80%)	38 (88.40%)	96 (83.50%)	Referent	Referent	Referent
3	*Disinfectant mat for cars*	Yes	31 (19.60%)	10 (23.30%)	21 (18.30%)	0.737	0.315-1.727	0.483
		No	127 (80.40%)	33 (76.70%)	94 (81.70%)	Referent	Referent	Referent
4	*Litter quality*	Good	12 (7.60%)	8 (18.60%)	4 (3.50%)	0.103	0.025-0.425	0.002
		Moderate	41 (25.90%)	12 (27.90%)	29 (25.20%)	0.496	0.180-1.369	0.176
		Bad	58 (36.70%)	15 (34.90%)	43 (37.40%)	0.588	0.225-1.538	0.279
		Very bad	47 (29.70%)	8 (18.60%)	39 (33.90%)	Referent	Referent	Referent
5	*Disposal of removed litter*	Next to the building	72 (45.60%)	21 (48.80%)	51 (44.30%)	0.835	0.414-1.685	0.614
		Outside the farm	86 (54.40%)	22 (51.20%)	64 (55.70%)	Referent	Referent	Referent
6	*Feed analysis*	Monthly	19 (12.00%)	6 (14.00%)	13 (11.30%)	0.722	0.235-2.215	0.569
		Once a week	38 (24.10%)	12 (27.90%)	26 (22.60%)	0.722	0.297-1.755	0.472
		Every 3 to 6 months	37 (23.40%)	9 (20.90%)	28 (24.30%)	1.037	0.405-2.656	0.940
		Never	64 (40.50%)	16 (37.20%)	48 (41.70%)	Referent	Referent	Referent
7	*Water analysis*	Monthly	19 (12.00%)	9 (20.90%)	10 (8.700%)	0.511	0.175-1.485	0.217
		Once a week	39 (24.70%)	8 (18.60%)	31 (27.00%)	1.780	0.677-4.680	0.242
		Every 3 to 6 months	46 (29.10%)	9 (20.90%)	37 (32.20%)	1.889	0.747-4.776	0.179
		Never	54 (34.20%)	17 (39.50%)	37 (32.20%)	Referent	Referent	Referent
8	*Water disinfection*	Yes	45 (28.50%)	13 (30.20%)	32 (27.80%)	0.890	0.413-1.918	0.766
		No	113 (71.50%)	30 (69.80%)	83 (72.20%)	Referent	Referent	Referent
9	*Water circuit control*	Yes	83 (52.50%)	27 (62.80%)	56 (48.70%)	0.562	0.274-1.154	0.116
		No	75 (47.5%)	16 (37.20%)	59 (51.30%)	Referent	Referent	Referent
10	*Feather condition*	Good	29 (18.40%)	4 (9.30%)	25 (21.70%)	1.838	0.430-7.852	0.411
		Moderate	44 (27.80%)	17 (39.50%)	27 (23.50%)	0.467	0.145-1.501	0.201
		Bad	63 (39.90%)	17 (39.50%)	46 (40.00%)	0.796	0.254-2.493	0.695
		Very bad	22 (13.90%)	5 (11.60%)	17 (14.80%)	Referent	Referent	Referent
11	*Legs condition*	Good	16 (10.10%)	5 (11.60%)	11 (9.60%)	0.931	0.261-3.316	0.912
		Moderate	73 (46.20%)	19 (44.20%)	54 (47.00%)	1.202	0.500-2.892	0.681
		Bad	32 (20.30%)	8 (18.60%)	24 (20.90%)	1.269	0.437-3.687	0.661
		Very bad	37 (23.40%)	11 (25.60%)	26 (22.60%)	Referent	Referent	Referent

12	*Allocation of wastewater*	Discharged directly into the environment	37 (23.40%)	8 (18.60%)	29 (25.20%)	0.582	0.222-1.525	0.271
		Collected and treated before discharge	56 (35.40%)	18 (41.90%)	38 (33.00%)	0.897	0.294-2.731	0.848
		Reused without treatment	34 (21.50%)	8 (18.60%)	26 (22.60%)	0.674	0.224-2.030	0.483
		Not applicable / No specific system in place	31 (19.60%)	9 (20.90%)	22 (19.10%)	Referent	Referent	Referent
13	*Period of veterinary control or visits*	Weekly	9 (5.70 %)	5 (11.60%)	4 (3.50%)	0.343	0.081-1.458	0.147
		Never	69 (43.70%)	14 (32.60%)	55 (47.80%)	1.684	0.725-3.910	0.226
		Every fifteen days	30 (19.00%)	9 (20.90%)	21 (10.6%)	1.000	0.372-2.685	1.000
		Critical cases	50 (31.60%)	15 (34.90%)	26 (30.6%)	Referent	Referent	Referent
14	*Hand washing when accessing the poultry area (s)*	On each occasion	45 (28.5%)	21 (48.80%)	24 (20.90%)	0.201	0.082-0.489	0.000
		Rarely	46 (29.1%)	12 (27.90%)	34 (29.60%)	0.497	0.194-1.273	0.145
		Never	67 (42.4%)	10 (23.30%)	57 (49.60%)	Referent	Referent	Referent
15	*Specific/clean clothing when accessing the poultry area(s)*	On each occasion	30 (19.00%)	18 (41.90%)	12 (10.40%)	0.121	0.042-0.346	0.000
		Rarely	76 (48.10%)	17 (39.50%)	59 (51.30%)	0.631	0.250-1.594	0.330
		Never	52 (32.90%)	8 (18.60%)	44 (38.30%)	Referent	Referent	Referent
16	*Rodent control and pest control programme*	Yes, regularly monitored and implemented by professionals	13 (8.20%)	2 (4.70%)	11 (9.60%)	2.292	0.452-11.610	0.316
		Yes, irregularly implemented by farm staff	56 (35.40%)	15 (39.90%)	41 (35.70%)	1.139	0.490-2.649	0.763
		No, but pests are occasionally controlled when noticed	38 (24.10%)	11 (25.60%)	27 (23.50%)	1.023	0.406-2.577	0.962
		No pest control measures in place	51 (32.30%)	15 (34.90%)	36 (31.30%)	Referent	Referent	Referent
17	*Isolate any poultry showing signs of disease from healthy poultry.*	On each occasion	42 (26.60%)	21 (48.80%)	21 (18.30%)	0.183	0.076-0.443	0.000
		Rarely	45 (28.50%)	11 (25.60%)	34 (29.600%)	0.567	0.222-1.444	0.234
		Never	71 (44.90%)	11 (25.60%)	60 (52.2%)	Referent	Referent	Referent
18	*The period between batches of the poultry*	Less than 3 days	39 (24.70%)	16 (37.20%)	23 (20.20%)	2.037	0.845-4.914	0.113
		Between 3 and 7 days	55 (34.80%)	14 (32.60%)	41 (35.70%)	2.319	0.762-7.059	0.139
		More than 7 days	26 (16.50%)	6 (14.00%)	20 (17.40%)	3.081	1.090-8.709	0.034
		No specific period / immediate restocking	38 (24.10%)	7 (16.30%)	31 (27.00%)	Referent	Referent	Referent

19	*Disinfect the housing between batches of poultry.*	Thorough cleaning followed by proper disinfection	61 (38.60%)	19 (44.20%)	42 (36.50%)	1.304	0.602-2.826	0.501
		Basic cleaning only, without disinfection	66 (41.80%)	17 (39.50%)	49 (42.60%)	1.629	0.526-5.038	0.397
		No cleaning or disinfection is done	23 (14.60%)	5 (11.60%)	18 (15.70%)	1.357	0.251-7.352	0.723
		Only floor cleaning, walls and equipment are ignored	8 (5.10%)	2 (4.70%)	6 (50.20%)	Referent	Referent	Referent
20	*Thoroughly clean and disinfect shared equipment before use.*	Thoroughly cleaned and disinfected every time	47 (29.70%)	11 (25.60%)	36 (31.30%)	0.722	0.272-1.917	0.514
		Occasionally cleaned, rarely disinfected	37 (23.40%)	11 (25.60%)	26 (22.60%)	0.535	0.215-1.332	0.179
		Used directly without cleaning	44 (27.80%)	16 (37.20%)	28 (24.30%)	1.528	0.472-4.941	0.479
		No equipment is shared between buildings	30 (19.00%)	5 (11.60%)	25 (21.70%)	Referent	Referent	Referent
21	*Vehicles used for transporting poultry are cleaned and disinfected after the journey.*	Yes, always after each transport	55 (34.80%)	14 (32.60%)	41 (35.70%)	0.743	0.322-1.714	0.486
		Sometimes, depending on the journey	54 (34.20%)	17 (39.50%)	37 (32.20%)	1.238	0.460-3.332	0.673
		Rarely or never	37 (23.40%)	8 (18.60%)	29 (25.20%)	0.683	0.178-2.621	0.578
		Not applicable (no transport vehicles used)	12 (7.60%)	4 (9.30%)	8 (7.00%)	Referent	Referent	Referent
22	*Disposal of dead poultry*	Collected daily and incinerated or composted	52 (32.90%)	14 (32.60%)	38 (33.00%)	0.967	0.417-2.242	0.938
		Thrown into an open pit	58 (36.70)	16 (37.20%)	42 (36.50%)	2.118	0.622-7.218	0.230
		Left in the open until natural decomposition	27 (17.10%)	4 (9.30%)	23 (20.00%)	0.491	0.170-1.417	0.188
		Buried without treatment	21 (13.30)	9 (20.90%)	12 (10.40%)	Referent	Referent	Referent
23	*The mortality record book is present*	Yes, up-to-date and regularly reviewed	50 (31.60%)	17 (39.50%)	33 (28.70%)	2.862	1.141-7.180	0.025
		Yes, but inconsistently filled	59 (37.30%)	9 (20.90%)	50 (43.50%)	0.987	0.397-2.455	0.978
		No, but mortalities are discussed verbally.	35 (22.20%)	12 (27.90%)	23 (20.00%)	0.927	0.268-3.204	0.905
		No record of mortality kept	14 (8.90%)	5 (11.60%)	9 (7.80%)	Referent	Referent	Referent

24	*Mortality rate of the last depopulated herd (%)*	0 – 2 %	24 (15.20%)	7 (29.20%)	17 (70.80%)	1.318	0.425-4.083	0.633
		> 2 – 5 %	42 (26.60%)	10 (23.80%)	32 (76.20%)	0.711	0.225-2.250	0.562
		> 5 – 10 %	30 (19.00%)	11 (36.70%)	19 (63.30%)	1.235	0.345-4.426	0.746
		> 10 – 15 %	24 (15.20%)	6 (25.00%)	18 (75.00%)	1.327	0.418-4.211	0.631
		> 15 %	38 (24.10%)	9 (23.70%)	29 (76.30%)	Referent	Referent	Referent
25	*Mortality rate of the last depopulated herd due to disease (%)*	0%	34 (21.50%)	8 (18.60%)	26 (22.60%)	0.852	0.309-2.351	0.757
		> 0 – 2 %	49 (31.00%)	13 (30.20%)	36 (31.30%)	0.923	0.200-4.255	0.918
		> 2 – 5 %	12 (7.60%)	3 (7.00%)	9 (7.80%)	1.846	0.339-10.043	0.478
		> 5 – 8 %	14 (8.90%)	2 (4.70%)	12 (10.40%)	1.046	0.293-3.739	0.945
		> 8 – 12 %	22 (13.90%)	5 (11.60%)	17 (14.80%)	0.385	0.128-1.152	0.088
		> 12 %	27 (17.10%)	12 (27.90%)	15 (13.00%)	Referent	Referent	Referent
26	*Drugs*	Antimicrobials	47 (29.70%)	13 (30.20%)	34 (29.60%)	0.945	0.403-2.215	0.896
		Antihelminthics	59 (37.3%)	17 (39.50%)	42 (36.50%)	1.311	0.456-3.773	0.616
		Anticoccidial	31 (19.60%)	7 (16.30%)	24 (20.90%)	0.956	0.305-2.996	0.938
		Traditional herbs	21 (13.30%)	6 (14.00%)	15 (13.00%)	Referent	Referent	Referent
27	*Drug choice based on*	Personal experience	56 (35.4%)	10 (23.30%)	46 (40.00%)	1.150	0.348-3.799	0.819
		Drug seller's advice	77 (48.7%)	28 (65.10%)	49 (42.60%)	0.438	0.148-1.294	0.135
		Veterinary advice	25 (15.8%)	5 (11.6%)	20 (17.40%)	Referent	Referent	Referent
28	*Anti-coccidial treatment*	Monensin	13 (8.20%)	3 (7.0%)	10 (8.7%)	1.020	0.200-5.209	0.981
		Salinomycin	22 (13.90%)	5 (11.6%)	17 (14.8%	0.300	0.057-1.581	0.156
		Narinsin	14 (8.90%)	7 (16.3%)	7 (6.1%)	0.900	0.143-5.646	0.910
		Lasalocid	12 (7.60%)	3 (7.0%)	9 (7.8%)	1.200	0.094-15.260	0.888
		Maduramicin	5 (3.20%)	1 (2.3%)	4 (3.5%)	0.100	0.007-1.353	0.083
		Semduramicin	4 (2.50%)	3 (7.0%)	1 (0.9%)	0.450	0.050-4.085	0.478
		Narasin + Nicarbazin	5 (3.20%)	2 (4.7%)	3 (2.6%)	0.450	0.050-4.085	0.478
		Nicarbazin	5 (3.20%)	2 (4.7%)	3 (2.6%)	48.46 x 10^{7}	0.000	0.999
		Robenidine	5 (3.20%)	0 (0.0%)	5 (4.3%)	1.200	0.094-15.260	0.888
		Clopidol	5 (3.20%)	1 (2.3%)	4 (3.5%)	1.200	0.094-15.260	0.888
		Halofugione	5 (3.20%)	1 (2.3%)	4 (3.5%)	48.46 x 10^{7}	0.000	0.999
		Toltrazuril	5 (3.20%)	0 (0.0%)	5 (4.3%)	0.900	0.067-12.179	0.937
		Diclazuril	4 (2.50%)	1 (2.3%)	3 (2.6%)	0.200	0.022-1.816	0.153
		Sulfadimethoxine	5 (3.20%)	3 (7.0%)	2 (1.7%)	48.46 x 10^{7}	0.000	0.999
		Sulfamerazine	5 (3.20%)	0 (0.0%)	5 (4.3%)	0.450	0.050-4.085	0.478
		Sulfaguanidine	5 (3.20%)	2 (4.7%)	3 (2.6%)	1.200	0.094-15.260	0.888
		Sulfaquinoxaline	5 (3.20%)	1 (2.3%)	4 (3.5%)	48.46 x 10^{7}	0.000	0.999

		Trimethoprim + Sulfadimethoxine	5 (3.20%)	0 (0.0%)	5 (4.3%)	48.46 x 10^7	0.000	0.999
		Ormetoprim + Sulfadimethoxine	4 (2.50%)	0 (0.0%)	4 (3.5%)	1.200	0.094-15.260	0.888
		Amprolium	5 (3.20%)	1 (2.3%)	4 (3.5%)	1.200	0.094-15.260	0.888
		Diaveridine	5 (3.20%)	1 (2.3%)	4 (3.5%)	0.075	0.006-0.954	0.046
		Framycetin	5 (3.20%)	4 (9.3%)	1 (0.9%)	48.46 x 10^7	0.000	0.999
		Hydroxyquinolines	5 (3.20%)	0 (0.0%)	5 (4.3%)	0.450	0.050-4.085	0.478
		Roxarsone	5 (3.20%)	2 (4.7%)	3 (2.6%)	Referent	Referent	Referent
29	*History of past Coccidiosis outbreaks*	Yes, within the last 6 months	54 (34.20%)	18 (41.90%)	36 (31.30%)	1.500	0.632-3.560	0.358
		Yes, but more than 6 months ago	48 (30.40%)	12 (27.90%)	36 (31.30%)	1.875	0.715-4.914	0.201
		Never	38 (24.10%)	8 (18.60%)	30 (26.10%)	1.300	0.401-4.215	0.662
		I do not know / No records available	18 (11.40%)	5 (11.60%)	13 (11.30%)	Referent	Referent	Referent

In commercial poultry production, we consider that coccidiosis impairs intestinal function and reduces performance (ADG, FCR), and is primarily controlled through the use of anticoccidials and/or vaccination with live coccidia, points consistently reported across systematic reviews and vaccine/control overviews (Ahmad et al., 2024; Coe et al., 2022; Freitas et al., 2023; Soutter et al., 2020). Authoritative, peer-reviewed reviews establish these principles.

The fourth category (D, Table 16), focused on production parameters, revealed that only one practice was statistically significantly associated with a higher prevalence of coccidiosis: weekly weighing (OR = 2.875; 95% CI, 1.123–7.361; p = 0.028). All other variables (daily/weekly data recording, feed-intake tracking, feed-conversion calculation, availability/estimation of average daily gain, slaughter age, and slaughter weight) did not show a significant association ($p > 0.05$). Biologically, repeated handling is known to induce stress responses in chickens**;** corticosterone alters immune function and can increase susceptibility to enteric disease, providing a plausible pathway linking weighing frequency to higher detected prevalence (Abo-Al-Ela et al., 2021; Graat et al., 1997; Ismail et al., 2018; J. Yang et al., 2015; Zaytsoff et al., 2022). We also recognise potential surveillance/detection bias: farms that are more health-attentive may weigh more often and detect more cases without weighing being a direct causal driver (Bartlett et al., 2010; O'Connor et al., 2016; Vergne & Drewe, 2019).

When examining performance parameters (FCR, ADG), we note that they reflect downstream consequences of intestinal damage. Under field prophylaxis (anticoccidials/vaccination), performance shifts may be attenuated or time-lagged relative to infection dynamics, which helps explain the lack of association with point prevalence in our dataset, even though the literature shows that *Eimeria* infection reduces growth and worsens feed efficiency (Choi et al., 2023; Freitas et al., 2023; Sharma et al., 2024; Taylor et al., 2022). For management, we avoid inferring a direct causal link. Instead, we recommend low-stress weighing (small, representative subsamples; gentle restraint; appropriate equipment; trained staff) and maintaining a robust prophylaxis programme (anticoccidials and/or live vaccination) integrated with structured clinical and post-mortem surveillance, the enduring cornerstones of control in commercial operations (Ahmad et al., 2023; Ismail et al., 2018; Nguyen et al., 2024; Soutter et al., 2020; Zaheer et al., 2022).

Finally, to gain further insight, we will test whether the “weekly weighing” signal persists after explicit adjustment for available health/management variables (e.g., prophylaxis protocols, vaccination status), following good epidemiological practice for confounding and bias control in observational studies (Berezowski et al., 2019; Haine et al., 2018; O’Connor et al., 2016).

Tableau 16. Association between farm production parameters and coccidiosis prevalence in broiler farms (Category D).

Nº	**Variables**	**Reference variable**	**Answered**	**Coccidiosis prevalence N (%)**		**ORR**	**95% Confidence interval (%)**	**p**
				Absente 43 (27.2%)	Absente 43 (27.2%)			
1	*Daily data registration*	Yes (daily)	56 (35.44%)	18 (32.1%)	38 (67.9%)	2.084	0.859-5.058	0.104
		Yes (weekly)	54 (34.18%)	10 (18.5%)	44 (81.5%)	1.042	0.455-2.387	0.922
		No	48 (30.38%)	15 (31.3%)	33 (68.8%)	Referent	Referent	Referent
2	*Feed intake*	Yes (measured)	56 (35.44%)	18 (32.1%)	38 (67.9%)	0.704	0.297-1.665	0.424
		No	54 (34.18%)	10 (18.5%)	44 (81.5%)	1.051	0.426-2.594	0.914
		Rough estimation	48 (30.38%)	15 (31.3%)	33 (68.8%)	Referent	Referent	Referent
3	*Water intake*	Yes (monitored daily)	52 (32.91%)	12 (23.1%)	40 (76.9%)	0.800	0.333-1.922	0.618
		No	55 (34.81%)	15 (27.3%)	40 (72.7%)	0.656	0.274-1.575	0.346
		Unknown	51 (32.28%)	16 (31.4%)	35 (68.6%)	Referent	Referent	Referent
4	*Bodyweight*	Weekly weighing	54 (34.18%)	18 (33.3%)	36 (66.7%)	2.875	1.123-7.361	0.028
		Only at slaughter	54 (34.18%)	8 (14.8%)	46 (85.2%)	0.971	0.430-2.190	0.943
		Not recorded	50 (31.65%)	17 (34.0%)	33 (66.0%)	Referent	Referent	Referent
5	*Feed conversion rate*	Calculated regularly	53 (33.54%)	12 (22.6%)	41 (77.4%)	0.878	0.357-2.157	0.777
		Not calculated	52 (32.91%)	13 (25.0%)	39 (75.0%)	0.569	0.241-1.343	0.198
		Unknown	53 (33.54%)	18 (34.0%)	35 (66.0%)	Referent	Referent	Referent
6	*Daily gain (gram/day)*	Yes (data available)	55 (34.81%)	14 (25.5%)	41 (74.5%)	0.951	0.402-2.250	0.909
		No	53 (33.54%)	14 (26.4%)	39 (73.6%)	0.797	0.338-1.877	0.603
		Estimated	50 (31.65%)	15 (30.0%)	35 (70.0%)	Referent	Referent	Referent
7	*Average age at slaughter (days)*	Less than 35 days	45 (28.48%)	11 (24.4%)	34 (75.6%)	0.878	0.352-2.193	0.781
		35–42 days	52 (32.91%)	14 (26.9%)	38 (73.1%)	0.773	0.322-1.853	0.564
		More than 42 days	61 (38.61%)	18 (29.5%)	43 (70.5%)	Referent	Referent	Referent
8	*Average weight at slaughter (kgs)*	Less than 1.5 kg	49 (31.01%)	14 (28.6%)	35 (71.4%)	0.988	0.428-2.284	0.978
		1.5 – 2.5 kg	59 (37.34%)	17 (28.8%)	42 (71.2%)	1.267	0.516-3.107	0.606
		More than 2.5 kg	50 (31.65%)	12 (24.0%)	38 (76.0%)	Referent	Referent	Referent

2.4.Overall Biosecurity Scores and Biosecurity Status

To assess the impact of overall biosecurity on the prevalence of coccidiosis, all 158 surveyed broiler farms were classified according to a standardised biosecurity scoring system (Figure 16). Each farm was evaluated across four categories: structural characteristics, external isolation, internal biosecurity, and production monitoring, with a cumulative score normalised to a 100-point scale. Farms with a total score of 70 or above were classified as having good biosecurity, while those scoring below 70 were deemed to have poor biosecurity. Among the 43 farms where coccidiosis was absent (27.2% of the total), 22 farms (51.2%) had good biosecurity, and 21 farms (48.8%) had poor biosecurity. In contrast, among the 115 farms with coccidiosis (72.8% of the total), none (0%) met the criteria for good biosecurity, and all 115 farms (100%) fell into the poor biosecurity category. This apparent divergence indicates a strong association between biosecurity performance and disease presence. The fact that all farms classified as having good biosecurity were free of coccidiosis suggests that exceeding a minimum threshold of sanitary practices may provide robust protection against infection. These findings underline the predictive value of structured biosecurity assessment tools and emphasise that enhancing daily practices, particularly those related to hygiene, access control, and monitoring, can effectively mitigate the risk of endemic protozoan infections in intensive poultry systems.

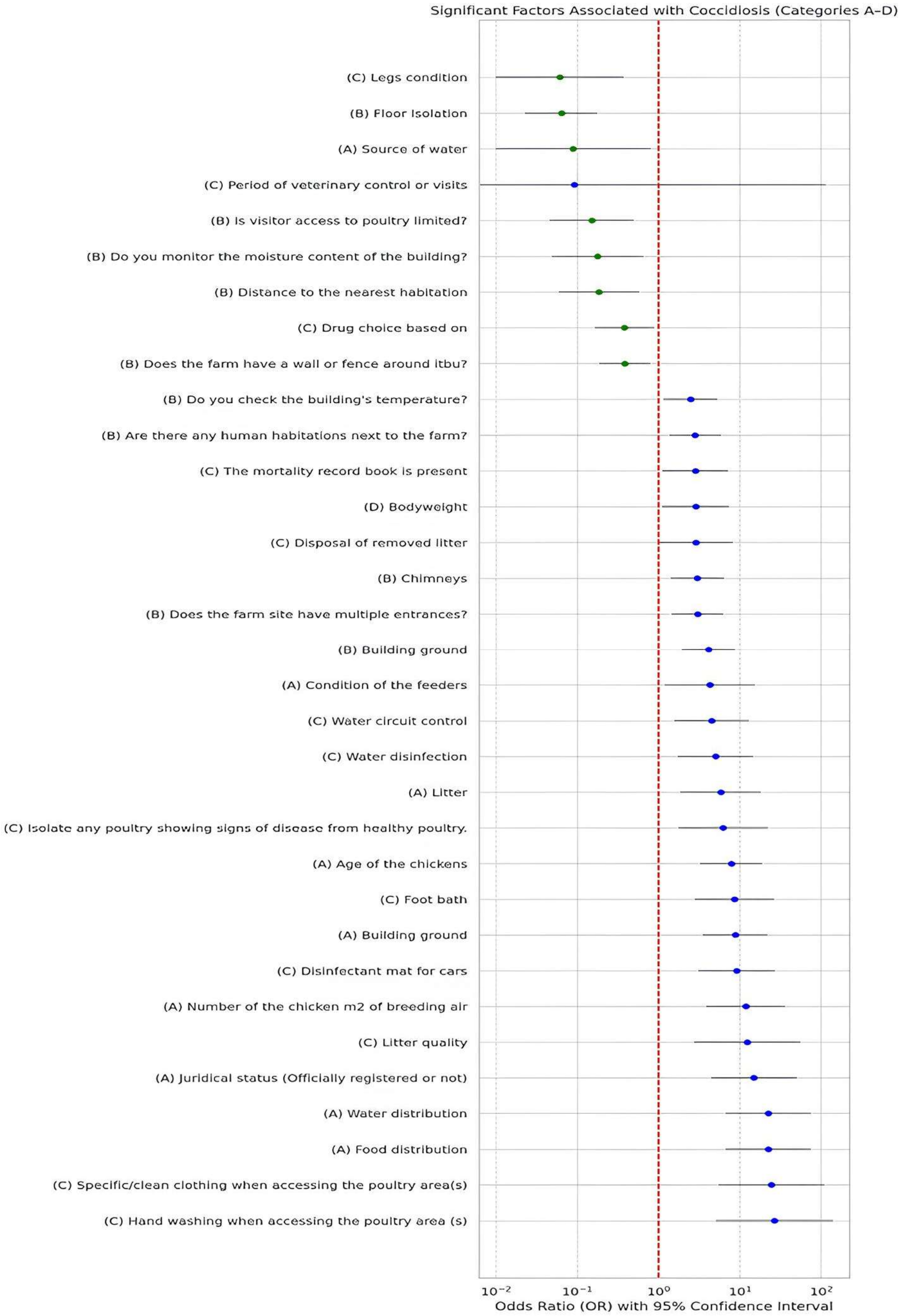

Figure 16. Forest plot of significant risk and protective factors associated with coccidiosis in broiler farms.

PARTII.

Phytochemical and pharmacological properties of the Algerian *Mentha aquatica* extracts

1. Materials and methods

1.1. Chemicals

All chemicals used in this study were of analytical grade. Cyanidin 3-O-glucoside (Cy3Gluc), cyanidin 3-O-rutinoside (Cy3Rut), pelargonidin 3-O-rutinoside (Pg3Rut) and peonidin 3-O-rutinoside (Pn3Rut) were from Extrasynthese (Genay, France). β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitrotetrazolium blue chloride (NBT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and α-glucosidase from Saccharomyces cerevisiae (type I, lyophilised powder) were obtained from Sigma-Aldrich (St. Louis, MO, USA). N-(1-Naphthyl) ethylenediamine dihydrochloride, sulfanilamide, 4-nitrophenyl-alpha-D-glucopyranoside, and sodium nitroprusside dihydrate (SNP) were obtained from Alfa Aesar (Karlsruhe, Trypsinethylenediaminetetraacetic acid (trypsin-EDTA) solution, 3-(4,5-dimethylthiazol-2-yl)-2,5- -2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and sodium nitroprusside dihydrate (SNP) were obtained from Alfa Aesar (Karlsruhe, Germany). Other phenolics and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and acetonitrile for HPLC (purity ≥ 99.9%) were from Fisher Chemical (Glenfield, Leicestershire, UK). Cell lines were from the American Type Culture Collection (ATCC, Manassas, VA, USA). Water was deionised using a Milli-Q water purification system (Millipore Ibérica, S.A.U., Madrid, Spain).

1.2. Plant material

The entire flowering plant *Mentha aquatica* was collected in August in the Medjana commune, Oued Sayed, Wilaya of Bordj Bou Arreridj (36°08′N 4°40′E). The plant taxonomist identified a specimen, Prof. Cherchour A. (Faculty of Medicine / Department of Pharmacy, University of Badji Mokhtar-Annaba, Algeria) (Figure 17). Voucher specimens (No. MA-1) were deposited in the Herbarium of the laboratory of VALCORE (Valorisation and Conservation of Biological Resources, Biology Department, Faculty of Science, University M'Hamed Bougara, Boumerdes, Algeria).

Figure 17. Water Mint (*Mentha aquatica*) in Bloom: Morphology and Ecosystem (INaturalist, 2025).

1.3. Infusion and hydroethanolic extracts preparation

The aerial parts of *Mentha aquatica* were carefully washed and dried at room temperature, in the dark, for 15 days. Then, the plants were powdered (mean particle size < 910 µm) and used to prepare infusions and hydroethanolic extracts.

1.3.1. Infusion

Infusions (INF) were extracted according to the method previously reported (Jesus et al., 2019a). One gram of the dried material was boiled in water for 5 minutes, mimicking the method used for its regular preparation for human consumption. The extract obtained was filtered through a Büchner funnel, frozen, lyophilised and stored in a desiccator in the dark until analysis. Extractions were performed in triplicate.

1.3.2. Hydroethanolic extract

Hydroethanolic extract (HE) was prepared based on the method described by (Teixeira and Silva, 2013). The dried material (10g) was extracted with 10 ml of a mixture of ethanol and water (70:30). The preparation was subjected to three extraction steps: a first sonication (30 min), then a maceration (2 hours with shaking) and finally, a second sonication (30 min) at room temperature. The extracts obtained were filtered through a Büchner funnel. The preparation was then evaporated under reduced pressure, freeze-dried, and stored at -20 °C until further analysis. Extractions were performed in triplicate.

1.4. Total phenolic compounds determination

The total phenolic content in the INF and HE of *Mentha aquatica* was measured using a colourimetric method, with modifications based on the procedure in reference (Nunes et al., 2021). In short, 50 µL of methanolic preparation (concentration: 2.5 mg/mL) was combined with 450 µL of distilled water and 2.5 mL of Folin-Ciocalteu reagent (0.2 N). After incubating for 5 minutes at room temperature, 2 mL of a saturated sodium carbonate solution was added, and the total volume was adjusted to 5 mL with distilled water. The mixture was then placed in a 30 °C water bath for 90 minutes in the dark. Absorbance was measured at 765 nm. Gallic acid was used for the standard curve (range: 50–500 mg/mL; equation: $y = 0.0011x + 0.0057$, $R^2 = 0.9958$, Table 16). Each measurement was conducted in triplicate, and the results were reported as mean values ± standard deviations, expressed as the molar concentration of gallic acid.

1.5. Profiling of chemical compounds by High-Performance Liquid Chromatography with Electrospray Ionisation and Tandem Mass Spectrometry Detection (HPLC-ESI-MS-MS)

The analysis was conducted using the method described by Kerboua et al. (Kerboua et al., 2021) on an Orbitrap Thermo q-Exactive mass spectrometer, coupled to a Vanquish HPLC. A Kinetex XB-C18 (Phenomenex) with a particle size of 2.6 microns, 100 mm of length, and a diameter of 2.1 mm was used as a column. The mobile phases were 0.1% formic aqueous solution (A) and acetonitrile (B). The gradient program (time (min), % B) was as follows: (0.00, 50); (20.00, 100); (25.00, 100); (26.00, 50). The flow rate was 0.200 mL min^{-1} and the injection volume was 10 µL. The ionisation electrospray in positive mode was used. The following analysis parameters were used: electrospray voltage, −3.8 kV; sheath gas flow rate, 30; auxiliary gas unit flow rate, 10; drying gas temperature, 310 °C; capillary temperature, 320 °C; S-lens and RF level, 55. The acquisition was performed in a mass range from 100 to 1000 a.m.u. An auto MS2 program was used with a fragmentation voltage of 30.

