Use of Diatomaceous Earth as a dietary supplement in organic laying hen production

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This dissertation is submitted for the degree of Master in Philosophy

September 2018

Institute of Biological, Environmental and Rural Sciences,
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ACKNOWLEDGEMENTS

This work was funded via the EU's Convergence European Regional Development Fund through the Welsh Assembly Government KESS program. I wish to thank Natural Feeds and Fertilisers Ltd. and Knowledge Economy Skills Scholarships *(*KESS*)* for providing financial support for the completion of this MPhil scholarship.

I am sincerely grateful to my supervisors Dr Justin Pachebat, Prof. Jamie Newbold and Ms Helen Edwards, for their advice, support and encouragement throughout this project.

I would like to express my thanks to Dr Emilio del Cacho for his valuable advice and to the Royal Veterinary College of London for the hospitality provided during a short training period, notably to Dr Damer Blake, Dr Virginia Marugán-Hernández and Dr Matthew Nolan for their support and guidance.

My appreciation to Dr Eva Ramos-Morales, Dr Alejandro Belanche, Dr Eli Saetnan and Dr Eric Pinloche from IBERS, Ms Liz Findlay, Ms Marianne Azevedo Teixeira, Dr Susan Girdwood and Mr Alan Cookson for their advice and technical support. I am grateful to the IBERS HGE group and laboratory staff, particularly to Ms Hilary Worgan, Ms Eleanor Jones, Dr Adrian S. Horton, Mr Vince Theobald and Ms Kate E. Waddams, for their inputs.

Finally, this project would have not been possible without Dr Gabriel de la Fuente, who has given me his trust and cared for me, and has been my inspiration and guide through this adventure, together with my family and friends.

I dedicate this work to my father José Ángel, who has always been proud of me.

With all my love, thank you.

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Academic year the work submitted for examination	2016
Full title of thesis:	USE OF DIATOMACEOUS EARTH AS A DIET SUPPLEMENT IN ORGANIC LAYING PRODUCTION

Summary:

Dietary supplementation with diatomaceous earth was proposed in this study in order to evaluate its effect on the concentration of parasites, egg production and egg quality of free-range organic laying hens. 440 Novogen Brown hens of 54 weeks of age were split in two groups of 220 hens each and fed a basal organic diet with (intervention group) and without (control group) supplementation of diatomaceous earth for five weeks. Faecal egg counts were performed in faecal samples once a week using the McMaster and Modified Stoll methods. External and internal egg quality tests were performed in eggs sampled on weeks one and five of the trial. Four DNA extraction methods for faeces were applied and compared, and DNA was tested for the presence of *Eimeria* species parasites by PCR; *Eimeria acervulina* was detected and quantified by qPCR. Dietary treatment did not have a significant effect on the concentration of parasites, but seemed to lower the parasite counts of *Ascaridia galli* and *Heterakis gallinarum.* Intervention daily egg production and laying index were increased, and even if they laid good quality eggs these failed to prove better quality eggshells or lower number of broken eggs. Further studies may want to consider a dietary intervention with diatomaceous earth in a flock of younger (pullets) or older hens and increasing the length of time of the trial.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

CHAPTER ONE

INTRODUCTION

1.1 POULTRY FARMING

1.1.1 SOCIAL AND ECONOMIC IMPORTANCE OF POULTRY FARMING

Poultry is one of the fastest growing agricultural sub-sectors (Mottet and Tempio, 2017). Poultry meat and eggs are common animal source food and are contributing to satisfy an increasing global demand. Poultry production is characterized by presenting a diversity of production systems, short production cycles and potential for development in both rural and urban areas (Mottet and Tempio, 2017). Backyard flocks and small holders are predominant in less developed and rural areas of the world, whereas production at large industrial scale is located in richer areas. In addition, poultry production plays an important role in developing countries, where livestock is key for poor people as source of income and asset representing, and it is one of the principal activities for women (Ahuja and Sen, 2007).

According to FAOSTAT data (http://www.fao.org/faostat/en/), global poultry meat production has undergone a linear increase from 1985 (31 M tonnes) to 2014 (113 M tonnes), with the Americas the largest chicken meat-producing region; the 2013 ranking revealed U.S., Brazil, Mexico and Argentina as the biggest producers (Evans, 2016a). Global poultry meat consumption is increasing due to higher demand from developing countries, which are experiencing a rapid population growth and urbanisation (Evans, 2016b). FAOSTAT calculates a poultry meat consumption from 11 kg per person and year (2000) to 15 kg per person and year (2013). It predicts a positive trend for the chicken meat production over the coming years as it accounts for

around 89% of poultry meat availability (Evans, 2016b). In Europe, poultry meat production has experienced a rapid increase from 2006 (13 M tonnes) to 2014 (19 M tonnes), although the non-European Union countries Russian Federation and Poland led the 2013 ranking of chicken meat production, followed by United Kingdom, France and Spain (Evans, 2015a).

Global egg production reached 70 M tonnes in 2014, with Europe showing a steady increase in egg production from 9 M tonnes (1996) to 11 M tonnes (2014), as reported by FAOSTAT. Europe is the region that dominated the global egg trade in the last decade, judged by the number of exports and imports from and to European countries, with minimal trade done with external countries (Evans, 2015b). However, since the modification of European legislation of the housing systems for laying hens in 2012, which banned the conventional cages (battery production) and demanded their replacement for other housing options, Europe's egg growth has slowed down (Evans, 2015c).

1.1.2 POULTRY FARMING SYSTEMS: CHICKEN MEAT PRODUCTION

Chicken meat production has been conventionally developed under intensive farming conditions, characterized by confined housing systems of broiler-type chickens (fast-growing birds). Animals are raised in littered floor up to 2-2.2 kg live weight in 38 to 42 days, with high stocking rates that can reach 16 birds and 33 kg per square meter (m^2) (Cepero, 2005). The European Council Directive 2007/43/CE compiled some rules for the welfare of chickens kept for meat production. For UK (England), DEFRA have produced the welfare code of recommendations for meat and breeding chickens in Welfare of Farmed Animals (England) Regulations 2000 (DEFRA, 2002b).

An alternative to the conventional production system is the free-range system, which has been developed in Europe following the French model of "Label" chicken. In brief, rearing conditions under the *Label Rouge Fermier*

model include the use of slow-growing breeds, raised up to 2-2.5 kg in at least double the time compared to conventional broiler chickens (81 days), with a smaller stocking rate (11 birds per m^2). Chickens have free access to external parks to exercise and to small indoor barns with natural lighting and ventilation, where they get a less concentrated cereal-based feed (Synalaf, 2013).

1.1.3 POULTRY FARMING SYSTEMS: LAYING HENS AND EGG PRODUCTION

The European Council Directive 1999/74/EC for laying hens, made a distinction between three types of rearing systems: enriched cages, non-enriched cages (battery cages - which were banned from 1st January 2012) and non-cage systems or alternative systems. For UK (England), DEFRA have produced the welfare code of recommendations for laying hens in Welfare of Farmed Animals (England)(Amendment) Regulations 2002 (DEFRA, 2002a). The standards for keeping laying hens were detailed in the cited directive as follows:

- Enriched cages allow a group of hens (colony) to be housed together with a minimum of 750 cm^2 of cage area per hen, being the total cage area of at least 2000 cm^2; ground should be covered with litter to allow pecking and scratching. Enriched cages must have a nest, perching space of at least 15 cm per hen and claw-shortening devices. Access to food and water must be unrestricted, allowing access to a minimum of 12 cm of feed trough length and 2 nipple or cup drinkers per hen.

- Non-cage systems must have a maximum stocking density of 9 hens per m^2 usable area, with a ground surface of at least 250 cm^2 per hen, which must be covered in litter at least one third of its surface. One nest must be provided per every 7 hens or a minimum nest space of 1 m^2 for every 120 hens when group nests are used. Access to feeders

and drinkers must be equally guaranteed to all hens, providing a minimum of 10 cm of feed trough length or 4 cm of circular feeder per hen and either 2.5 cm continuous drinking trough length or 1 cm of circular drinker per hen. If nipple drinkers are used, at least one nipple must be available per 10 hens.

Under an alternative system, hens can be raised on floor in an enclosed facility (aviary or floor pen) or with outdoor access in a free-range system. The latter allows hens to have free all-day access through pop holes to external parks or open runs, which must accommodate a maximum of 2500 hens per hectare (4 m² per hen). Open runs must be of an area appropriate for the stocking density, must provide shelter for adverse weather conditions and protection from predators and other risks.

Some European countries use other models of housing for free-range hens, adding multi-tier aviaries to the housing facility in order to accommodate a larger flock size. These are structures built above the house ground that add usable floor space, which allows an increase in the number of birds per meter (stocking rate) (Terraz, 2011).

1.1.4 ORGANIC FARMING AND ORGANIC POULTRY PRODUCTION

From the middle of the 20th century, agriculture and livestock production have undergone progressive intensification, which in return has induced negative impact in the environment and thus in society. Soils have reduced their fertility, as a consequence of contamination through the use of chemical products for agriculture and overexploitation. Forestry areas and biodiversity have been reduced, including diversity of farm animal breeds. Air pollution derived from these activities, methane (CH_4) and carbon dioxide (CO_2) emissions, are contributing largely to global heating and climate change. Furthermore, institutions and public opinion show concern about sanitary

issues regarding residues from drugs or chemical substances in edible products and animal welfare issues (García, 2009).

Organic farming is based on the principles of preservation of the natural environment, its habitats and biodiversity, supporting the use of indigenous breeds in order to maintain genetic variability and to benefit from rustic animals and maintaining high standards of animal welfare under non intensive rearing conditions. Animal and environmental health balance is ensured through preventive medicine and natural complementary therapies (such as herbal medicine and homeopathy) for animal health care, avoiding the application of medical drugs and the use of contaminant chemicals in the soils (García, 2009; Soil Association, 2014).

The objectives of organic farming are the production of differentiated, healthy and safe quality food; the protection of the environment and the natural integrated systems through the application of modern farming and agricultural techniques and a sustainable use of natural resources. In addition, the creation of social networks based on the fair trade of services and products and broadening knowledge about all the aspects related to organic production and living are important aspects (García, 2009).

Organic poultry production is an alternative option for producing meat and eggs. Requirements include the regulations that apply to free-range chicken and layer production together with specific husbandry, nutrition and health care principles, which are different for meat or egg production. Organic egg production characteristics are detailed in chapter 1.2.1.

1.1.5 CHALLENGES FOR THE ALTERNATIVE AND ORGANIC EGG PRODUCTION INDUSTRY

Future perspectives for these production systems are optimistic, although some technical difficulties have arisen and some challenges have to be faced, such as bird sanitary and animal welfare issues, higher costs of

alternative production and response to an increasing customer awareness and demand of alternatives to conventional products (Cepero, 2005).

Animal health and welfare issues

Promotion of alternatives for housing layer hens was initiated prior to the 2012 ban of non-enriched cages (battery cages) in Europe (Buttow Roll *et al*., 2009). Animal welfare aspects of the systems of keeping laying hens were assessed and motivated the legislative modification. An analysis made by the Scientific Panel on Animal Health and Welfare on a request from the EU Commission (Blokhuis *et al*., 2005), suggested that the possibilities for the hens to show species natural behaviours like dust-bathing, foraging, nesting and perching differed between hen housing systems and, if hens were prevented from doing those, it would result in frustration and injury. Having sufficient space to allow movement, exercise and other activities was identified as a priority, facilitated by complex environments rather than space restricted environments.

Birds with outdoor access have an increased risk of exposure to greater range and different infectious agents compared to birds kept indoors. Lay Jr. *et al.* (2011) found that non-cage systems facilitated nest-dwelling ectoparasites such as red mite, soft ticks and bed bugs, but offered a better control of insects like flies and beetles. In addition, mortality and some welfare problems like cannibalism were worse in these systems. Blokhuis *et al*. (2005) considered injurious pecking a serious problem difficult to control, that should be addressed by management measures and control of bird genetics rather than by beak trimming, deemed to be a painful procedure.

Enriched cage systems showed lower risk of bone breakage, lower incidence of footpad problems and reduced mortality rates compared to non-cage systems. In spite of that, both systems seem to have a higher number of downgraded eggs (grade B: dirty, broken or cracked eggs) and higher bacterial eggshell contamination compared to eggs from non-enriched cages (battery cages) (Lay Jr. *et al.*, 2011).

Cost of non-conventional production systems

Matthews and Sumner (2015) compared the costs of egg production in a commercial farm, with three different housing systems: conventional battery cages, enriched cages and aviary. Egg production costs were higher for the aviary system than the other two systems, with operating costs (including feed, labour, pullets, energy and miscellaneous costs) about 23% higher compared to conventional system, while costs of enriched cage system were about 4% higher. Labour was the main source of costs in the aviary, as workers dealt with a more disperse area for hen care, covering egg collection and recovery of dead birds; these were time consuming tasks that entailed more dedication as the laying cycle progressed.

Nevertheless, European legislation has progressively introduced restrictions to the poultry farming industry, such as limitation of use of antibiotics as growth promoters, a ban of animal derivate ingredients in the feed and regulations applicable to production systems and management. As a result, costs and prices have pushed up to a level that differences are not so large compared to alternative products (Cepero, 2005).

With regard to organic egg production, costs are assumed to be higher than conventional production. This is explained by the higher cost of organic feedstuffs for animal feed, difficulties in making a balanced feed formulation and higher total costs in a smaller production scale (García-Trujillo *et al*., 2009; Cepero, 2005). Commercial production would require a large starting-up inversion to cover the costs of larger and mechanised facilities, able to provide housing to larger flocks but simplified to limit the cost per hen. In order to make the farm profitable, higher costs of feed and labour could be compensated by the higher sale price of organic eggs, provided that a suitable market for the product is found. On the other hand, direct sale production model, from farm to customer, would be profitable using small flocks and indigenous crossed breeds, keeping the birds for two production cycles with induced moult (Pont, 2006).

Demand and consumer awareness of organic products

Demand of organic farming products in Europe has experimented a rapid growth in the last two decades, with France, Germany and UK leading the retail sales from 2002 to 2014 (Cepero, 2005; Meredith and Willer, 2016). Although the number of organic producers has increased by 60% in the last decade, and the European market is in dynamic growth, organic production moves at much slower rate. In 2014, organic farmland in EU represented 7% of the total agricultural land (2.4% in Europe), with 11.2 million hectares of organic land, and Liechtenstein, Austria and Sweden the countries with largest organic area (Meredith and Willer, 2016).

Social and mass media pressure has increased after food security crises, raising concern about industrial food production and demanding a change in agricultural and animal production models (Cepero, 2005). People's awareness of obtaining quality food, free from chemicals and derivates of genetically modified organisms (GMO), under farming conditions of animal welfare and respect to the environment, has helped to increase the demand and broaden the market of organic food. However, informative campaigns, improvement of the labelling and development of marketing strategies for organic products need to be implemented to enhance public interest and sales (Meredith and Willer, 2016).

European countries have progressively established legislative policies for the promotion of sustainable agriculture and farming. Still, a supportive policy environment and private sector investment could help to stimulate the further development of the organic food and farming sector (Meredith and Willer, 2016).

1.2 EGG PRODUCTION AND QUALITY

1.2.1 ORGANIC EGG PRODUCTION

LAYING HEN BREEDS

The Soil Association allows the use of traditional hen breeds such as Light Sussex and Rhode Island Red or commercial hybrids. Some of these hybrid breeds are Isa Brown, Essex Brown, Hy-line Brown, Shaver 579, Hubbard Golden Cornet, Lohman and Hendrix Bovans (Soil Association, 2006).

Hybrid breeds have been selected for superior egg production and feed efficiency in a cage-housing system, compared with traditional breeds (Soil Association, 2006). They also reach bigger size and start laying eggs earlier, around 18 weeks of age. Due to the high production costs of organic farming, the use of these commercial hybrid breeds may remain the only viable option for the organic farmer (Pont, 2009).

However, hybrid breeds pose some challenges with regard to their characteristics and ability to adapt to the outdoors. They are less rustic and less resistant to outdoor weather variations, which make them more vulnerable to health issues (Pont, 2009). In addition, normal behavioural traits seem to be reduced or suppressed and are linked to a genetic component. For instance, Jensen (2006) compared jungle fowl and Leghorn hens, finding the latter less active (reduced foraging and exploratory behaviour), less social and showing tamed reaction to predators.

Several factors influence husbandry issues like feather pecking and cannibalism. These undesirable behaviours relate to the ability of the flock to adapt to their environment, and it seems to be harder to control in free-range systems (Blair, 2008).

PRODUCTION SYSTEM AND HOUSING IN ORGANIC FARMING

The organic farming standards compiled by the Soil association (2014) are based on the principles of enhancement and utilization of the natural biological cycles in soils, crops and livestock. Livestock living conditions must accommodate the health and natural behaviour of the animal. For these reasons, organic production of laying hens requires free-range systems.

Permitted flock size ranges from small (below 100 birds) to medium (up to 500 birds) and large flocks (up to 2000 birds), although the maximum number of hens can vary depending on the country; for instance, Spain sets a limit number at 3000 hens and European countries like Austria, Germany and Netherlands can permit up to 18000 hens, separated in smaller size groups (Terraz, 2011). A flock of limited size, composed by birds of unimproved breeds or breeds acclimatised to the region would be the ideal scenario for outdoor production, as these may be hardier, healthier and rely more on foraging as a food resource (Blair, 2008).

The two most popular housing options are fixed and mobile hen houses, which must be large enough to comply with a stocking rate of six hens per square meter (m^2) in indoor facilities (Soil Association, 2006).

Fixed houses can be large and are usually set up fixed in a location. Their inconvenience is that they give access to the same piece of field, where pasture gets scarce with time and results in an accumulation of pathogens and dirt. An option is to make subdivisions of the field with shade provided with trees and bushes. This would help to move the flock around, taking it further away from the entrance of the house (Fanatico, 2007). Land should be rested for nine to twelve months between flocks or paddocks rotated before being used again.

Mobile houses are generally preferred, as they can be moved within the field frequently to allow grazing areas to be rotated; paddocks are suggested to be used for periods of six to eight weeks (DEFRA, 2001). Houses are small to medium in size therefore accommodate smaller flocks (*Figures 1.1 and 1.2*).

Figure 1.1. Mobile hen house (tunnel). This kind of house is easy to set up and inexpensive; it is frequently used for housing small flocks of young hens or pullets. Source: Fanatico (2007).