1.6. Profiling and quantification of individual phenolic compounds by High Performance Liquid Chromatography with Diode Array Detector (HPLC-DAD) and High-Performance Liquid Chromatography with Electrospray Ionisation and Tandem Mass Spectrometry Detection (HPLC-ESI-MSn)

The phenolic compounds identification was performed in an Agilent HPLC 1100 series model equipped with a photodiode array detector (model G1315B), a mass detector in series (Agilent Technologies, Waldbronn, Germany), a binary pump (model G1312A), a de-gasser (model G1322A) and an autosampler (model G1313A), based on a method described by Gonçalves et al. (Gonçalves et al., 2021a). Injections (20 μL) of plant extracts were performed in triplicate. The mass detector was an ion trap spectrometer (model G2445A) with an electrospray ionisation interface. It was controlled by LC/MS software (Esquire Control Ver. 6.1, Build No. 534.1, Bruker Daltonik GmbH, Bremen, Germany). A Nucleosil® 100–5 C18 column (25.0 cm × 0.46 cm; 5 μm particle size Waters; Macherey-Nagel, Düren, Germany) was used, and the mobile phase was composed of two solvents: eluent A consisted of water/formic acid (99:1, v/v), and eluent B of acetonitrile. The solvent system started at 8% B and reached 15% B at 25 min, 22% at 55 min, and 40% at 60 min, with a 5 min wash-out period, after which it returned to its initial conditions. Mass spectra were acquired over the m/z range 100-1200. The MS parameters were set as follows: the capillary temperature was 350 °C, the capillary voltage was set at 4 kV, the nebuliser pressure was 65.0 psi, and the nitrogen flow rate was 11 L/min. The flow rate was 0.8 mL/min during the run, and all gradients were linear. Collision-induced fragmentation experiments were performed in an ion trap using helium as collision gas, with voltage ramping cycles from 0.3 to 2 V. Mass spectrometry data were acquired in a negative ionisation mode for non-coloured phenolics. MSn was carried out automatically on more abundant fragment ions in MS(n-1). ChemStation controlled HPLC system for LC 3D Systems software Rev. B.01.03- SR2 (204) (Agilent Technologies Spain S.L., Madrid, Spain). Phenolic compounds were tentatively identified based on their elution order, retention times, and ultraviolet-visible and mass spectra features as compared to authentic standards analysed under the same conditions (Table 16) and data available in the literature (Gonçalves et al., 2021a, 2018; Jesus et al., 2019b; Martins et al., 2024).

Table 16. Calibration curves of external standards (concentrations range of 1.5–100 µg/mL) used in the quantification of phenolic compounds of *Mentha aquatica* extracts.

Phenolic Compound	Calibration Curve Equation		R^2
Syringic acid	$y = 9077x - 8779.4$	Also used to quantify Salvianolic acid B, B/E, and similar compounds	0.999
Rosmarinic acid	$y = 3869.8x - 12610$	Used to quantify galloyl derivatives and *p*-hydroxybenzoic acid derivatives	0.999
p-Hydroxybenzoic acid (4-hydroxybenzoic acid)	$y = 24.8x - 4376.7$	Used to quantify naringenin derivatives	0.999
Hesperidin	$y = 16.884x - 22.233$	Used to quantify luteolin and apigenin derivatives	0.999
Luteolin	$y = 2726.8x - 4670$	Used to quantify luteolin derivatives	0.999
Quinic acid	$y = 25.540x + 124.292$	Also used to quantify ferulic acid derivatives	0.999
Caffeic acid	$y = 74.093x + 53.692$	Used to quantify 4-CQA and sinapoyl hexoside	0.999
Clorogénico	$y = 47.383x - 12.104$	Used to quantify kaempferol derivatives	0.999
Kaempferol 3-O-glucoside	$y = 13.085x + 13.444$	Used to quantify ferulic derivatives	0.999
Ferulic acid	$y = 21.172x - 9478.7$	Used to quantify p-coumaroyl hexoside	0.999
trans-Cinnamic acid (m-Coumaric acid)	$y = 17.721x + 12.1678$	Also used to quantify Salvianolic acid B and B/E, and similar	0.999

1.7. Antioxidant activity

1.7.1. 2.2-Diphenyl-1-Picrylhydrazyl Radical (DPPH$^\bullet$)-Scavenging Activity

The effect of *Mentha aquatica* INF and HE extracts on the DPPH$^\bullet$ was estimated by a microplate spectrophotometer (Bio-Rad Laboratories; Hercules, CA, USA) method. All sample dilutions were previously prepared in methanol. For each extract, seven different dilutions were prepared. 25 µL of each concentration was placed in the 96-well microplate, followed by the addition of 200 µL of methanolic DPPH (150 mM). Then, the plates were incubated in the dark at room temperature for 30 minutes. After that, the absorbance of samples was noted at 515 nm against a blank containing only DPPH solution. The radical scavenging potential of the samples was compared with Ascorbic acid solutions of varying strengths. All experiments were performed in triplicate. The results were expressed as half maximal inhibitory concentration (IC_{50}) values (µg/mL). IC_{50} corresponding to the concentration of the sample required to reduce the absorbance of the DPPH solution by 50% was determined from the inhibition curves.

1.7.2. Nitric oxide ($^\bullet$NO) radical assay

$^\bullet$NO scavenging capacity was determined by measuring the accumulation of nitric oxide generated by sodium nitroprusside (SNP) in the preparations with or without the extracts or ascorbic acid (positive control) using the Griess reagent (Silva and Teixeira, 2015). The two extracts were prepared and serially diluted in phosphate buffer (100 mM, pH 7.4) to achieve concentrations ranging from 100 to 1000 µg/mL. The absorbances were read at 562 nm. The experiments of each extract were performed in triplicate, and the results were expressed as IC_{50} values (µg/mL) calculated by plotting the percentage of inhibition against various concentrations of the extract and determining the concentration required to inhibit 50% of the activity.

1.7.3. Superoxide radical (O2$^{\bullet-}$) assay

The $O_2^{\bullet-}$ scavenging capacity was determined as described by Gonçalves et al. (Gonçalves et al., 2021b). *Mentha aquatica* extracts were tested at concentrations ranging from 100 to 1000 µg/mL. The reaction was monitored at 560 nm for 2 min, at room temperature. The results were expressed as IC_{50} values (µg/mL), identifying the concentration required to achieve 50% inhibition.

1.8. *In vitro* $ROO^{\bullet -}$ induced oxidative damage in human erythrocytes

The *in vitro* $ROO^{\bullet -}$ induced oxidative damage in human erythrocytes was evaluated according to the Gonçalves et al. study (Gonçalves et al., 2020). The extracts were dissolved in PBS (1 mg/mL), and six concentrations were prepared. Four experiments were triplicated per microplate, and results were expressed as IC_{50} values (µg/mL).

1.8.1. Isolation of human erythrocytes

Venous human blood was collected from randomised patients in the Centro Hospitalar of Cova da Beira (Covilhã) by antecubital venipuncture into K3EDTA vacuum tubes. Erythrocytes were isolated based on the procedure described by Gonçalves et al. (Gonçalves et al., 2021b, 2020)

1.8.2. Inhibition of haemoglobin oxidation

The inhibition of haemoglobin oxidation (Hbox) was determined by monitoring the lyophilised extracts' ability to prevent methemoglobin formation (Chisté et al., 2014). The decomposition of AAPH (dissolved in PBS) is achieved by the water-bath temperature (37ºC), which reacts with oxyhemoglobin, forming methemoglobin. Six dilutions of each extract were prepared with PBS. The sample solution was mixed with the erythrocyte solution to create the reaction mixture. Controls and blanks were performed by replacing the sample with PBS. The reaction mixtures were incubated in a water bath at 37°C for 30 minutes, under slow agitation (50 rpm). After incubation, AAPH was added to the mixture (except in the blank), and the mixture was further incubated under the same conditions described above for 4 hours. The entire volume was transferred to a conical Eppendorf tube and centrifuged. The supernatant (300 µL) was transferred to a 96-well plate, and absorbance was measured at 630 nm. The results were expressed as IC_{50} values (µg/mL). Five experiments were performed in duplicate. Quercetin was used as a positive control.

1.9. Inhibition of lipid peroxidation

Lipid peroxidation (LPOX) in erythrocytes was indirectly assessed by the formation of thiobarbituric acid-reactive substances (TBARS) (Chisté et al., 2014). Six different concentrations of infusion and hydroethanolic extracts were dissolved in PBS and mixed with human cell suspension at 37 ºC for 30 min with slow agitation (≈ 50 rpm). After incubation, tert-butyl hydroperoxide (tBHP) was added to the media, which was then further incubated at 37 °C under slow agitation for 30 min. After incubation, the entire contents were collected and transferred to conical Eppendorf tubes, and trichloroacetic acid (TCA) was added to promote protein precipitation, followed by centrifugation. The reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) to form TBARS was achieved by inserting the supernatant in a 2 mL-conical test tube (with screw cap), followed by the addition of TBA 1% (w/v). The resulting mixture was heated for 15 min at 100 ºC in a water bath. Finally, the test tubes were cooled at room temperature, and the absorbance was measured at 532 nm. The final results were expressed as IC_{50} value (µg/mL). Five experiments were performed in duplicate. Quercetin was used as a positive control.

1.10. α-Glucosidase inhibition assay

The α-glucosidase inhibition assay was carried out using a previously established protocol (Silva and Teixeira, 2015). A total of seven different concentrations of the extract were prepared. For each well, 150 µL of potassium phosphate buffer (19 mM, pH 7.4) was mixed with 50 µL of the sample dissolved in the same buffer and 100 µL of 4-nitrophenyl-α-D-glucopyranoside (PNP-G) as the substrate. The control contained only phosphate buffer and PNP-G. The reaction began by adding 25 µL of α-glucosidase enzyme to each well, followed by a 10-minute incubation at 37°C. After incubation, the absorbance of the released 4-nitrophenol was measured at 405 nm to assess enzyme activity. Acarbose was used as a positive control. Each experiment was conducted in triplicate, with three independent replicates performed to ensure accuracy.

1.11. Cell Viability Assays

1.11.1. Cell Culture

The N27 and NHDF cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in 75 cm^2 culture flasks at 37 °C in a humidified air incubator with 5% CO_2 (Brito et al., 2023). N27 cells were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS) and 1% antibiotic mixture of 10,000 U/mL penicillin G and 100 mg/mL streptomycin (Sp). NHDF cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, and 1% of the antibiotic/antimycotic Ab. The medium was renewed every 24 hours (N27 cells) or every 2 days (NHDF cells) until the cells reached near confluence. When cells reach approximately 90–95% confluence, they are gently detached by trypsinisation (trypsin-EDTA solution: 0.125 g/L of trypsin and 0.02 g/L of EDTA). Before each experiment, viable cells were counted using a Neubauer chamber by a trypan blue exclusion assay, then diluted to an appropriate concentration in the appropriate complete cell culture medium. During the assays, NHDF cells were used between passages 22 and 25, and for N27 cells, between passages 10 and 14.

1.11.2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay

After reaching a confluence state, cells were trypsinised and counted by the trypan-blue exclusion assay and then seeded with an initial density of 2×10^4 cells/mL (for NHDF) and 1×10^5 cells/mL (for N27) in 96-well culture plates (Nunc, Apogent, Denmark) and left to adhere and grow for 48 h. Subsequently, the medium was removed, and the cells were treated with INF and HE solutions (5-1200 μg/mL) in complete culture medium for 24, 48, and 72 hours. Untreated cells were used as the negative control. Each experiment was performed in quadruplicate and repeated at least two times independently. The *in vitro* antiproliferative effects were evaluated by the MTT assay. After the incubation period, the medium was removed, and the cells were washed with 100 μL of phosphate-buffered saline (PBS). PBS contains 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4 in deionised water, with a pH adjusted to 7.4. Then, 100 μL of the MTT solution (5 mg/mL) was prepared in the appropriate serum-free medium and added to each well, followed by incubation for four hours at 37 °C.

The MTT-containing medium was removed, and the formazan crystals were dissolved in DMSO. Then, the absorbance was measured at 570 nm using an xMarkTM microplate spectrophotometer (BIO-RAD Laboratories). Cell proliferation was expressed as a percentage of the relative absorbance measured in the treated wells compared with the control wells.

1.12. Statistical Analysis

GraphPad Prism 9 (GraphPad, La Jolla, CA, USA) was used for statistical analysis, including a one-way analysis of variance (ANOVA) followed by a Tukey multiple-comparison test. A Student's t-test was used to compare two distinct samples, with a significance level of $p < 0.05$. Differences between groups were considered statistically significant at a p-value lower than 0.05 ($p < 0.05$).

2. Results and discussion

2.1. The yield and the total Phenolic Content of *Mentha aquatica* Extracts

Yield and total phenolic content (TPC) are crucial metrics that provide complementary insights into the efficiency and quality of extraction methods for *Mentha aquatica* (Table 17). A higher yield indicates a more efficient extraction process, suggesting that a larger quantity of the plant material has been successfully converted into extract. This is particularly important for ensuring the economic viability of the extraction process in large-scale applications. TPC, on the other hand, is a key indicator of the extract's quality. Phenolic compounds are well-known for their antioxidant properties, and a higher TPC typically correlates with greater potential health benefits (Diep et al., 2020). These compounds play a significant role in neutralising free radicals, thereby contributing to the plant extract's therapeutic properties. Therefore, an extraction method that yields a high TPC along with a good yield is considered superior, as it maximises both the quantity and quality of the extract.

Table 17. Yield extraction (%) and total phenolic contents (mg GAE/g DW extract) of infusion (INF) and hydroethanolic extracts of *Mentha aquatica*.

Sample extract	Yield (%)	TPC (mg GAE/g DW)
INF	16,210 ± 6,690	170.290 ± 8.505
HE	12,113 ± 0,021	177.078 ± 4.842

In our study, the INF has a higher extraction yield of 16.210 ± 6.690%, compared to the hydroethanolic extract HE, which has a yield of 12.113 ± 0.021% (Table 17). This indicates that the infusion process is more effective at extracting a larger overall amount of material from the plant, likely due to the high solubility of many plant constituents in water. However, the higher yield does not necessarily correlate with a significantly higher concentration of specific bioactive compounds, such as phenolics. When we look at the total phenolic content, HE showed a slightly higher value (177.078 ± 4.842 mg GAE/g DW) compared to INF (170.290 ± 8.505 mg GAE/g DW), indicating that the hydroethanolic extraction process has a marginally better capacity to extract phenolic compounds than simple infusion (Table 17).

However, the differences are not statistically significant. When comparing our results to other studies, the TPC values obtained for both HE and INF are much higher than those reported by Fidan et al. (Fidan et al., 2023) and Benabdallah et al. (Benabdallah et al., 2016), where TPC values for ethanolic and hydromethanolic extracts were 43.36 mg GAE/g and 43.21 ± 1.09 mg GAE/g DW, respectively (Benabdallah et al., 2016; Fidan et al., 2023). Interestingly, Pereira (Pereira et al., 2019b) reported a significantly higher TPC of 307 ± 29 µg/mg for a hydroethanolic extract of *Mentha aquatica*, highlighting the substantial variability in phenolic content depending on the specific extraction conditions, such as solvent concentration, plant material, and extraction protocol (Pereira et al., 2019b). This difference underscores the effectiveness of hydroethanolic extraction for optimising phenolic recovery, while also illustrating the variability that can arise across different experimental setups. Additionally, the study by Silva et al. (Silva et al., 2023) found that for *Mentha spicata*, both the infusion and hydroethanolic extract had relatively close TPC values (38.79 mg/g and 57.92 mg/g, respectively). In contrast, the decoction showed the highest TPC (77.20 mg/g). This suggests that decoction can extract more phenolic compounds than either infusion or hydroethanolic extraction, regardless of the plant used (Silva et al., 2023). Similarly, Tourabi et al. (Tourabi et al., 2023) demonstrated that for *Mentha longifolia*, aqueous extracts had the lowest TPC (17.90 ± 0.49 mg GAE/g DW), while hydroethanolic extracts exhibited the highest TPC (23.52 ± 0.14 mg GAE/g DW) (Tourabi et al., 2023).

2.2. Phytochemical Profiling by HPLC-ESI-MS-MS

The chemical analysis of *Mentha aquatica* extracts reveals a diverse array of bioactive compounds with significant potential for pharmacological applications. A total of 136 compounds were detected in both the infusion (INF) and hydroethanolic extract (HE) using HPLC/ESI/MS/MS. The identification of the key compounds was achieved by analysing their MS/MS spectra (Figures 2 and 3), supported by our system's resources and cross-referencing with published studies (Tables 18 and 19).

2.2.1. Infusion preparation

The compounds identified in INF comprise 62 diverse chemical compounds, including nitrogen-containing compounds, phenolics, glycosides, and various esters, reflecting the plant's rich chemical diversity and significant bioactive potential (Table 18, Figure 18). Of particular interest are 10 biologically active compounds (Table 19, C1 to C10): alkaloids (elaeokanine C), two coumarins (5-hydroxycoumarin, 7-methoxycoumarin), two flavonoids (kaempferol 3-O-rutinoside and creoside I), one ascorbic acid derivative (6-O-acetylascorbic acid), one aromatic ester (vanillyl nonanoate), one benzoquinone derivative (5-O-methyl embelin), and one sesquiterpene ketone (farnesylacetone). These compounds were selected based on their known pharmacological activities, structural uniqueness, and relevance to antioxidant, antimicrobial, and anti-inflammatory properties. These compounds represent key categories of bioactive constituents in Mentha aquatica that have demonstrated efficacy in targeting oxidative stress and supporting cellular health. Focusing on these compounds enables a targeted exploration of Mentha aquatica's therapeutic potential, as they represent the plant's functional diversity and are relevant to health-promoting applications.

Kaempferol 3-O-rutinoside, detected at a retention time of 8.34 minutes, has a molecular formula of $C_{27}H_{30}O_{15}$ with a molecular ion at m/z 594.1572 (C1, Table 18). The MS/MS spectra reveal key fragment ions at m/z 287.0544, 449.1071, and 85.0287. The fragment at m/z 287.0544 corresponds to the aglycone kaempferol, indicating the loss of the rutinoside moiety. The fragment at m/z 449.1071 represents the loss of a rhamnose sugar unit from the molecule, and the ion at m/z 85.0287 is likely due to a small fragment from the sugar structure. These fragmentation patterns are characteristic of kaempferol glycosides and confirm the identity of the compound as kaempferol 3-O-rutinoside, a flavonoid glycoside known for its antioxidant, antimicrobial and anti-diabetic properties (Gülçin et al., 2020a; Soukaina et al., 2024).

The analysis of 5-hydroxycoumarin, identified at a retention time of 8.65 minutes, revealed a molecular ion at m/z 162.0310, corresponding to its molecular formula $C_9H_6O_3$ (C2, Table 18). The MS/MS fragmentation pattern showed key ions at m/z 135.044, 95.049, 89.039, and 63.024. The ion at m/z 135.044, resulting from the loss of a hydroxyl group, is characteristic of coumarin derivatives and suggests the stability of the core structure during fragmentation.

The smaller ions at m/z 95.049, 89.039, and 63.024 are likely the result of the breakdown of aliphatic chains or functional groups attached to the coumarin backbone, indicating stepwise degradation of the molecule. Similarly, 7-methoxycoumarin, observed at a retention time of 10.09 minutes, exhibited a molecular ion at m/z 176.0465, consistent with its molecular formula $C_{10}H_8O_3$ (C5, Table 18). The MS/MS spectra displayed significant fragments at m/z 149.023, 121.029, 111.044, and 65.039. The ion at m/z 149.023 indicates the loss of the methoxy group, confirming its position on the coumarin ring. The ion at m/z 121.029 reflects further fragmentation of the core structure, while the ion at m/z 111.044 suggests the presence of a stable aromatic fragment. This pattern highlights the characteristic fragmentation of methoxylated coumarins, providing insights into the molecular structure.

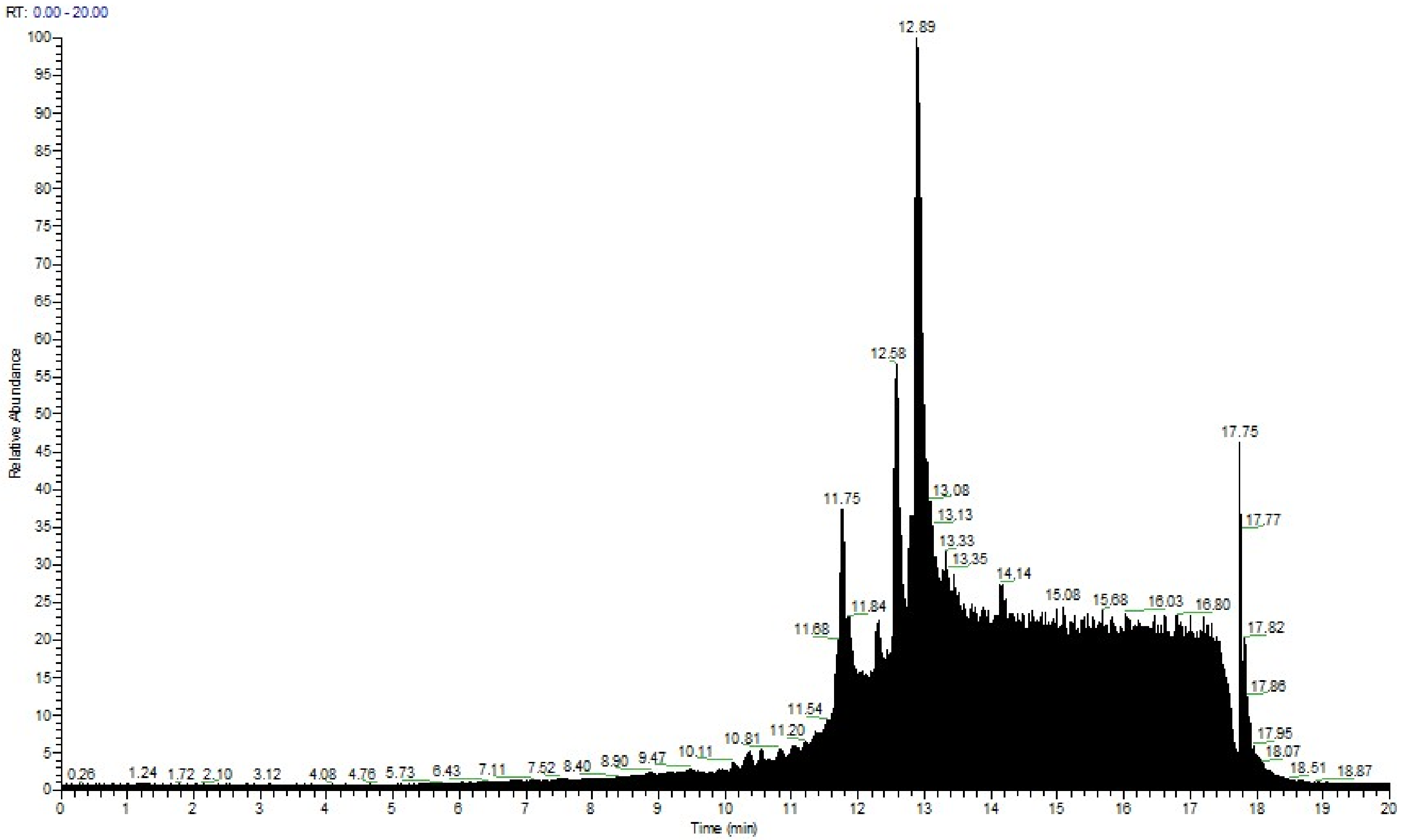

Figure 18. High-performance liquid chromatography-electrospray ionisation mass spectrometry (HPLC-ESI-MS/MS) total ion chromatogram of *Mentha aquatica* infusion showing key peaks and retention times for identified compounds.

The identification of coumarins such as 5-hydroxycoumarin and 7-methoxycoumarin in *Mentha aquatica* aligns with previous reports of coumarins being present across various species within the *Mentha* genus (Sharma et al., 2024; Yousefian et al., 2023b). Coumarins are a class of naturally occurring aromatic compounds known for their diverse biological activities, including anticoagulant, anti-inflammatory, and antioxidant properties (Annunziata et al., 2020).

6-O-Acetylascorbic acid, detected at a retention time of 9.44 minutes, exhibited a molecular ion at m/z 218.0393, consistent with its molecular formula $C_8H_{10}O_7$ (C3, Table 18). The MS/MS fragmentation pattern included key ions at m/z 95.049, 105.045, 77.039, and 51.024. The ion at m/z 95.049 corresponds to the loss of the acetyl group, leaving the ascorbate core intact. The ion at m/z 105.045 suggests the presence of a stable fragment associated with the ascorbic acid structure, while the ions at m/z 77.039 and 51.024 represent smaller, stable fragments. This fragmentation pattern is typical of ascorbic acid derivatives, reflecting their structural features and stability under MS/MS conditions. Interestingly, 6-O-Acetylascorbic acid has also been reported in *Verbascum betonicifolium*, suggesting that this compound may play a broader role in the antioxidant defence mechanisms of different plant families (Fadel et al., 2020).

Compound 4 (Table 18), detected at a retention time of 9.87 minutes, exhibited a molecular ion at m/z 211.1564. The MS/MS spectra revealed product ions at m/z 55.055, 91.055, 79.055, and 141.070. These spectral characteristics indicate that the compound corresponds to elaeokanine C, an alkaloid with the molecular formula $C_{12}H_{21}NO_2$. This compound was reported as an active compound in *Averrhoa bilimbi* (Ahmed et al., 2018).

Creoside I, an acyclic monoterpenoid, exhibits a fragmentation pattern that provides valuable insights into its structural characteristics (C6, Table 18). The ion at m/z 68.9976 indicates a small, stable aliphatic fragment, possibly representing a simple segment of the monoterpenoid structure. The fragment at m/z 129.0181 suggests a larger substructure, likely associated with the core backbone of the monoterpenoid, reflecting a key component of the molecule that remains intact during fragmentation. The ion at m/z 185.0804 may correspond to a significant portion of the monoterpenoid chain, potentially involving a functional group such as an ester or ether bond.

Lastly, the fragment at m/z 255.4873 corresponds to a substantial subunit, possibly involving multiple functional groups, such as hydroxyls or ethers, suggesting cleavage of a larger portion of the molecule while retaining its core structure. These fragmentation patterns provide crucial information for understanding the breakdown pathways and structural elements of creoside I, contributing to the elucidation of its detailed molecular composition and potential bioactive properties as an acyclic monoterpenoid. The detection of creoside I in *Mentha aquatica* is significant, especially considering that it has previously been reported in *Rhodiola crenulata*, a plant known for its adaptogenic and medicinal properties (Nakamura et al., 2008).

The detection of antiarol (3,4,5-trimethoxyphenol) at a retention time of 10.54 minutes, with a molecular formula of $C_9H_{12}O_4$ and a molecular ion at m/z 184.0728, supports its phenolic nature (C7, Table 18). The fragmentation ions at m/z 68.998 and 129.018 indicate a stable aromatic ring structure, with the fragment at m/z 129.018 likely corresponding to the core phenolic group. Previously, it has been reported in *Vitis vinifera*, *Diospyros eriantha* and *Tarenna attenuata*, (Chen et al., 1994; Stefanini et al., 2017; Yang et al., 2007).

Vanillyl nonanoate, found at a retention time of 10.79 minutes, has a molecular formula of $C_{17}H_{26}O_4$ and a molecular ion at m/z 294.1822 (C8, Table 18). The significant fragments at m/z 221.1167 and 133.101 provide crucial structural information. The ion at m/z 221.1167 suggests the loss of the nonanoic acid side chain. In contrast, the fragment at m/z 133.101 corresponds to the vanillyl core structure, which is recognised for its sensory and biological activities. Vanillyl nonanoate is likely to exhibit analgesic and anti-inflammatory effects, as aromatic esters often interact with sensory receptors to alleviate pain and inflammation (Luo et al., 2011).

Table 18. Retention time (Rt), mass spectral data and identification of the chemical compounds found in *Mentha aquatica* infusion (INF) and hydroethanolic extract (HE) by HPLC-ESI-MS-MS.

No	RT	Molecular formula	[M+H]+ (m/z)	MS/MS fragments (m/z)	Compound name	Nature of compound
***Mentha aquatica* infusion (INF)**						
1	8.34	$C_{27}H_{30}O_{15}$	594.1572	287.0544, 449.1071, 85.0287	Kaempferol 3-O-rutinoside	Flavonoid Glycoside
2	8.65	$C_9H_6O_3$	162.0310	63.024, 89.039, 95.049, 135.044	5-hydroxycoumarin	Coumarin
3	9.44	$C_8H_{10}O_7$	218.0393	95.049, 51.024, 105.045, 77.039	6-O-Acetylascorbic acid	Ascorbic Acid Derivative
4	9.87	$C_{12}H_{21}NO_2$	211.1564	55.055, 91.055, 79.055, 141.070	Elaeokanine C	Alkaloid
5	10.09	$C_{10}H_8O_3$	176.0465	65.039, 149.023, 121.029, 111.044	7-methoxycoumarin	Coumarin
6	10.53	$C_{14}H_{24}O_7$	304.1512	68.9976, 129.0181, 185.0804, 255.4873	Creoside I	Acyclic monoterpenoids
7	10.54	$C_9H_{12}O_4$	184.0728	68.998, 129.018	Antiarol	Phenol
8	10.79	$C_{17}H_{26}O_4$	294.1822	57.0705, 73.0289, 221.1167, 133.101	Vanillyl nonanoate	Aromatic Ester
9	11.18	$C_{18}H_{28}O_4$	308.1977	57.071, 221.117, 15.039, 107.049	5-O-methyl embelin	Benzoquinone Derivative
10	12.55	$C_{18}H_{30}O$	262.2287	67.0548, 81.0703, 95.0858, 105.0702	Farnesylacetone	Sesquiterpene Ketone
***Mentha aquatica* hydroethanolic extract (HE)**						
11	7.96	$C_{27}H_{32}O_{15}$	595.1657	289.0702, 85.0287, 195.0286, 435.1274	Neoeriocitrin	Flavanones
12	7.98	$C_{15}H_{12}O_6$	287.0552	153.018, 89.0389, 163.0387, 135.0439	Eriodictyol	Flavanones
13	8.00	$C_{21}H_{22}O_{10}$	433.1131	195.0287, 85.0288, 245.0437, 135.044	Naringenin-8-O-glucoside	Flavanones
14	8.34	$C_{27}H_{30}O_{15}$	593.1501	287.0544, 449.1071, 85.0288	Nictoflorin	Dihydroxyflavanone
15	8.36	$C_{15}H_{10}O_6$	285.0394	153.018, 89.0389, 68.9976, 135.0439	Kaempferol	Tetrahydroxyflavone
16	8.46	$C_{21}H_{18}O_{12}$	461.0714	287.0544, 153.0179	Scutellarin	Glycosyloxyflavone
17	8.65	$C_9H_6O_3$	161.0238	63.0235, 89.0389, 117.0335	4-Hydroxy coumarin	Coumarins
18	8.69	$C_{27}H_{30}O_{14}$	577.1551	271.0594, 433.1123	Kaempferitrin	Glycosyloxyflavone
19	9.68	$C_{19}H_{20}O_8$	375.1074	213.039, 216.0261, 198.0157, 139.0025	Hyperinone	Polyketides
20	10.09	$C_{10}H_8O_3$	175.0393	65.0391, 149.0232, 121.0286, 111.0444	7-Methoxycoumarin	Coumarins
21	10.17	$C_{17}H_{16}O_6$	315.0865	183,0286, 133.0647, 161.0595, 168.0048	Persicogenin	Flavanones
22	10.20	$C_{18}H_{18}O_7$	345.0968	213.0389, 216.0259, 198.0156, 55.0185	Amorphaquinone	Isoflavonoid
23	10.75	$C_{18}H_{16}O_7$	343.0813	315.0494, 169.0129, 154.9973, 201.0025	Ayanin	Trimethoxyflavone
24	10.78	$C_{17}H_{26}O_4$	293.1748	57.0705, 73.0289, 221.11164, 101.0236	Nordihydrocapsiate	Phenols

25	10.84	$C_{19}H_{20}O_7$	359.1125	227.0545, 197.0078, 169.0129, 215.0182	Elaeocyanidin	Leucoanthocyanidins
26	11.09	$C_{20}H_{20}O_8$	387.1074	359.0755, 169.0128, 341.0647, 197.0076	Combretol	Pentamethoxyflavone
27	11.42	$C_{19}H_{18}O_7$	357.0979	329.065, 169.0129, 311.0546, 197.0079	Retusin	Tetramethoxyflavone

Compound 9 belongs to the class of monohydroxy-1,4-benzoquinones, specifically known as 5-O-Methyl embelin, where the hydroxy group at position 5 in embelin is substituted with a methoxy group. It was isolated from *Lysimachia punctata* and *Embelia ribes* (Bøgh et al., 1996; Podolak et al., 2007). The mass spectrum confirms its presence in the INF of Mentha, with a retention time (RT) of 11.18 minutes, a molecular ion peak at m/z 308.1977, and fragment ions at m/z 221.117 and 107.049, suggesting a complex benzoquinone structure. The fragment at m/z 221.117 likely represents the benzoquinone core, while the fragment at m/z 107.049 indicates a methoxy-substituted aromatic ring. Benzoquinone derivatives, such as 5-O-methyl embelin, have been reported to possess various pharmacological activities, including potent cytotoxic, contraceptive, antimicrobial, antiparasitic, analgesic, and anti-inflammatory effects (Wróbel-Biedrawa et al., 2020).

Farnesylacetone, observed at a retention time of 12.55 minutes, has a molecular formula of $C_{18}H_{30}O$ and a molecular ion at m/z 262.2287. The fragmentation pattern includes ions at m/z 105.0702, 95.0858, 81.0703, and 67.0548, characteristic of the typical fragmentation of sesquiterpene ketones. The fragment at m/z 105.0702 likely corresponds to the central ketone structure, while the smaller fragments at m/z 95.0858 and 81.0703 indicate the loss of aliphatic side chains, which is common for terpenoid fragmentation. Sesquiterpene ketones, such as farnesylacetone, are known for their anti-inflammatory and antifungal properties and are often utilised in traditional medicine and cosmetic products for their therapeutic benefits. has been reported in *Ononis angustissima* Lam. *subsp. filifolia* Murb, *Artemisia annua* and *Marrubium globosum* subsp. *globosum* (Benmeddour et al., 2024; Ćavar et al., 2012; Sarikurkcu et al., 2008).

2.2.2. Hydroethanolic extracts

The compounds identified in the hydroethanolic extract (HE) of *Mentha aquatica* exhibit a diverse chemical profile, comprising 63 distinct compounds (Table 19, Figure 19), including flavonoids, alkaloids, phenolics, and various esters. Of particular interest are 17 biologically active compounds (Table 19, C11-C27), grouped into distinct chemical classes, including flavanones, flavones, coumarins, and phenols. Each class contributes uniquely to the plant's potential pharmacological benefits, particularly in antioxidant, anti-inflammatory, and enzyme-inhibitory activities.