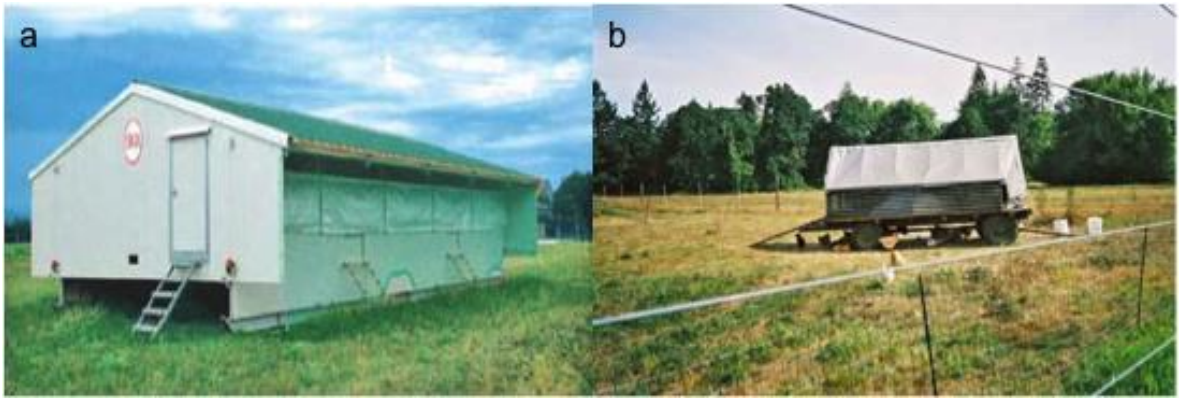


Figure 1.2. Mobile hen houses: a larger model on rails (a) and simpler model with wheels (b). Houses provided with wheels are more frequently moved than railed houses because it is easier to move them. Source: Fanatico (2007).

Housing should provide roost perches, nest boxes, feeders (linear or circular) and drinkers (nipple or bell). These should comply with certain requirements of size per hen. One nest must be provided per every 7 hens or a minimum nest space of 1 m² for every 120 hens when group nests are used. Access to feeders and drinkers must be provided at a minimum of 10 cm of feed trough length or 4 cm of circular feeder per hen and either 2.5 cm continuous

drinking trough length or 1 cm of circular drinker per hen. If nipple drinkers are used, at least one nipple must be available per 10 hens (García-Trujillo *et al.*, 2009; DEFRA, 2002a).

Bedding material like straw or wood shavings should be used to cover floors at least one third of its surface, at an adequate depth. The house can be designed installing perches above an area with slatted platform, with a dropping belt beneath. This way floors and housing equipment is kept cleaner (*Figures 1.3a and 1.3b*). Perch space must be of at least 18 cm per bird (García-Trujillo *et al.*, 2009; DEFRA, 2002a).

Figures 1.3a and 1.3b. Circular feeders and bell drinkers. This equipment can be relocated within the house and its height must be adapted to the size of the hens. Source: García-Trujillo *et al.* (2009).

MANAGEMENT OF THE LAND

Hens should have continuous and easy daytime access to land covered with vegetation and a fresh supply of water. Shade and shelter from wind, rain, cold and sun should be provided in the field, by planting trees, shrubs or tall hedges. An electrified fence can be useful as a measure for protection from predators, like foxes and other wildlife.

It has been observed that hens usually prefer to forage from the area closer to the house than more distant areas, and frequently not all the field is used. Another important element for field usage is the location of the house (Hegelund *et al*., 2005; Pont, 2013). Some strategies like keeping outdoor feeders and drinkers or small sheds distributed within the field can help to attract the hens to use the land and forage (Fanatico, 2007; Pont, 2013). In addition, having a protected and well-sheltered field is key for encouraging hens to move away from the house (Zeltner and Hirt, 2003).

The stocking rate or maximum number of hens per hectare of land is defined by the certification body, being 1000 hens per hectare of land the limit set by the Soil Association organic standards. Besides, EU regulations limit an excess of 170 kg of nitrogen per hectare a year of manure coming from livestock of the agricultural area used. In practice, the producer would need a larger total organic managed area to meet these requirements.

Paddock rotation is a required strategy to allow vegetation cover to re-grow and reduce the excessive load of pathogens and nutrients in the soil. Flocks should change to a fresh paddock every two to three months, and this is easier to achieve with mobile houses (Fanatico, 2007). General recommendations suggest not mixing animals of different ages or species in the same plots. In order to reduce disease risks, the “all in-all out” management system should be followed, as depopulation reduces the pathogen load if no host is present (Blair, 2008).

MANAGEMENT OF THE FLOCK

Parent stock is not required to be organic, meaning that if organic pullets are not available, non-organic birds under 18 weeks of age can be used. Nevertheless, the Soil Association establishes a conversion period for the non-organic flocks to become organic. Once the farmland has completed a conversion period of 24 months to be recognised as organic, the flock must complete six weeks managed under organic standards before selling their eggs as organic eggs (Soil Association, 2014).

Natural and artificial lighting are permitted, but light hours should not exceed sixteen hours per day and eight hours of darkness should be provided at night (García-Trujillo *et al.*, 2009). Hens reach maturity to lay when they have the appropriate age and bodyweight, and are stimulated by light. Hence the photoperiod is generally managed to give light-stimulation in a progressive way, from eight to sixteen hours a day, and remain stable during the laying period (Barroeta *et al.*, 2010).

The laying cycle is divided in several periods: light-stimulation until 5% production (from week 20-21 to week 24-25), peak egg production (week 30-31) and depletion. An egg production curve is detailed in Figure 1.4.

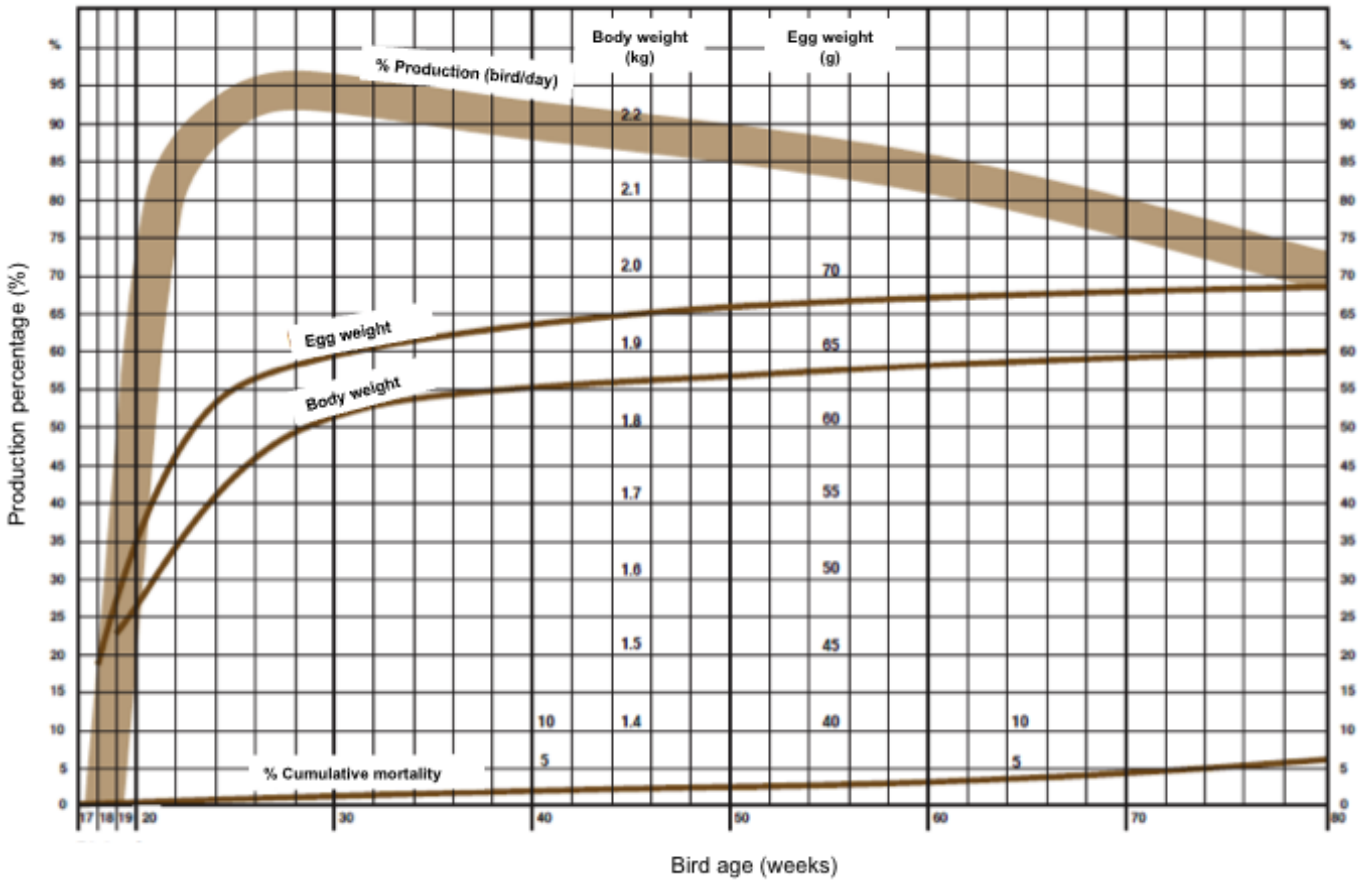


Figure 1.4. Egg production curve, egg weight, body weight and mortality in brown-egg laying hens. Adapted from Barroeta *et al.* (2010).