Within the flavanones, neoeriocitrin (C11, molecular ion at m/z 595.1657) and Eriodictyol (C12, molecular ion at m/z 287.0552) play crucial roles. Neoeriocitrin, with key MS/MS fragments at m/z 289.0702 and 435.1274. Recent studies suggest that neoeriocitrin may be a promising candidate for treating osteoporosis, as its bioactive components have demonstrated the potential to enhance bone health by promoting osteogenic differentiation and inhibiting bone resorption (Li et al., 2011). Eriodictyol, with its key fragmentation ions at m/z 285.0387 and 135.0439, contributes to the plant's antioxidant profile by scavenging reactive oxygen species (ROS) (Buranasudja et al., 2022). Naringenin-8-O-glucoside (C13, molecular ion at m/z 433.1113), another flavanone, shows potential as an inhibitor of enzymes such as α-glucosidase, making it relevant for diabetes management. Its glycosylated form enhances its bioavailability and increases its effectiveness in controlling postprandial blood sugar levels (Hartogh and Tsiani, 2019).

In the flavone group, kaempferol (C15, molecular ion at m/z 285.0394) and scutellarin (C16, molecular ion at m/z 461.0714) are highly prominent. Kaempferol exhibits anti-inflammatory and anticancer activities, with MS/MS fragments at m/z 255.0298 and 135.0439 (de Morais et al., 2024). Scutellarin, a glucosylflavone, displays neuroprotective and anti-inflammatory properties, with key fragmentation ions at m/z 285.0394, making it beneficial in treating neurodegenerative diseases (Wang et al., 2011). Nictoflorin (C14, molecular ion at m/z 359.1501) adds to the antioxidant and antidiabetic properties of the extract. Its MS/MS fragmentation pattern, including key ions at m/z 287.0447, reflects its dual role in scavenging ROS and reducing inflammation (Patel, 2022).

Kaempferitrin (C18), classified as a flavonol glycoside, has a retention time of 8.36 minutes and a molecular formula of C27H30O15. Its key MS/MS fragments appear at m/z 287.0552, 433.1113, and 285.0394. Known for its antioxidant and anti-inflammatory properties, kaempferitrin contributes to the extract's capacity to manage oxidative stress (Jiang et al., 2018).

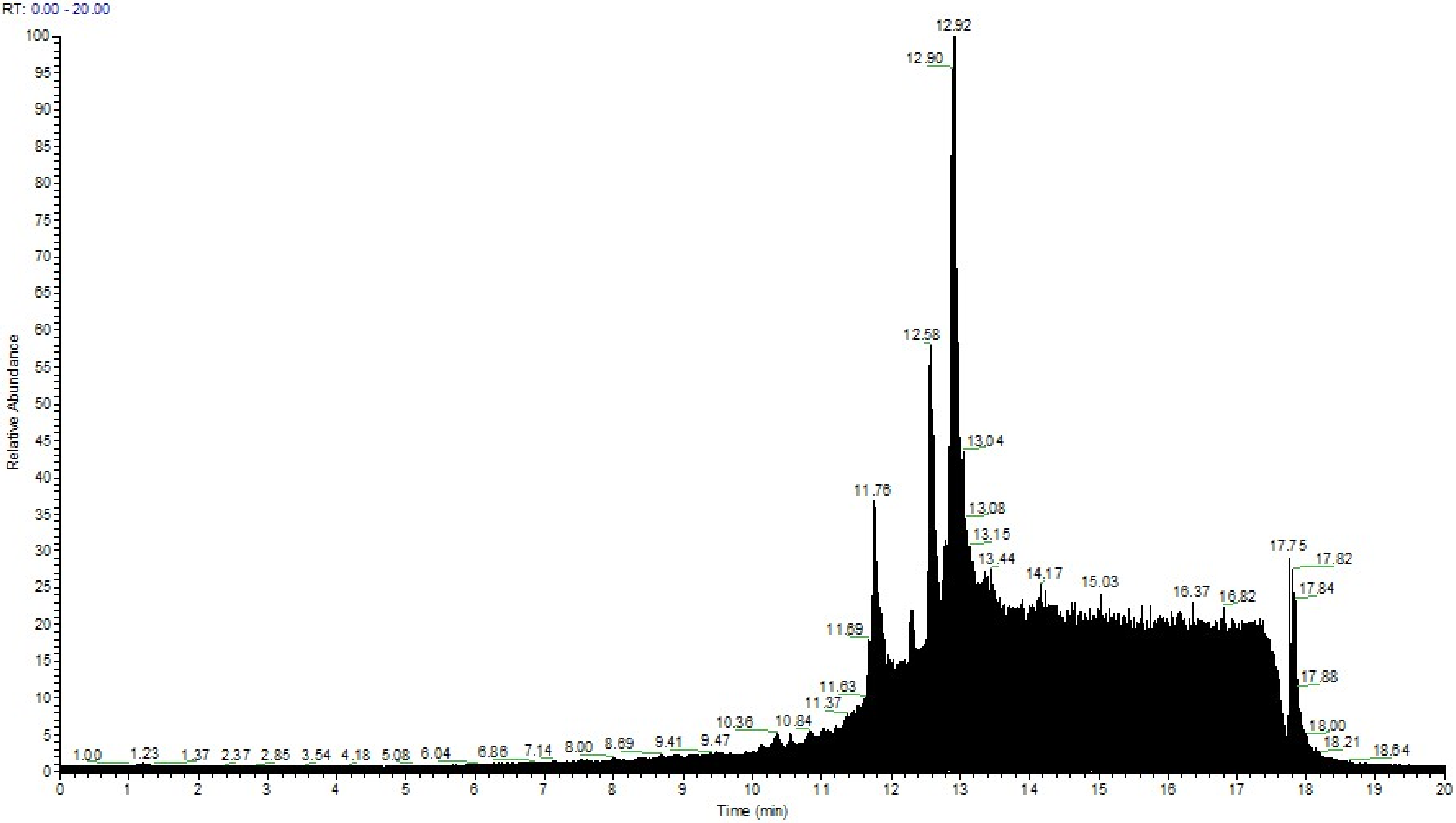

Figure 19. High-Performance Liquid Chromatography-Electrospray Ionisation Mass Spectrometry (HPLC-ESI-MS/MS) Total ion chromatogram of *Mentha aquatica* hydroethanolic extract (HE) showing key peaks and retention times for identified compounds.

Persicogenin (C21), a 3',5-dihydroxy-4',7-dimethoxyflavanone, shows a retention time of 10.17 minutes with a molecular formula of $C_{17}H_{16}O_6$, and MS/MS fragments at m/z 315.0863 and 287.0552. This compound has been reported as an anti-mutagenic, anticancer, and anti-mycobacterial agent (Saquib et al., 2020). Amorphaquinone (C22) is identified with a retention time of 10.20 minutes and a molecular formula of C18H18O7. Its MS/MS fragments are at m/z 345.0984 and 271.0595. It has antiparasitic and antimicrobial properties (Su et al., 2015). Combretol (C26), a pentamethoxyflavone known for its leishmanicidal activity, has a retention time of 11.42 minutes and a molecular formula of C19H18O7, with MS/MS fragmentation at m/z 357.0979 and 329.0654 (Kennedy et al., 2011).

The coumarin family is represented by 4-hydroxycoumarin (C17, molecular ion at m/z 163.0383) and 7-methoxycoumarin (C20, molecular ion at m/z 195.0393). Both compounds contribute anti-inflammatory properties and show strong antioxidant activities, protecting biological tissues from oxidative stress (Kang and Hyun, 2020).

Elaeocyanidin (C25), a leucoanthocyanidin with a molecular ion at m/z 359.1125, exhibits a series of characteristic fragmentation ions that help confirm its structure. The fragment at m/z 227.0545 likely corresponds to a cleavage of part of the flavonoid core, indicating the loss of a specific structural group. The ion at m/z 197.0078 suggests further fragmentation within the polyphenolic backbone, while m/z 169.0129 is commonly associated with the formation of a stable aromatic ring structure. Finally, the fragment at m/z 215.0182 indicates another cleavage that may represent the loss of specific hydroxyl or methoxy groups, consistent with the structural characteristics of leucoanthocyanidins.

Retusin, a tetramethoxyflavone (C27), is another significant compound. With a molecular ion at m/z 357.0979, it exhibits key MS/MS fragments at m/z 329.065, 311.0546, 197.0079, and 169.0129. The fragment at m/z 329.065 corresponds to the loss of a methoxy group ($–OCH_3$), while the ion at m/z 311.0546 indicates further cleavage within the flavonoid structure. The fragment at m/z 197.0079 likely results from the breakdown of the flavonoid core, and the ion at m/z 169.0129 suggests the formation of a stable aromatic structure, further confirming the presence of a highly substituted flavonoid. Retusin is known for its potent antioxidant properties, protecting cells from oxidative damage and contributing to overall cellular health (Pshenichnyuk et al., 2015).

2.3. Profiling and quantification of individual phenolic compounds by HPLC-DAD and HPLC-ESI-MSn

Phenolic compounds present in *Mentha aquatica* were analysed in the plant dried treated (by Methanol 50% + Formic acid 1%) and both types of extracts: infusion (INF) and hydroethanolic (HE), and quantified using HPLC-DAD-ESI-MSn (Table 20). The results show significant differences in the quantities and types of compounds extracted, depending on the method used (Compounds C1-C30). The HE proves particularly effective for isolating certain phenolic compounds such as dimethyl caffeic acid and its derivatives. Specifically, dimethyl caffeic acid derivatives (C17) are found at 3100.73 ± 13.78 µg/g in the HE, while dimethyl caffeic acid hexose (C19) reaches 4744.83 ± 34.00 µg/g in the extract. These high concentrations suggest that the hydroethanolic solvent is more suitable for extracting these compounds, likely due to their higher solubility in this solvent mixture, which combines the polar properties of both water and ethanol (Islam et al., 2024; Petkovska et al., 2016; Plaskova and Mlcek, 2023).

In contrast, ferulic acid derivatives also showed a strong preference for hydroethanolic extraction, with concentrations reaching 98324.02 ± 783.94 µg/g for ferulic acid derivative 2 (C22), 70297.35 ± 392.71 µg/g for ferulic acid derivative 1 (C21). These results indicate that the HE is superior for isolating these specific molecules, which are known for their antioxidant and anti-inflammatory properties (Shi et al., 2021). The absence of these compounds in the INF suggests that water alone is not as efficient in breaking down plant cell walls or in dissolving these compounds, possibly due to their lower polarity or stability in purely aqueous solutions (Gortzi et al., 2024; Islam et al., 2024). However, the efficiency of extracting these bioactive compounds is not solely dependent on the choice of solvent. Several factors can significantly influence the extraction yield and composition of the extracts (Gil-Martín et al., 2022). The drying method and temperature are particularly crucial, as they can alter the chemical structure and stability of phenolic compounds (Djamila et al., 2021b). High temperatures can lead to the degradation of sensitive compounds, while freeze-drying can help preserve the integrity of these bioactive molecules (Safaiee et al., 2019). Similarly, extraction time, especially when using techniques such as sonication, plays a vital role (Liyana-Pathirana and Shahidi, 2005).

Optimising sonication time is essential to strike a balance between maximising yield and preserving compound integrity, as prolonged sonication can cause degradation of specific compounds due to heat and ultrasound energy (Shen et al., 2023).

In your study, certain phenolic compounds (Table 20), Syringic acid (C1), Apigenin-7-O-rutinoside (C13), Hesperetin-7-O-rutinoside (C15), Dimethyl caffeic acid (C17), Feruloyl derivative (C20), Quercetin (C28), and Quercetin derivative (C29), were quantified only in the *Mentha aquatica* samples treated with 50% methanol and 1% formic acid (A). The absence of these compounds in INF and HE extracts suggests that the methanol-based treatment was more effective at removing these particular phenolic compounds, likely due to the solvent's specific polarity and ability to disrupt plant cell walls more effectively than water or hydroethanolic mixtures alone (Kaczorová et al., 2021). This selective extraction highlights the importance of solvent choice in maximising the yield of certain phenolics, as methanol may better solubilise less polar phenolic compounds like those mentioned (Dorta et al., 2012). Interestingly, certain phenolic compounds show a greater affinity for the aqueous environment of the INF. Galloyl derivative (C26), for example (Table 20), is found at a concentration of 35273.99 ± 45.58 µg/g but is absent in the HE. This suggests that water is a better solvent for these compounds, possibly due to their higher hydrophilicity (Molino et al., 2023). Similarly, quinic acid (C30), quantified at 21151.90 ± 83.26 µg/g (Table 20), and the *p*-hydroxybenzoic acid derivative at 24588.37 ± 103.65 µg/g in the INF extract (Table 20), indicate that these compounds are more efficiently extracted in a purely aqueous environment (Islam et al., 2024).

Additionally, the geographical origin and habitat of *Mentha aquatica* significantly impact its phytochemical composition (Hassanpouraghdam et al., 2022). Environmental factors such as soil type, altitude, climate, and exposure to biotic and abiotic stressors can influence the concentration and diversity of secondary metabolites in the plant (Qaderi et al., 2023). Plants grown in regions with high sunlight exposure or nutrient-rich soils may produce higher levels of certain phenolic compounds as a defence mechanism against environmental stressors. Furthermore, the plant's habitat can also affect the balance between primary and secondary metabolites, potentially altering its medicinal properties and effectiveness (Pant et al., 2021).

The total phenolic content, represented by the sum of all identified compounds, is higher in the HE (184347.57 μg/g) compared to the INF (96593.60 μg/g) and *Menth*a-treated (A) sample (21403.55 μg/g). This significant difference highlights the broader extraction capacity of the hydroethanolic solvent, which can isolate a wider range of phenolic compounds, including those that are less polar and thus poorly soluble in water alone. These variations underscore the importance of carefully selecting the extraction method and considering the environmental factors influencing plant composition to optimise the yield and efficacy of phenolic compounds in *Mentha aquatica* extracts (Petkovska et al., 2016).

Table 20. Quantification of phenolic compounds (µg/g of plant extract) identified in plants treated with Methanol 50% + Formic acid 1%(A), Infusion (INF) and hydroethanolic extracts (HE) of *Mentha aquatica* by HPLC-DAD-ESI-MSn.

N°	**Compounds Identification**	**Nature of compound**	**Formula**	**λmax (nm)**	**Molecular Ion [M-H] (m/z)**	**Fragments MS/MS (m/z)**	**Quantification (µg/g)**		
							Mentha treated (A)	**INF**	**HE**
1	Syringic acid	Methoxybenzoic acid	$C_9H_{10}O_5$	276	197	182, 153	2032.25 ± 29.99	n.q	n.q
2	3-O-Caffeoylquinic acid	Hydroxycinnamic acid ester	$C_{16}H_{18}O_9$	326	353	193,181	n.q	n.q	n.q
3	Caffeic acid hexoside (1)	Hydroxycinnamic acid glycoside	$C_{15}H_{18}O_9$	238, 328	341	179, 163	n.q	n.q	n.q
4	Caffeic acid hexoside (2)		$C_{15}H_{18}O_9$	238, 328	341	179, 163	n.q	n.q	n.q
5	Caffeic acid hexoside (3)		$C_{15}H_{18}O_9$	238, 328	341	179, 163	n.q	n.q	n.q
6	Icariside b	Flavonoid glycoside	$C_{19}H_{32}O_8$	-	506	507, 147	n.q	n.q	n.q
7	Medioresinol	Lignan	$C_{21}H_{23}O_7$	-	387	207	n.q	n.q	n.q
8	Eriodictyol-7-O-rutinoside	Flavanone glycoside	$C_{27}H_{32}O_{15}$	285	595	287	n.q	n.q	n.q
9	Luteolin-7-O- rutinoside		$C_{27}H_{30}O_{15}$	240, 288, 328	593	285	n.q	n.q	n.q
10	Luteolin-7-O-glucoside		$C_{21}H_{20}O_{11}$	234, 328	447	285	n.q	n.q	n.q
11	Luteolin-7-O-glucuronide		$C_{21}H_{18}O_{12}$	256, 348	461	287	n.q	n.q	n.q
12	Naringenin-7-O-rutinoside		$C_{27}H_{32}O_{14}$	280, 330	579	435, 419, 273	n.q	n.q	n.q
13	Apigenin-7-O-rutinoside		$C_{27}H_{30}O_{14}$	268, 334	577	269, 225, 201	1776.62 ± 13.48	n.q	n.q
14	Salvianolic acid B/E isomer	Polyphenolic acid	$C_{36}H_{30}O_{16}$	238, 330	717	555, 519, 475, 357, 295	n.q	n.q	n.q
15	Hesperetin-7-O-rutinoside	Flavanone glycoside	$C_{28}H_{34}O_{15}$	285, 332	609	286, 177, 151	852.88 ± 0.71	n.q	n.q
16	Rosmarinic acid	Caffeic acid ester	$C_{18}H_{16}O_8$	238, 328	359	197, 179, 161	n.q	n.q	n.q
17	Dimethyl caffeic acid	Hydroxycinnamic acid derivative	$C_{11}H_{12}O_4$	323, 294	207	135	n.q	n.q	3100.73 ± 13.78

18	Dimethyl caffeic acid derivative		$C_{11}H_{12}O_4$	-	-	-	n.q	n.q	4744.83 ± 34.00
19	Dimethyl caffeic acid hexose		$C_{17}H_{22}O_9$	280	371	209, 191, 163	n.q	2620.23 ± 18.12	nq
20	Ferloyl derivative		$C_{10}H_{10}O_4$	310, 325	193-195	178, 149, 134	n.q	485.20 ± 10.39	nq
21	Ferulic acid derivative 1		$C_{10}H_{10}O_4$	-	-	-	n.q	n.q	70297.35 ± 392.71
22	Ferulic acid derivative 2		$C_{10}H_{10}O_4$	-	-	-	n.q	n.q	98324.02 ± 783.94
23	Ferulic acid derivative 3		$C_{10}H_{10}O_4$	-	-	-	n.q	n.q	7880.64 ± 255.60
24	Feruloyl Hexose		$C_{16}H_{20}O_9$	320	355	193,160	535.87 ± 4.14	n.q	n.q
25	Feruloyol derivative		$C_{10}H_{10}O_4$	-	-	-	n.q	12473.91 ± 165.60	n.q
26	Galloyl derivative	Gallic acid derivative	$C_7H_6O_5$	-	-	-	n.q	35273.99 ± 45.58	n.q
27	*p*-Hydroxybenzoic acid derivative	Phenolic acid derivative	$C_7H_6O_3$	-	-	-	n.q	24588.37 ± 103.65	n.q
28	Quercetin	Flavonol	$C_{15}H_{10}O_7$		301	227	7119.73 ± 4.33	n.q	n.q
29	Quercetin derivative	Flavonol derivative	$C_{15}H_{10}O_7$	-	-	-	9086.20 ± 22.60	n.q	n.q
30	Quinic acid	Cyclohexanecarboxylic acid	$C_7H_{12}O_6$	330	191	171, 173	n.q	21151.90 ± 83.26	n.q
Σ							21403.55	96593.60	184347.57

(A): Methanol 50% + Formic acid 1%

n.q: Detected but not quantified

2.4. Biological Activities

The biological activities of medicinal plants, namely their antioxidant and anti-inflammatory properties, are attracting increasing interest due to their therapeutic potential in the prevention and treatment of various diseases (Sun and Shahrajabian, 2023). Bioactive plant secondary metabolites are abundantly present in medicinal plants and possess remarkable therapeutic potential. Oxidative stress and drug resistance are key contributors to contemporary health issues such as diabetes, atherosclerosis, cardiovascular disorders, cancer, and inflammation (Riaz et al., 2023). The bioactive compounds present in plant extracts, such as flavonoids, polyphenols, and alkaloids, play a crucial role in neutralising free radicals, modulating enzymatic activity, and protecting against oxidative damage. Evaluating these activities allows for a better understanding of the mechanisms of action of these extracts and the identification of those with significant beneficial properties, potentially suitable for pharmacological applications (Atanasov et al., 2021).

In this study, the INF and HE of *Mentha aquatica* were analysed to evaluate their antioxidant, antiradical, and antidiabetic potential, as well as their ability to protect human erythrocytes against oxidative stress, in comparison with quercetin and ascorbic acid as reference standards. The results are summarised in Table 21.

Table 21. *In vitro* biological activities of the infusion (INF) and hydroethanolic extracts (HE) of *M. aquatica*. The results are presented as mean IC_{50} (μg/mL) ± standard deviation (n = 3).

	DPPH	SO	NO	A-GLUC	Hb_ox	LP_ox
INF	20.353 ± 0.563	96.484 ± 3.251	11.073 ± 1.237	20.917 ± 1.632	52.638 ± 0.168	267.662 ± 15.827
HE	38.875 ± 0.693*	281.417 ± 21.019	12.73 ± 0.123*	354.179 ± 19.275*	128.711 ± 13.274*	490.611 ± 25.305*
ASC	6.720 ± 0.123	142.4228 ± 6.356	112.567 ± 8.407	-	-	-
ACA	-	-	-	17.0175 ± 0.977		-
QUE	-	-	-	-	369.701 ± 18.185	2.607 ± 0.064

DPPH: 2,2-diphenyl-1-picrylhydrazyl (DPPH•) assay, NO: nitric oxide (•NO) assay, SO: superoxide radical scavenging ($O_2^{\bullet-}$) assay, Hb_ox: Haemoglobin oxidation assay, LPox: lipid peroxidation assay, A-GLUC: α-glucosidase activity, ASC: Ascorbic acid, ACA: Acarbose; QUE: Quercetin.

* Indicates significant differences between extracts ($p < 0.05$), using Student's t-test analysis.

2.4.1. Antioxidant activity

The antioxidant activities of plant extracts are typically evaluated using various assays, each targeting different reactive oxygen species (ROS) or reactive nitrogen species (RNS) to provide a comprehensive understanding of the extract's ability to combat oxidative stress (Munteanu and Apetrei, 2021). The most commonly used assays include 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), nitric oxide ($^{\bullet}$NO), and superoxide radical scavenging assays ($O_2^{\bullet -}$). These assays measure the extract's capacity to neutralise specific free radicals, thus preventing cellular damage associated with oxidative stress. The results of this study demonstrate varying levels of antioxidant activity for the INF and HE of *Mentha aquatica* across different assays, highlighting the complex interplay between extraction methods, bioactive compound concentration, and antioxidant efficacy (Table 21 and Figure 20).

a. DPPH Radical Scavenging Activity

The DPPH assay is widely used to evaluate the free radical scavenging ability of antioxidants in plant extracts. This assay measures the capacity of the extract to donate an electron or hydrogen to stabilise the synthetic radical, which is DPPH$^{\bullet}$ (Munteanu and Apetrei, 2021).

In our study, all extracts exhibited antioxidant activity in a concentration-dependent manner (Figure 20). INF shows superior activity with an IC$_{50}$ of 20.353 ± 0.563 µg/mL, compared to the HE, which has a significantly higher IC$_{50}$ of 38.875 ± 0.693 µg/mL (Table 21). Although HE is more concentrated in bioactive compounds, as indicated by the previous phytochemical analysis, its lower efficacy in the DPPH• test could be due to the specific composition and interactions of the compounds in the INF, which enhance its antioxidant properties. The standard used in this assay, ascorbic acid, exhibits an IC$_{50}$ of 6.720 ± 0.123 µg/mL, confirming its superior free radical scavenging ability. This result aligns with the findings in previous studies, as ascorbic acid is a well-known potent antioxidant (Gęgotek and Skrzydlewska, 2022). When compared to other studies on species of the Mentha genus, the IC$_{50}$ for DPPH• radical scavenging activity is 19.98 ± 0.91 µg/mL for the hydroethanolic extract (70/30 v/v) of *Mentha pulegium*, suggesting a slightly higher antioxidant activity for this species (Tacherfiout et al., 2022).

Another study by Boualam et al. (Boualam et al., 2023) showed that the aqueous extract of *Mentha rotundifolia* has an IC_{50} value of 26.47 µg/mL in the DPPH assay and exhibits neuroprotective properties by reducing the effects of hydrogen peroxide (H_2O_2) in rats. The high content of kaempferol glucuronide is cited as being responsible for these beneficial effects (Boualam et al., 2023). Such comparisons highlight variations in antioxidant potential among different Mentha species, which are attributed to differences in the phytochemical composition of each extract.

b. Superoxide Radical Scavenging Activity

The superoxide radical scavenging (SO) assay evaluates the extract's ability to neutralise superoxide anions, which are highly reactive oxygen species that can cause oxidative damage to cells and tissues. Superoxide anions are typically generated as a byproduct of cellular respiration and are involved in various physiological and pathological processes. If not adequately neutralised, these radicals can lead to oxidative stress, which is linked to numerous chronic diseases, including cardiovascular disorders, neurodegenerative diseases, and cancer (Munteanu and Apetrei, 2021). The mechanism of action for superoxide radical scavenging involves the extract donating an electron or hydrogen atom to the superoxide anion ($O_2^{-}\bullet$), thereby neutralising it and converting it into a less reactive species, such as hydrogen peroxide (H_2O_2). This process prevents the superoxide anion from reacting with other molecules, such as nitric oxide (NO), to form more harmful species, such as peroxynitrite ($ONOO^-$). Concerning *M. aquatica* extracts, they showed a slight concentration-dependent behaviour (Figure 20). The INF again outperforms the HE, with an IC_{50} of 96.484 ± 3.251 µg/mL compared to 281.417 ± 21.019 µg/mL for the HE (Table 21). The significant difference between these two extracts indicates that the compounds present in the INF are more effective in scavenging superoxide radicals. The SO assay specifically evaluates the ability to neutralise superoxide anions, highly reactive species that contribute to oxidative stress.

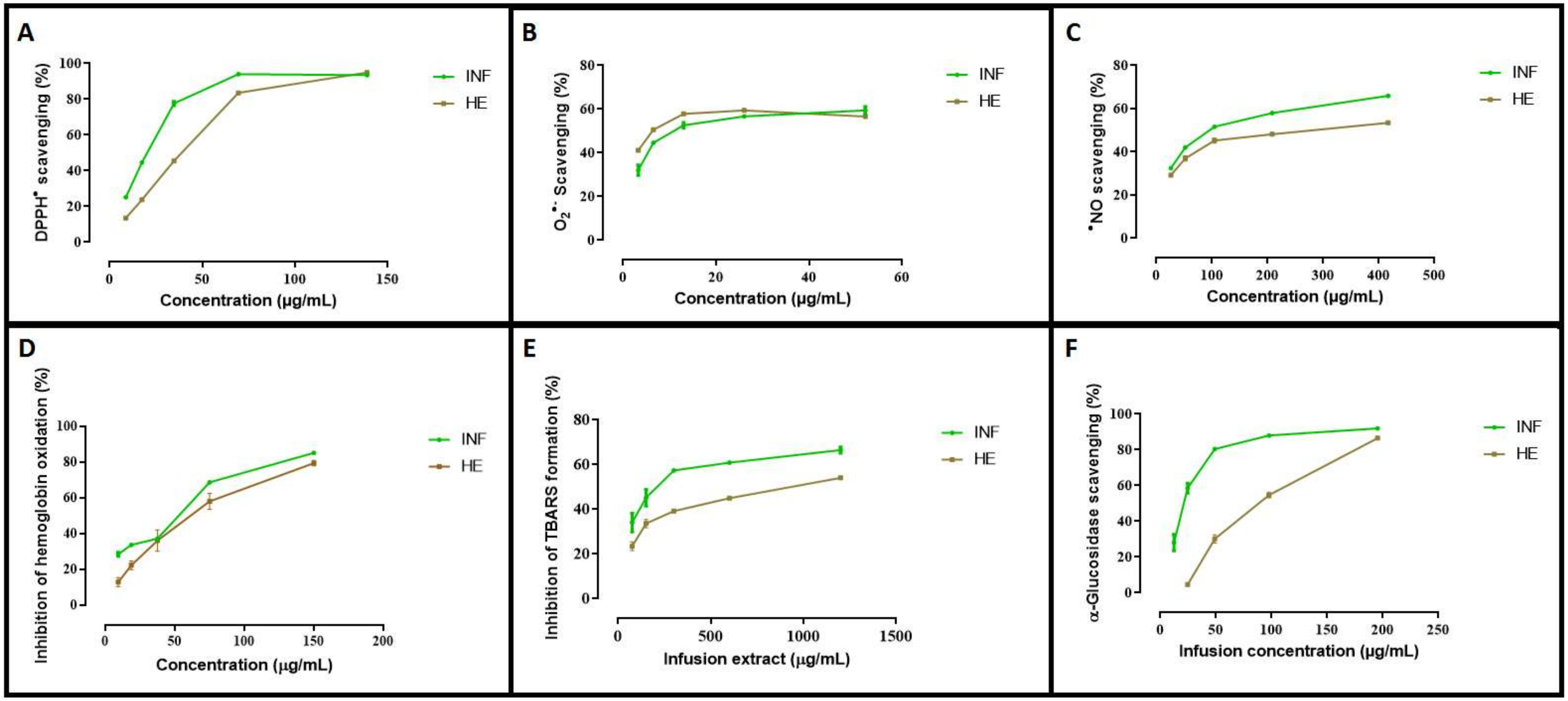

Figure 20. Scavenging activity against DPPH$^{\bullet}$ radical (A), superoxide anion ($O_2^{-\bullet}$) (B), radical NO$^{\bullet}$ (C), and the inhibition of Haemoglobin oxidation (D), Lipid peroxidation (E) and α-Glucosidase activity (F) of *Mentha aquatica* Infusion (INF) and Hydroethanolic (HE) extracts. Values expressed as mean ± SD; n = 3.

The fact that the INF performs better despite the HE's higher concentration of bioactive compounds suggests that the qualitative nature of these compounds and their synergistic interactions are crucial. Ascorbic acid, used as a reference standard in this test, has an IC_{50} of 142.4228 ± 6.356 µg/mL, which places it between the two extracts in terms of efficacy. This indicates that while ascorbic acid is effective, the INF of *Mentha aquatica* is even more potent in neutralising superoxide radicals. Previous studies have shown that the superoxide radical scavenging activity of different solvent fractions of *Mentha spicata* was investigated, demonstrating that the ethyl acetate and aqueous fractions of the ethanol extract exhibited higher superoxide radical scavenging activity compared to other fractions (Anwar et al., 2017). Another study examined the effect of heat stress on the essential oil composition and antioxidant enzyme activity, including SO, in *Mentha piperita* and *Mentha arvensis*. It was found that heat stress significantly affected the SO activity in both species, and the use of salicylic acid and melatonin helped alleviate oxidative stress, thereby enhancing the SO activity (Haydari et al., 2019). In addition to these findings, research on other *Mentha* species, such as *Mentha longifolia*, has demonstrated that different extraction methods and plant parts can yield varying levels of SO activity. For instance, the essential oil of *Mentha longifolia* has demonstrated significant superoxide radical scavenging capacity, attributed to its rich phenolic and flavonoid content(Bahadori et al., 2018). Furthermore, the environmental conditions in which these plants are grown play a crucial role in their antioxidant potential. A study conducted on six wild Mentha species from Algeria highlighted the influence of geographical and climatic factors on the total phenolic content and antioxidant activity, suggesting that these external conditions can significantly modulate the bioactive properties of the extracts (Benabdallah et al., 2016). Overall, these studies emphasise that the antioxidant efficacy of Mentha extracts depends not only on the type of solvent used for extraction but also on environmental conditions, plant species, and the specific part of the plant used. Such factors must be considered when evaluating the potential therapeutic applications of Mentha extracts, particularly for managing oxidative stress-related conditions.

c. Nitric Oxide (NO) scavenging activity

Nitric oxide (NO) scavenging activity refers to the ability of an extract or compound to neutralise nitric oxide radicals. Nitric oxide is a reactive nitrogen species involved in various physiological processes, including vasodilation, neurotransmission, and the immune response. However, excessive production of NO can lead to the formation of peroxynitrite ($ONOO^-$) when it reacts with superoxide anions ($O_2^{-}\bullet$), contributing to oxidative stress and tissue damage, which are associated with conditions like inflammation, cancer, and neurodegenerative diseases (Munteanu and Apetrei, 2021).

In our study, the INF and HE show comparable activities, with IC_{50} values of 11.073 ± 1.237 µg/mL and 12.73 ± 0.123 µg/mL, respectively (Figure 20 and Table 21). The lack of a statistically significant difference between the two suggests that both extracts possess similar abilities to neutralise peroxynitrite radicals. Despite the higher concentration of bioactive compounds in the HE, its performance is nearly equivalent to that of the INF, indicating that the specific compounds effective in this assay are present in comparable amounts or that their reactivity is similar in both extracts. Ascorbic acid, on the other hand, has an IC_{50} of 112.567 ± 8.407 µg/mL, indicating much lower efficacy in this test. The slightly lower IC_{50} value of the INF indicates a marginally higher potency in scavenging NO radicals compared to the HE. This could be due to improved extraction of certain water-soluble compounds with potent NO-scavenging activity in the INF. On the other hand, the HE, while slightly less powerful, may contain a broader spectrum of bioactive compounds, contributing to a comparable overall antioxidant effect.

These results are consistent with findings from other studies, in which different Mentha species, such as *M. spicata* and *M. longifolia*, have demonstrated varying degrees of NO scavenging activity depending on the extraction method and solvent used. For *M. spicata*, the IC_{50} for NO scavenging was reported to be 210.6 ± 7.7 µg/mL, which demonstrates moderate effectiveness in neutralising NO radicals at relatively low concentrations (Ebrahimzadeh et al., 2010). In the case of Mentha longifolia, different extracts exhibited varying degrees of NO scavenging activity. The hexane extract of *Mentha longifolia* showed the highest potency with an IC_{50} of 0.010 ± 0.002 mg/mL, followed by the essential oil (IC_{50} = 0.032 ± 0.002 mg/mL) and the methanol extract (IC_{50} = 0.052 ± 0.004 mg/mL) (Karimian et al., 2013).

These results highlight that the choice of solvent can significantly influence NO scavenging efficiency, with some solvents extracting more potent compounds than others (Safaiee et al., 2019). The similarity in the NO scavenging activities of INF and HE in our study suggests that either method could be used to obtain extracts with strong antioxidant properties. However, the choice between them might depend on factors such as the desired profile of bioactive compounds, extraction efficiency, and the intended application.

2.4.2. Protective effects of *Mentha aquatica* extracts against oxidative damage in human blood erythrocytes

Plant extracts protect human blood erythrocytes from oxidative damage through mechanisms that inhibit haemoglobin oxidation and lipoperoxidation (Félix et al., 2020). The antioxidants present in these extracts, such as flavonoids and phenolic acids, neutralise reactive oxygen species, preventing the oxidation of haemoglobin into methemoglobin, which compromises its oxygen-carrying capacity (Salehi et al., 2020). Additionally, these compounds can interrupt the chain reactions that lead to lipid peroxidation in cell membranes, maintaining erythrocyte integrity and function. By scavenging free radicals and chelating metal ions that catalyse oxidative reactions, plant extracts help preserve the structural stability of erythrocytes and protect against hemolysis, highlighting their potential therapeutic use in conditions associated with oxidative stress (Félix et al., 2020).

The protective effects of plant extracts against oxidative damage in human blood erythrocytes can be evaluated using assays such as haemoglobin oxidation (Hb_ox) and lipoperoxidation (LP_ox). These tests are crucial in understanding how plant-derived antioxidants can mitigate oxidative stress, which is known to damage cellular structures, including lipids, proteins, and DNA.

For haemoglobin oxidation (Hb_ox), the INF displays greater efficacy, with an IC50 of 52.638 ± 0.168 µg/mL, compared to the HE, which has an IC_{50} of 128.711 ± 13.274 µg/mL, indicating a significantly lower effectiveness (Figure 20 and Table 21). This statistically significant difference suggests that the INF is more potent in preventing haemoglobin oxidation, potentially due to the presence of more effective compounds or a more favourable composition for this specific activity.

Quercetin, with an even higher IC_{50} of 369.701 ± 18.185 µg/mL, is much less effective at inhibiting haemoglobin oxidation compared to *Mentha aquatica* extracts. This indicates that the combination and synergy of bioactive compounds in the INF and HE are more effective at scavenging the specific reactive oxygen species (ROS) involved in haemoglobin oxidation.

In the lipoperoxidation (LP_ox) test, which measures the ability to inhibit lipid peroxidation, the INF again shows superior performance with an IC_{50} of 267.662 ± 15.827 µg/mL, significantly outperforming the HE, which has an IC_{50} of 490.611 ± 25.305 µg/mL (Figure 3 and Table 21). This significant difference indicates that the INF is more effective in preventing lipid peroxidation, suggesting that the compounds extracted through the INF are better at protecting lipids from oxidative damage. However, quercetin exhibits an extremely low IC_{50} of 2.607 ± 0.064 µg/mL, demonstrating extreme antioxidant activity in preventing lipid peroxidation.

The observed differences in the effectiveness of *Mentha aquatica* extracts in preventing haemoglobin oxidation and lipid peroxidation can be attributed to the nature and interactions of the bioactive compounds present in these extracts. Haemoglobin oxidation occurs when reactive oxygen species (ROS), such as superoxide anions ($O_2^{-}\bullet$) or hydrogen peroxide (H_2O_2), react with the iron in haemoglobin, converting it from its functional ferrous (Fe^{2+}) state to the non-functional ferric (Fe^{3+}) state, forming methemoglobin (Nagababu and Rifkind, 2000). This oxidative modification impairs the haemoglobin's oxygen-carrying capacity, potentially leading to cellular dysfunction and reduced oxygen delivery to tissues. The significantly lower IC_{50} value of the INF compared to the HE in inhibiting haemoglobin oxidation suggests that the compounds in the INF are more effective at neutralising the ROS responsible for this process. Water-soluble compounds present in the INF may directly scavenge these ROS, thereby preventing their interaction with haemoglobin. Additionally, these compounds might chelate transition metal ions like iron, which catalyse the formation of more harmful radicals like hydroxyl radicals ($^{\bullet}OH$) through the Fenton reaction (Salehi et al., 2020). By sequestering these metal ions, the INF reduces their availability to catalyse these oxidative reactions, thereby offering a double layer of protection.

When comparing these results with those of other species, such as *Mentha pulegium*, *Salvia moorcroftiana*, and *Ocimum sanctum*, distinct differences in efficacy against oxidative damage are observed (Tacherfiout et al., 2022; Yasir et al., 2022).

The hydroethanolic extract of *Mentha pulegium* demonstrated significant protective effects against AAPH-induced oxidative damage to erythrocyte membranes, with an IC_{50} of 129.52 ± 2.15 µg/mL. While effective, this value is notably higher than that of the *Mentha aquatica* INF (IC_{50} = 52.638 ± 0.168 µg/mL), suggesting that INF is more potent in preventing oxidative damage, likely due to the presence of more efficient water-soluble antioxidants (Tacherfiout et al., 2022). Similarly, hydrolysed extracts of Salvia moorcroftiana and Ocimum sanctum displayed IC_{50} values of 168.46 ± 2.78 µg/mL and 198.65 ± 2.45 µg/mL, respectively, which are significantly higher than the IC_{50} of *Mentha aquatica* (Yasir et al., 2022). This indicates that *Mentha aquatica* provides superior protection against haemoglobin oxidation. The enhanced effectiveness of the INF could be attributed to the preservation of sensitive antioxidant compounds during the infusion process, which may be partially lost or altered in hydroethanolic extractions or during hydrolysis.

Lipid peroxidation, on the other hand, is initiated when ROS attack the polyunsaturated fatty acids in cell membranes, leading to the formation of lipid radicals and hydroperoxides, which compromise membrane integrity and can propagate oxidative damage to other cellular components (Endale et al., 2023). The INF's superior performance in the lipoperoxidation (LP_ox) test, with a lower IC_{50} than the HE, indicates that its bioactive compounds are more effective at halting the chain reactions of lipid peroxidation. These compounds can donate hydrogen atoms or electrons to stabilise lipid peroxyl radicals, thereby terminating the chain reaction that would otherwise result in extensive damage to the lipid bilayer of cellular membranes (Martemucci et al., 2022).

Quercetin, with an exceptionally low IC_{50}, demonstrates even greater potency in preventing lipid peroxidation due to its strong radical-scavenging abilities. It can donate hydrogen atoms to neutralise lipid radicals and interrupt the peroxidation chain, highlighting its high reactivity and efficacy in this specific context. The stark difference between quercetin and *Mentha aquatica* extracts suggests that while the latter contains a diverse range of bioactive compounds, their collective effectiveness in this assay is not as high as that of pure quercetin. Although *Mentha aquatica* extracts still provide substantial antioxidant protection, their effectiveness is significantly lower than that of other plants. In the study of Spréa et al. (Mascoloti Spréa et al., 2022), the extracts from *Origanum vulgare*, *Thymus vulgaris*, *Ocimum basilicum*, *Salvia officinalis*, *Melissa* *officinalis*, and *Matricaria chamomilla* exhibited exceptional antioxidant activity in TBARS assays, with IC_{50} values under 26 µg/mL. This indicates their strong capacity to inhibit lipid peroxidation at very low concentrations, likely due to their high levels of phenolic compounds and flavonoids, which efficiently neutralise lipid peroxyl radicals. Another study of Barros et al. (2010) reported remarkably low IC_{50} values for TBARS inhibition in brain homogenates for *Origanum vulgare* (0.01 ± 0.00 mg/mL), *Glechoma hederacea* (0.11 ± 0.01 mg/mL), and *Thymus mastichina* (0.43 ± 0.02 mg/mL) (Barros et al., 2010). The difference could be attributed to the distinct phytochemical compositions of these plants, where the concentration and type of phenolic compounds, flavonoids, and other bioactive molecules play a crucial role in their antioxidant efficacy (Barros et al., 2010).

2.4.3. α-Glucosidase inhibitory activity

Diabetes is a chronic condition characterised by high blood sugar levels, often managed by inhibiting A-GLUC, which slows the breakdown of carbohydrates into glucose. This helps reduce postprandial blood sugar spikes. Natural plant extracts show promising A-GLUC inhibitory activity, offering a complementary approach to conventional treatments. These natural inhibitors not only help control blood glucose but also provide additional health benefits through their antioxidant properties.

In this study, the hydroethanolic extract (HE) exhibited significantly lower inhibitory activity, with an IC_{50} of 354.179 µg/mL, compared to the infusion (INF), which had an IC_{50} of 20.917 µg/mL (Figure 20 and Table 21). This significant difference suggests that the INF extract, with its high inhibitory activity, could be a key component in the development of more effective antidiabetic treatments. By inhibiting the A-GLUC enzyme, which breaks down carbohydrates into glucose, the INF extract could potentially revolutionise the management of post-meal blood glucose levels. Additionally, the standard used in this test, acarbose, has an IC_{50} of 17.017 µg/mL, indicating slightly higher efficacy than the INF extract (Figure 20 and Table 21).

A-GLUC inhibitory activities have also been previously reported for the methanol extract of Mentha pulegium from Turkey, with an IC_{50} value of 20.38 µg/mL, demonstrating strong inhibition (Gülçin et al., 2020b).

This is closely comparable to the INF of *Mentha aquatica* in the present study, indicating a similar potency in inhibiting A-GLUC activity. When compared to other studies, the root extracts of *Mentha rotundifolia* from the Mila region (IC_{50} = 17.21 ± 0.11 µg/mL) showed even higher A-GLUC inhibition than both the INF and methanol extract of *Mentha pulegium* (Kecis et al., 2023c). However, the aerial parts of *Mentha rotundifolia* had much lower inhibitory activity, with IC_{50} values ranging from 107.176 ± 1.49 µg/mL to 181.253 ± 3.02 µg/mL, which are still more effective than the HE of *Mentha aquatica* (Kecis et al., 2023c). Moreover, the ethyl acetate fraction of *Mentha pulegium* demonstrated an IC_{50} of 61.85 ± 1.69 µg/mL, which, while effective, is not as potent as the INF of *Mentha aquatica* or the methanol extract of *Mentha pulegium* from Turkey (Abbou et al., 2022; Gülçin et al., 2020b). This suggests that the choice of extraction solvent and method significantly influences the concentration and efficacy of active compounds in inhibiting A-GLUC.

The statistically significant difference between the extracts underscores the INF's superior performance in inhibiting A-GLUC activity. These results can be attributed to several mechanisms. The INF process typically extracts a higher concentration of bioactive compounds that can effectively bind to the enzyme's active site, preventing it from interacting with and hydrolysing carbohydrate substrates. Indeed, several experiments have reported high correlation between phenolic contents in plant extracts and A-GLUC inhibitory activities (Ouattara et al., 2019). Additionally, the specific combination of compounds in the INF may exhibit synergistic effects, enhancing their collective inhibitory activity. This synergy could increase the binding affinity of these molecules for the enzyme, making the inhibition more potent compared to the HE, where such interactions might be less pronounced. Furthermore, some components in the INF may induce conformational changes in A-GLUC, altering its structure and reducing its catalytic efficiency. This mechanism, known as non-competitive inhibition, can significantly reduce the enzyme's activity even when substrate levels are high. The water-based extraction method used in the INF may also preserve or enhance the bioavailability and solubility of these inhibitory compounds, allowing them to interact more efficiently with the enzyme. In contrast, the HE may contain less soluble or less bioavailable inhibitory molecules, resulting in lower efficacy.

2.5. Cytotoxicity of *Mentha* extracts

2.5.1. Normal Human Dermal Fibroblasts (NHDF)

Figure 21 demonstrates the effects of various concentrations of *Mentha aquatica* infusion (INF) and hydroethanolic extract (HE) on the proliferation of NHDF cells (Normal Human Dermal Fibroblasts) across 24, 48, and 72-hour time points. The tested concentrations ranged from 9.375 µg/mL to 1200 µg/mL. The results illustrate how cell proliferation relative to the control changes with increasing concentration and exposure time, revealing both the cytotoxic and proliferative potential of these extracts.

At 24 hours, both the infusion and the hydroethanolic extract show an initial increase in cell proliferation at lower concentrations (9.375-150 µg/mL). The highest proliferation is observed around 75 µg/mL for both extracts. However, at concentrations above 300 µg/mL, cell viability declines. At the highest concentrations (600 to 1200 µg/mL), cytotoxicity is significant, with the extracts inhibiting cell proliferation almost completely. After 48 hours, the trend remains consistent: low to moderate concentrations (up to 150 µg/mL) promote cell growth, while concentrations above 300 µg/mL exhibit cytotoxic effects. The extracts exhibit similar behaviour, with higher concentrations increasingly inhibiting proliferation. Cytotoxicity becomes increasingly evident at 600 µg/mL and above. By 72 hours, the cytotoxic effects of both the infusion and the hydroethanolic extract are even more pronounced. While lower concentrations still promote cell proliferation, the highest concentration (1200 µg/mL) nearly completely inhibits cell growth, indicating potent cytotoxicity with prolonged exposure.

Based on the IC_{50} values, a clear difference in cytotoxicity is evident. The HE consistently shows lower IC_{50} values across all time points, indicating greater cytotoxicity than the INF. Specifically, at 24 hours, the IC_{50} of the HE is 560.3 ± 14.42 µg/mL, which decreases significantly to 449.5 ± 15.56 µg/mL at 48 hours, before slightly increasing to 471.2 ± 21.45 µg/mL at 72 hours. This reduction in IC_{50} over time indicates that the HE becomes increasingly cytotoxic with prolonged exposure, particularly at 48 hours. Hydroethanolic extraction yields a broader range of compounds, including both polar and non-polar substances, such as flavonoids, phenolic acids, terpenoids, and other secondary metabolites. These compounds are present in higher concentrations in the HE and contribute to its stronger biological activity. However, at high doses, these compounds can disrupt cell function and cause cytotoxicity. Some of these potent compounds may affect critical cellular pathways, increasing oxidative stress or inducing apoptosis, which explains the stronger cytotoxic effects observed with the HE at higher concentrations (Braicu et al., 2022).

In contrast, the IC_{50} values for the INF remain more stable over time, with 586.1 ± 15.87 µg/mL at 24 hours, 605.1 ± 23.68 µg/mL at 48 hours, and 599 ± 26.56 µg/mL at 72 hours. These values suggest that the cytotoxic effects of the INF do not increase significantly over time, making it a less aggressive option compared to the HE. The relatively stable IC_{50} values for the INF indicate that it has a more consistent, milder impact on cell viability, particularly over extended periods.

Overall, the data suggest that while both extracts exhibit some cytotoxicity, the HE is considerably more potent, especially at longer exposure times. The INF, on the other hand, shows a more moderate cytotoxic profile, potentially making it a safer choice for therapeutic applications that require lower toxicity. The choice between the HE and the INF would therefore depend on the intended use and the desired balance between efficacy and safety, with the INF offering a more stable and less toxic alternative.

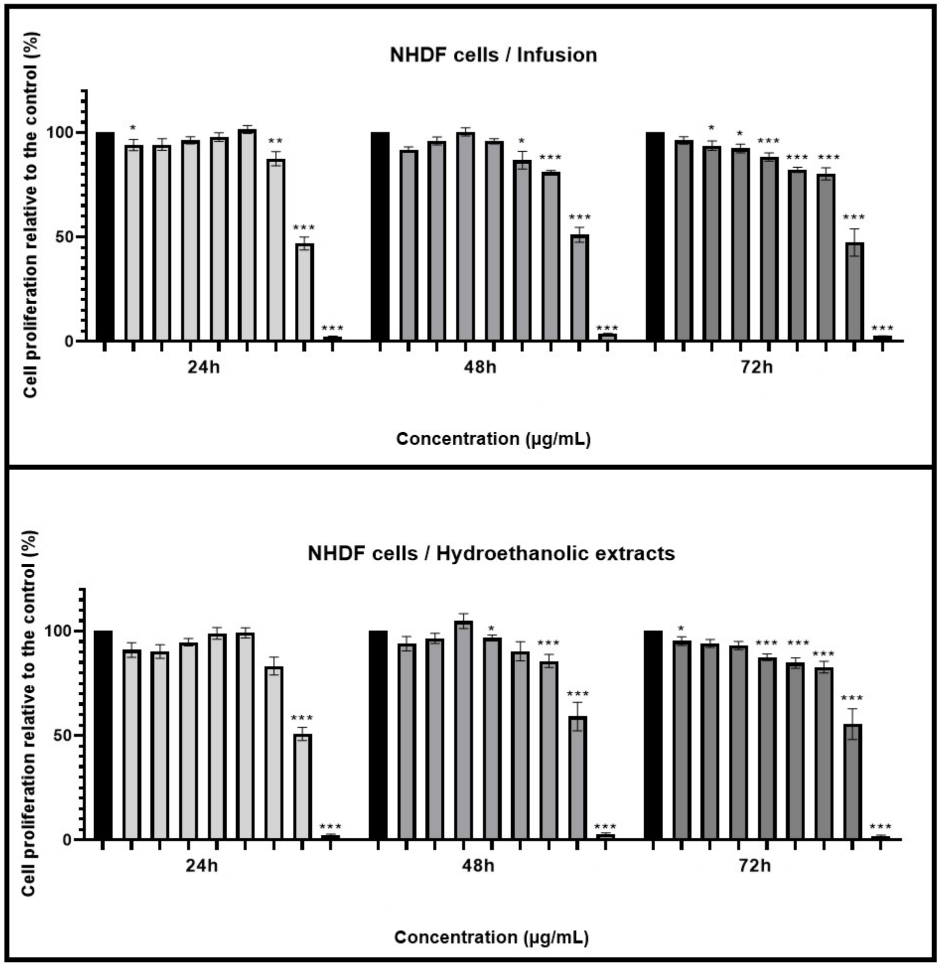

Figure 21. Effect of infusion and hydroethanolic extract on NHDF viability after 24 h, 48h and 72h of exposure, assessed by MTT reduction. Values show mean ± SEM. One-way ANOVA was used to determine statistical significance between NHDF cells and the control (Black bar). A two-way ANOVA was used in NHDF to compare the two extracts at different concentrations (9.375, 18.75, 37.5, 75, 150, and 300 µg/mL). (* $p < 0.05$, ** $p < 0.01$,*** $p < 0.0001$).

2.5.2. N27 dopaminergic cells

The effects of *Mentha aquatica* INF and HE on N27 dopaminergic cells were evaluated at concentrations ranging from 9.375 to 300 µg/mL over 24, 48, and 72 hours (Figure 22). Both extracts exhibited dose- and time-dependent cytotoxicity, with the hydroethanolic extract showing stronger overall cytotoxicity.

For the INF, cell proliferation at 24 hours remained relatively high at lower concentrations. At 9.375 µg/mL, proliferation was 94.8% ± 2.94, decreasing slightly to 92.39% ± 3.48 at 18.75 µg/mL, and maintaining 95.9% ± 4.72 at 37.5 µg/mL. However, as the concentration increased, cytotoxicity became more pronounced, with cell proliferation dropping to 82.09% ± 10.26 at 150 µg/mL and then declining steeply to 27.37% ± 6.28 at 300 µg/mL.

At 48 hours, a similar pattern emerged, with higher proliferation at lower concentrations (99.77% ± 10.29 at 9.375 µg/mL and 87.09% ± 9.46 at 18.75 µg/mL), while higher concentrations showed greater cytotoxicity. At 150 µg/mL, cell proliferation was reduced to 62.76% ± 14.87, and at 300 µg/mL, only 17.19% ± 6.05 of the cells remained viable. After 72 hours, cytotoxic effects were more pronounced, with proliferation reduced to 45.29% ± 4.07 at 150 µg/mL and only 9.32% ± 3.87 at 300 µg/mL. The IC_{50} values for the infusion decreased over time, reflecting increasing cytotoxicity: 236.2 ± 6.25 µg/mL at 24 hours, 178.7 ± 9.26 µg/mL at 48 hours, and 143.8 ± 6.38 µg/mL at 72 hours.

For the HE, the effects were more pronounced across all concentrations and time points. At 24 hours, proliferation at the lowest concentration (9.375 µg/mL) was 91.78% ± 8.65, and at 18.75 µg/mL, it dropped to 85.89% ± 6.77. At 37.5 µg/mL, proliferation was 92.09% ± 6.71, but at 150 µg/mL and 300 µg/mL, proliferation fell sharply to 58.67% ± 9.99 and 22.07% ± 8.54, respectively. After 48 hours, the cytotoxicity of HE increased significantly, with cell viability of 81.91% ± 10.51 at 9.375 µg/mL, 72.06% ± 8.75 at 18.75 µg/mL, and 64.11% ± 6.30 at 75 µg/mL. At higher concentrations, 150 µg/mL reduced proliferation to 40.18% ± 4.28, and at 300 µg/mL, only 16.25% ± 5.64 of the cells remained viable. At 72 hours, cytotoxicity was even more severe, with 90.80% ± 21.71 cell viability at 9.375 µg/mL, but only 8.40% ± 2.58 remaining at 300 µg/mL.

The IC_{50} values for the hydroethanolic extract declined steeply over time, reflecting its potent cytotoxicity: 183.1 ± 9.31 µg/mL at 24 hours, 93.11 ± 14.90 µg/mL at 48 hours, and 80.34 ± 9.65 µg/mL at 72 hours.

When comparing the two extracts, it becomes evident that the hydroethanolic extract (HE) is significantly more cytotoxic than the infusion (INF). The HE's IC_{50} values are lower across all time points, indicating that it reduces cell viability more effectively at lower concentrations. For example, at 72 hours, the IC_{50} for the HE is 80.34 ± 9.65 µg/mL, whereas for the INF, it is 143.8 ± 6.38 µg/mL, indicating that the HE becomes increasingly toxic over time. Additionally, at the highest concentration of 300 µg/mL, the HE causes almost complete cell death by 72 hours (8.40% ± 2.58 viability). In contrast, the INF, though still toxic, retains slightly more viable cells (9.32% ± 3.87 at 72 hours).

When comparing the effects of *Mentha aquatica* INF and HE on N27 dopaminergic cells and on normal human dermal fibroblasts (NHDF), differences in sensitivity and cytotoxicity become apparent. Both cell types show dose- and time-dependent responses to the extracts; however, the neural cells (N27) exhibit significantly greater vulnerability, especially to the HE.

In N27 cells, the HE shows significantly lower IC50 values across all time points, indicating more potent cytotoxicity. For example, at 72 hours, the IC_{50} for HE is 80.34 ± 9.65 µg/mL, while for INF, it is 143.8 ± 6.38 µg/mL. This indicates that the HE exerts more severe cytotoxicity at lower concentrations. At 300 µg/mL, HE nearly wipes out the N27 cells, with only 8.40% ± 2.58 cell viability remaining at 72 hours, compared to 9.32% ± 3.87 for the INF. The fact that both extracts are significantly more toxic to N27 cells reflects the heightened sensitivity of neural cells to oxidative stress and mitochondrial dysfunction, which are likely induced by the bioactive compounds in the HE. Additional studies would help clarify the underlying molecular mechanisms of this toxicity and confirm whether these effects are directly attributable to specific compounds in the extracts. This further investigation would enable a more robust validation of the hypothesis and provide greater insight into the safety and therapeutic potential of the extracts in sensitive cellular models, such as neurons.

When we compare these results with the NHDF cells, the cytotoxic effects of both extracts are less pronounced. For NHDF cells, the IC_{50} values are higher, indicating greater resistance to the toxic effects of both INF and HE. For example, NHDF cells exposed to the HE for over 72 hours have a higher IC_{50} than N27 cells, and cell viability remains higher at corresponding concentrations. The NHDF cells retain higher proliferation rates even at high concentrations of HE and INF, suggesting that dermal fibroblasts are more resilient to oxidative stress or apoptotic triggers that are more harmful to neural cells. While both extracts still demonstrate some toxicity to NHDF cells, the damage is not as immediate or severe as in the more sensitive dopaminergic neurons.

The difference in cellular response can be attributed to the fact that neural cells, such as N27 cells, have higher metabolic activity and are more susceptible to mitochondrial dysfunction and oxidative stress. Neural cells, including N27 cells, are characterised by their elevated metabolic rates, which are crucial for maintaining neurotransmission and overall cellular function. This high metabolic demand necessitates a well-functioning mitochondrial network, as mitochondria are the primary source of ATP production through oxidative phosphorylation (Casanova et al., 2023). However, this reliance on mitochondrial health also makes neural cells more susceptible to disruptions caused by bioactive compounds. Mitochondrial dysfunction can lead to increased production of reactive oxygen species (ROS), contributing to oxidative stress and cellular damage (Guo et al., 2013). In contrast, NHDF cells exhibit a different metabolic profile, which may include a greater reliance on glycolysis and alternative energy pathways. This metabolic flexibility allows NHDF cells to maintain cellular functions even under conditions of oxidative stress (Pinho et al., 2025; Zhang et al., 2012). Additionally, dermal fibroblasts have been shown to possess more robust antioxidant defence mechanisms, enabling them to mitigate the effects of oxidative stress more effectively than neural cells (Pinho et al., 2025). These insights highlight the importance of considering cell-type-specific responses when developing therapeutic strategies targeting oxidative stress-related conditions.

Furthermore, cytotoxicity assays conducted on NHDF cells and N27 dopaminergic neurons indicated that INF is less toxic than HE, especially at higher concentrations and prolonged exposure times. While the HE demonstrated more potent cytotoxic effects, particularly in neural cells, the INF exhibited a safer profile, maintaining higher cell viability. This suggests that the infusion may be better suited for long-term use or as part of a daily regimen in functional foods or herbal therapies aimed at reducing oxidative stress and managing diabetes.

Mentha aquatica, especially in its infusion form, emerges as a promising natural agent with hypoglycemic, antioxidant, and cytoprotective properties. Its ability to inhibit key enzymes involved in carbohydrate metabolism, combined with its relatively low cytotoxicity, positions it as a viable candidate for further development as a therapeutic, particularly for diabetes and oxidative damage mitigation. Further research should explore the clinical potential of *Mentha aquatica* extracts, particularly the INF, to confirm its efficacy in vivo for the management of diabetes and related oxidative stress disorders. Studies assessing the long-term effects of *Mentha aquatica* extracts on glycemic control, oxidative markers, and inflammation could provide valuable insights. Additionally, isolating and characterising individual bioactive compounds from the infusion may help identify those with the most potent effects, paving the way for the development of targeted therapeutic agents. Investigating optimal extraction conditions, formulation techniques, and delivery methods could also enhance bioavailability and maximise therapeutic efficacy. The influence of environmental factors on the phytochemical composition of *Mentha aquatica* should be further examined to support sustainable cultivation practices that preserve or enhance its bioactive potential.

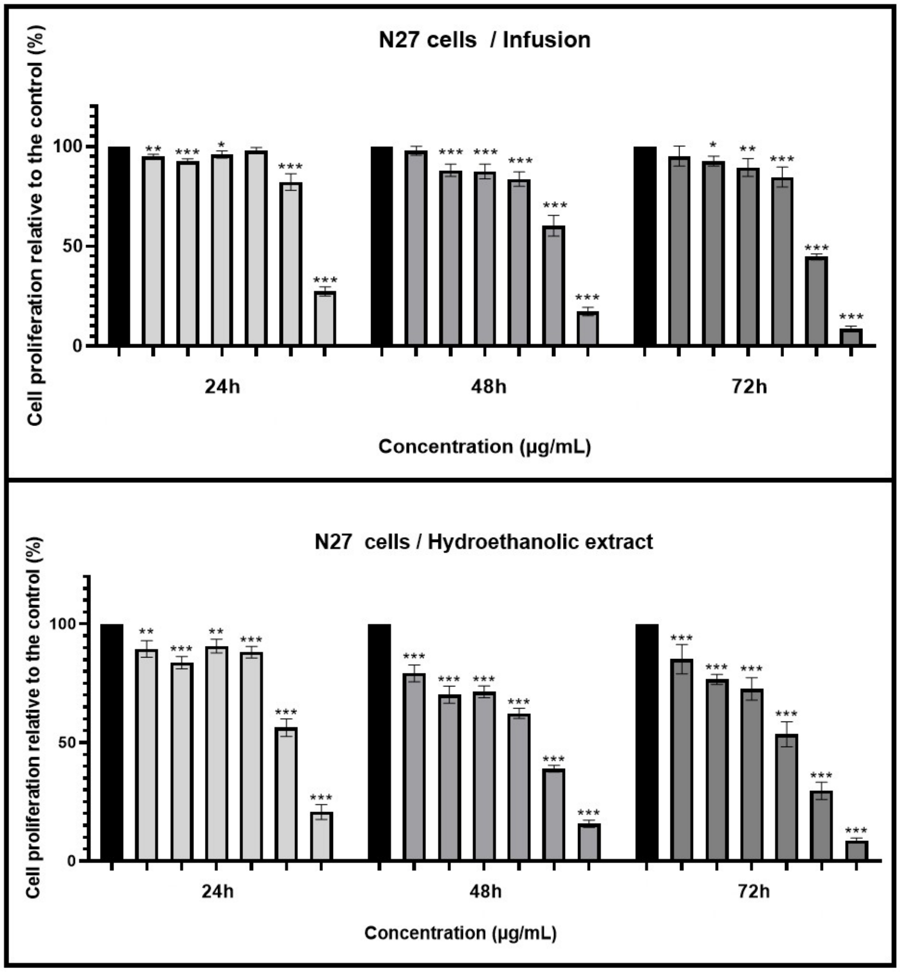

Figure 22. Effect of infusion and hydroethanolic extract on N27 viability after 24 h, 48h and 72h of exposure, assessed by MTT reduction. Values show mean ± standard deviation performed in triplicate. One-way ANOVA was used to determine statistical significance between N27 cells and the control (Black bar). A two-way ANOVA was used in N27 to compare the two extracts at different concentrations (9.375, 18.75, 37.5, 75, 150, and 300 µg/mL). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$)

PART III.
Anticoccidial Effects of Natural Phytogenic Additives in Broiler Chickens: An *In Vitro* and *In Vivo* Evaluation.

1. Introduction

Coccidiosis is one of the most common and economically devastating diseases in poultry farming, particularly in broiler production systems (Mesa-Pineda et al., 2021). Caused by several *Eimeria* species, it affects the intestinal epithelium, completing a 4–6-day life cycle that results in diarrhoea (sometimes bloody), dehydration, weight loss, poor feed conversion, and increased susceptibility to secondary infections (Latha & Srikanth, 2022). In 2016, the global economic cost of coccidiosis in chickens was estimated at €11.91 billion, including both productivity losses and prevention/treatment costs (Blake, 2025). The formation of resilient oocysts, highly resistant to disinfectants and persistent in the environment, complicates biosecurity and sanitation measures (Avi et al., 2023).

In Algeria, *Eimeria tenella*, *E. acervulina*, and *E. maxima* are the predominant species affecting broiler flocks (Avi et al., 2023; Bora et al., 2024). Poor hygiene, overcrowding, and inadequate vaccination contribute to disease prevalence (Amina et al., 2025; Ayadi et al., 2024; Benabdelhak et al., 2024; Debbou-Iouknane et al., 2018; Djemai et al., 2016, 2022; Rahmani et al., 2024). Furthermore, resistance to commonly used anticoccidials such as monensin and diclazuril is increasingly reported, with mechanisms involving altered membrane permeability, drug efflux, target site modifications, and alternative biochemical pathways (Noack et al., 2019; Xie et al., 2020; H. Zhang et al., 2022, 2023; Zhao et al., 2024). This resistance may also be linked to the emergence of antimicrobial resistance in other pathogens, including *Campylobacter* spp., raising public health concerns (Q. Zhang & Plummer, 2014).

These challenges have driven the search for alternative, sustainable control strategies. Botanical products, including essential oils, polyphenols, tannins, and alkaloids, have demonstrated antimicrobial, antiparasitic, antioxidant, anti-inflammatory, and immunomodulatory properties (Ahmad et al., 2024; Aitfella Lahlou et al., 2021; El-Shall et al., 2022). Unlike conventional drugs that often target specific parasite stages, plant-derived products act through multiple mechanisms, improving gut integrity, modulating microbiota, and reducing oxidative stress (Al-Garadi et al., 2025; Jamil et al., 2017; Saleem et al., 2024; Saleh et al., 2018).

Within the Lamiaceae family, species such as *Mentha aquatica, Rosmarinus officinalis*, and *Thymus algeriensis* are rich in bioactive compounds with potential anticoccidial effects (Aitfella Lahlou et al., 2021; Lahlou et al., 2022, 2024). While their chemical composition and antioxidant properties are well documented, their direct link to anticoccidial performance in poultry remains unexplored. This study investigates whether extracts with high phenolic and flavonoid contents and strong in vitro antioxidant capacity can improve oocyst inactivation, reduce intestinal lesions, and enhance zootechnical performance in broilers. By integrating phytochemical profiling with *in vitro* and *in vivo* assays, we aim to establish a clear relationship between the extract composition and its anticoccidial efficacy, thereby contributing to the development of sustainable phytogenic alternatives to conventional drugs.