Hens are usually kept one production year (around 54 weeks of lay), up to 72 weeks of life. Another option is to keep them for a second production year, until 120 weeks of life. This would involve moulting the flock around week 70 or when production falls to 50%, and re start the laying period afterwards (about 28 to 30 days after initiating the moult) (Pont, 2009). Moulting is the process where the birds renew their plumage and stop laying; this would naturally happen after the first year. Moulting is used as a management practice because it increases the productive use of the batch of hens, obtaining a better percentage of egg laying and higher shell quality and albumen height (Barroeta *et al.*, 2010). In addition, producers prefer to do so because organic pullets are not easy to find in the market (García-Trujillo *et al.*, 2009).

DIET

Ingredients, formulation and manufacturing

Grain should be of high quality with good levels of moisture for safe storage, in order to avoid mould growth and mycotoxins. Whole grain is preferred as it has some advantages for the development of the gizzard; this seems to play role in the prevention of pathogen bacteria entering the distal digestive tract (Blair, 2008).

The laying period feed should be energetic and high in calcium, as hens deposit 2 to 2.3 grams of calcium per egg laid. This is offered in the form of limestone or oyster shells (big size particle, 3-4 mm) and ideally administered late afternoon as shell is formed overnight in the oviduct (Barroeta *et al.*, 2010). Standard feed should be available to hens at all times (*ad libitum*).

Feedstuffs should be produced on the farm or in the local region, and must be 100% organic. In addition, some restrictions apply: declaration of non-genetically modified grain or grain by-products, prohibition of chemical products, drugs and pure/synthetic amino acids or vitamins. Some feed

sources are deficient in essential amino acids like methionine and lysine, therefore approved protein sources such as rapeseed, soybean cake or skim milk powder should be provided to compensate this limitation, especially in the first weeks of life (Sundrum, 2005).

Feed can be manufactured on-farm with a mix-mill, to get a mash meal. Milling feedstuffs allows uniform mixing of all ingredients and a final product (mash) of small particle size but high surface area. This is positive with regard to digestion efficiency and utilization of nutrients in the digestive tract (Blair, 2008). Amerah *et al.* (2007) found that offering mashed diets improved poultry performance compared to pelleted or crumbled diets, thanks to more uniform ingredient intake plus the effect of particle size on gizzard development.

Forage

Birds in free-range systems have the opportunity to supplement their diet with the nutrients they need as they have freedom to range. Biodiversity in organic farms is greater than in conventional farms; depending on the outdoors environment, there can be found vegetation, fruit, seeds and macro invertebrates (spiders, beetles, earthworms), all these being potential feed sources for organic birds (Van de Weerd *et al*., 2009).

Grassland contribution to the bird's nutrition is very seasonal, as the nutritional value of grass changes throughout the year (higher protein in spring and summer) and the amount of sward available varies. Intake from outdoor's area is not well documented, although studies from Horsted and colleagues (Horsted *et al*., 2006; Horsted and Hermansen, 2007) are inclined to confirm that hens feed a considerable amount of herbage and other food items, and this was a way to fulfil their nutrient requirements.

Because having access to good-quality forages has the potential to supply a significant proportion of the nutrient needs of poultry, some reduction from

formulated feeds could be considered (Blair, 2008). A restriction in nutrient supply seems to increase forage intake (Fuller, 1962), but this should be monitored for nutritional deficiencies.

Arthropods present in the soil (insects and earthworms) are highly palatable and provide good amount of protein with composition in amino acids similar to fishmeal (Ravindran and Blair, 1993). However, they may accumulate contaminants, are low in calcium/phosphorus ratio and may be potential disease vectors; for instance, earthworms may act as paratenic hosts of *Histomonas meleagridis*, the protozoan parasite responsible for blackhead disease. Novel sources of protein such as houseflies, silkworm pupae, adult field crickets and spent bees have been studied to replace fish or soya meal, with positive results (Blair, 2008).

Feeding programme

Hens have different feeding needs to meet their requirements for body development and egg production (*Table 1.1*). These stages are classified according to the production stage: as starters, they require feed with high nutrient and protein content; as growers, feed is less rich in energy and protein, and higher in fibre given as whole grain, which helps to develop the muscular gizzard and increases the calcium intake (Blair, 2008). By the end of the pre-laying period, feed must increase in nutrients again to stimulate starting of lay, together with an increasing lighting programme; in the laying period and until lay drops to 85%, hens require high protein content feed, but when lay declines feed has lower energy and protein content. The calcium (Ca) content of the feed must be high in the whole laying period, because the 90% of the calcium used for the eggshell comes from the feed (García-Trujillo *et al*., 2009).

Table 1.1. Feeding needs of free-range laying hens for an optimal body development and egg production according to the production stage (Benedí *et al.*, 1999).

Production stage	Age (weeks)	Crude protein (CP) %	Metabolizable energy (ME) Kcal/kg feed	ME/CP	Lysin %	Methionine %	g Ca/kg feed	g P/kg feed
Starter	0 - 8	19.5-21	2750-2970	139-146	1.07	0.4	9	6
Grower	9 - 14	15-17	2640-2970	170-190	0.86	0.32	9	5
Pre-laying period	15 - 20	12-14	2640-2970	> 190	0.65	0.25	9	5
Laying period	21 - 40	16.5-18.5	2750-3080	165-169	0.62-0.68	0.31-0.34	27	7
Final stage	40 - 72	14-15.5	2640-2970	187-191	0.60-0.66	0.30-0.33	27	7

OTHER ASPECTS

Birds are sensitive animals, so stress should be minimized and any change (feed, environment) introduced progressively. Young hens in their pre-lay period (up to week 15) are usually kept in a separate shed; it is a good practice to move them into the laying house before the laying period starts (Barroeta *et al.*, 2010). Once they start laying, closing them up in the houses during the morning and performing twice-daily egg collection are good strategies for adaptation (Pont, 2009).

Feather pecking and cannibalism are welfare problems identified as contributory factors for mortality, which can occur in any poultry production system (cage and non-caged) (Van de Weerd *et al*., 2009). Causes that may favour it are bird genetics, high density of animals, low protein diet, lack of some minerals (sodium), high light intensity, parasitism, stress and boredom about feeds (Pont, 2009). Even if a free-range system offered greater opportunity to perform natural behaviour, once feather pecking occurs it may

spread quickly amongst the flock. For this reason, prevention during rearing is important to minimise the problem for when the laying period starts.

Beak trimming is one of the husbandry measures not permitted under organic standards. In addition, health care of the organic flock must follow accepted veterinary practices established by the Soil Association, i.e. the use of complementary therapies and trace elements instead of synthetic chemicals.

Free-range poultry have higher energy requirements, due to increased demand for movement and thermoregulation (Van de Weerd *et al*., 2009). Thermo neutral temperature range is 18-24 °C, thus feed intake varies if temperatures are higher (decreasing) or lower (increasing). Feed composition may be modified and adjusted depending on the season in order to balance the nutrients or the energy intake (Blair, 2008).

Integrated systems with agriculture and other livestock (sheep, etc.) can be considered. Keeping a diversity of farm animals to forage the fields allows recycle of nutrients, control of herbages and has an effect of fertilisation (Fanatico, 2007). This may be cost-effective for the producer since the area needed to meet requirements is larger than standard specifications and also gives the option of producing home grown feedstuffs.

1.2.2 EGGS AS QUALITY PRODUCTS FOR HUMAN NUTRITION

Eggs are considered basic food and are a common ingredient in cooking: they are cheap, easily available and versatile. Eggs have a balanced proportion of nutrients, moderate energy content (150 kcal per 100 g edible egg) and high nutrient density, being actually better compared to other protein animal-sourced products like meat. For instance, the content of unsaturated fatty acids, phospholipids, some minerals and vitamins like A, B_{12}. E and D (usually scarce in food), makes eggs an excellent source to cover daily needs (Applegate, 2000).

However, the amount of cholesterol found in eggs has been a major reason of its devaluation and descent on its consummation as part of a healthy diet. Nevertheless, it has been found that the quantity and type or quality of fat is a factor more important than cholesterol content for the control of human cholesterolemia (Ortega, 2002).

More recently and as research findings advance, eggs have been classified as 'functional foods' thanks to their presumed antioxidant, antimutagenic and anticarcinogenic effects (pigment properties) and contribution to regularization of arterial pressure and blood cholesterol levels (Barroeta, 2008; Ortega, 2002).

Egg nutritional value

A 60-gram egg contains around six grams of protein, found in both albumen and yolk. Albumen is made of 88% water and 11% protein (containing ovoalbuminin, ovotransferrin, ovomucin and lysozyme, among others) and yolk consists of 49% water and 16% lipoproteins. Egg protein is of high biological value, highly digestible (95%) and ready available for the human organism. This means that the profile of amino acids and their proportions are ideal for human nutritional needs, containing all of the essential amino acids (Mine, 2008).

Eggs contain around 10% w/w of lipids (6 grams of a 60 gram egg), only present in the yolk. These are triglicerydes, phospholipids (lecitin and phosphatidylcholine) and cholesterol. Unsaturated fatty acids (mono and polyunsaturated) are in higher proportion than saturated fatty acids. Cholesterol content is 385 mg per 100 g of edible egg (yolk only), thus eggs are a major contributor of this component in the human diet (Barroeta *et al.*, 2008; Ortega, 2002).

Eggs contain all vitamins except for vitamin C. Fat-soluble vitamins (A, D, E and K) are only present in yolk, but water-soluble vitamins (B1 to B12, biotin, choline and inositol) are found in both albumen and yolk. Minerals are

present in the form of organic chelates highly bioavailable. Eggs are especially rich in phosphorus, potassium, selenium iodine and zinc. Pigments responsible for the egg yolk colour are luthein and zeaxantine, which are only found in vegetables too. (Barroeta *et al.*, 2008; Ortega, 2002).