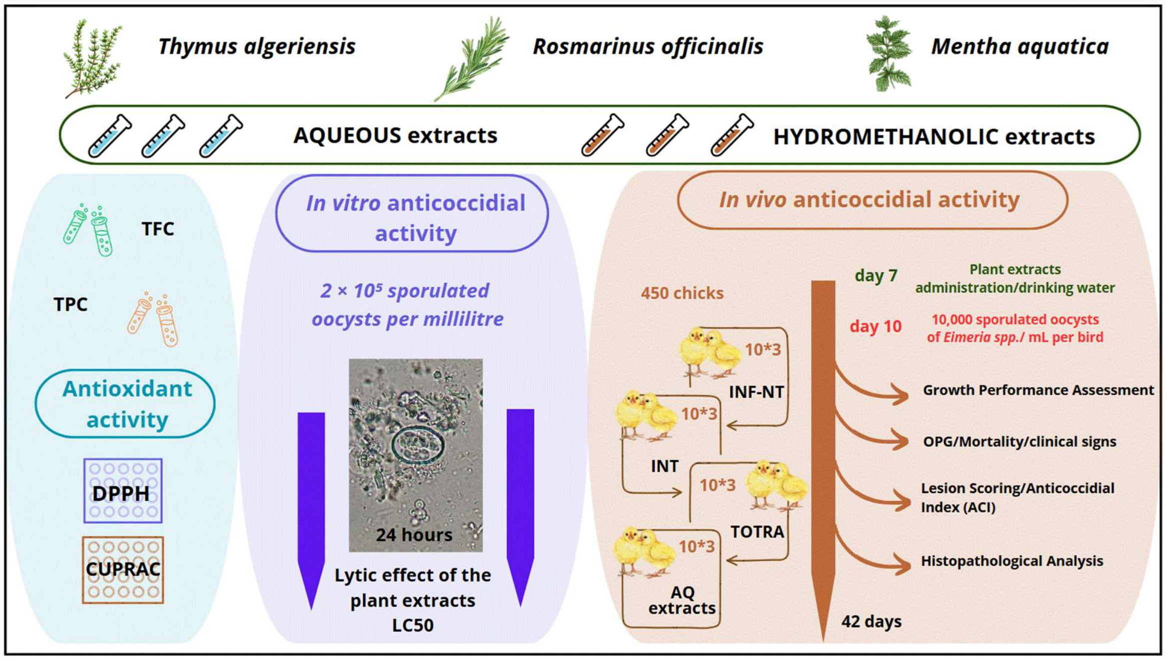

Figure 23. Antioxidant and anticoccidial effects of *Thymus algeriensis*, *Rosmarinus officinalis*, and *Mentha aquatica* Extracts: *in-vitro* oocyst lysis and *in-vivo* broiler challenge.

2. Materials and methods

2.1. Plant extractions

The aerial parts of *Mentha aquatica* (ME), *Rosmarinus officinalis* (RO) and *Thymus algeriensis* (TA) were collected in the district of Bordj Bou Arréridj, north-eastern Algeria. Specimens of ME and TA were harvested in the Oued Sayed area (Medjana), at 1050 m altitude, whereas RO was gathered in the Hammam El Biban area (Mansoura). The geographic coordinates of each site are listed in Table 17. After taxonomic verification, voucher specimens were deposited in the Herbarium of the VALCOR Laboratory, Faculty of Science, University M'Hamed Bougara, Boumerdes, Algeria. For extraction, the air-dried aerial parts were ground into a fine powder, and 10 g of each sample was macerated at room temperature for 24 hours in 100 mL of pure water (aqueous extract, AQ) or in a methanol: water (80:20, v/v) solution (hydromethanolic extract, HM). The macerates were filtered, and the solvents were removed under reduced pressure with a rotary evaporator at a controlled temperature. The concentrates were then lyophilised to give the crude extracts; the corresponding extraction yields are reported in Table 17.

Table 17. Geographic coordinates and yields of aqueous and hydromethanolic extracts of three Lamiaceae species.

Plants	**Geographic coordinates**	**Yield (%)**
Rosmarinus officinalis	36°11'00.4"N 4°25'53.7"E	AQ: 18.577 ± 3.696
		HM: 15.913 ± 4.110
Thymus algeriensis	36°10'49.1"N 4°36'43.6"E	AQ: 11.675 ± 0.625
		HM: 11.455 ± 0.010
Mentha aquatica	36°10'49.1"N 4°36'43.6"E	AQ: 21.640 ± 1.619
		HM: 19.300 ± 0.080

2.2. Total polyphenol content

Total polyphenols were quantified with the Folin–Ciocalteu (FC) assay, following Singleton et al. (1999), and expressed as gallic-acid equivalents (GAE). Briefly, 20 µL of each plant extract (1000 ppm in methanol) was mixed with 100 µL of 10 % (v/v) Folin–Ciocalteu reagent (Singleton et al., 1999). After 5 min, 75 µL of 7.5 % (w/v) Na_2CO_3 were added. A reagent blank was prepared under identical conditions, substituting methanol for the extract. The mixtures were kept in the dark at room temperature for 2h, and absorbance was then read at 765 nm. All measurements were performed in triplicate, and results are reported as µg GAE/mg extract (mean ± SD).

2.3. Total flavonoid content

Flavonoid content was quantified by the aluminium-chloride colourimetric assay described by Türkoğlu et al. (2007). The method relies on the formation of a yellow Al^{3+}–flavonoid complex, which shows a maximum absorbance at 415 nm. In each well of a 96-well microplate, 50 μL of plant extract, 130 μL of methanol, 10 μL of 1 M potassium acetate, and 10 μL of 10 % (w/v) $AlCl_3{\cdot}6H_2O$ were mixed (Türkoğlu et al., 2007). A reagent blank was prepared by replacing the extract with methanol. After incubation for 40 min at room temperature in the dark, absorbance was measured at 415 nm. A quercetin calibration curve (0–200 μg/mL) was used for quantification, and results were expressed as milligrams of quercetin equivalents per milligram of extract (mg QE/mg).

2.4. Antioxidant activity

2.4.1. DPPH assay

The hydrogen- - or electron-donating capacity of the samples was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, following the method of Blois (1958). A 0.04% (w/v) DPPH solution was prepared in methanol (4 mg DPPH/100 mL), stored in the dark at 4 °C, and then brought to room temperature before use (Blois, 1958). In a 96-well plate, 40 μL of each sample at the desired concentration was mixed with 160 μL of the DPPH solution (0.1 mM). A negative control consisted of 40 μL of methanol and 160 μL of DPPH. The plate was incubated for 30 min in the dark at room temperature, after which absorbance was read at 517 nm. Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) served as positive standards for antioxidants.

$$Inhibition\ (\%) = \frac{A\ control - A\ sample}{A\ control} \times 100$$

A Control is the initial concentration of the DPPH

A Sample is the absorbance of the remaining concentration of DPPH in the presence of the extract and positive control.

Results are expressed as IC_{50} values (mg/mL), the sample concentration required to scavenge 50% of DPPH radicals under the assay conditions.

2.4.2. CUPRAC assay

The cupric-ion reducing capacity of each fraction was evaluated using the CUPRAC assay, as described by Apak et al. (2004). In a test tube, 1.0 mL of 10 mM $CuCl_2 \cdot 2H_2O$, 1.0 mL of ammonium-acetate buffer (1 M, pH 7.0), and 1.0 mL of 7.5 mM neocuproine were mixed with 0.5 mL of plant extract or reference standard at the desired concentration (Apak et al., 2006). The reaction volume was brought to 4.1 mL by adding 0.6 mL of distilled water. All samples were prepared in triplicate. The mixtures were incubated for 60 min at room temperature, after which absorbance was read at 450 nm against a reagent blank (buffer replacing the extract) (Apak et al., 2006). A CUPRAC solution containing 50 µg/mL of standard antioxidant served as the assay control; BHT and BHA were used as positive references. An increase in absorbance reflected a greater reducing (antioxidant) capacity. Results are expressed as IC_{50} values (µg/mL), i.e., the extract concentration required to produce an absorbance of 0.50 under the assay conditions.

2.5. Anticoccidial activity

2.5.1. Parasite Source

Field sampling was carried out on deceased broiler chickens. Farms were selected in coordination with the Wilaya of Boumerdès' Agricultural Services Department (DSA); the selection criteria included prior veterinary reports indicating a suspected coccidiosis. A total of ≈ 80 carcasses were collected. After necropsy, the entire intestinal tract was removed, and both external and internal surfaces were inspected. Intestinal contents were recovered by gentle scraping at multiple sites along the tract to ensure comprehensive parasite collection (Conway & McKenzie, 2007). The resulting material was pooled in conical tubes, placed in insulated containers, and transported immediately to the VALCOR laboratory at the Faculty of Science, University M'hamed Bougara, Boumerdes. Samples were stored at 5–10 °C until further processing and preparation of parasite suspensions.

2.5.2. Parasite preparation

Direct microscopic examination confirmed the presence of coccidia in intestinal scrapings. Using sterile cotton swabs, material was collected from both the surface and deeper layers, smeared onto glass slides, covered with coverslips, and examined under a light microscope at ×40 and ×100 magnification (Carvalho et al., 2011). All positive scrapings were pooled and weighed. A 2% (w/v) potassium dichromate ($K_2Cr_2O_7$) solution was added as a preservative (10 mL per 2 g of sample); the mixture was homogenised in a mortar, and coarse debris was removed with a fine sieve. The suspension was incubated at 28–30 °C and ≈80 % relative humidity, with aeration for 7 days to allow oocyst sporulation. Pre- and post-incubation oocyst counts, made with a Malassez haemocytometer, yielded a sporulation rate of 96.66% (Conway & McKenzie, 2007). Sporulated oocysts were concentrated by standard flotation. The suspension was mixed with 6.1 M NaCl in a conical tube, incubated for 10 min to facilitate flotation, and centrifuged at 1200 rpm for 5 min. The upper 1 mL layer was transferred to Eppendorf tubes, repeatedly washed with distilled water (following centrifugation) to remove salt, and then filtered. Duplicate Malassez counts (third count if >10 % variance) gave $(1.93 \pm 0.07) \times 10^8$ total oocysts. For morphometric species identification, 100 sporulated oocysts were measured in triplicate (n = 300) under a microscope equipped with a calibrated ocular micrometre. Length, width, and the length/width ratio were recorded for each oocyst (Table 18). These values were then compared to established morphometric references to determine species. Data from the comparative chart below were used to confirm identification. The resulting species composition was as follows: *Eimeria maxima* (37.0%), *Eimeria brunetti* (19.4%), *Eimeria necatrix* (15.7%), *Eimeria praecox* (13.9%), *Eimeria tenella* (8.3%), and *Eimeria mitis* (5.6%).

Table 18. Comparative oocyst sizes of *Eimeria* spp. in chickens (Adapted from Jean-Michel Reperant, ANSES, 2018).

Species	Length (µm)	Width (µm)
Eimeria acervulina	17.7–20.2	13.7–16.3
Eimeria brunetti	20.7–30.3	18.1–24.2
Eimeria maxima	21.5–42.5	16.5–29.8
Eimeria mitis	11.7–18.7	11.0–18.0
Eimeria necatrix	13.2–22.7	11.3–18.3
Eimeria praecox	19.8–24.7	15.7–19.8
Eimeria tenella	19.5–26.0	16.5–22.8

2.5.3. *In vitro* anticoccidial activity

The anticoccidial activity of the plant extracts against *Eimeria* spp. Oocysts were assessed using the procedure described by Remmal et al. (2011), with minor modifications. A sterile 96-well microplate was first filled with 140 µL per well of an agar-supplemented HBSS medium (Hank's balanced salt solution lacking sodium bicarbonate and phenol red) (Remmal et al., 2011). The liquid phase was fortified with penicillin (100 IU/mL) and fluconazole (17 mg/mL) and then sterilised by filtration through a 0.2 µm membrane. Each plant extract was dissolved in phosphate-buffered saline (PBS) to give stock solutions ranging from 0.2 to 20 mg/mL. For every concentration tested, 40 µL of the extract was added to a well, followed by 20 µL of a parasite suspension containing 2×10^5 sporulated oocysts per millilitre.

Two commercial anticoccidials, Sulfaquinoxaline/trimethoprim (Vetacox) and Toltrazuril (Baycox ND), were included at the same concentrations, while untreated wells served as negative controls. Plates were left uncovered to ensure adequate aeration and incubated for 24 hours at 25–30°C under 60–80% relative humidity with gentle shaking. After incubation, oocysts in each well were enumerated with a Malassez hemacytometer, and both the percentage of viable oocysts and any wall deformations were recorded. The LC_{50}, the extract concentration required to eliminate or deform 50 % of the oocysts present in the negative control, was calculated for each treatment. All tests were performed in triplicate at each concentration.

The lytic effect of the plant extracts and reference anticoccidials on *Eimeria* oocysts was further evaluated by monitoring the release of intracellular material at 273 nm (Remmal et al., 2011). Aliquots (1 mL) were prepared in sterile Eppendorf tubes containing a washed and filtered oocyst suspension, 700 µL of PBS (pH 7.4), and 200 µL of each plant extract at concentrations ranging from 0.2 to 20 mg/mL (Remmal et al., 2011). Tubes were incubated for 24h at 25°C. Sulfaquinoxaline/trimethoprim (SUS) and Toltrazuril (TOTRA) were included as positive controls at the same concentration levels. After incubation, samples were centrifuged at 3200 rpm for 5 min at 4°C. Supernatants (500 µL) were collected, and their absorbance was measured at 273 nm using a UV spectrophotometer (Remmal et al., 2011). Baseline correction was performed using PBS containing the corresponding extract concentration, but without the addition of oocysts.

The resulting pellets were resuspended, and oocysts were counted in a Malassez hemacytometer to correlate absorbance changes with oocyst lysis. Data are presented as changes in absorbance at 273 nm and corresponding oocyst viability versus extract concentration. Each assay was tested in triplicate.

2.5.4. *In vivo* evaluation of plant-extract efficacy against coccidiosis

a. Farm characteristics and animal husbandry

The study was conducted at a broiler facility within the AVIGA complex (Industrial Poultry Accumulation and Fattening) in the Wilaya of Boumerdès. The farm is privately owned and specialises in broiler production. Although the selected house has a total surface area of 940 m^2, only a portion, stocked at roughly 10 birds m^{-2}, was used for the entire 42-day trial. A total of 450 Cobb 500 (*Gallus gallus domesticus*) chicks were placed on straw bedding for the first ten days; they were then transferred to 21 floor pens (100 × 120 cm, wire-mesh sides, no cover) containing wood shavings, at 10 birds per pen. Each pen was equipped with a feeder, a drinker and a butane-gas radiant heater, all of which had been disinfected with bleach. Two exhaust fans provided ventilation. Chicks were weighed on day 0 to establish a uniform average hatch weight and again on day 7, immediately before being randomly allocated to pens. All birds were vaccinated against Newcastle disease, infectious bronchitis and infectious bursal disease (Gumboro) and received an anti-stress supplement before and after each vaccination or transfer. Any chick that died during the first two weeks was weighed and replaced with one of similar weight. The feed was provided as mash, formulated with a multivitamin premix and without anticoccidial additives. Starter and grower diets were offered on days 0-21 and 22-42, respectively (Table 19), with both feed and water supplied ad libitum. A work schedule was established to record body weight at the end of each feeding phase.

Table 19. Ingredients and nutrient composition of the basal diets administered during the trial.

Ingredients (%)	**Starter (1–21 d)**	**Grower (22–42 d)**
Corn	55.00	55.00
Soybean meal	25.00	20.00
Wheat bran	10.00	10.00
Vegetable oil	4.00	5.00
Dicalcium phosphate	1.80	1.60
Limestone	1.10	1.00
DL-Methionine (98%)	0.32	0.26
L-Lysine HCl (78%)	0.35	0.30
Salt	0.20	0.25
Vitamin–mineral premix[1]	1.00	1.00
Total	**100.00**	**100.00**
Calculated nutrient composition		
Metabolizable energy (kcal/kg)	2850	3100
Crude protein (%)	21.70	20.00
Crude fiber (%)	4.00	4.00
Crude fat (%)	3.50	5.00
Calcium (%)	1.20	1.00
Available phosphorus (%)	0.50	0.45
Lysine (%)	1.30	1.10
Methionine (%)	0.50	0.45
Methionine + cysteine (%)	0.94	0.85
Threonine (%)	0.80	0.75
Tryptophan (%)	0.20	0.20
Arginine (%)	1.30	1.20
Sodium (%)	0.20	0.25

b. Safety dose assessment

Before the main experimental trial, a preliminary study (10 days) was conducted to assess the safety of the different plant extracts on broiler chickens (Not published). The animals used in this study were 10 days old (10 subjects for each group), clinically healthy, and free of coccidial infection, as confirmed by parasitological examination of faecal samples. The aqueous extract stock solutions were prepared by suspending 100 g of dried, ground plant material in 1 L of distilled water and allowing the mixtures to stand at room temperature for 24 hours. From these stock solutions, four working concentrations, 5 mL, 15 mL, 50 mL, 100 mL, and 200 mL, were diluted in 1 L of drinking water and freshly prepared each day. The hydro-methanolic extract was initially prepared, dried, and then reconstituted in water before being administered orally via gavage.

However, at all tested concentrations, the hydro-methanolic extract induced an unacceptably high mortality rate during the first 24 hours. Clinical signs of toxicity were observed, including lethargy, ruffled feathers, reduced feed and water intake, loss of balance, and severe prostration, indicating acute systemic effects. As a result, only the aqueous extract was retained for the *in vivo* study. It was administered through drinking water to ensure consistent intake and to minimise stress related to handling. Based on the safety outcomes, only the concentrations of 15 mL/L and 50 mL/L of the aqueous extract were selected for continuation in the experimental phase. All procedures were conducted in accordance with current European regulations and ethical standards for animal experimentation, including the EMA-CVMP guidelines for target animal safety (Albretsen, 2024; FDA, 2009; Medicines Agency, 2022, 2024).

c. Experimental design

From day 7 onward, the 450 chicks were allocated to nine experimental groups based on the plant species and administered dose (Table 20). Each group consisted of 30 birds, housed in three replicate pens of 10 birds. Plant treatments also began on day 7. The aqueous and hydroethanolic extract stock solutions were prepared by suspending 100 g of dried, ground plant material in 1 L of distilled water and allowing the mixtures to stand at room temperature for 24 hours. Two working concentrations (15 mL/L and 50 mL/L) of the extract preparation were mixed with drinking water and prepared fresh each day. On day 10, birds in the six challenged groups received 10,000 sporulated oocysts of *Eimeria* spp. by oral gavage (1 mL per bird). Unchallenged groups were given 1 mL of sterile 0.9 % saline.

The toltrazuril group (I) was treated when clinical signs of coccidiosis first appeared (25 mg toltrazuril in 200 L of drinking water for 5 consecutive days, equivalent to 7 mg/kg body weight). Groups G (uninfected, untreated) and H (infected, untreated) received no anticoccidial medication. To rule out pre-existing infections, all chicks underwent routine health checks, including coprological screening, during a pre-trial adaptation period.

Table 20. Broiler group treatments for botanical natural products and anticoccidial tests.

Group	**Infection status**	**Treatment description**	**Dosage oral oocysts**
A (TH-AQ1)	Infected	*Thymus algeriensis* aqueous extract, 15 mL/L (1.5g/L)	10000
B (TH-AQ2)	Infected	*T. algeriensis* aqueous extract, 50 mL/L (5g/L)	10000
C (RO-AQ1)	Infected	*Rosmarinus officinalis* methanolic extract, 15 mL/L (1.5g/L)	10000
D (RO-AQ2)	Infected	*R. officinalis* methanolic extract, 50 mL/L (5g/L)	10000
E (ME-AQ1)	Infected	*Mentha aquatica* methanolic extract, 15 mL/L (1.5g/L)	10000
F (ME-AQ2)	Infected	*M. aquatica* methanolic extract, 50 mL/L (5g/L)	10000
G (NINT)	Uninfected	Untreated (blank control)	-
H (INT)	Infected	Untreated (negative control)	10000
I (Toltrazuril)	Infected	Toltrazuril, 7 mg/kg bw for 5 days (positive control)	10000

d. Studied parameters

- Growth Performance Assessment

Throughout the trial, growth was monitored by weighing all birds in each pen at placement and weekly on days 0, 10, 20, and 32 post-infection (PI). The difference between consecutive weighings provided body weight gain (BWG) for each interval. In contrast, feed intake for the same intervals was calculated by subtracting residual feed and the feed estimated for any birds that died from the total feed offered. Feed-conversion ratio (FCR) was expressed as feed intake divided by weight gain and adjusted for mortality. Daily checks were made to record mortality, and every deceased bird was weighed and examined to establish the cause of death. Together, body weight (BW), feed intake (FI), feed conversion ratio (FCR), and mortality provided an overview of growth performance and overall flock health. To summarise these variables into single techno-economic efficiency metrics, two composite indices were computed using the method of Marcu et al. (2013): the European Production Efficiency Factor (EPEF) and the European Broiler Index (EBI). Both indices combine growth rate, feed efficiency and livability, offering an integrated appraisal of each treatment's overall performance across the 32-day experimental period (Marcu et al., 2013).

- **Survival and Weight-Gain Indices**

A comprehensive evaluation was performed on day 32 PI. Clinical symptoms and mortality were assessed and recorded each day after infection. The survival rate for each treatment was calculated as the proportion of birds alive relative to the initial number placed. The weight-gain rate for each bird corresponded to its net weight increase between the time of infection and day 32. The relative weight-gain (RWG) rate expresses this increase as a percentage of the animal's pre-infection weight. These indices allowed direct comparison of post-infection growth and viability among the various experimental groups.

- **Oocyst Enumeration**

The parasitic load was monitored by counting oocysts at various time points. Faecal samples (500g) were collected from each pen on repeatedly measured days at 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, and 32 days post-infection (dpi) (n = 6 replicates), homogenised, and examined microscopically using a Malassez hemacytometer to determine oocysts per gram of faeces (OPG). Subsequently, the OPG was converted to the oocyst value (OV%). The reduction rate of oocyst production and oocyst value was calculated as indicated by Chauhan et al. (2017). The results were expressed as OPG output (Chauhan et al., 2017; Holdsworth et al., 2004).

- **Lesion Scoring**

At day 10 post-infection (PI), a standardised sampling protocol was carried out on fifteen broiler chickens (five birds per treatment group) (Chang et al., 2021a; Chauhan et al., 2017; Johnson et al., 1970). Before sampling, these chickens were fasted for 24 hours to clear their gastrointestinal tract, while water was provided ad libitum to maintain hydration. On day 10 PI, the birds were humanely slaughtered by approved animal welfare guidelines. Slaughter was performed by exsanguination via the jugular vein, and immediately afterwards, each carcass was de-feathered and eviscerated. The entire intestinal tract was then carefully removed and collected for gross examination of lesions. Gross intestinal lesions were scored using the established Johnson & Reid scoring system, which assigns a numerical severity grade from 0 to 4. A score of 0 indicates no visible lesions, whereas a score of 4 reflects the most severe intestinal damage, characterised by extensive haemorrhage and large caseous cores. Intermediate scores (1, 2, and 3) denote progressively increasing levels of lesion severity, as defined by the standard criteria.

This grading method provides a consistent, objective assessment of lesion severity across all treatment groups, enabling reliable comparisons of disease impact across different experimental conditions.

- **Anticoccidial Index (ACI)**

The ACI was calculated for each treatment group as an integrated measure of anticoccidial efficacy, incorporating four parameters: survival rate (SR), relative weight-gain rate (RWG), oocyst value (OV), and lesion score (LS) (Chang et al., 2021b). This index provides a comprehensive assessment of how effectively the treatment controls coccidiosis. Interpretation of ACI values followed standard thresholds: ACI < 120 indicates no anticoccidial effect; values of 120–159 reflect low efficacy; 160–179 denotes moderate efficacy; and ACI ≥ 180 is considered indicative of excellent anticoccidial efficacy.

- **Histopathological Analysis**

Intestinal tissue sampled at day 10 PI was fixed in neutral-buffered 10 % formalin, processed routinely, embedded in paraffin, sectioned at 5 μm and stained with haematoxylin–eosin (S. Khan et al., 2023). Microscopic examination focused on mucosal integrity, inflammatory infiltrates, haemorrhage and the presence of parasitic stages within epithelial cells. Histopathology thus complemented gross lesion scoring by revealing cellular-level alterations induced by infection and by any protective effects of the administered treatments.

2.6. Statistical analysis

Statistical analyses were performed on triplicate measurements for each assay. GraphPad Prism (version 8) was used to conduct a one-way ANOVA ($\alpha = 0.05$). Post-hoc comparisons among treatments were carried out with Tukey's and Dunnett's multiple-comparison tests at the 95 % confidence level. Correlation analyses employed Pearson correlation coefficients (r), simple linear regression, and analysis of covariance (ANCOVA).

3. Results and discussion

3.1. Polyphenols and flavonoids contents

The total phenolic content (TPC) and total flavonoid content (TFC) of *Mentha aquatica* (ME), *Rosmarinus officinalis* (RO), and *Thymus algeriensis* (TH) extracts are summarised in Table 21. Overall, the hydromethanolic (HM) extracts yielded higher total phenolic content (TPC) than the aqueous (AQ) extracts for all three species (Figure 24). For instance, TH showed a TPC of 220.35 ± 5.92 mg GAE/g DW in the HM extract, compared to 128.11 ± 10.56 mg GAE/g in the AQ extract, representing a nearly 1.7-fold increase. RO similarly exhibited a higher TPC in HM (215.50 ± 17.19 mg GAE/g) compared to water extract (160.96 ± 6.55 mg/g). MA had the highest TPC among aqueous extracts (167.13 ± 26.48 mg/g), which further rose to 199.26 ± 12.26 mg/g in the HM extract. Statistical analysis confirmed that TPC was significantly higher ($p < 0.05$) in HM extracts compared to AQ extracts for each plant (Figure 24). Thus, adding 80% methanol markedly enhanced polyphenol extraction efficiency, consistent with the general notion that polar organic solvents recover more phenolics than water alone. Among the species, TH and RO-HM extracts exhibited the highest phenolic levels (~215–220 mg GAE/g), while ME was only slightly lower (~199 mg GAE/g) (Figure 24). In the aqueous preparations, ME retained the highest total phenolic content (TPC), followed by RO and TH. These results indicate that both species identity and solvent type influenced the polyphenol yield.

Table 21: Total phenolic and flavonoid content in different plant extracts.

Samples	**Aqueous extracts**		**Hydromethanolic extracts**	
Parameters	**TPC***	**TFC***	**TPC***	**TFC***
Rosmarinus officinalis	160.96 ± 6.55	49.26 ± 0.40	215.50 ± 17.19	33.26 ± 1.40
Thymus algeriensis	128.11 ± 10.56	15.20 ± 2.30	220.35 ± 5.92	28.05 ± 1.31
Mentha aquatica	167.139 ± 26.48	29.56 ± 2.58	199.26 ± 12.26	59.77 ± 4.07

*All values are significant at ($p<0.05$). TPC: Total phenolic content (mg GAE/g DW), TFC: Total flavonoid content (mg QE/g DW).

The trends in flavonoid content (TFC) were more variable among the extracts. ME stood out with the highest flavonoid levels in its HM extract (TFC = 59.77 ± 4.07 mg QE/g DW), which was roughly double that of its water extract (29.56 ± 2.58 mg/g) (Figure 24). TH also showed a substantially higher TFC in the HM extract (28.05 ± 1.31 mg/g) compared to the AQ extract (15.20 ± 2.30 mg/g) (Figure 24). In contrast, RO displayed the opposite pattern: its AQ extract had a notably greater TFC (49.26 ± 0.40 mg/g) than the HM extract (33.26 ± 1.40 mg/g). This anomaly suggests that certain flavonoids in rosemary may be more efficiently extracted by water (perhaps due to highly water-soluble glycosides) or that some compounds in the methanol-water mixture could interfere with flavonoid yield (Sultana et al., 2009). Despite this species-specific divergence, the differences in TFC between extract types were statistically significant ($p < 0.05$) for each plant (Figure 24). Overall, ME had the richest flavonoid content in the HM form, whereas RO led in flavonoid content for the aqueous extracts. These data suggest that the choice of extraction solvent can differentially affect flavonoid recovery, depending on the plant's phytochemistry. Hydromethanol (80% MeOH) tends to extract more flavonoids in ME and TH, whereas pure water is surprisingly effective for RO flavonoids. Such variations could be linked to the distinct profiles of flavonoid compounds in each species (e.g. rosemary may contain more polar flavonoid glycosides, while mint and thyme have flavonoids that are better solubilised by aqueous methanol).

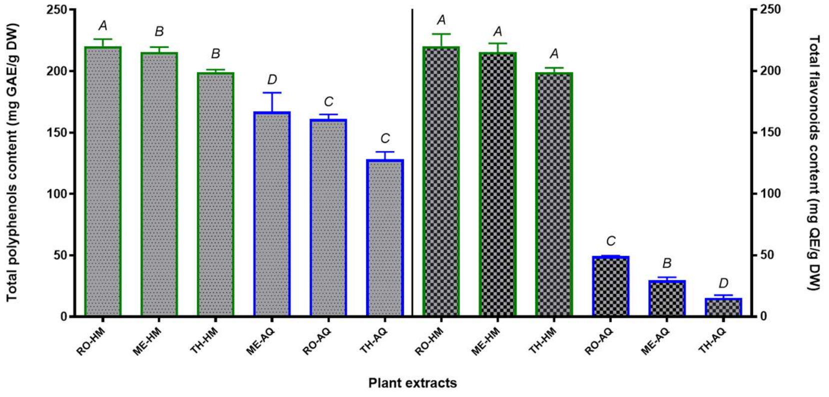

Figure 24. Total polyphenol content (TPC) expressed as gallic acid equivalents (µg GAE/mg DW) and the total flavonoids (TFC) content as quercetin equivalents (µg QE/g DW) of hydromethanolic (HM) and aqueous (AQ) extracts of *Mentha aquatica* (ME), *Rosmarinus officinalis* (RO), *Thymus algeriensis* (TH). Values show means ± standard deviation (SEM) of three independent assays. Statistical analysis: A one-way ANOVA with Tukey's multiple comparisons test was performed for each extract compared to the others. Different letters above the bars indicate significant differences between groups at $p < 0.05$. Each letter denotes a homogeneous group; bars sharing the same letter are not significantly different, while bars with different letters indicate statistically significant differences.

Across the three Lamiaceae herbs, comparative work keeps converging on two consistent messages: (i) extraction and post-harvest handling can swing phenolic and flavonoid yields by an order of magnitude, and (ii) even modest quantitative differences matter, because antioxidant capacity tracks very closely with the totals. *Mentha aquatica* illustrates the first point vividly. In Safaiee et al.'s (2019) study, which systematically varied drying strategies and ultrasound conditions, freeze-drying followed by a short 5-minute sonication at 60 °C delivered the highest recovery. However, this peak still reached only 0.245 mg gallic acid equivalents (GAE) per gram of dry leaf, equivalent to approximately 245 µg GAE/g. Microwave- and oven-dried samples came in lower, indicating that water-mint phenolics are delicate once moisture is removed. Sonication time itself, interestingly, had almost no statistical weight on the final yield (Safaiee et al., 2019). Our yields eclipse or at least equal the best figures reported so far. In Benabdallah et al. (2016), six wild *Mentha* species were recorded at 43.21 ± 1.09 mg GAE/g (Benabdallah et al., 2016), while Fidan et al. (2023) confirmed a comparable value of 43.36 mg GAE/g; both are roughly one-quarter of our hydromethanolic value (Fidan et al., 2023).

Rosmarinus officinalis behaves quite differently. Afonso et al (2013) prepared a gentle aqueous infusion (INF) and a non-esterified phenolic fraction (NEPF) from dried rosemary leaves. The infusion retained a respectable 16.67 ± 0.40 mg GAE/g, while the more selective NEPF held 8.59 ± 0.31 mg GAE/g. Although that study did not report a separate total-flavonoid figure, its chromatographic profile confirmed rosmarinic acid and carnosic acid as dominant compounds, and the authors linked the higher phenolic load of the INF directly to stronger *in vivo* antioxidant endpoints (lower TBARS and boosted catalase/GPx activities) (Afonso et al., 2013). Our results, therefore, sit an order of magnitude above the infusion and about double the strongest alcoholic benchmarks, reflecting both our late-summer harvest and the higher affinity of methanol for diterpenic acids such as rosmarinic and carnosic.

Thymus algeriensis has long been considered modest in its soluble polyphenol content. A recent work optimised ultrasound-assisted extraction with a Box–Behnken design. Under the best conditions (60 °C, 36.7 min, 32.8 mL solvent), flowering-stage material yielded 2.961 g GAE 100/g (≈ 29.6 mg GAE/g) and 0.887 g quercetin equivalents (QE) 100/g (≈ 8.9 mg QE/g).

Those totals, coupled with IC_{50} values of 0.016 mg/mL for the DPPH assay and 0.426 mg/mL for the FRAP assay, underscore how ultrasound can coax out a chemically potent but extraction-sensitive phenolic pool in this North African thyme (Souadia et al., 2025). The leap very likely comes from the exceptionally arid provenance of our stand (a known trigger for caffeic-acid biosynthesis), combined with a 24-h maceration that penetrates both leaf and woody bract tissues. Ziani et al. (2019) data on T. algeriensis follow the same pattern, peaking near 34 mg GAE/g, which is well below our 220 mg plateau (Ziani et al., 2019).

These comparisons show that extraction chemistry and ecological stress can outweigh species identity when the aim is to maximise antioxidant density. Where the literature typically quotes double-digit milligram values, our hydromethanolic protocol routinely climbs past 200 mg GAE/g. That potency, however, comes with trade-offs: more bitter diterpenes in rosemary, a darker hue in mint, and resinous notes in thyme that formulators will have to balance against shelf-life or health-claim targets.

3.2. Antioxidant activity

Oxidative stress is known to exacerbate intestinal damage and immune dysfunction in poultry affected by coccidiosis, a primary parasitic disease that compromises gut integrity, growth performance, and overall welfare in broiler chickens (Ghareeb et al., 2022; Griss et al., 2019; Tompkins et al., 2023). Given the increasing restrictions on synthetic anticoccidials and growing consumer demand for natural alternatives, plant-based extracts with antioxidant and anti-inflammatory properties are gaining interest as supportive agents in poultry health management. In this context, evaluating the antioxidant potential of selected medicinal plants can provide valuable insights into their capacity to mitigate oxidative damage associated with *Eimeria* infections. To this end, we assessed the radical-scavenging activity of aqueous (AQ) and hydromethanolic (HM) extracts prepared from three aromatic species. Both extraction methods reflect realistic applications, infusions mimic traditional uses, and methanol–water mixtures enhance the recovery of bioactive compounds. Two well-established *in vitro* assays were used to quantify the antioxidant activity of the extracts, providing a dual perspective on their ability to quench free radicals and reduce oxidative burden in broiler models exposed to coccidial stress.

Table 22 compares the radical-scavenging power of aqueous (AQ) and hydromethanolic (HM) extracts prepared from *Mentha aquatica* (MA), *Thymus algeriensis* (TH) and *Rosmarinus officinalis* (RO). Activity was quantified with the well-established DPPH assay, which measures the capacity of an antioxidant to reduce the purple $DPPH^{\bullet}$ radical, and with the CUPRAC test, which monitors the reduction of the Cu(II)–neocuproine complex at physiological pH. In both methods, a lower IC_{50} denotes a more potent antioxidant, i.e., less extract is required to quench 50% of the radicals (Apak et al., 2004; Brand-Williams et al., 1995).

Hydromethanolic extraction enhanced antioxidant potency. The ME-HM extract was the most powerful of all plant samples (IC_{50}^{DPPH} = 10.19 ± 0.51; IC_{50}^{CUPRAC} = 9.47 ± 0.19 µg/mL), outperforming every other extract and even surpassing the synthetic antioxidant BHT in the DPPH test, while approaching BHT in CUPRAC. Only BHA remained markedly stronger (IC_{50}^{DPPH} = 5.73 ± 0.41; IC_{50}^{CUPRAC} = 3.64 ± 0.19 µg/mL). The second-best sample was the ME-AQ extract (12.79 ± 0.05; 12.06 ± 0.60 µg/mL), followed by TH-HM (15.56 ± 0.63; 11.72 ± 0.85 µg/mL) and RO-HM (15.74 ± 0.69; 14.50 ± 0.14 µg/mL). In every species, the HM fraction showed lower IC_{50}s than its aqueous counterpart, confirming that a 50 % $MeOH/H_2O$ solvent retrieves a broader spectrum of redox-active metabolites.

The concordant rankings from DPPH and CUPRAC suggest that the extracts contain both hydrogen-donating and electron-donating antioxidants, primarily polyphenols. Mixed methanol–water systems are known to extract these compounds more efficiently than water alone, because methanol disrupts cell walls and solubilises moderately polar phenolics while still keeping highly polar compounds in solution (Babbar et al., 2012). Therefore, the superior activity of ME-HM likely reflects a richer load of flavonoids, rosmarinic acid derivatives and other phenolics contributing additionally to radical scavenging.

Table 22: Antioxidant capacities of aqueous and hydromethanolic extracts by CUPRAC and DPPH methods.

	IC_{50} (µg/ml) *			
Extracts	**Aqueous extracts**		**Hydromethanolic extracts**	
	DPPH	**CUPRAC**	**DPPH**	**CUPRAC**
Rosmarinus officinalis	16.37 ± 0.01	18.80 ± 0.02	15.74 ± 0.69	14.50 ± 0.14
Thymus algeriensis	17.18 ± 0.09	15.65 ± 0.58	15.56 ± 0.63	11.72 ± 0.85
Mentha aquatica	12.79 ± 0.05	12.06 ± 0.60	10.19± 0.51	9.47 ± 0.19
BHA	5.73 ± 0.41	3.64 ± 0.19	5.73 ± 0.41	3.64 ± 0.19
BHT	22.32 ± 1.19	9.62 ± 0.87	22.32 ± 1.19	9.62 ± 0.87

*All values are Significant at ($p<0.05$). IC_{50} (µg/mL) indicates the concentration at which 50% of the scavenging activity is observed.

The antioxidant ranking obtained in our study (ME-HM > ME-AQ > TH-HM ≈ RO-HM > RO-AQ > TH-AQ) is broadly consistent with the recent literature on the same Lamiaceae species, while highlighting how solvent choice, chemotype and processing can modulate absolute IC_{50} values. For *Mentha aquatica*, Pereira et al. (2019) reported a DPPH EC_{50} of 8.1 ± 1.3 µg/mL for an 80 % hydroethanolic extract, very close to the 10.2 µg/mL we measured for the hydromethanolic fraction and likewise identified the mint as the most potent antioxidant among the plants they compared (Pereira et al., 2019). Another study comparing an infusion (INF) with a 70 % hydroethanolic extract (HE) showed the DPPH IC_{50} of the HE to be approximately six-fold lower than the INF, mirroring the solvent-driven gain we observed for the HM fraction over the aqueous one (Lahlou et al., 2024). A more recent study encapsulating a pure methanolic extract of *M. aquatica* in alginate hydrogels showed that the entrapped extract retained low-micromolar DPPH IC_{50} values and superior oxidative stability, confirming that the strong radical-scavenging power of this species is preserved across formulation types (Solhtalab et al., 2024). Elsewhere, a drying-method investigation (microwave, oven, shade) demonstrated that essential oil–rich fractions exhibit up to a 4-fold reduction in antioxidant capacity compared with the corresponding ethanolic extracts, reinforcing the solvent-polarity effect we observed (HM < AQ).

A Tunisian survey of *Thymus algeriensis* wild populations found that methanolic leaf extracts scavenged DPPH radicals with an IC_{50} of ≈ approximately 7 µg/mL, outperforming BHT (11.5 µg/mL) and confirming the strong radical-quenching capacity of this species when extracted with polar organic solvents (Megdiche-Ksouri et al., 2015). An investigation of Algerian populations reported that a phenolic-rich hydromethanolic fraction (50% MeOH) scavenged DPPH radicals with an IC_{50} of ≈ approximately 7 µg/mL and markedly enhanced plasma antioxidant status *in vivo* (Righi et al., 2020). Our hydromethanolic extract (IC_{50} = 15.6 µg/mL) retains the same activity range. However, the slightly higher value is expected because we analysed the entire aerial parts rather than just the phenolic-rich leaves, and used a milder 50% MeOH/H_2O system. By contrast, the hydro-distilled essential oil (rich in terpenoids but almost devoid of polyphenols) displayed very weak radical-scavenging power (DPPH IC_{50} = 83.8 mg/mL), roughly four orders of magnitude higher than our HM extract (15.6 µg/mL) (Hamza et al., 2015). The result highlights how removing the phenolic fraction in favour of volatile monoterpenes can drastically reduce antioxidant efficacy.

For *Rosmarinus officinalis*, a multi-approach study on Algerian rosemary reported IC_{50} values of 6.82 µg/mL (DPPH) and 1.59 µg/mL (CUPRAC) for an 80% methanolic extract, which is substantially stronger than our HM fraction (15.7 and 14.5 µg/mL, respectively). The gap highlights the impact of chemotype (Mediterranean vs. local ecotype) and intensification techniques (sonication, higher MeOH content) on polyphenol yield; however, both studies concur in ranking rosemary as a top natural antioxidant under CUPRAC conditions (Bencharif-Betina et al., 2023). Sahunie (2024) showed that switching from 80 % methanol to ethyl-acetate (low-polarity) extraction more than doubled the IC_{50} (DPPH and CUPRAC), confirming that semi-polar solvents recover fewer redox-active diterpenes and phenolic acids than hydro(methanol) systems (Sahunie, 2024). Conversely, the study of Doozakhdarreh et al (2022) on rosemary essential oils harvested and dried under different regimes reported IC_{50} values as low as 6–10 µg/mL for shade-dried material, comparable to our HM extract and showing that an optimised terpene profile can rival polyphenol-rich solvent extracts (Doozakhdarreh et al., 2022). An additional recent study using super-critical CO_2 coupled with natural deep-eutectic solvents (NADES) demonstrated that cleaner, terpene-enriched fractions still display measurable radical-quenching activity, although they remain less potent than hydro-methanolic counterparts because key phenolic acids (rosmarinic, carnosic) partition poorly into CO_2 (Ayyildiz et al., 2024). Martín-Sampedro et al. (2016) demonstrated that super-critical CO_2 antisolvent fractionation can further concentrate carnosic and rosmarinic acids; however, DES or hydro-methanol still yield the lowest IC_{50}s overall (Sánchez-Camargo et al., 2016). According to Bejenaru et al. (2024), ethyl-acetate, ethanolic and aqueous extracts gave DPPH IC_{50} values of 272, 387 and 534 µg/mL, respectively, far weaker than our HM fraction (15.7 µg/mL) and illustrating the limited solubility of carnosic acid/carnosol in those solvents (Bejenaru et al., 2024). Conversely, an agrifood study found that the essential oil scavenged DPPH with an IC_{50} of 15.0 µg/mL, essentially matching our HM value and underscoring that diterpenes in the oil can, when sufficiently concentrated, rival polyphenol-rich solvent extracts (Gokbulut et al., 2022).

Taken together, these external datasets reinforce the qualitative hierarchy observed here and demonstrate that hydromethanolic extraction systematically boosts the recovery of redox-active phenolics, thereby lowering IC_{50} values. Variations across studies primarily reflect geographic chemotypes, plant parts, and extraction intensity; yet, the core message remains consistent: *M. aquatica* and *T. algeriensis* possess exceptional electron- and hydrogen-donating capacities, while rosemary extracts remain highly effective, mainly when richer methanolic systems or process intensification are employed. The MeOH/H_2O system we used thus remains a robust, food-compatible strategy for maximising antioxidant efficacy in *M. aquatica*, *T. algeriensis* and *R. officinalis*.

3.3. *In vitro* anticoccidial activity

Maintaining effective biosecurity in broiler production is essential to controlling coccidiosis, a parasitic disease that remains one of the most economically damaging and welfare-compromising conditions in poultry farming. The infective oocysts of *Eimeria* are incredibly resilient in the environment, resisting standard cleaning and surviving for extended periods in poultry housing, where they can rapidly reinfect flocks. Traditional chemical disinfectants often fail to destroy the fully sporulated, infective form of the parasite, and their repeated use raises concerns about environmental and health impacts.

In light of this, we explored sustainable, plant-based alternatives that can target *Eimeria* oocysts outside the host. The aim was to evaluate the direct oocysticidal activity of selected plant extracts as potential natural disinfectants. To this end, fully sporulated oocysts (Figure 25), the most resistant developmental stage, were incubated for 24 hours with aqueous and *hydromethanolic* (HM) extracts of *Rosmarinus officinalis* (RO), *Mentha aquatica* (ME), and *Thymus algeriensis* (TH).

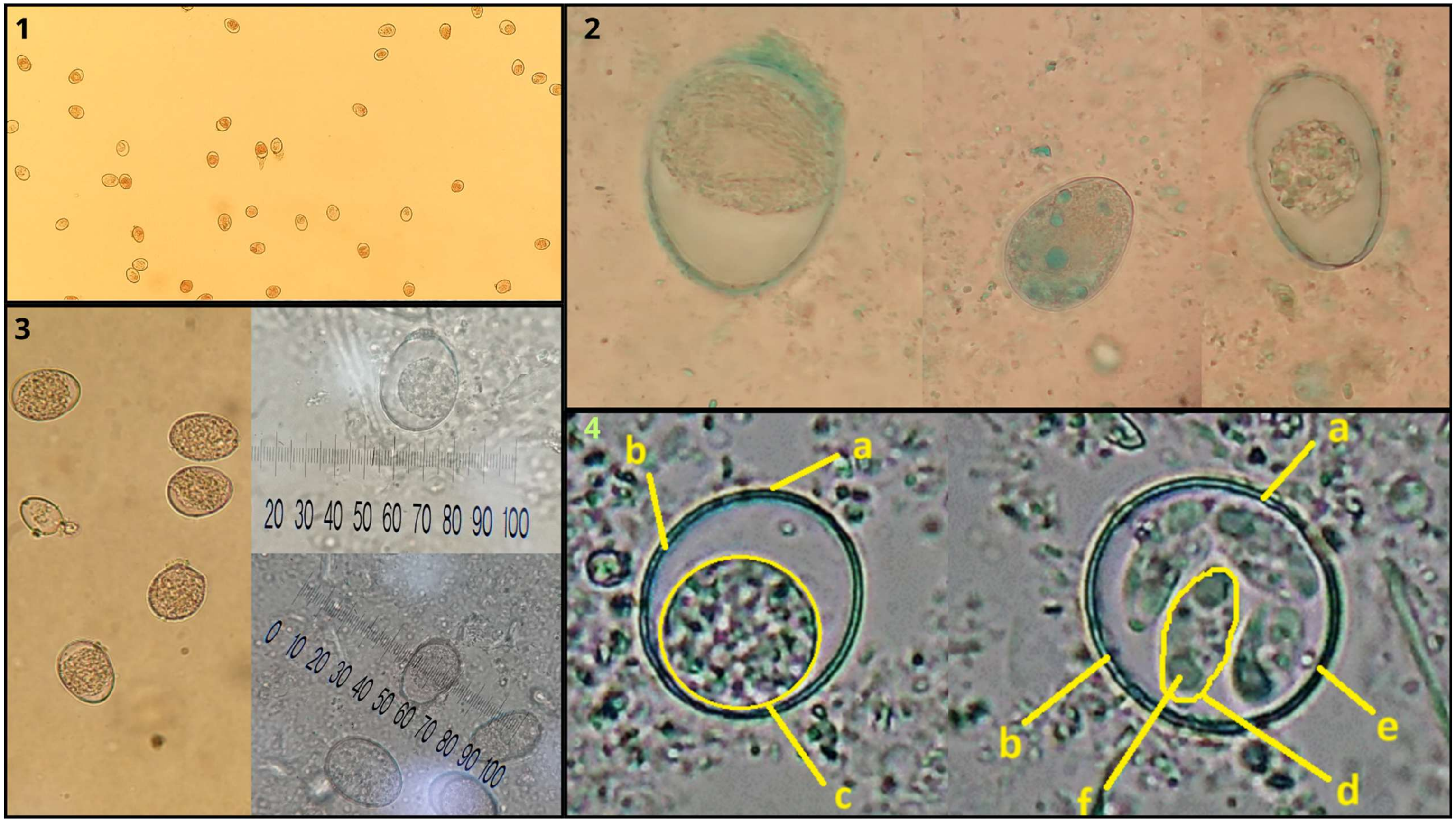

Figure 25. Microscopic evaluation of *Eimeria spp.* oocysts isolated from broiler chicken intestinal contents. (1) Sporulated oocysts observed under light microscopy at low magnification. (2) Morphological diversity of oocysts after differential staining, revealing internal sporocysts and wall characteristics. (3) Oocyst morphometry using an ocular micrometre for species identification. (4) Structural features of sporulated oocysts: (a) outer wall, (b) inner wall, (c) sporocyst, (d) sporozoites, (e) micropyle cap, (f) residual body.

According to Table 23, the most effective treatment was RO-HM, with an LC_{50} of 233.14 ± 3.56 μg/mL, followed by ME-HM (967.11 ± 2.58 μg/mL) and RO-AQ (699.11 ± 4.61 μg/mL). The aqueous extract ME-AQ also showed relevant activity (329.86 ± 2.78 μg/mL). In comparison, both *T. algeriensis* extracts, TH-HM (2621.62 ± 2.90 μg/mL) and TH-AQ (3449.56 ± 2.53 μg/mL), were markedly less active, indicating limited oocysticidal potential.

Table 23. Anticoccidial activities of plant extracts and anticoccidial drugs.

	LC_{50} (μg/ml) *	
Samples	**Aqueous extracts**	**Hydromethanolic extracts**
Rosmarinus officinalis	699.11 ± 4.61	233.14 ± 3.56
Thymus algeriensis	3449.56 ± 2.53	2621.62 ± 2.90
Mentha aquatica	329.86 ± 2.78	967.11 ± 2.58
Sulfaquinoxalin sodic trimethoprim	NA	
Toltrazuril	NA	

*All values are significant ($p < 0.0001$)

Conventional anticoccidial drugs, sulfaquinoxaline/trimethoprim and toltrazuril, showed no measurable activity under these experimental conditions, underscoring the need to develop effective, environmentally friendly alternatives (Table 23). They are engineered to act inside the bird, where *Eimeria* schizonts or gamonts are actively synthesising folate or generating ATP; they therefore leave entirely sporulated oocysts, metabolically quiescent and shielded by a thick, dityrosine-cross-linked wall, completely untouched (Chapman & Rathinam, 2022; Walker et al., 2016). The impermeability and biochemical inertia of that wall mean that even high drug concentrations cannot penetrate or find a vulnerable target (Bushkin et al., 2013; Walker et al., 2016). As a result, the environmental reservoir of infective oocysts persists despite in-host medication, underscoring the need for complementary strategies that break the cycle outside the bird, rigorous litter management, and the use of alkaline or cresolic disinfectants, as well as novel agents that block sporulation altogether (Chapman & Rathinam, 2022; Mai et al., 2009). In this context, plant-derived phenolics such as thymol and carvacrol have demonstrated the ability to deform or even lyse sporulated oocysts and to inhibit new sporulation in vitro, suggesting phytogenic additives as promising adjuncts to conventional treatments (Arafa et al., 2020; El-Shall et al., 2021; Felici et al., 2020).

Marked differences in oocysticidal efficacy were observed among the tested extracts. The hydromethanolic extracts of RO-HM and ME-HM exhibited the most vigorous activity, reducing oocyst viability by more than 65% compared to the untreated control (Figure 26). These two extracts demonstrated comparable potency, suggesting similar effectiveness in disrupting oocyst integrity. In contrast, the hydromethanolic extract of TH-HM was significantly less effective, with a viability reduction approximately 25% lower than that achieved with ME-HM, indicating limited antiparasitic activity (Figure 26). Among the aqueous extracts, ME-AQ showed the highest efficacy, significantly decreasing oocyst viability and surpassing the effects of both RO-AQ and TH-AQ. TH-AQ, in particular, exhibited the weakest activity, with oocyst viability remaining relatively high even at the highest concentrations tested (Figure 26).

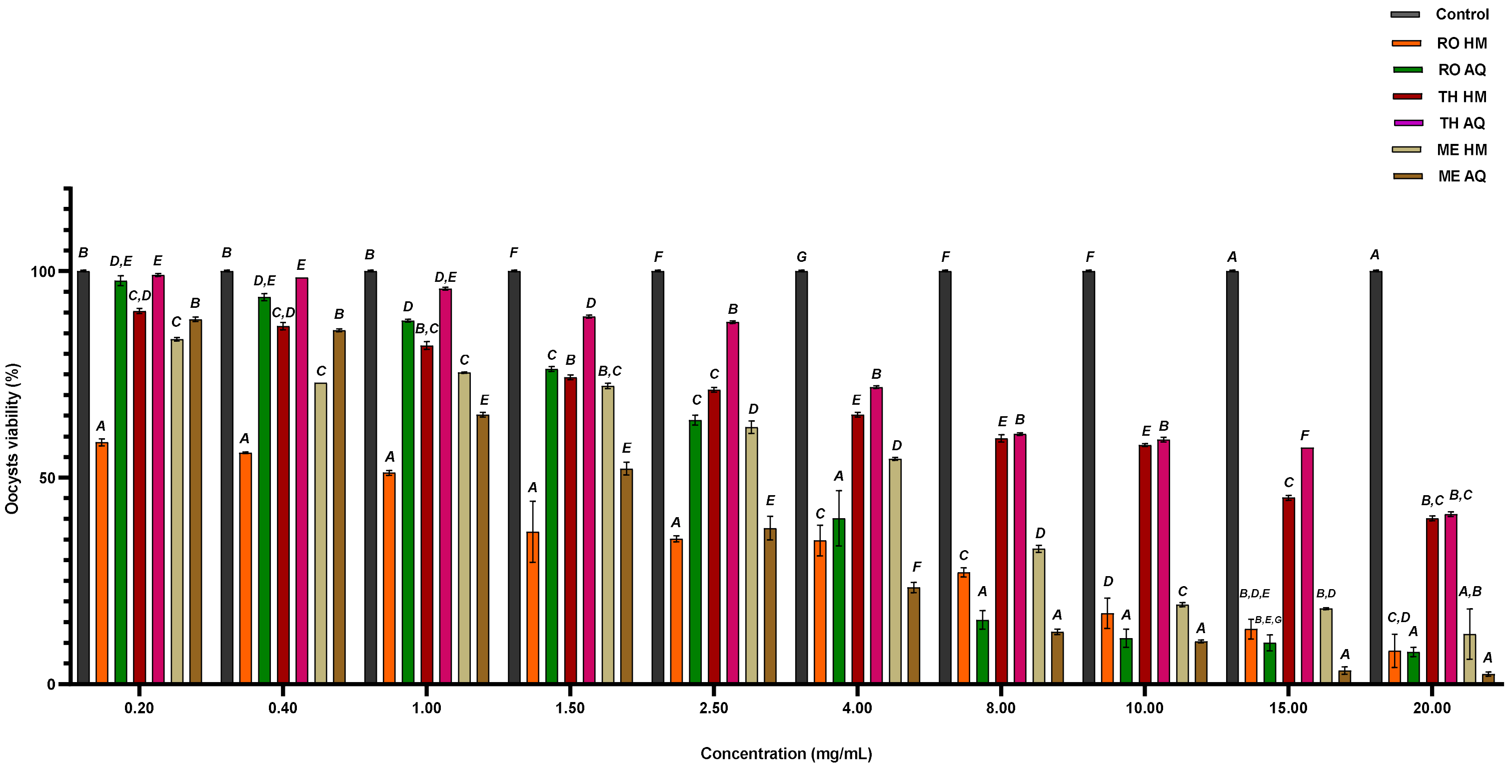

Figure 26. Viability of *Eimeria* oocysts after exposure to hydro-methanolic (HM) and aqueous (AQ) extracts of *Rosmarinus officinalis* (RO), *Thymus algeriensis* (TH), and *Mentha aquatica* (ME), across a concentration gradient (C1 to C10). Oocyst viability (%) was assessed following incubation with plant extracts at increasing concentrations ranging from 0.2 to 20 mg/mL. The bar plots show mean viability ± standard error (n = 3). Black bars indicate the untreated control. Hydro-methanolic and aqueous extracts are represented by orange and green (RO), red and magenta (TH), and beige and brown (ME). Statistical differences between groups were determined using two-way ANOVA followed by Tukey's multiple comparisons test. For each concentration point (C1–C10), treatments that do not share a common letter differ significantly ($p < 0.05$). The analysis revealed significant effects of concentration, extract type, and their interaction on oocyst viability ($p < 0.0001$ for all factors).

Our findings accord with earlier *in vitro* studies. Remmal et al. (2011) demonstrated that four essential oils (thyme, artemisia, tea tree, and clove) effectively destroyed sporulated *Eimeria* oocysts within hours, accompanied by the release of 273-nm-absorbing compounds (Remmal et al., 2011). Jitviriyanon et al. later reported Thai essential oils of *Ocimum basilicum* and *Boesenbergia pandurata* with IC_{50} values of 0.101 and 0.134 mg/mL, respectively, against *E. tenella* sporulation (Jitviriyanon et al., 2016). More recently, Gadelhaq et al. found that aqueous garlic and moringa extracts blocked chicken oocyst sporulation almost completely (up to 100% at 70% v/v), whereas plain hypochlorite achieved only ~50% inhibition under identical conditions (Gadelhaq et al., 2018). Maodaa *et al.* observed that a methanolic extract of *Thymus daenensis* inhibited *E. papillata* sporulation by >90 % at 300 mg/mL (S. N. Maodaa et al., 2024). Orange-peel essential oil, an agro-industrial co-product, damaged both sporulated and unsporulated *E. tenella* oocysts, causing up to 64 % structural loss at 30 % v/v (Kalita, 2022).

Pomegranate peel, another processing by-product, blocked oocyst sporulation and reduced shedding *in vivo* (Dkhil, 2013). Compared with conventional disinfectants, these extracts perform favorably: Lee et al. determined that ≥ 7.5 % hydrogen-peroxide solutions had to act for three hours to inactivate fully (Lee & Lee, 2007) *E. acervulina* oocysts, and H_2O_2 gas plasma were required for rapid eradication. Against that benchmark, the LC_{50}s of RO-HM (0.23 mg/mL) and ME-AQ (0.33 mg/mL) place the botanical extracts among the most potent agents, rivalling reference oxidants while being less corrosive and safer for operators.

RO-HM's superiority stems from its high content of phenolic diterpenes (carnosic acid, carnosol) and rosmarinic acid, which can permeabilise parasite membranes and inhibit calcium-dependent kinases essential to the oocyst life-cycle (Remmal et al., 2011; Sidiropoulou et al., 2020). The intense activity of ME-AQ highlights the role of highly hydrophilic polyphenols (rosmarinic-acid glycosides, flavonoids) that, like essential-oil components, destabilise the oocyst's double chitinous wall. Thymus extract is weaker, reflecting its lower thymol/carvacrol content; studies on thyme and oregano oils show that boosting these monoterpenes can increase oocysticidal efficacy by an order of magnitude (Felici, Tugnoli, Ghiselli, et al., 2023).

Mortality was paralleled by a rise in supernatant absorbance at 273 nm, indicating leakage of nucleotides and aromatic amino acids and confirming a membrane-disruptive mode of action (Figure 27). Lysis of the Eimeria population was described by a linear regression equation (Table 24) and showed uniformly negative slopes ($p \leq 0.003$), confirming a dose-dependent coccidiocidal effect across all samples tested. Among aqueous extracts, the potency hierarchy was RO > ME > TH, with rosemary displaying the steepest decline (slope = −0.024 ± 0.005), whereas the hydromethanolic series favoured *M. aquatica* (−0.018 ± 0.002). Goodness-of-fit values (R^2 = 0.77–0.90) indicated that linear models accounted for most of the variance, although Mentha-AQ showed hints of non-linearity at sub-inhibitory doses (R^2 = 0.70) (Figure 27). The divergence between chemical richness (the highest TPC in HM fractions) and biological potency (often higher in AQ fractions) suggests that rapidly diffusing polar metabolites, rather than bulk phenolics, dominate early oocyst inactivation. Because each concentration was assayed only once, these slopes should be considered provisional potency indices pending full biological replication.

Table 24. Linear-regression statistics describing the concentration-dependent fall in *Eimeria* oocyst viability produced by aqueous (AQ) and hydromethanolic (HM) extracts of *Rosmarinus officinalis* (RO), *Thymus algeriensis* (TH) and *Mentha aquatica* (ME). Ten concentrations (0–20 mg mL^{-1}) were tested once each; simple linear fits were generated. DF = 1, 8 for all regressions. Sy.x = standard error of the regression. All slopes differ significantly from zero.

Extract	**Slope ± SE (10^{-3} % mL mg^{-1})**	**95 % CI slope**	**Y-intercept ± SE (%)**	**R^2**	**Sy.x**	**Equation**	***p* (slope)**
RO-AQ	−24.07 ± 4.63	−34.74 – −13.40	80.56 ± 8.35	0.772	19.03	y = -4.814301x + 80.56027	0.0008
TH-AQ	−14.24 ± 1.73	−18.22 – −10.26	93.82 ± 3.12	0.895	7.10	y = -2.848292x + 93.82187	<0.0001
ME-AQ	−20.11 ± 4.71	−30.97 – −9.25	63.32 ± 8.50	0.695	19.36	y = -4.022062x + 63.31536	0.0027
RO-HM	−11.83 ± 1.84	−16.08 – −7.58	48.63 ± 3.32	0.838	7.57	y = -2.365649x + 48.63178	0.0002
TH-HM	−11.70 ± 1.37	−14.85 – −8.55	81.90 ± 2.47	0.902	5.62	y = -2.340540x + 81.90387	<0.0001
ME-HM	−18.64 ± 2.39	−24.14 – −13.14	73.66 ± 4.31	0.884	9.81	y = -3.727003x + 73.66176	<0.0001

Linear-regression analysis leaves no doubt that oocyst viability decreases as extract concentration increases (Figure 27): every slope is negative, and, according to the F-tests ($p \leq 0.003$), each differs significantly from zero. The magnitude of the slope is a direct measure of potency on a mass basis. The aqueous rosemary extract (RO-AQ) shows the steepest slope in absolute terms (−0.024 % mL/mg) and is therefore the most aggressive, followed by the aqueous mint extract (ME-AQ) and, lastly, the aqueous thyme extract (TH-AQ) (Figure 27). In the hydromethanolic series, the pattern is reversed: mint (ME-HM) is now the most active, while thyme (TH-HM) and rosemary (RO-HM) bring up the rear with slopes of roughly −0.012 % mL/mg. This ranking does not accurately reflect the total phenolic content, which is typically higher in the hydromethanolic fractions; instead, it suggests that rapid oocysticidal activity is driven by highly polar metabolites that diffuse readily through the oocyst wall. Model quality supports this view: coefficients of determination range from 0.77 to 0.90 (with the sole exception of ME-AQ, $R^2 = 0.70$) (Figure 27), meaning that a straight line explains between 77% and 90% of the observed variance, while the standard error of the regression (Sy. x) remains below 20%. The y-intercept, which varies between 44% and 94% across batches, remains close to the expected spontaneous mortality and does not compromise slope comparisons.

From an application standpoint, aqueous rosemary stands out as the leading candidate because it combines rapid action, technological simplicity and regulatory acceptability. The other aqueous extracts also display appreciable potential, whereas the hydromethanolic fractions, despite their richer chemistry, are less effective on a per-unit-mass basis. Taken together, the results call for biological replication, the targeted identification of active water-soluble small molecules, and the optimisation of an aqueous formulation suited for veterinary or agri-food use.

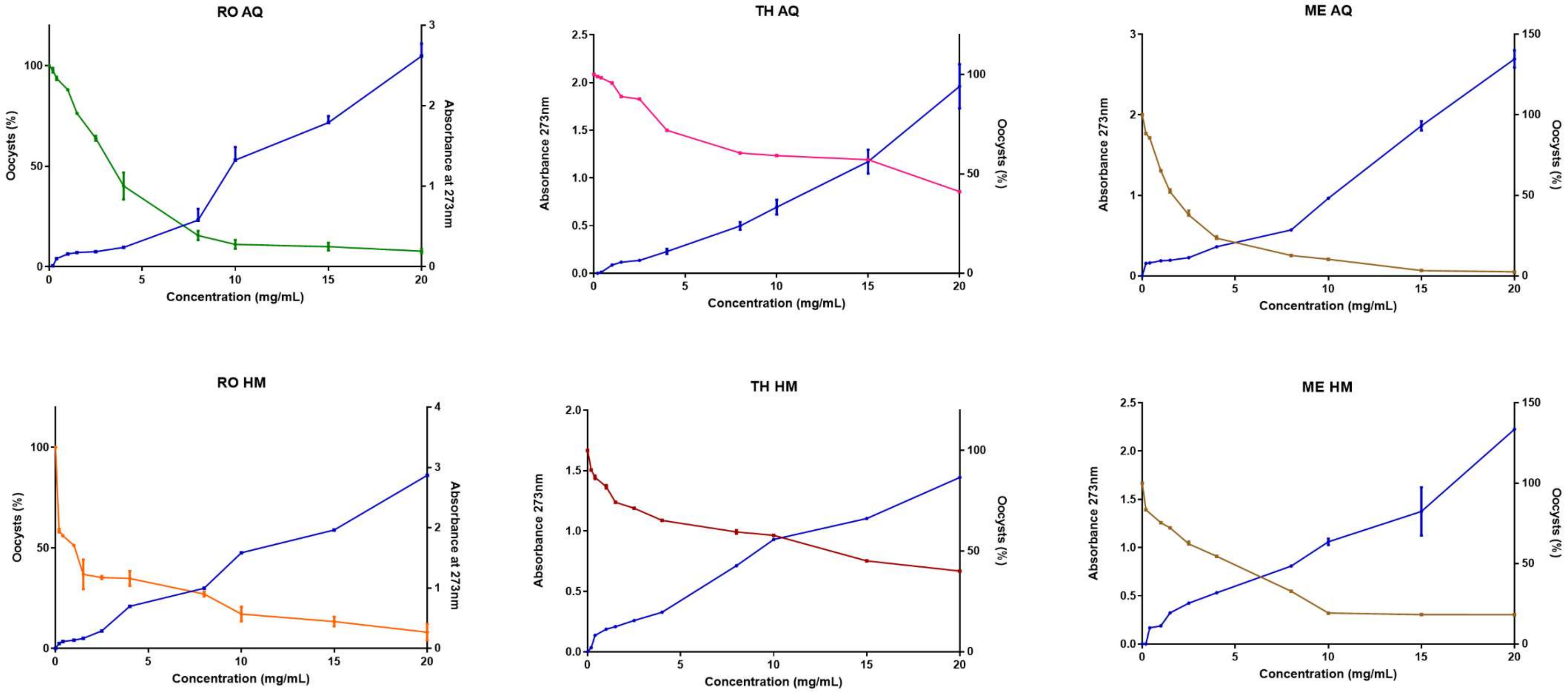

Figure 27. Dose-response profiles of aqueous (AQ) and hydromethanolic (HM) extracts from *Rosmarinus officinalis*, *Thymus algeriensis* and *Mentha aquatica* showing oocyst inhibition (%) and corresponding UV absorbance at 273 nm.