1.2.3 EGG COMPOSITION AND METHODS TO TEST FOR QUALITY

EGG FORMATION AND COMPOSITION

An egg is formed in the female reproductive tract of the chicken, over a period of 24-26 hours (*Figure 1.5*). Yolk starts forming within the ovary, then is released (ovulation) to the first part of the oviduct, the infundibulum, where the egg white begins to form. The immature egg goes through the magnum and isthmus adding components, to finally get to the uterus where spends a great number of hours and forms the shell, a protective but complex structure made of three layers (mammillary or inner layer, spongy layer and cuticle). The fully formed egg enters the vagina, the cloaca and vent to be laid (Barroeta *et al.*, 2010).

The proportions of components of the fresh egg are: 31% yolk, 58% albumen and 11% shell (*Figure 1.6*). The chemical composition is illustrated in Table 1.2.

Egg quality deteriorates from once the egg is laid, even if the chemical composition does not change much. Modifications include loss of water, access of CO_2 through eggshell pores and a pH increase of both albumen and yolk. Alkalinisation of albumen is temperature dependant and can alter protein structure (Coutts *et al.*, 2007).

EGG EXTERNAL QUALITY

Egg grading and external examination

Egg grading involves a classification of eggs according to weight and similar characteristics, grouping them within weight ranges or classes. Countries have established quality standards to classify the eggs of the domesticated chicken, i.e. the USDA egg grading standards.

The evaluation of the external egg quality relies on the factors of egg shape, texture, soundness and cleanliness (USDA, 2000). The normal egg has an oval shape and smooth texture, with no ridges or rough areas affecting its shape. Abnormalities include ridges or “body checks” which result from shell repair after damage caused by stress when the egg is at the shell gland, and thin spots or “pimples” (small shell aggregates). Eggs may have foreign material adhered to their surface such as blood, faeces and cage marks. Depending on the proportion of shell surface affected they are considered clean or dirty eggs (USDA, 2000).

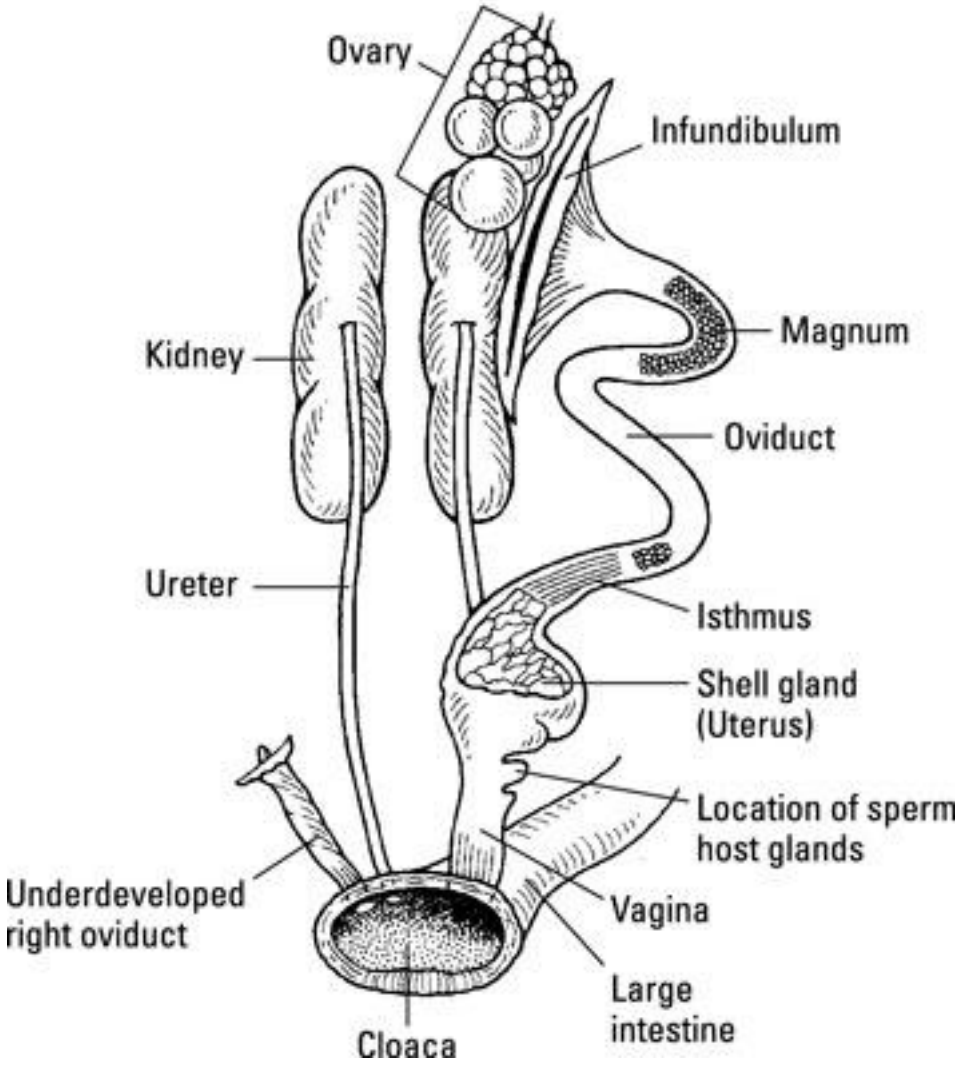


Figure 1.5. Female urinary and reproductive tract of a chicken. illustration by Kathryn Born.

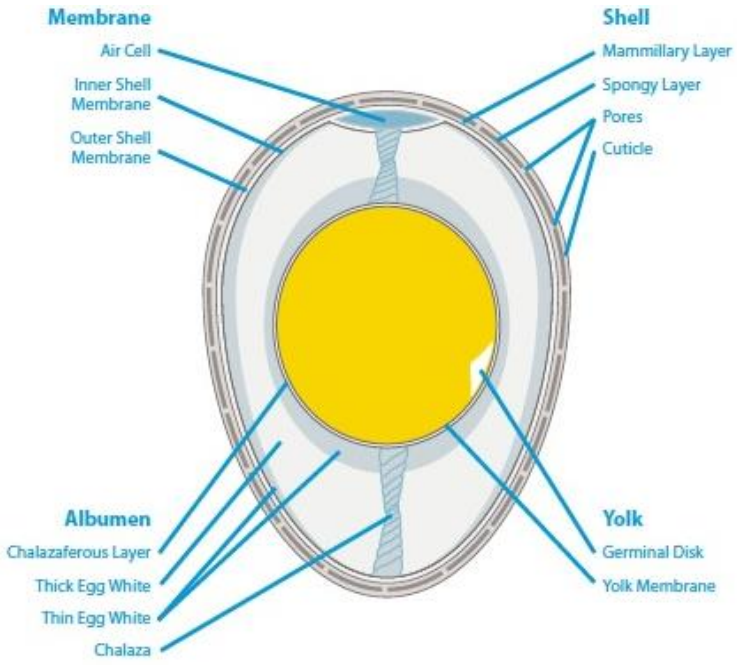


Figure 1.6. Egg internal structure (AVIAGEN, 2013).

Candling

Candling examination is a classic method for the evaluation of eggs, still widely used in the commercial setting and minimally modified throughout the years (Overfield, 1996). It is a manual process, done by hand, where the operator brings the egg to a bright light with a rotation movement. Shell defects are easily identified with this technique, e.g. fractures (hairline, star and pinhole), repaired fractures and shell-density aspects like weak shell areas or very porous “mottled” shells. In addition, candling can detect some internal features of the egg, although this requires certain level of expertise so other broken-egg tests can be performed to complement.

Table 1.2. Chemical composition of the egg (USDA, 2000).

	Percent	Water	Protein	Fat	Ash
Whole egg	100	65.5	11.8	11	11.7
Albumen	58	88	11	0.2	0.8
Yolk	31	48	17.5	32.5	2
		Calcium carbonate	*Magnesium carbonate*	*Calcium phosphate*	*Organic matter*
Shell	11	94	1	1	4

Eggshell quality

Many countries use existing classification standards for commercial eggs as a way of product quality control. The detection of problems on the eggshell is the major cause of low classification, although it is argued that an intact eggshell does not exclude a poor internal egg quality (Overfield, 1996).

For the commercial layer, there is an estimated 10% loss of egg produce before reaching the end user (2-5% uncollectible or broken eggs, plus 3-8% lost in the way from producer to seller). Thus, poor eggshell quality means a higher cost for the producer.

Traditional methods to study eggshell quality are direct methods, based on measuring shell strength; these require specific equipment and need of high number of measures. Indirect methods evaluate eggshell quantity or density and are easier to perform (*Figure 1.7*).

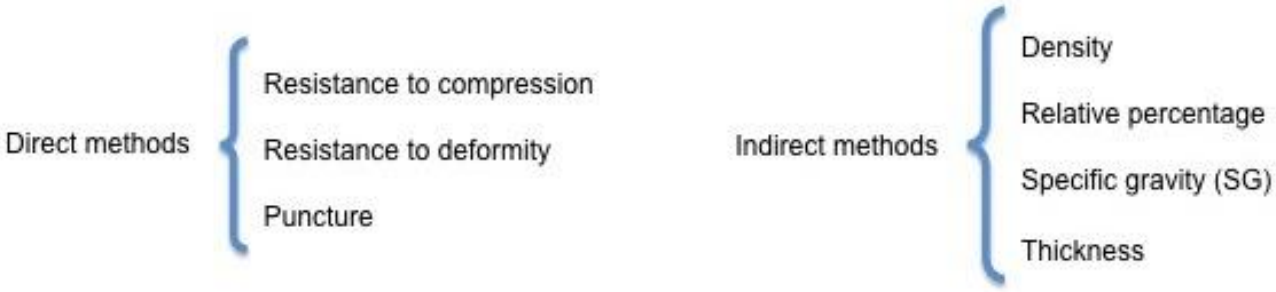


Figure 1.7. Methods for the evaluation of the eggshell quality (Hamilton, 1982).

The specific gravity (SG) of an object equals the weight of its volume relative to the weight of an equal volume of water, being both at the same temperature. If this is applied to an egg, its SG is equal to the egg's density relative to water. The egg has four components, each of them accounting for different SG (shell: 2.325, Yolk: 1.032, albumen: 1.038, shell membranes: 1.075), with the shell SG more than two times higher than the other parts of the egg. It is therefore assumed that the percentage of the shell has a big influence on the SG of the whole egg, and the two values keep a positive correlation (as shell amount increases, egg SG is higher) (Gupta, 2008).

As a conclusion, the specific gravity represents the amount of shell present relative to the amount of albumen, yolk, and shell membranes and is highly related to the incidence of breaks and cracks (Wineland, 1992).

Egg internal quality

Yolk colour is affected by its content in liposoluble pigments (carotenoids) and it is strongly influenced by the composition of hen's diet. The consistency is more dependant on other factors such as age of the hen, storage time and conditions, and relays in the permeability of the vitellin membrane (Soler *et al.*, 2011). Not surprisingly, yolk colour is one of the consumer's main criteria to judge the quality of eggs. Perception of colour may change depending on

geographical location, culture and tradition but a golden-yellow yolk is preferred for most consumers (DSM, 2014).

Yolk colour is evaluated by direct visual examination in homogeneous natural light conditions, comparing the yolk colour with a standardized colour fan, the DSM Yolk Colour Fan. It has fifteen coloured blades, ranging from pale yellow to dark orange/red (Beardsworth and Hernández, 2004)

Albumen controls the position of the egg yolk, and that has great influence in the microbiological defence of the egg (Overfield, 1996). Albumen consistency is a quality factor dependant on the age of the hen and freshness of the egg, but it can also be modified by the protein content, amino acidic profile and other components of the hen's diet (Soler *et al.*, 2011).

The standard method for albumen evaluation is in Haugh Units (Haugh, 1937), whose values integrate the height of the thick albumen with the weight of the whole egg (Overfield, 1996). Callipers or preferably three-footed digital micrometers are used to take the measurements (*Figure 1.8*). The USDA describes three quality grades: AA, A and B (*Table 1.3*).

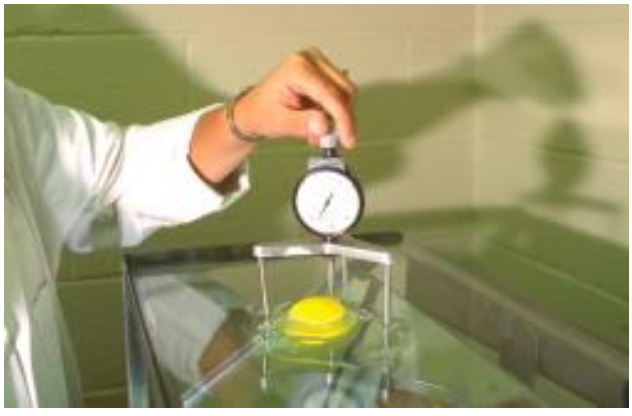


Figure 1.8. Demonstration of the use of a Haugh meter (USDA, 2000).

Quality grade	Albumen texture	Haugh Units	Spots
AA	Clear and firm (thick)	72 or higher	-
A	Reasonable firm (less thick)	60 - 71	-
B	Weak, thin and watery	< 60	Can be present

Table 1.3. U.S. Standards for quality of egg albumen (USDA, 2000).

Haugh Units measured at a t[a] between 45-60 ◦F (7.2 - 15.6 ◦C).

Blood spots are caused during the process of ovulation, when the mature yolk is released from the ovary previous rupture of the yolk sac. They appear as red spots on the yolk's surface. Meat spots, usually found in the chalazeae, albumen or yolk, may be either blood spots that have oxidized its colour or debris of tissue from the reproductive tract of the hen. They appear as reddish brown to paler brown coloured spots (USDA, 2000).

Both types of inclusions seem to be influenced not only by genetics, but also by factors such as flock age and stress. It is not clear that diet plays a role in the frequency of inclusions found in eggs, but they can appear more frequently after drastic diet protein increase or the presence of dietary toxics (Soler *et al.*, 2011).

1.3 PARASITES AND PARASITIC INFECTIONS RELEVANT TO POULTRY

1.3.1 HELMINTHS

Endoparasitic infections in poultry have become less common due to modern farm practices, in which the birds are reared in confinement with reduced or null access to the outdoors. Nevertheless, helminth infections are still relevant for backyard flocks, floor-reared layers, and free-range production systems. These have a higher risk of parasitic infections, due to the access to pastures (Permin *et al.,* 1999). Pastures can be potentially infected with parasites and support intermediate hosts (like earthworms, slugs, snails and arthropods), which are essential for the transmission of parasites with indirect life cycle. Seasonal abundance of invertebrates can also play a role in parasite prevalence (Macklin, 2013). Arthropods able to breed in contaminated litter like beetles are becoming a problem for floor-housed flocks, as they may act as vectors of cestodes (tapeworms) (Van de Weerd *et al.*, 2009). In addition, ranging birds generally have a greater bird-to-bird contact and exposure to faecal material, which increases the chances of faecal-oral infection through parasite eggs present in faeces.

NEMATODES – Phylum Nematoda

ORDER ASCARIDIDA

- *Ascaridia galli*

The size of adults is: females 72-116 mm, males 50-76 mm long. Adults are present in the small intestine. The size of eggs is 77-94 x 43-55 µm, elliptical shaped and thick-shelled.

The life cycle is direct, the infective stage is a larvated egg (containing L2) found in the environment. An indirect cycle via intermediate host is possible, as earthworms can ingest eggs and become infective (L2). Transmission is faecal-oral, the birds pass parasite eggs on faeces, and these develop in the environment and become infective by ingestion. Eggs hatch in the intestinal lumen, larval development occurs within the intestinal mucosa and they mature in the lumen. Prepatent period is 6-8 weeks.

Clinical importance is moderate, being more serious in young birds of 3-4 weeks old. It may cause mucous enteritis (during larvae development in mucosa) and intestinal obstruction with heavy burdens, due to the large size of the adult worm. Incubation period is 3-5 days. It is diagnosed by finding eggs on faecal flotation or adult worms in small intestine at necropsy (Atkinson *et al.*, 2009; Foreyt, 2001; Gibbons, 2010; Kassai, 1999).

- *Heterakis gallinarum* (caecal worm)

The size of adults is: females 10-15 mm, males 7-13 mm long. Adults are present in caeca. The size of eggs is 66-79 x 41-48 µm, elongated shape with a thick and smooth shell. They are similar in size and morphology to *Ascaridia galli* eggs and therefore not easily differentiated.

The life cycle is direct (larvated egg with L2) or indirect via paratenic host (an earthworm with encysted L2) with faecal-oral transmission. Prepatent period is 4-5 weeks.

Clinical importance is mild, although heavy infections can cause marked alteration of the intestinal wall and reduce egg production in laying hens. In addition, *H. gallinarum* eggs/larvae act as vector of the protozoan parasite *Histomonas meleagridis*, which causes ‘blackhead’ disease in turkeys. It is diagnosed by finding eggs on faecal flotation or adult worms in caeca at necropsy (Atkinson *et al.*, 2009; Foreyt, 2001; Gibbons, 2010; Kassai, 1999).

ORDER ENOPLIDA

- *Capillaria spp.* (hairworm)

Several species can infect chickens. Eggs are lemon-shaped with bipolar plugs, with some morphological differences depending on species (Wakelin, 1965) (*Table 1.4*). Some have direct life cycle (larvated egg with L2), other species indirect cycle (earthworm as intermediate host, L2). Transmission is faecal-oral and prepatent period is 3-4 weeks. It can cause severe inflammation of digestive tract and heavy infections can be fatal. Diagnosis is based in the identification of eggs on faecal flotation, or the finding of adult worms in the crop lining or small intestine at necropsy (Urquhart *et al*., 1996; Zajac and Conboy, 2012).