3.4. *In vivo* experimentation

3.4.1. Growth Performance and Production Efficiency

The inclusion of extracts or essential oils from Lamiaceae plants has emerged as a promising nutritional strategy for broiler chickens. Beyond their well-known antioxidant and anti-inflammatory properties, many of these botanicals exhibit direct anticoccidial effects, resulting in faster growth, improved feed conversion efficiency, and enhanced gut health in commercial flocks (Arczewska-Włosek et al., 2017; Arczewska-Wlosek et al., 2018; Hosseinzadeh et al., 2023; L. M. Pop et al., 2019; Shah et al., 2023). Systematic reviews and controlled experiments demonstrate that incorporating these phytogenic supplements, either alone or in combination with standard anticoccidials, consistently yields heavier finishing weights and more efficient feed conversion (Abbas, 2012; Abdelli et al., 2021; Aitfella Lahlou et al., 2021; Aminullah et al., 2025; Biswas et al., 2024; El-Shall et al., 2021; Koorakula et al., 2022; Upadhaya & Kim, 2017; J. Wang et al., 2024). In this context, our experimental trial aimed to assess both the prophylactic and therapeutic efficacy of aqueous extracts from *Rosmarinus officinalis*, *Thymus algeriensis*, and *Mentha aquatica* against avian coccidiosis in broiler chickens. To evaluate their potential, we conducted a controlled trial using aqueous extracts of the plants at two concentrations (15 and 50 mL/L). From day 7, 450 chicks were distributed into nine groups (n = 30), with extract administration via drinking water. On day 10, six groups were challenged with *Eimeria* spp. (10,000 oocysts/bird), while others remained uninfected or untreated. A toltrazuril-treated group served as a positive control. The aim was to monitor *in vivo* anticoccidial and performance-related effects. Table 25 summarises the post-infection evolution of performance parameters: body weight gain (BWG), Total Weight Gain (TWG), Average Daily Gain (ADG), Feed intake (FI), Feed Conversion Ratio (FCR), the European Production Efficiency Factor (EPEF) and European Broiler Index (EBI).

Both the plant species and the extract dosage markedly influenced broiler performance. The group treated with *Thymus algeriensis* at 15 mL/L (TH-AQ1) exhibited the highest cumulative body weight gain (BWG) at 32 days post-infection (2319.55 ± 43.56 g), significantly outperforming all other groups ($p < 0.05$) (Table 25). This superior growth was further reflected in total weight gain (TWG) (2098.40 ± 35.05 g) and average daily gain (ADG) (65.58 ± 1.10 g), underscoring the efficacy of this treatment in sustaining growth even under parasitic pressure (Table 25).

These trends are visually reinforced in Figure 28, which tracks the evolution of BWG over time. The graph clearly shows that broilers receiving *T. algeriensis* extracts (both TH-AQ1 and TH-AQ2) maintained a consistent upward trajectory in weight gain post-infection, exceeding both the positive control group (TOTRA) and the infected untreated group (INF-NT). By contrast, broilers treated with *Mentha aquatica*, especially at 50 mL/L (ME-AQ2), showed a marked growth stagnation following parasite inoculation, suggesting poor resilience and possibly toxicity or suboptimal efficacy.

FI and FCR results mirrored the growth patterns (Table 25). Birds from the TH-AQ1 group consumed 2933.52 ± 60.22 g of feed during the whole rearing period (0–32 days) with an FCR of 1.40 ± 0.01, indicating efficient feed utilisation. The TOTRA group, while recording a slightly higher FI (3073.28 ± 30.07 g), exhibited a less efficient FCR (1.60 ± 0.01). Meanwhile, the INF-NT group presented very low feed intake (469.90 ± 5.38 g) and artificially favourable FCR (0.69 ± 0.00), which were associated with severe growth inhibition and increased mortality (Table 25).

Table 25. Growth performance parameters of broiler chickens during the experimental period.

	Period	**TH-AQ1**	**TH-AQ2**	**RO-AQ1**	**RO-AQ2**	**ME-AQ1**	**ME-AQ2**	**NINF-NT**	**INF-NT**	**TOTRA**
BWG	**1d**	44.39 ± 7.60	43.37 ±5.51	43.38 ± 6.78	43.36 ± 7.20	43.35 ± 0.07	43.38 ± 0.06	42.66 ± 2.21	43.31 ± 0.58	43.14± 0.39
	7d[A]	182.72 ± 0.04	187.80 ± 6.98	183.76 ± 1.36	183.39 ± 0.52	182.67 ± 0.90	182.95 ± 0.09	182.68 ± 0.07	180.75 ± 6.14	182.58± 0.73
	10d[B]	221.15 ± 1.25	221.15 ± 1.26	221.15 ± 1.27	221.15 ± 1.28	221.15 ± 1.29	221.15 ± 1.30	221.15 ± 1.31	221.15 ± 1.32	221.15± 1.33
	10d PI	877.97 ± 3.25^{a}	788.35 ± 1.70^{b}	718.20 ± 3.84^{c}	757.82 ± 1.99^{d}	641.50 ± 8.00^{e}	643.38 ± 5.53^{e}	685.12 ± 1.25^{f}	448.75 ± 4.18^{g}	835.74± 1.25^{h}
	20d PI	1987.74 ± 2.21^{a}	1777.37 ± 2.17^{b}	1237.94 ± 3.91^{c}	1525.97 ± 0.17^{d}	714.48 ± 2.57^{e}	658.59 ± 4.34^{f}	985.43 ± 1.67^{g}	583.02 ± 3.09^{h}	1722.06± 1.78^{i}
	32d PI	2319.55 ± 43.56^{a}	2182.15 ± 2.52^{b}	1674.40 ± 0.75^{c}	1770.24 ± 7.93	987.97 ± 12.26^{e}	757.82 ± 1.99^{f}	1131.39 ± 5.41^{g}	902.17 ± 9.12^{h}	2143.27± 7.64^{i}
TWG	**C**	656.82 ± 3.13^{a}	567.20 ± 1.53^{b}	497.05 ± 2.62^{c}	536.68 ± 2.38^{b}	421.02 ± 7.92^{d}	421.83 ± 4.62^{d}	463.98 ± 1.69^{e}	227.89 ± 2.89^{f}	614.59 ± 1.39^{g}
	D	1109.77 ± 1.18^{a}	989.02 ± 3.14^{b}	519.74 ± 1.30^{c}	768.14 ± 1.59^{d}	72.32 ± 8.03^{e}	15.60 ± 7.46^{f}	300.30 ± 2.16^{g}	133.98 ± 5.87^{h}	886.32 ± 1.28^{i}
	E	331.81 ± 37.35^{b}	404.79 ± 0.91^{a}	436.46 ± 3.80^{a}	244.27 ± 6.37^{c}	273.49 ± 11.28^{c}	99.24 ± 5.17^{d}	145.96 ± 4.98^{e}	319.15 ± 5.36^{b}	421.20 ± 5.92^{a}
	F	2098.40 ± 35.05^{a}	1961.01 ± 2.29^{b}	1453.26 ± 1.57^{c}	1549.09 ± 7.00^{d}	766.82 ± 9.20^{e}	536.68 ± 2.38^{f}	910.24 ± 5.06^{g}	681.02 ± 8.46^{h}	1922.12 ± 7.09^{i}
ADG	**C**	65.68 ± 0.31^{a}	56.72 ± 0.15^{b}	49.71 ± 0.26^{c}	53.67 ± 0.24^{d}	42.10 ± 0.79^{e}	42.18 ± 0.46^{e}	46.40 ± 0.17^{f}	22.79 ± 0.29^{g}	61.46± 0.14^{h}
	D	110.98 ± 0.12^{a}	98.90 ± 0.31^{b}	51.97 ± 0.13^{c}	76.81 ± 0.16^{d}	7.23 ± 0.80^{e}	1.56 ± 0.75^{f}	30.03 ± 0.22^{g}	13.40 ± 0.59^{h}	88.63± 0.13^{i}
	E	27.65 ± 3.11^{a}	33.73 ± 0.08^{b}	36.37 ± 0.32^{b}	20.36 ± 0.53^{c}	22.79 ± 0.94^{d}	8.27 ± 0.43^{e}	12.16 ± 0.41^{f}	26.60 ± 0.45^{a}	35.10± 0.49^{b}
	F	65.58 ± 1.10^{b}	61.28 ± 0.07^{a}	45.41 ± 0.05^{c}	48.41 ± 0.22^{d}	23.96 ± 0.29^{e}	16.77 ± 0.07^{f}	28.45 ± 0.16^{g}	21.28 ± 0.26^{h}	60.07± 0.22^{a}
FI	**C**	826.63 ± 15.39^{a}	901.86 ± 6.80^{a}	606.37 ± 3.87^{b}	658.34 ± 12.16^{b}	362.13 ± 11.35^{c}	293.58 ± 1.75^{d}	685.33 ± 16.15^{b}	130.37± 2.72^{e}	885.01± 5.16^{a}
	D	1489.30 ± 12.76^{a}	1549.49 ± 19.63^{a}	751.89 ± 0.61^{c}	979.64 ± 11.49^{d}	69.38 ± 7.54^{b}	10.96 ± 5.24^{b}	489.49 ± 3.71^{e}	91.24± 4.00^{b}	1503.80± 5.65^{a}
	E	528.52 ± 58.92^{c}	659.76 ± 18.69^{a}	704.17 ± 7.80^{a}	354.97 ± 20.60^{d}	269.48 ± 11.24^{b}	98.05 ± 5.82^{e}	256.62 ± 14.76^{b}	260.75± 4.35^{b}	699.19± 20.46^{a}
	F	2933.52 ± 60.22^{d}	3128.91 ± 44.48^{a}	2073.32 ± 9.08^{b}	2041.83 ± 38.04^{b}	717.00 ± 10.86^{e}	426.78 ± 3.69^{c}	1475.83 ± 30.85^{f}	469.90± 5.38^{c}	3073.28± 30.07^{a}
FCR	**C**	1.26 ± 0.03^{a}	1.59 ± 0.01^{c}	1.22 ± 0.01^{a}	1.23 ± 0.02^{a}	0.86 ± 0.02^{d}	0.70 ± 0.00^{e}	1.48 ± 0.03^{b}	0.57± 0.01^{f}	1.44± 0.01^{b}
	D	1.34 ± 0.01^{b}	1.57 ± 0.02^{c}	1.45 ± 0.00^{d}	1.28 ± 0.01^{e}	0.96 ± 0.00^{f}	0.70 ± 0.00^{a}	1.63 ± 0.01^{g}	0.68± 0.00^{a}	1.70± 0.00^{h}
	E	1.59 ± 0.00^{a}	1.63 ± 0.05^{a}	1.61 ± 0.01^{a}	1.45 ± 0.05^{c}	0.99 ± 0.00^{b}	0.99 ± 0.01^{b}	1.76 ± 0.04^{d}	0.82± 0.00^{e}	1.66± 0.04^{f}
	F	1.40 ± 0.01^{a}	1.60 ± 0.02^{b}	1.43 ± 0.00^{a}	1.32 ± 0.02^{c}	0.94 ± 0.01^{d}	0.80 ± 0.00^{e}	1.62 ± 0.03^{b}	0.69± 0.00^{f}	1.60± 0.01^{b}
EPEF	**-**	395.08 ± 6.06^{a}	325.63 ± 0.31^{b}	279.44 ± 0.10^{b}	319.79 ± 1.17^{c}	251.58 ± 2.55^{d}	226.90 ± 0.49^{e}	166.16 ± 0.65^{f}	311.31 ± 2.57^{g}	319.16 ± 0.93^{g}
EBI	**-**	469.10 ± 7.84^{a}	384.08 ± 0.45^{c}	318.32 ± 0.34^{b}	367.29 ± 1.66^{c}	179.40 ± 2.15^{d}	189.81 ± 0.84^{d}	175.45 ± 0.98^{d}	277.59 ± 3.45^{e}	375.68 ± 1.39^{c}

Values in a row with different letters are significantly different at $P < 0.05$. Groups sharing the same letter are not significantly different.

A: 3d before parasite inoculation; B: Inoculation Day of the parasite; C: 0–10 d PI; D: 11–20 d PI; E: 21–32 d PI; F: 0-32 d PI.

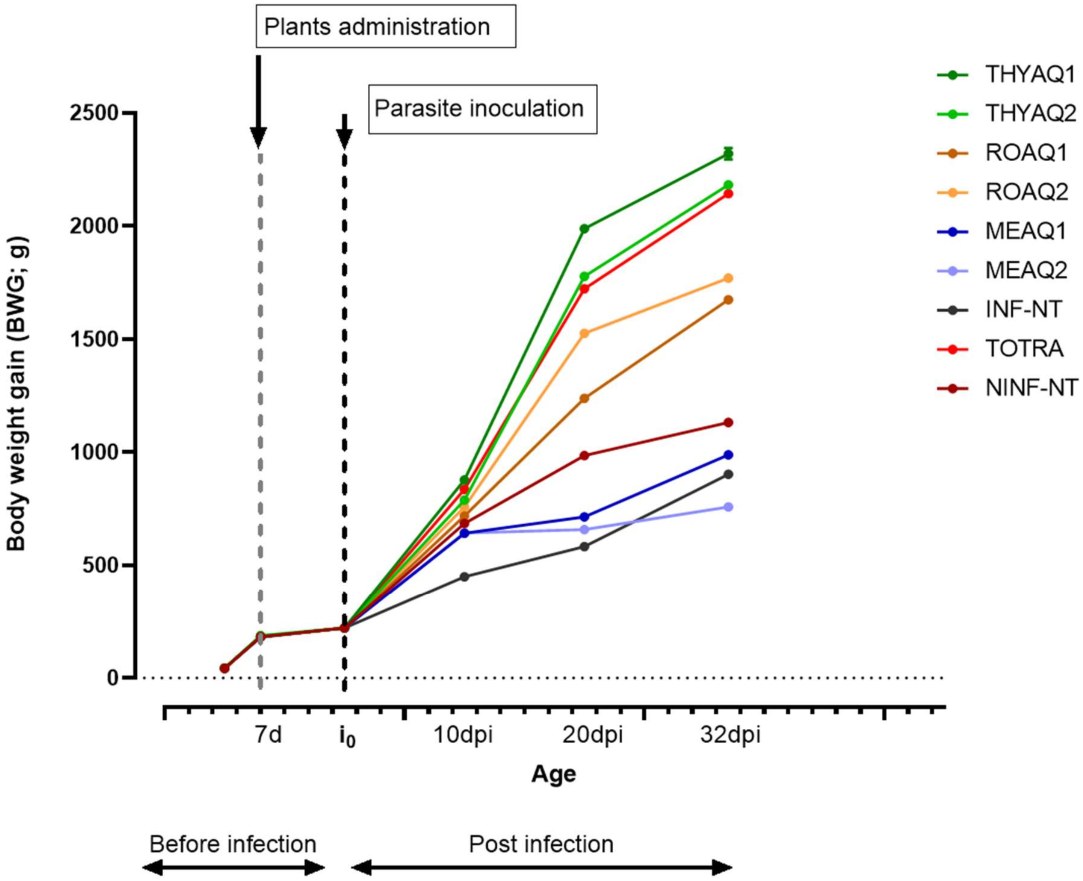

Figure 28. Evolution of body weight gain (BWG) in broiler chickens from pre-infection (day -10) to the end of the experiment (day 32) in response to treatment with aqueous plant extracts. Arrows indicate the day of *Eimeria* inoculation. THAQ1 and THAQ2 = *Thymus algeriensis*; ROAQ1 and ROAQ2 = *Rosmarinus officinalis*; MEAQ1 and MEAQ2 = *Mentha aquatica*; INF-NT = infected untreated; NINF-NT = uninfected untreated; TOTRA = toltrazuril-treated group.

Performance indices confirmed these observations. The European Production Efficiency Factor (EPEF) and European Broiler Index (EBI) were highest in the TH-AQ1 group (395.08 ± 6.06 and 469.10 ± 7.84, respectively), surpassing even the toltrazuril-treated group (TOTRA: EPEF 319.16 ± 0.93; EBI 375.68 ± 1.39). These results suggest that *Thymus algeriensis* may not only serve as a viable natural alternative but could also complement or enhance the efficacy of conventional anticoccidial agents or probiotics, particularly by reducing inflammation and improving resilience in broiler chickens. Although further targeted research is necessary to elucidate its mechanisms of action fully, current findings support the potential integration of *T. algeriensis* into poultry health management strategies. While specific studies on the effects of *T. algeriensis* in broilers remain limited, evidence from related Lamiaceae species indicates that their phytochemicals may modulate the gut microbiota, preserve intestinal barrier integrity, and reduce inflammatory responses during coccidial infections (Madlala et al., 2021a). For instance, supplementation with essential oil blends containing *T. vulgaris* has been shown to significantly enhance growth performance, reduce oocyst shedding, and improve intestinal health in broilers infected with *Eimeria* spp., often surpassing the efficacy of conventional anticoccidials, such as amprolium (Abou-Elkhair et al., 2014). The mode of action is usually attributed to antimicrobial and anti-inflammatory properties of thymol and carvacrol, which improve gut morphology and microbial balance. Furthermore, studies on *T. daenensis* have shown increased weight gain and enhanced villus height and crypt depth ratios in the jejunum, indicating improved nutrient assimilation (Malekzadeh et al., 2018). Similarly, *T. vulgaris* supplementation was shown to enhance immunity markers, such as antibody titers post-vaccination against Newcastle Disease, as well as reduce mortality under viral challenge, suggesting a potential immunostimulatory effect of thyme-based phytogenics (Hassanin et al., 2024). Supplementation with thyme at 2–5 g/kg of feed improves body weight gain, feed efficiency, and production indices (Ashour et al., 2025; Hassan & Awad, 2017a). Even in low-protein diets, thyme helps maintain growth and litter quality by enhancing protein and energy utilisation (Golshahi et al., 2025).

Thyme also supports health and immunity. It boosts immunoglobulin levels (IgY, IgM, IgG), increases lymphocyte counts, and improves humoral immunity (Hashemipour et al., 2013; Yalçin et al., 2020). Its antioxidant properties are notable, enhancing SOD and GPx activity and lowering malondialdehyde (MDA) levels. It reduces inflammatory cytokine expression and upregulates mucin expression in the intestine, improving gut integrity (Hassan & Awad, 2017b). Carcass and metabolic quality are also enhanced. Thyme reduces abdominal fat, liver weight, and improves carcass yield (Abdulkarimi et al., 2011). It improves lipid profiles by reducing total cholesterol, LDL, VLDL, and triglycerides, while increasing HDL levels. In laying hens, thyme lowers yolk cholesterol and enriches the egg in omega-3 fatty acids without impairing productivity (Yalçin et al., 2020). Importantly, thyme and its active compounds (thymol and carvacrol) present antimicrobial, antiviral, and anti-inflammatory properties, offering a natural alternative to antibiotics in poultry production (Gholami-Ahangaran et al., 2022; R. U. Khan et al., 2012).

When combined with other herbs such as garlic or rosemary, or with probiotics, these effects may be amplified (Ashour et al., 2025; Golshahi et al., 2025). Compared to probiotics, which have well-documented benefits on gut morphology, microbial balance, lesion reduction, and immunity (Mohsin et al., 2022; Y. Wang et al., 2021), thyme exhibits promising yet indirect evidence of similar effects. Combining thyme with probiotics could offer a powerful synergy for improving gut health, performance, and disease resistance under coccidial pressure (Elbaz et al., 2025), a promising avenue for future research.

Studies suggest *Thymus algeriensis* contains antioxidant and antimicrobial compounds that can support gut integrity, modulate the gut microbiota, and enhance immune function, especially under parasitic or microbial stress (Lahlou et al., 2022). This plant has been identified as a rich source of bioactive compounds, including flavonoids, phenolic acids, and monoterpenes such as thymol and carvacrol that may help maintain tight junction integrity, reduce oxidative stress, and suppress the growth of pathogenic bacteria, all of which are critical in mitigating the damaging effects of *Eimeria* infections on the intestinal barrier (Ben El Hadj Ali et al., 2015; Righi et al., 2020; Ziani et al., 2019).

Conversely, groups treated with *Mentha aquatica* and *Rosmarinus officinalis* demonstrated inferior performance, both in growth and feed efficiency. The ME-AQ2 group, which received a high dose of *M. aquatica* aqueous extract, showed significantly reduced performance indices, including the lowest European Production Efficiency Factor (EPEF) and European Broiler Index (EBI) among all treatments (EPEF = 91.71 ± 6.56; EBI = 109.97 ± 7.86). These poor scores were associated with reduced body weight gain and an elevated feed conversion ratio, suggesting that high concentrations of *M. aquatica* may compromise growth under coccidial stress. Several studies on closely related species, such as *Mentha pulegium*, have reported similar patterns. For example, Ghalamkari et al. (2012) found that supplementation with pennyroyal did not significantly enhance broiler growth performance compared to antibiotic controls (Ghalamkari et al., 2012). Similarly, Goodarzi and Nanekarani (2014) noted that while *M. pulegium* could improve some carcass traits, higher doses did not lead to better growth or feed conversion efficiency (Goodarzi & Nanekarani, 2014). Other research by Ölmez and Yörük (2021) demonstrated that although *M. pulegium* had antimicrobial and antioxidant effects, its impact on performance metrics was inconsistent and dose-dependent (Ölmez & Yörük, 2021).

Likewise, birds treated with *R. officinalis* extracts (RO-AQ1 and RO-AQ2) exhibited moderate performance, with complete survival and some reduction in oocyst shedding and lesion severity; however, their EPEF and EBI remained substantially lower than those of both *Thymus algeriensis* and the toltrazuril-treated group. For example, RO-AQ2 recorded an EPEF of 227.80 ± 2.74 and EBI of 272.24 ± 3.27, in contrast to TH-AQ1 (EPEF = 395.08; EBI = 469.10). These findings align with those of Rasouli et al. (2021), who reported that *R. officinalis* exhibits partial anticoccidial effects, reducing intestinal lesion scores, but is less effective than conventional anticoccidials or certain essential oils in improving feed efficiency and growth performance (Rasouli et al., 2021). Additionally, studies have shown that although rosemary possesses potent antioxidant and antimicrobial properties, its influence on performance indicators such as EPEF or FCR remains modest compared to synthetic drugs or more potent phytogenics (Kadhim, 2023)).

3.4.2. Clinical Protection, Lesion Scores and Anticoccidial Index

The evaluation of anticoccidial efficacy in broiler chickens relies on a series of standardised clinical performance indicators. The survival rate directly reflects the severity of the infection and the treatment's protective potential. In contrast, the relative weight-gain percentage indicates the product's capacity to maintain growth under pathogenic stress. Oocyst output (oocysts per gram of faeces, OPG) quantifies intestinal replication of the parasite, and the lesion score measures intestinal damage. These four variables are consolidated in the Anticoccidial Index (ACI); values > 160 denote adequate protection, whereas lower scores indicate partial or poor efficacy (Lan et al., 2016; Morisawa et al., 1977). Together, these parameters, summarised in Table 26 and Figure 29, offer a comprehensive assessment of the clinical protection conferred by the tested plant extracts against *Eimeria* infection in broilers.

As expected, the uninfected, untreated group (NINF-NT) maintained optimal parameters across all metrics, serving as the physiological benchmark (100 % survival and relative weight gain, zero oocysts, and no lesions) (Table 32, Figure 29). Conversely, the infected untreated group (INF-NT) showed typical signs of severe coccidiosis, with a reduced survival rate (90 %), significantly lowered relative weight gain (74.82 %), maximal oocyst output (1.0639 × 10^5), and the highest lesion score (2.85 ± 0.57), resulting in a low ACI of 28.50 ± 5.71.

Table 26. Survival Rate, Relative Weight Gain, Oocyst Shedding, Lesion Severity, and Anticoccidial Index (ACI) in broiler chickens experimentally infected with *Eimeria* spp. and treated with medicinal plant extracts or toltrazuril: comparison between NINF-NT (Non-Infected, Non-Treated), INF-NT (Infected, Non-Treated), TH-AQ1/2 (*Thymus algeriensis* aqueous extract, low/high dose), RO-AQ1/2 (*Rosmarinus officinalis* aqueous extract, low/high dose), ME-AQ1/2 (*Mentha aquatica* aqueous extract, low/high dose), and TOTRA (Toltrazuril-treated).

Group	Survival rate (%)	Relative weight gain rate (%)	Oocyst number x10^4	Oocyst value	Lesion score	Lesion value	ACI
NINF-NT	100	100	0	-	-	-	-
INF-NT	90	74.818	10.639	100	2.85± 0.57	28.50± 5.71	-
TH-AQ1	100	230.532	1.917	18.016	1.43± 0.52	14.33± 5.23	298.183
TH-AQ2	100	287.951	3.722	34.987	1.08± 0.22	10.83± 2.24	342.130
RO-AQ1	100	75.607	8.500	79.896	1.17± 0.73	11.67± 7.32	83.711
RO-AQ2	100	170.184	5.111	48.042	2.18± 0.33	21.83± 3.31	200.309
ME-AQ1	70	84.243	5.917	55.614	2.50± 0.00	25.00± 0.00	73.630
ME-AQ2	90	58.960	7.139	67.102	2.65± 0.56	26.50± 5.61	55.358
TOTRA	100	211.166	3.194	30.026	1.75± 0.62	17.50± 6.21	263.639

The aqueous extract of *Thymus algeriensis* showed the most robust protection (Table 26, Figure 29). At the lower dose (TH-AQ1) (Figure 29), birds achieved 100 % survival, a remarkable 230.5 % relative weight gain, minimal oocyst shedding (median ≈ 1.9 × 10^4 OPG; box-plot whiskers remain below 3 × 10^4) (Figure 29) and the lowest lesion score (1.43 ± 0.52). These metrics translate into the highest ACI (342.1), surpassing even toltrazuril (TOTRA), which recorded an ACI of 263.6 despite a strong performance (211.2% weight gain and 3.19 × 10^4 OPG) (Figure 29). The higher thyme dose (TH-AQ2, light green) produced slightly more oocyst output (median ≈ 3.7 × 10^4 OPG) but still delivered excellent overall protection (100% survival, ACI = 298.2), indicating dose flexibility without loss of efficacy (Figure 29).

Rosmarinus officinalis provided moderate, dose-dependent control (Table 26, Figure 29). The high dose (RO-AQ2) lowered median oocyst counts to ~6 × 10^4 OPG, improved relative weight gain to 170.2 % and raised the ACI to 200.3, whereas the low dose (RO-AQ1, orange) clustered near the infected controls in both the box-plot and clinical metrics (75.6 % weight gain, ACI = 83.7). Although both rosemary doses achieved 100% survival, their growth suppression and higher shedding rates indicate weaker anticoccidial potency than thyme and toltrazuril.

Mentha aquatica was the least effective. Both doses (ME-AQ1, ME-AQ2) generated broad, right-skewed OPG distributions (medians 7–8 × 10^4) (Table 26, Figure 29), substantial lesion scores (up to 2.65 ± 0.56), and pronounced growth depression. The higher dose (ME-AQ2) yielded the worst ACI (55.4), despite a 90% survival rate; the lower dose improved slightly (ACI = 73.6) but remained far below the protection afforded by the other treatments.

Figure 29 thus reinforces the numerical conclusions: TH-AQ1 is not only statistically but also visually superior, with the tightest and lowest oocyst-count distribution, even better than toltrazuril. Meanwhile, rosemary offers intermediate, dose-responsive benefits, and mint provides only marginal suppression of parasite shedding. Overall, aqueous *T. algeriensis* emerges as the most promising botanical anticoccidial *in vivo*, delivering consistent, high-level protection that rivals or surpasses that of a standard chemotherapeutic drug.

Similar high levels of efficacy have been reported with other botanicals; however, most do not surpass that of toltrazuril. For instance, a tannin-rich herbal blend comprising *Artemisia annua*, Quercus infectoria, and *Allium sativum* demonstrated lesion reduction and oocyst suppression comparable to toltrazuril, whilst also improving feed conversion and weight gain in broilers experimentally infected with *Eimeria* spp. (Ghafouri et al., 2023). Likewise, *Artemisia sieberi* extract reduced oocyst output and enhanced growth performance to a degree comparable to that of monensin, particularly when combined with other plant components (Mousavinasab et al., 2022). The methanolic extract of *Artemisia vestita*, a traditional herb from the Himalayas, significantly decreased oocyst output and enhanced feed efficiency, suggesting the presence of highly active compounds (Ahad et al., 2017).

Several studies have explored the efficacy of essential oils and their active compounds. Oils derived from *Thymus vulgaris*, *Origanum vulgare*, and *Allium sativum* have been shown to disrupt early intracellular development of *Eimeria tenella*, thereby reducing parasite pathogenicity (Felici, Tugnoli, Ghiselli, et al., 2023). In a study involving garlic, sage, thyme, echinacea, and oregano, dietary supplementation with this herbal blend restored growth performance to levels comparable to those observed in birds treated with coccidiostats or in uninfected birds. However, lesion scores remained only moderately improved (Arczewska-Włosek & Świątkiewicz, 2015).

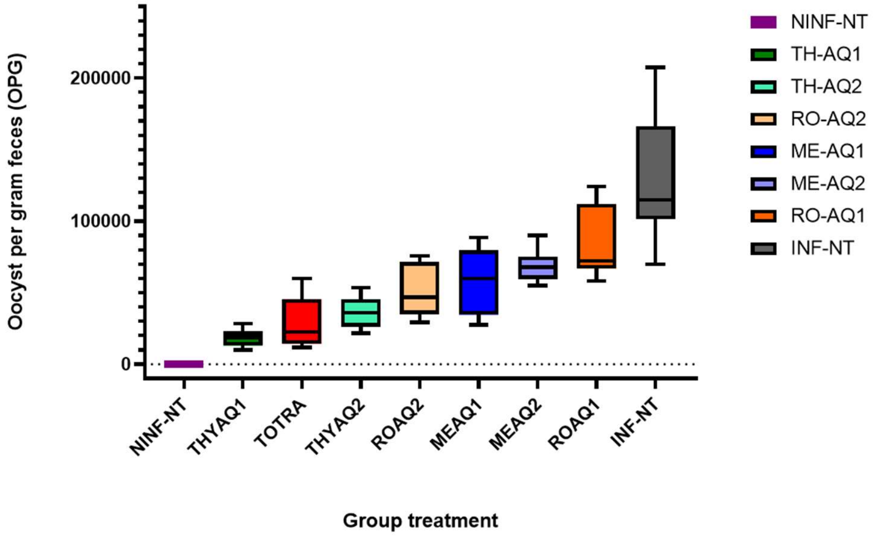

Figure 29. Longitudinal shedding of *Eimeria* oocysts in broiler chickens following different treatments. Box-and-whisker plots show faecal oocyst counts (oocysts per gram, OPG) measured every 2 days from 10 to 32 days post-infection (i.e., 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, and 32 dpi). For each treatment group (n = 6), all time-point values were pooled to depict overall shedding pressure during the patent period. Boxes represent inter-quartile ranges (25th–75th percentiles); horizontal lines mark medians; whiskers indicate minima and maxima. Colour codes: NINF-NT (non-infected, non-treated; purple), TH-AQ1 and TH-AQ2 (low and high doses of aqueous *Thymus algeriensis*; dark- and light-green), TOTRA (toltrazuril; red), RO-AQ2 and RO-AQ1 (high and low doses of aqueous *Rosmarinus officinalis*; beige and orange), ME-AQ1 and ME-AQ2 (low and high doses of aqueous *Mentha aquatica*; dark- and light-blue), and INF-NT (infected, non-treated; grey). Lower, more compact boxes indicate stronger suppression of oocyst shedding across the 10–32 dpi window; TH-AQ1 achieves the most significant overall reduction, surpassing toltrazuril, whereas mint extracts and low-dose rosemary show limited control.

In contrast, rosemary and mint exhibited limited protective effects in our study. These results are supported by other studies, which show that while Artemisia annua improved weight gain, it did not significantly reduce oocyst output or lesion severity (L. Pop et al., 2021). Other plant-based solutions, such as *Citrus sinensis* essential oil or artemisinin-containing extracts, have demonstrated efficacy *in vitro* or as litter treatments; however, their *in vivo* performance against systemic infection remains inferior to that of leading botanical candidates like *T. algeriensis* (Imran & Alsayeqh, 2022; Sprenger et al., 2015).

The findings of the current study align with broader research on the Lamiaceae family, which comprises several herbs recognised for their antiparasitic properties. For example, a comprehensive review by Lahlou et al. (2021) documented the efficacy of *Rosmarinus officinalis* and *Thymus vulgaris*, two other Lamiaceae members, in improving weight gain, reducing lesion severity, and supporting overall gut health in broiler chickens experimentally infected with *Eimeria* spp. These plants contain essential oils, phenolic acids, and flavonoids that provide antioxidant, immunomodulatory, and antimicrobial effects, contributing to their anticoccidial action (Aitfella Lahlou et al., 2021).

The current data, when considered in light of these findings, suggest that *T. algeriensis* may not only share but also exceed the bioactivity reported in other Lamiaceae plants. While rosemary and thyme are effective natural growth promoters and moderately anticoccidial, *T. algeriensis* distinguishes itself by providing consistent protection, even surpassing synthetic drugs under similar experimental conditions. The bioefficacy of Lamiaceae species appears closely linked to the presence and concentration of secondary metabolites, which vary among species and chemotypes.

3.4.3. Intestinal histopathology

Histopathology is the "gold standard" read-out in experimental coccidiosis. Because *Eimeria* spp. Develop intracellularly, and each species targets a characteristic intestinal segment. Microscopic examination reveals not only the extent of tissue destruction but also the identity and developmental stage of the parasite. Lesion scoring systems such as Johnson & Reid (Johnson & Reid, 1970) (0 – 4 per segment) have therefore become indispensable for (i) benchmarking new anticoccidials, (ii) explaining performance losses that gross necropsy may miss, and (iii) linking field indicators (weight gain, oocyst output, litter quality) to concrete epithelial damage. Typical coccidial lesions include villus blunting or collapse, crypt hyper-regeneration (the "honey-comb" pattern), fibrino-purulent exudate, heterophil swarms, and the presence of schizonts, gamonts, or developing oocysts within enterocytes (Alhotan & Abudabos, 2019; Hafeez et al., 2023; Maratea & Miller, 2007). When these microscopic features are mapped along the gut, they mirror the tropism of the challenge inoculum, thus providing a high-resolution measure of how well each treatment interrupts the parasite's life cycle (Alhotan & Abudabos, 2019; Hafeez et al., 2023).