	Crop capillariosis		Intestinal capilllariosis			Caecal capillariosis
	C. annulata	*C. contorta*	*C. bursata*	*C. caudinflata*	*C. obsignata*	*C. anatis*
Parasite location (adult)	Mucosa of crop and oesophagus		Small intestine			Caeca
Adult size (length)	F: 25-60 mm M: 18-26 mm	F: 15-60 mm M: 8-17 mm	F: 16-35 mm M: 11-20 mm	F: 12-25 mm M: 9-18 mm	F: 10-18 mm M: 7-13 mm	F: 11-28 mm M: 8-15 mm
Egg appearance	Irregular-shaped Operculated ends		Long-shaped Fine longitudinal ridges and a collar formed by a curl of the inner layer at the poles	Thin-bodied Thick outer shell layer Inner shell layer reflexed at the poles	Round-bodied with a reticulate surface Inner shell layer forming a small collar at the poles but not flexed	Long-shaped Thick, coarse outer shell layer Wide collar formed by a curl of the inner layer at the poles
Egg size (µm)	70-100 x 43-46		51-62 x 22-24	47-58 x 20-24	44-56 x 22-29	46-67 x 22-29
Disease characteristics	Highly pathogenic species, which can cause mortality in heavy infections. These are characterized by tissue wall thickening, catharral inflammation and sloughing off the mucosa.		It can affect birds of all ages, causing diarrhoea and emaciation with signs of inflammation and thickening of intestinal walls.			

Table 1.4. Characteristics of *Capillaria* species infecting chickens. Adapted from Urquhart *et al*. (1996), Wakelin (1965) and Zajac and Conboy (2012).

ORDER STRONGYLIDA

Syngamus trachea (gapeworm)

The size of adults is: females 5-20 mm, males 2-6 mm long. Adults are present in trachea and bronchi. The size of eggs is 77-94 x 43-55 µm, ellipsoidal with a thick operculum on each pole.

Life cycle is direct; the infective stage is a larvated egg (L3) or free L3 found in the environment, or indirect via ingestion of paratenic hosts (earthworms, slugs, snails and flies, encysted L3). Transmission is faecal-oral and prepatent period is 2 weeks. Female worms in trachea are coughed up and swallowed, once in the small intestine they produce eggs and are passed in faeces; birds ingest L3, which migrates to the upper airways and develops to L5, located in trachea.

Clinical importance is moderate to severe as adults found in trachea can cause obstruction of the airways due to number of parasites and exhudate produced. Signs of gasping, shaking of the head, emaciation and suffocation are commonly seen, and young birds more severely affected.

Diagnosis is based in the identification of eggs on faecal flotation or the finding adult worms in trachea and bronchi, usually in copulation with the female and male parasites joined in pair with a characteristic Y-shaped appearance (Atkinson *et al.*, 2009; Foreyt, 2001; Gibbons, 2010; Kassai, 1999).

Trichostrongylus tenuis

The size of adults is: females 6.5-11 mm, males 5.5-9 mm long. Adults are present in caecum and intestines. The size of eggs is 65-75 x 35-42 µm, thin-shelled strongylid-type eggs, with dissimilar poles. Life cycle is direct, the infective stage is a free L3 found in the environment.

Clinical importance is severe, as heavy infections cause enteritis with diaorrhea, weight loss and anaemia. This species also affects severely grouse and geese. Diagnosis is based in the identification of eggs on faecal flotation; adults may be found in small intestine at necropsy (Atkinson *et al.*, 2009; Kaufmann, 1996).

CESTODES - Phylum Platyhelminthes

Common parasites in free range systems but rarely found in confined birds, as their life cycle requires an intermediate host found in the environment. Cestode proglottids are hermaphroditic and are passed in faeces. Diagnosis is usually made at necropsy, but eggs and gravid proglottids can be detected by faecal flotation and concentration techniques (Zajac and Conboy, 2012).

ORDER CYCLOPHYLLIDEA

Davainea proglottina

Small tapeworm of 1.5-4 mm long, eggs are round with embrional hooks and measure approx. 55 x 36 µm. Adults are present in duodenum. It has an indirect life cycle with a gastropod mollusk as intermediate host.

This species is highly pathogenic: worms are found in high numbers in the duodenum and can penetrate deeply in the tissue, causing necrosis and haemorragic enteritis (Urquhart *et al*., 1996).

Choanotaenia infundibulum

The size of adults is 23-25 cm long, eggs measure approx. 54 x 47 µm. Adults are present in the small intestine. The life cycle is indirect via ingestion of intermediate hosts (beetles and flies). Clinical importance is moderate (Atkinson *et al.*, 2009; Foreyt, 2001; Urquhart *et al*., 1996).

Raillietina spp. (R. cesticillus, R. echinobothrida and R. tetragona)

The size of adults can go up to 25 cm long, eggs are identical in size (approx. 93 x 74 µm). Adults are present in various locations of the intestine. The life cycle is indirect via ingestion of intermediate hosts (ants, beetles and flies), which carry the infective stage cysticercoid. Clinical importance is moderate to severe, being *R. echinobothrida* a serious pathogen (Urquhart *et al*., 1996; Zajac and Conboy, 2012).

TREMATODES - Phylum Platyhelminthes

ORDER ECHINOSTOMIDA

Echinostoma spp (avian fluke)

The size of eggs is 97-126 x 60-70 µm. Adults are found in caecum and rectum. It has a complex indirect life cycle involving 2 intermediate hosts: a mollusc and other water snail or frog tadpoles. The pathogenicity of this species is low and the infection may cause enteritis (Zajac and Conboy, 2012).

1.3.2 PREVALENCE, PREVENTION AND CONTROL

Low rates of parasitic infection may not cause major effects in the flock but pose a risk, as some parasites can act as vectors for other pathogens, i.e. *Histomonas meleagridis* carried by *Heterakis gallinarum*, or increase the susceptibility to other infections facilitated by immunosuppresion, i.e. co-infection of *Ascaridia galli* and *Pasteurella multocida* (Dahl *et al.*, 2002). High infection rates cause direct damage in the bird's health and poor performance (reduced bird growth and laying rate). In addition, even if it is not a factor considered as a criterion in breeding, there are differences with regard to parasite resistance between laying hen's breeds. Observations using commercial breeds show that white-laying hens are more susceptible to ascarids than brown-laying hens (Permin and Ranvig, 2001; Gauly *et al.*, 2002; Benett *et al.*, 2011b).

Although there is a lack of scientific data, practical experience in UK organic systems do not suggest that poultry endoparasites are a major problem (Van de Weerd *et al*., 2009). In contrast, some studies of nematode prevalence across farms carried out in countries like Denmark (Permin *et al*., 1999), the

Nederlands (Iepema *et al.*, 2006) and Sweden (Jansson *et al.,* 2010), revealed this as an emerging problem in organic and free-range farms; high prevalence of nematodes *(A. galli*, *H. gallinarum* and *Capillaria spp.*) with higher egg counts at peak of laying were found. A survey by Pennycott and Steel (2001) in 27 free-range farms across England and Wales showed similar results in parasite prevalence, but noticed a large faecal egg count variation between flocks. Nonetheless, significance of parasitism for production losses, disease and animal welfare implications are still to be assessed in EU countries (Van de Weerd *et al.*, 2009).

Organic farming standards restrict the use of drugs and other synthetic chemicals in organic production, such as anti-coccidials, parasiticides, antibiotics and pest control products/biocides (García, 2003). When veterinary medicines other than complementary therapies or trace elements are used, an established withdrawal period must be respected in order to produce or sell products destined for human consumption, which in organic systems is at least double the time than the legal period (Soil Association, 2014). Only flubendazole is registered for use in most EU countries, being effective for roundworms but not for tapeworms. Due to the extensive use of this drug, an increased parasite resistance has been identified and residues in edible eggs are a current issue for human health; this denotes that prevention should rely more in management than chemotherapy in organic farming (Maurer *et al.,* 2007). Some factors contributing to parasite drug resistance are related to the genetics and biology of the parasite: if there is a resistant mutant population it can become predominant even in the presence of a drug, and this can happen rapidly depending on the multiplication of the parasite. Using a drug too frequently, for a long period of time or under-dosing are also management factors involved in the development of drug resistance (Agrinews, 2015).

In this context, an integrated approach combining different strategies is applied with the aim of controlling the parasite burden in the environment, as their elimination is not intended due to their close connection with the natural system through the exogenous phase of the life cycle (García, 2003).

1.3.3 PROTOZOA - PHYLUM APICOMPLEXA

❖ *Crystosporidium spp*

Relevant species to fowls are: *Cryptosporidium baileyi* (chickens) and *C. meleagridis* (turkey and fowls). *Cryptosporidium spp.* have affinity for epithelial cells from a variety of organs, so the infection can reach different locations. It causes respiratory and intestinal disease, with the symptoms: diarrhoea, dehydration, anorexia, dyspnoea, coughing, airsacculitis, etc. Infection by *C. meleagridis* is clinically more severe in turkey and quail (de Graaf *et al*., 1999)

The parasite life cycle has asexual and sexual stages. A particular characteristic is that intracellular stages are confined to the microvilli (apical part) of the host epithelial cells, thus there is not so much disruption of the cell as seen in other coccidia infections. Oocysts are small (4-6 µm) and two types are produced: thick-walled, passed on faeces, and thin-walled, which sporulate in the cell and are able to auto-infect the adjacent tissues after releasing the sporozoits. This feature makes the infection not self-limiting. The prepatent period is short (4-7 days). Oocysts are very resistant in the environment (2 to 6 months) and not susceptible to all anti-coccidial drugs used in poultry (de Graaf *et al*., 1999: Sréter and Varga, 2000).

Diagnosis can be done by identification of oocysts in faeces using saturated sugar flotation or concentration techniques, acid-fast staining of faecal smears and examination of tissue scrapings (Atkinson *et al.*, 2009; Casemore *et al.*, 1985).

❖ *Eimeria spp.*

Economic importance

Eimeria parasites infect the intestinal tract of poultry, causing the disease coccidiosis, which is characterised by enteritis and bloody diarrhoea. The

global economic impact of coccidiosis is estimated in $3 billion USD (£1.8 billion) per annum, counting production losses, costs of prevention and treatment (Dalloul and Lillehoj, 2006; Williams, 1999).