In the present trial, a mixed inoculum containing six species, *E. praecox* (duodenum), *E. maxima* (jejunum), *E. necatrix* and *E. brunetti* (mid-intestine), *E. tenella* (caeca) and *E. mitis* (diffuse), generated a spectrum of lesions that could be ranked with the Johnson & Reid scoring system (Johnson & Reid, 1970). In our study, across all experimental groups (Figures 30 and 31, Table 27), the caecum-to-duodenum histology revealed a clear continuum that mirrored the clinical endpoints and the known intestinal tropism of the six *Eimeria* species in the challenge inoculum.

The negative control (Figure 31A, NINF-NT) displayed entirely normal mucosa, characterised by tall, tapering villi, orderly crypts, and a virtually acellular lamina propria; the global Johnson & Reid score was 0.00 ± 0.00. By contrast, untreated infection (Figure 31C and D, INF-NT) produced the classic picture of uncontrolled coccidiosis. Villi had collapsed into mats of necrotic debris; crypts were ballooned and lake-like, with diffuse coagulative necrosis filling the lamina propria. Heterophils formed dense carpets, and all parasite stages, schizonts, gamonts, and oocysts, were abundant. The composite score climbed to 2.85 ± 0.57, the highest of the study, which corresponded to the poorest weight gain and the heaviest faecal shedding (Table 27).

Toltrazuril (Figure 31B, TOTRA) almost normalised the tissue. Only mild villus blunting and a sparse mononuclear sprinkling hinted at prior infection; no intracellular parasites could be detected. The mean lesion index of 1.75 ± 0.62 aligned with the drug's high anticoccidial index and near-physiological growth. Low-dose aqueous *Thymus algeriensis* (Figure 31 J–K, TH-AQ1) achieved a similar outcome: villi and crypts were preserved, inflammation was mild, and parasite forms were exceedingly rare, yielding a score of 1.43 ± 0.52 and the lowest oocyst output among the plant preparations. Doubling the thyme dose (Figure 31 L–M, TH-AQ2) did not enhance protection; crypt hyperplasia became slightly more pronounced, but parasites remained sporadic, and the score plateaued at 1.08 ± 0.22, indicating that efficacy had already reached its ceiling at the lower concentration (Table 27).

Aqueous *Rosmarinus officinalis* produced an intermediate picture. At dose 1 (Figure 31 N–P, RO-AQ1), large necrotic fields and abundant macrogamonts persisted, giving a mean score of 1.17 ± 0.73. Increasing the dose (Figure 31 Q–R, RO-AQ2) halved the parasite burden and restricted damage to patchy villus stumps supported by hyperplastic crypts; however, the score remained moderate at 2.18 ± 0.33, and growth performance was only partly restored. *Mentha aquatica* was the least effective extract. The low dose (Figure 31 E–F, ME-AQ1) produced heterogeneous damage, characterised by alternating residual crypts and carpets of necrotic, fusiform enterocytes; the score was fixed at 2.50 ± 0.00. The high dose (Figure 31 G–I, ME-AQ2) worsened the picture: villi were obliterated, crypts formed a honeycomb of hyperplasia, fibrino-purulent pockets dissected the lamina propria and macrogamonts or early oocysts were still plentiful, raising the score to 2.65 ± 0.56 and leaving performance indices scarcely better than the untreated infection (Table 27).

Across groups, the microscopic findings directly translated into improved digestive function. Toltrazuril and both thyme doses preserved absorptive villus surface and barrier integrity, sustaining almost normal weight gain. Rosemary allowed for partial villus loss, and the mint extract resulted in extensive loss, diverting nutrients into costly crypt regeneration and provoking heterophil-rich inflammation that correlates with wet litter and increased bacterial risk. The prevalence of macrogamonts in the tissue paralleled the faecal oocyst curve, ranging from $<2 \times 10^4$ OPG under TH-AQ1 to $>1 \times 10^5$ OPG in INF-NT.

Segment-specific damage mirrored the tropism of the six challenge species: duodenal blunting marked *E. praecox*, massive jejunal crypt carpets flagged *E. maxima*, mid-gut necrosis betrayed *E. necatrix*, and caecal gamonts revealed *E. tenella*. When the lesion matrix is read alongside the slides, protection ranks unambiguously as follows: toltrazuril ≈ TH-AQ1 > TH-AQ2 > RO-AQ2 > RO-AQ1 > ME-AQ1 ≈ ME-AQ2 ≫ INF-NT. Low-dose aqueous *Thymus algeriensis*, therefore, emerges as the most convincing botanical substitute for toltrazuril, conferring near-chemotherapeutic mucosal protection. In contrast, rosemary affords only partial rescue and mint extracts, regardless of dose, fail to prevent severe lesions along the digestive tract.

Infection with *Eimeria* species disrupts the intestinal ecosystem's equilibrium, causing significant damage to the mucosal barrier. The parasite invades epithelial cells, triggering inflammation, villus atrophy, crypt hyperplasia, and disruption of tight junction proteins, which in turn increase intestinal permeability and reduce nutrient absorption (Chen et al., 2025; Schneiders, 2020; Sharma et al., 2024a). These pathological changes also disrupt the gut microbial composition, leading to a decline in beneficial bacteria, such as Lactobacillus and *Faecalibacterium*, while promoting the proliferation of opportunistic pathogens, including *Clostridium perfringens*, *Campylobacter*, and *Escherichia coli* (Campos et al., 2024; Huang et al., 2018; Liu et al., 2024; Madlala et al., 2021b). During an acute infection, there is a marked reduction in short-chain fatty acid-producing bacteria, which are crucial for gut health. At the same time, facultative anaerobes and potential pathogens become more abundant (Campos et al., 2024; Huang et al., 2018; Liu et al., 2024). The disruption of the gut barrier and microbial balance can persist beyond the acute phase, leading to prolonged adverse effects on growth, nutrient utilisation, and overall animal health (Choi et al., 2023; Sharma et al., 2024b). These findings highlight the complex interplay between *Eimeria*, the intestinal barrier, and the microbiota, emphasising the importance of strategies that protect or restore gut integrity and microbial balance to mitigate the impact of coccidiosis (Campos et al., 2024; Chen et al., 2025; Huang et al., 2018; Liu et al., 2024; Madlala et al., 2021b; Sharma et al., 2024a).

Phytogenic plant extracts, rich in polyphenols, essential oils, and flavonoids, can counteract the intestinal disturbances caused by *Eimeria* infection through several complementary mechanisms. These bioactive compounds have been shown to enhance the expression of tight junction proteins, such as occludin and ZO-1, thereby restoring barrier integrity and reducing intestinal permeability (S. Maodaa et al., 2024; Murshed et al., 2023). They also upregulate antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase, which help mitigate oxidative stress and tissue damage induced by the parasite (S. Maodaa et al., 2024; Murshed et al., 2023). By modulating key inflammatory pathways, including NF-κB and MAPK, phytogenics reduce the production of pro-inflammatory cytokines and limit the inflammatory response in the gut (Jiao et al., 2018; Qasem et al., 2020). Many plant extracts also exhibit direct antimicrobial activity, suppressing the overgrowth of pathogenic bacteria, such as *Clostridium perfringens*, while promoting beneficial microbes and the production of short-chain fatty acids, which are crucial for gut health (Han et al., 2022; M. M. Khan et al., 2024; Muthamilselvan et al., 2016). *In vivo* studies confirm that these effects translate into reduced oocyst shedding, improved gut histology, increased goblet cell numbers, and better overall animal performance during *Eimeria* infection (Han et al., 2022; S. Maodaa et al., 2024; Murshed et al., 2023; Qasem et al., 2020). Collectively, phytogenic extracts help restore the balance of the gut microbiota, reinforce the mucosal barrier, and reduce inflammation, making them promising natural alternatives or adjuncts to conventional anticoccidial drugs (Han et al., 2022; Jiao et al., 2018; S. Maodaa et al., 2024; Murshed et al., 2023; Muthamilselvan et al., 2016; Qasem et al., 2020).

In this context, the present study demonstrates that low-dose aqueous *Thymus algeriensis* extract offers remarkable mucosal protection against *Eimeria* infection, with performance metrics closely resembling those of toltrazuril. This is particularly notable given its natural origin and low dosage, suggesting that specific phytoconstituents in *T. algeriensis* possess strong anticoccidial properties capable of modulating host–parasite interactions, maintaining epithelial integrity, and limiting parasite replication (Lahlou et al., 2022). These effects are likely attributable to the presence of thymol, carvacrol, and other bioactive terpenoids, which are known to exert antimicrobial, antioxidant, and immunomodulatory effects (Lahlou et al., 2022).

In contrast, rosemary extract exhibited only partial efficacy. While it modestly improved weight gain and reduced lesion severity in a dose-dependent manner, it failed to match the consistent protection observed with *T. algeriensis*. These findings align with those of Peng et al. (2024), who reported that rosemary increased the abundance of Lactobacillus and improved cecal health. However, it did not entirely suppress oocyst shedding or epithelial damage (Peng et al., 2024). The partial rescue effect of rosemary may stem from lower concentrations or less potent ratios of active constituents, such as rosmarinic acid or 1,8-cineole, which may not be sufficient to disrupt Eimeria's complex life cycle *in vivo.*

Mint extracts, on the other hand, showed negligible protective effects, with high lesion scores and oocyst outputs observed across both tested doses. The limited efficacy of mint may be due to the predominance of menthol and menthone, which lack the broad-spectrum antiparasitic mechanisms found in other Lamiaceae plants, such as *Thymus* or *Origanum* (Bailén et al., 2023; Maciel et al., 2022; Tjitraresmi et al., 2020).

Phytogenic feed additives, including essential oils, polyphenols, and flavonoid-rich plant extracts, have demonstrated strong potential to mitigate these adverse effects and restore gut microbial balance. In a recent study, a mixed botanical formula enhanced growth performance and reduced oocyst shedding, while increasing Lactobacillus abundance in the ceca of coccidia-infected broilers (Ghafouri et al., 2023). Similarly, dietary supplementation with rosemary extract helped modulate the cecal microbiota and alleviate inflammatory damage by increasing beneficial bacteria and enhancing the production of short-chain fatty acids (Peng et al., 2024). Further supporting this, polyherbal formulations containing *Artemisia annua* and *Allium sativum* not only reduced *Eimeria* lesions and oocyst output but also increased microbial diversity and improved the Firmicutes-to-Bacteroidetes ratio, suggesting enhanced gut resilience (Ghafouri et al., 2023). Additionally, thyme and oregano essential oils have been shown to restore tight-junction integrity, inhibit the growth of pathogenic bacteria, and stimulate the secretion of mucins and antimicrobial peptides, thereby supporting mucosal recovery (Felici, Tugnoli, De Hoest-Thompson, et al., 2023; Felici, Tugnoli, Ghiselli, et al., 2023).

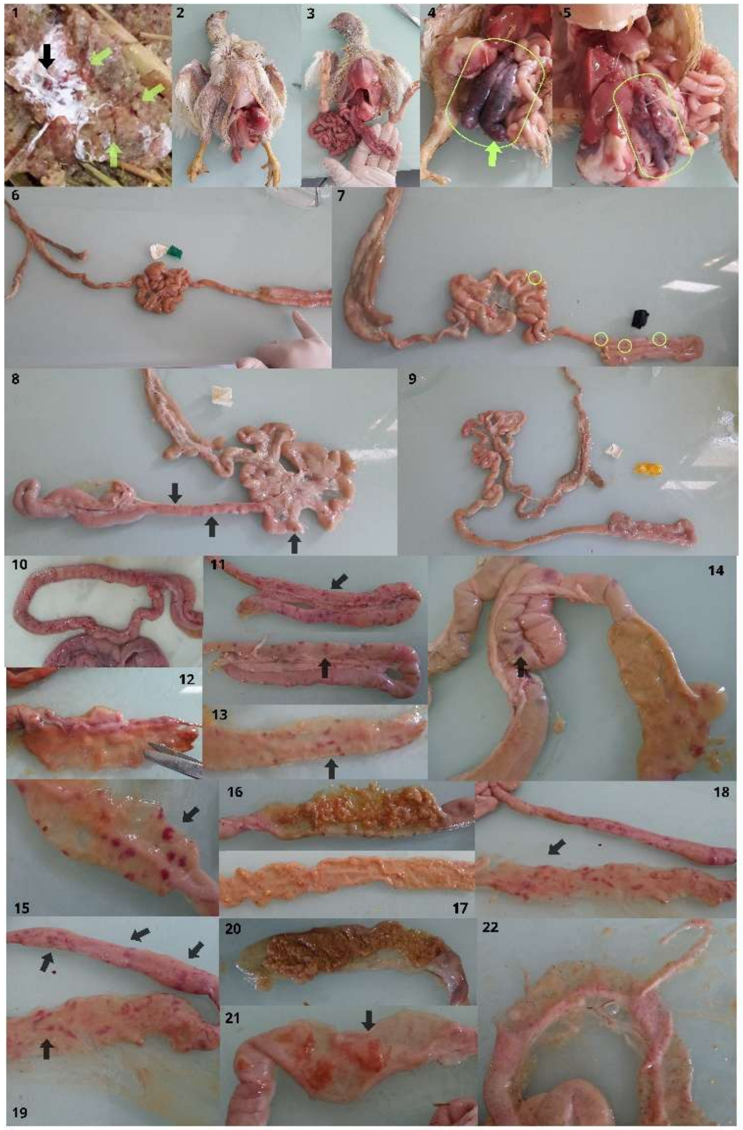

Figure 30. Representative images of severe intestinal lesions (lesion scores 3 and 4) observed in broiler chickens at day 10 post-infection with a mixed *Eimeria* spp. infection. Panels 1–22 show intestinal samples collected at necropsy from experimentally infected broiler chickens. Lesions were evaluated on day 10 post-infection following exposure to a mixed suspension of *Eimeria* spp. Macroscopic alterations correspond to scores 3 and 4 according to the Johnson & Reid scoring system, reflecting moderate to severe intestinal pathology.

- Panel 1: Clinical signs in vivo showing bloody, mottled faeces (arrow), indicative of active intestinal haemorrhage.
- Panels 2–3: External views of infected birds showing abdominal distension.
- Panels 4–7: Caeca with severe haemorrhagic lesions and caseous cores, characteristic of *Eimeria tenella* infection.
- Panels 8–12: Jejunum and ileum with thickened, congested mucosa and fibrinous exudates, suggestive of *E. maxima* and *E. necatrix*.
- Panels 13–17: Mid- to lower intestine showing petechiae, mucosal erosion, and exudative lesions, consistent with *E. brunetti*.
- Panels 18–22: Mixed caecal and intestinal damage with overlapping haemorrhagic and necrotic lesions, reflecting the synergistic impact of multiple *Eimeria* species.

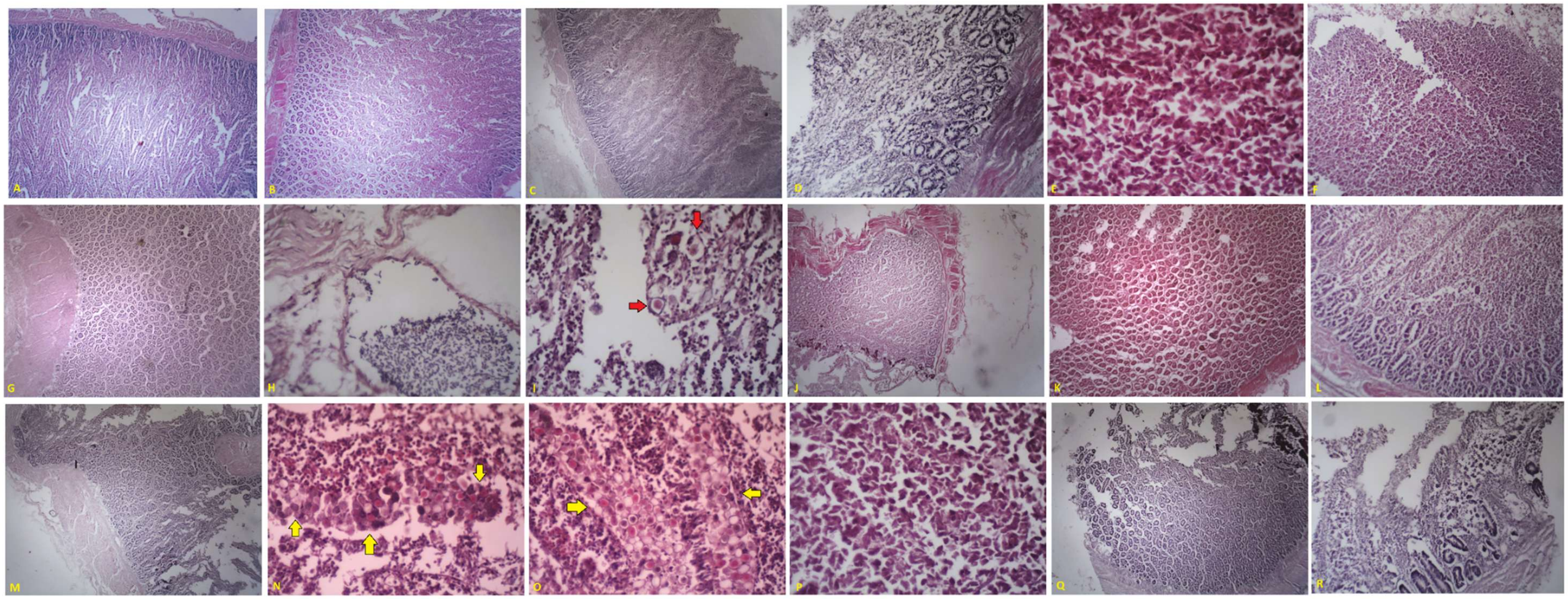

Figure 31. Representative intestinal histological sections for all groups (A–R). A—NINF-NT (non-infected, non-treated): intact villi and crypts, score 0. B—TOTRA (toltrazuril): slight villus blunting, no parasites, score ≈ 1.7. C, D—INF-NT (infected, non-treated): villus collapse, necrotic crypt lakes, abundant schizonts/gamonts; score ≈ 2.9. E, F—ME-AQ1 (*Mentha aquatica*): mixed fields, residual crypts and carpets of necrotic fusiform enterocytes, score ≈ 2.5. G, H, I—ME-AQ2 (*M. aquatica*): honeycomb crypt hyperplasia (G), fibrino-purulent pocket (H) and numerous macrogamonts/oocysts (red arrows, I); score ≈ 2.6. J, K—TH-AQ1 (*Thymus algeriensis*): largely preserved architecture, mild lamina propria infiltrate, parasites rare; score ≈ 1.4. L, M—TH-AQ2 (*T. algeriensis*): similar to TH-AQ1 with slightly greater crypt hyperplasia, score ≈ 1.1. N, P—RO-AQ1 (*Rosmarinus officinalis*): marked villus loss, focal necrosis, abundant macrogamonts (yellow arrows), score ≈ 3. O (inset, RO-AQ1, higher power): degenerating enterocytes and inflammatory debris. Q, R—RO-AQ2 (*R. officinalis*): patchy villus stumps with regenerating crypts (Q) and scattered parasite stages (R); score ≈ 2.2.

Table 27. Mean intestinal lesion scores and key microscopic findings for each treatment group. Scores are Johnson & Reid composites (duodenum, jejunum, ileum, caeca). The table also lists the segment most affected, provides a concise histological summary, indicates the presence or absence of parasite stages, and offers an interpretation in the context of the mixed *Eimeria* challenge.

Group	**Mean composite lesion score ± SD ***	**Segment(s) most affected †**	**Concise histological description**	**Parasite stages seen**	**Interpretation vs. mixed *Eimeria* challenge ‡**
NINF-NT	0.00 ± 0.00	–	Long slender villi, orderly crypts, intact epithelium, minimal lamina propria cells.	None	True physiological baseline. Confirms sampling artefacts are negligible.
INF-NT	2.85 ± 0.57	Duo > Jej ≈ Ile > Cae	Massive villus loss, dilated crypts packed with necrotic debris, extensive coagulative necrosis, dense heterophil infiltrate.	Numerous schizonts, gamonts, and oocysts	Unrestricted replication of all challenge species: *E. praecox* (duodenum), *E. maxima* (jejunum), *E. necatrix* (mid-gut), *E. tenella* (caeca) etc.
TOTRA	1.75 ± 0.62	Jej > Duo	Slight villus blunting, crypts intact, continuous epithelium, sparse mononuclear infiltrate; parasites absent.	None	Near-complete protection, matching the broad anti-*Eimeria* spectrum of toltrazuril.
TH-AQ1	1.43 ± 0.52	Duodenum	Largely intact architecture, mild lamina propria infiltrate, very occasional vacuolated parasite residua.	Very rare	Best-performing plant extract, strong suppression of *E. praecox/maxima* and reasonable control of *E. tenella*.
TH-AQ2	1.08 ± 0.22	Duo ≈ Jej	Blunted villi, moderate crypt hyperplasia, light mixed infiltrate, parasites extremely sparse.	Sporadic	Higher dose offers no meaningful histological gain over TH-AQ1—efficacy already near plateau.
RO-AQ1	1.17 ± 0.73	Duo ≈ Jej	Marked villus loss, crypt destruction, focal necrosis, dense inflammation; many gamonts/oocysts.	Abundant gamonts and oocysts	Incomplete protection: only moderate impact on *E. praecox/maxima*; caeca relatively spared.

RO-AQ2	2.18 ± 0.33	Duo > Jej ≈ Ile	Patchy villus absence, crypt hyperplasia, focal fibrin, parasites fewer than RO-AQ1.	Scattered gamonts	A higher dose lowers the parasite load somewhat, yet leaves moderate lesions, resulting in intermediate efficacy.
ME-AQ1	2.50 ± 0.00	All segments (even)	Carpets of necrotic exfoliated enterocytes, partial crypt preservation, diffuse inflammation, and scattered parasites.	Moderate gamonts/oocysts	Variable, only partial protection, limited activity versus *E. maxima* and *E. brunetti*.
ME-AQ2	2.65 ± 0.56	All, esp. Jej/Ile	Villus obliteration, "honey-comb" hyperplastic crypts, fibrino-purulent exudate, oocysts still numerous.	Numerous macrogamonts/oocysts	A higher dose fails to improve outcome; lesions approach INF-NT severity, explaining the poor ACI.

* Composite average of Johnson & Reid lesion scores for duodenum, jejunum, ileum and caeca, derived from the spreadsheet you supplied.

† Rank order of median segmental scores (Duo = duodenum, Jej = jejunum, Ile = ileum, Cae = caeca).

‡ Lesion distribution reflects the known tropism of species in the oral inoculum (37 % *E. maxima*, 19 % *E. brunetti*, 16 % *E. necatrix*, 14 % *E. praecox*, 8 % *E. tenella*, 6 % *E. mitis*).

CONCLUSIONS

CONCLUSIONS

This study reveals an overall prevalence of coccidiosis of 72.8% in 158 broiler farms in Boumerdès Province, Algeria, with seven *Eimeria* species identified: *E. tenella* (the dominant species, accounting for up to 49% of isolates in Tidjelabine), E. maxima (predominant in Zemouri), *E. necatrix*, *E. acervulina*, *E. brunetti*, E. mitis, and *E. praecox*. Multivariate analysis reveals that infection is strongly correlated with a biosecurity index below 70/100, stocking densities exceeding 12 birds/m^2, worn litter, earthen floors, and unsecured water supplies. Each of these parameters increases the relative risk of infection by a factor of six to fifteen. Faced with this parasitic pressure, screening of medicinal plants from the Lamiaceae family indicates that only the aqueous fraction of *Thymus algeriensis* reduces oocyst shedding by more than 80%, while maintaining 100% survival and preserving intestinal villous architecture, thereby offering a rational phytotherapeutic alternative to synthetic anticoccidials.

Building on these findings, five integrated research-to-action avenues emerge. First, a nationwide "turn-key" biosecurity programme for Algerian poultry farms, combining facility audits, certified farmer training, and financial incentives for litter renewal and water-line protection, will help to elevate operations to the identified threshold rapidly. Second, micro- or nano-encapsulation of the aqueous *T. algeriensis* extract in biopolymeric matrices is planned to stabilise volatile metabolites, extend shelf life, and enable targeted release in the jejunum. Third, a combined meta-omics strategy (metagenomics, transcriptomics, metabolomics) will dissect microbiota–phytochemical–parasite interactions and map the immune pathways modulated by treatment. Fourth, portable multiplex- or digital-PCR (ddPCR) devices will provide real-time molecular diagnostics to quantify parasite load, distinguish Eimeria species, and detect resistant genotypes early, supplying veterinarians with immediate, data-driven decision support. Finally, expanding the screening to additional Algerian flora, especially Apiaceae, Asteraceae, and Fabaceae, and testing rational combinations of extracts could yield synergistic formulations with broader efficacy against parasite burden and oxidative stress.

Integrating these approaches should result in measurable reductions in the use of chemical antiparasitics, improved farm profit margins, and decreased environmental levels of pharmaceutical residues, all while aligning Algerian poultry production with One-Health principles for food safety, animal welfare, and ecological sustainability.

PERSPECTIVES

- **Validate and scale the biosecurity baseline.** Run multi-site, longitudinal studies to confirm the **≥70/100** operational threshold and quantify the causal impact of key levers (≤12 birds·m^{-2}, sealed floors, high-quality litter, treated/public water) on incidence, intensity (OPG), and performance (ADG, FCR, EPEF). Use stepped-wedge or cluster RCT designs that are both farm-friendly and robust.
- **Map *Eimeria* at the micro-regional scale.** Combine municipal-level risk mapping with molecular typing (qPCR/amplicon sequencing) to link species/strains to lesion patterns and severity, then tailor vaccination/rotation and advisory messages by locality.
- **Advance *Thymus algeriensis* from proof-of-concept to field use.**

1. Standardise the aqueous extract (chemical markers, lot-release specs);
2. Test stability in drinking systems (pH, temperature, biofilm);
3. Run multi-farm randomised trials with decision-grade endpoints (mortality, lesion scores, OPG, ADG, FCR, EPEF), dose–timing windows, and compatibility with vaccines/anticoccidiostats.

- **Position rosemary for environmental control.** Evaluate hydro-alcoholic rosemary against sporulated oocysts on litter/surfaces versus reference disinfectants, and prototype safe, farm-practical protocols that integrate into cleaning/disinfection workflows.
- **Open the black box: mechanisms.** Utilise bio-guided fractionation to link constituents to targets, including oocyst wall/sporulation, epithelial barrier protection, immune modulation, and microbiome shifts, across in vitro, ex vivo, and in *vivo* tiers. This will de-risk formulation choices and regulatory dossiers.
- **Engineer formulations for real barns.** Develop microencapsulated, waterline-compatible formats; verify shelf life and line integrity under shelf conditions; monitor palatability/water intake; document safety margins and interactions at supra-effective doses.
- **Turn evidence into decisions.** Deploy a lightweight farm dashboard, biosecurity score, density, litter quality, water hygiene logs, early OPG trends, ADG/FCR, to trigger timely corrections and quantify ROI of "biosecurity alone" vs "biosecurity + *Thymus*".
- **Prepare knowledge transfer.** Co-create practical SOPs and checklists with farmers and vets (training modules, pictograms), anchored in your risk factors and workable on family-scale and commercial farms alike.

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ملخص

أجرينا دراسة لتقدير معدل انتشار داء الكوكسيديا الطيري في ولاية بومرداس، ولاختبار بدائل نباتية لمضادات الكوكسيديا الاصطناعية. بين ديسمبر 2017 وأبريل 2018، فَحصت 158 مزرعة دجاج، وجاءت نتائج فحوصاتها إيجابية لبكتيريا الإيميريا في 72.8% منها. حصلت كل وحدة على درجة أمان حيوي (0-100) بناءً على 75 مؤشرًا؛ ولم تكن أي من المزارع الـ 22 التي حصلت على درجة ≤ 70 خالية من العدوى، بينما حصلت جميع المزارع الإيجابية على درجة أقل. أكد الانحدار اللوجستي الدور المحوري للأمن الحيوي: حيث تزيد عدة ، عوامل (الوضع القانوني غير الرسمي، وكثافة التخزين > 12 طائرًا/م^2 وأرضيات ترابية، ونفايات متحللة) من الخطر بشكل كبير، في حين أن مياه الشبكة العامة لها تأثير وقائي. تم تحديد سبعة أنواع من الإيميريا، ولكل منها نمط توزيع خاص بكل منطقة دقيقة. وبالتوازي مع ذلك، وصفنا النعناع المائي كمصدر لمضادات الأكسدة ومضادات الكوكسيديا. تم تحليل مستخلصين (النقع المائي والنقع الهيدروإيثانولي). كان المستخلص الهيدروإيثانولي أغنى بالمركبات، ولكنه أيضًا أكثر سمية للخلايا، بينما أظهر المستخلص المنقوع تحملًا خلويًا أفضل، وحماية متزايدة لكريات الدم الحمراء، وتثبيطًا قويًا لإنزيم آلفا-غلوكوزيداز، مما يشير إلى إمكاناته الغذائية العلاجية. ومع ذلك، لم يسمح لنا الثراء الكيميائي ولا القدرة المضادة للأكسدة بالتنبؤ بفعالية مضادات الكوكسيديا في المختبر وفي الجسم الحي. من بين المستخلصات المختبرة، يبدو آن هو الأكثر فعالية في المستخلص المائى لـ Thymus algeriensis تقليل استخدام مضادات الكوكسيديا الاصطناعية، شريطة أن يكون مصحوبًا بمستوى أدنى من السلامة الحيوية.

الكلمات المفتاحية: داء الكوكسيديا الطيري، الأمن الحيوي، أنواع الأيميريا، الانتشار، المستخلصات النباتية.

RESUME

Nous avons mené une étude visant à estimer la prévalence de la coccidiose aviaire dans la wilaya de Boumerdès et à tester des alternatives phytogéniques aux anticoccidiens de synthèse. Entre décembre 2017 et avril 2018, 158 élevages de poulets ont été inspectés : 72,8 % étaient positifs à *Eimeria*. Chaque unité a reçu un score de biosécurité (0–100) basé sur 75 indicateurs ; aucun des 22 élevages notés ≥ 70 n'était pas infecté, alors que tous les élevages positifs présentaient un score inférieur. La régression logistique a confirmé le rôle central de la biosécurité : plusieurs facteurs (statut juridique informel, densité > 12 volailles/m^2, sol en terre battue, litière dégradée) augmentent fortement le risque, tandis qu'une eau issue du réseau public exerce un effet protecteur. Sept espèces d'*Eimeria* ont été identifiées, chacune présentant un profil de distribution propre à chaque microrégion. Parallèlement, nous avons caractérisé la menthe aquatique (*Mentha aquatica* L.) comme source d'antioxydants et d'anticoccidiens. Deux extraits (infusion aqueuse et macération hydroéthanolique) ont été analysés par HPLC-ESI-MS/MS. L'extrait hydroéthanolique était plus riche en composés, mais aussi plus cytotoxique, tandis que l'infusion présentait une meilleure tolérance cellulaire, une protection accrue des érythrocytes et une inhibition marquée de l'α-glucosidase, ce qui suggère un intérêt nutraceutique. Toutefois, ni la richesse chimique ni le potentiel antioxydant n'ont permis de prédire l'efficacité anticoccidienne, *in vitro* comme *in vivo*. Parmi les extraits testés, l'extrait aqueux de *Thymus algeriensis* apparaît comme le plus prometteur pour réduire le recours aux anticoccidiens de synthèse, à condition d'être associé à un niveau minimal de biosécurité.

Mots-clés : Coccidiose aviaire, biosécurité, *Eimeria* spp., prévalence, extraits phytogéniques.

ABSTRACT

We investigated avian coccidiosis in Boumerdès Province (Algeria) and evaluated phytogenic alternatives to synthetic anticoccidials. From December 2017 to April 2018, 158 broiler farms were inspected; 72.8% were positive for *Eimeria*. Each farm received a biosecurity score (0–100) based on 75 indicators; none of the 22 farms scoring ≥ 70 were infected, whereas all positive farms scored below this threshold. Multivariate logistic regression confirmed biosecurity as the key determinant of infection: informal legal status, stocking density > 12 birds m^2, earth floors and degraded litter markedly increased risk, while using public-supply water had a strong protective effect. Seven Eimeria species were detected, with distinct micro-regional distribution patterns. In parallel, water mint (*Mentha aquatica* L.) was characterised as a source of antioxidants and as a potential anticoccidial agent. HPLC-ESI-MS/MS analysed two extracts (aqueous infusion, hydro-ethanolic macerate). The hydro-ethanolic extract contained higher levels of phenolics. Still, it was more cytotoxic, whereas the infusion showed better cell tolerance, greater protection of human erythrocytes and strong α-glucosidase inhibition, indicating promising nutraceutical properties. However, neither chemical richness nor antioxidant capacity predicted anticoccidial efficacy *in vitro* and *in vivo*. Among the plant extracts tested, the aqueous extract of *Thymus algeriensis* showed the best in vivo performance, with high survival, improved lesion scores, and reduced oocyst shedding. At the same time, rosemary provided intermediate protection, and mint remained ineffective. Overall, achieving a minimal biosecurity threshold and combining optimised husbandry with selected phytogenics emerge as complementary strategies to control coccidiosis and reduce reliance on synthetic drugs in poultry production.

Keywords: Avian coccidiosis; biosecurity; *Eimeria* spp.; prevalence; phytogenic extracts.