In the UK, anti-coccidial drugs used for the control of coccidiosis represent more than the 40% of all antimicrobials used in animals (277 tonnes of active ingredient in 2011; mostly for control of *Eimeria* parasites), being ionophore antibiotics more than 70% of these (VMD, 2012).

Morbidity and mortality

Eimeria is a highly prevalent parasite, known to be ubiquitous wherever chickens are kept (McDougal and Reid, 1997). High morbidity and mortality is encountered when uncontrolled outbreaks occur, but subclinical disease is more common and considered to be relevant economically, as it causes reduced egg production and poor feed conversion (Blake and Tomley, 2014).

In broiler flocks, the species with higher incidence are *Eimeria acervulina* and *E. maxima*, followed by *E. tenella* and *E. mivati*, and the less likely to be encountered are *E. mitis*, *E. brunetti* and *E. necatrix* (Conway and McKenzie, 2007).

Species, pathogenicity, clinical signs and diagnosis

The *Eimeria* parasite (Phylum Apicomplexa, Genus *Eimeria* containing at least 120 species) causes coccidiosis disease in chickens but also infects other farm animals (cattle, sheep, rabbits, fish) and other animal species (cats, dogs, seals and others) and it is highly host-specific. Parasites belonging to the Apicomplexa phyllum include species relevant to farm animals such as *Cryptosporidium, Sarcocystis, Neospora, Toxoplasma, Besnoitia, Babesia* and *Theileria* (Muller and Hemphill, 2013). *Eimeria* species

are more closely related to *Toxoplasma, Neospora* and *Isospora* than to *Babesia*, *Plasmodium* and *Theileria* (Beck *et al*., 2009).

There are seven species of *Eimeria* that infect chickens, each of them with particular affinity for a location in the intestine and different pathogenicity characteristics (*Tables 1.5, 1.6a and 1.6b*). Two additional species are under review: *E. hagani* has not been further described after Levine (1938), and *E. mivati* is being evaluated to confirm its validity as a separate species from *E. mitis* (Conway and McKenzie, 2007).

Table 1.5. Characteristics of *Eimeria* species infecting chickens (adapted from Witcombe and Smith, 2014).

Species	Site of development	Pathogenicity	Immunogenicity
E. acervulina	Duodenum, ileum	Low to moderate	Moderate
E. brunetti	Ileum, low intestine, rectum	Moderate to high	High
E. maxima	Jejunum, ileum	Moderate to high	High to very high
E. mitis	Jejunum, Ileum	Low	Moderate
E. necatrix	Jejunum, ileum, caeca	High to very high	Low
E. praecox	Duodenum, jejunum	Low	Moderate
E. tenella	Caeca	High to very high	Low

Table 1.6a. Characteristics of *Eimeria* species infecting chickens (Conway and McKenzie, 2007)

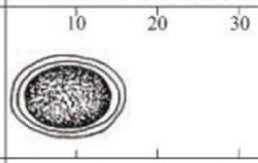
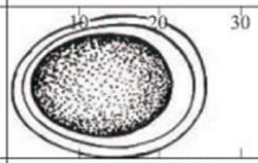
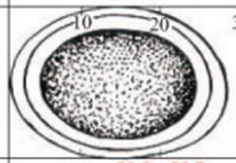
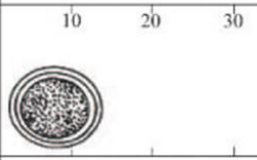
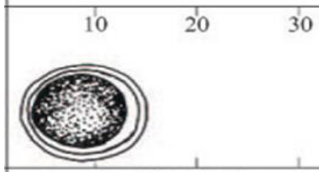
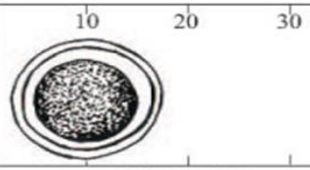
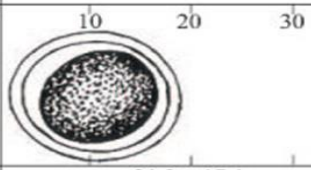
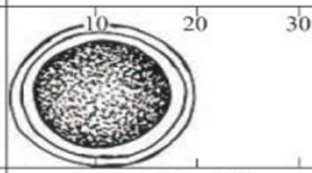
	CHARACTERISTICS	*E. acervulina*	*E. brunetti*	*E. maxima*	*E. mivati*
MACROSCOPIC LESIONS	ZONE PARASITIZED				
	MACROSCOPIC LESIONS	Light infection: whitish round lesions sometimes in ladder-like streaks. Heavy infection: plaques coalescing; thickened walls	Coagulation necrosis mucoid, bloody enteritis in lower intestine	Thickened walls, mucoid, blood-tinged exudate, petechiae	Light infection: rounded plaques of oocysts. Heavy infection: thickened walls coalescing plaques
MICROSCOPIC CHARACTERISTICS	MILLIMICRONS OOCYSTS REDRAWN FROM ORGINALS				
	LENGTH x WIDTH µm LENGTH = WIDTH =	AV = 18.3 x 14.6 17.7 - 20.2 13.7 - 16.3	24.6 x 18.8 20.7 - 30.3 18.1 - 24.2	30.5 x 20.7 21.5 - 42.5 16.5 - 29.8	15.6 x 13.4 11.1 - 19.9 10.5 - 16.2
	OOCYST SHAPE AND INDEX - LENGTH/WIDTH	ovoid 1.25	ovoid 1.31	ovoid 1.47	ellipsoid to broadly ovoid 1.16
	SCHIZONT, MAX IN MICRONS	10.3	30.0	9.4	17.3
	PARASITE LOCATION IN TISSUE SECTIONS	epithelial	2nd generation schizonts subepithelial	gametocytes subepithelial	epithelial
LIFE HISTORY CHARACTERISTICS	MINIMUM PREPATENT PERIOD-HR	97	120	121	93
	SPORULATION TIME MINIMUM (HR)	17	18	30	12

Table 1.6b. Characteristics of *Eimeria* species infecting chickens (Conway and McKenzie, 2007)

E. mitis	*E. necatrix*	*E. praecox*	*E. tenella*
	large schizonts, no oocysts 		
No discrete lesion in intestine Mucoid exudate	Ballooning, white spot (schizonts), petechiae, mucoid blood – filled exudate	No lesions, mucoid exudate	Onset: hemorrhage into lumen Later: thickening, whitish mucosa, cores clotted blood
			
15.6 x 14.2 11.7 - 18.7 11.0 - 18.0	20.4 x 17.2 13.2 - 22.7 11.3 - 18.3	21.3 x 17.1 19.8 - 24.7 15.7 - 19.8	22.0 x 19.0 19.5 - 26.0 16.5 - 22.8
subspherical 1.09	oblong ovoid 1.19	ovoid 1.24	ovoid 1.16
15.1	65.9	20	54.0
epithelial	2nd generation schizonts subepithelial	epithelial	2nd generation schizonts subepithelial
93	138	83	115
15	18	12	18

All *Eimeria* species are pathogenic, although to a different degree. It is common to encounter mortality with infections by *E. tenella* and *E. necatrix*, due to the extensive damage they cause to the intestinal epithelium. Other *Eimeria* species like *E. acervulina*, *E. maxima*, *E. brunetti*, *E. praecox* and *E. mitis* affect the birds as a subclinical disease, causing reduction in weight gain, malabsorption of nutrients and favouring secondary bacterial infections (e.g. by *Clostridium perfringens* or *Salmonella enterica* serovars *Typhimurium* and *Enteritidis*). These *Eimeria* species reproduce more abundantly within the intestine than the more pathogenic ones (McDougal, 1998).

Al-Sheikhly and Al-Saieg (1980) suggested that birds affected with coccidiosis are more likely to suffer necrotic enteritis by *Clostridium perfringens*. It seems that the mucosal damage caused by the parasite replication is needed for the bacteria to colonise and multiply, thus worsening the lesions and exacerbating mortality within the flock.

Clinical signs can be linked to specific stages of parasite development and include mucus to bloody diarrhoea, dehydration, loss of appetite and listlessness; birds can appear huddling with ruffled feathers and paler combs, wattles and shanks (Fanatico, 2006). The severity of the intestinal lesions depends on the exposure to the parasite, measured by the number of oocysts ingested by the host (McDougal, 1998) and will also be influenced by the degree of re-infection. There is nonetheless a non-linear relationship between the oocyst dose and the degree of lesions in the intestine (Conway and McKenzie, 2007).

The lesion scoring method, described by Johnson and Reid (1970), provides a numerical ranking of the gross lesions caused by each *Eimeria* species, with a full description of the different grades of pathogenicity (from 0, meaning no lesions to 4, meaning extremely severe damage). It is performed by macroscopic examination of the whole intestine divided in four areas, screening both serosal and mucosal surfaces, and confirmed by microscopic examination of tissue scrapings. It is not recommended to attempt species identification in a mixed species infection, although presumptive diagnostics can be made with the information given by the intestinal regions affected and

the presence of pathognomonic signs (Johnson and Reid, 1970). This method has been widely used as a tool for diagnostics, as a standardised practice in coccidiosis research (as found in Eckert *et al.,* 1995) and as a regular procedure for the evaluation of the efficacy of anti-coccidial drugs (Holdsworth *et al*., 2004; FDA, 2012; Chapman, 1998).

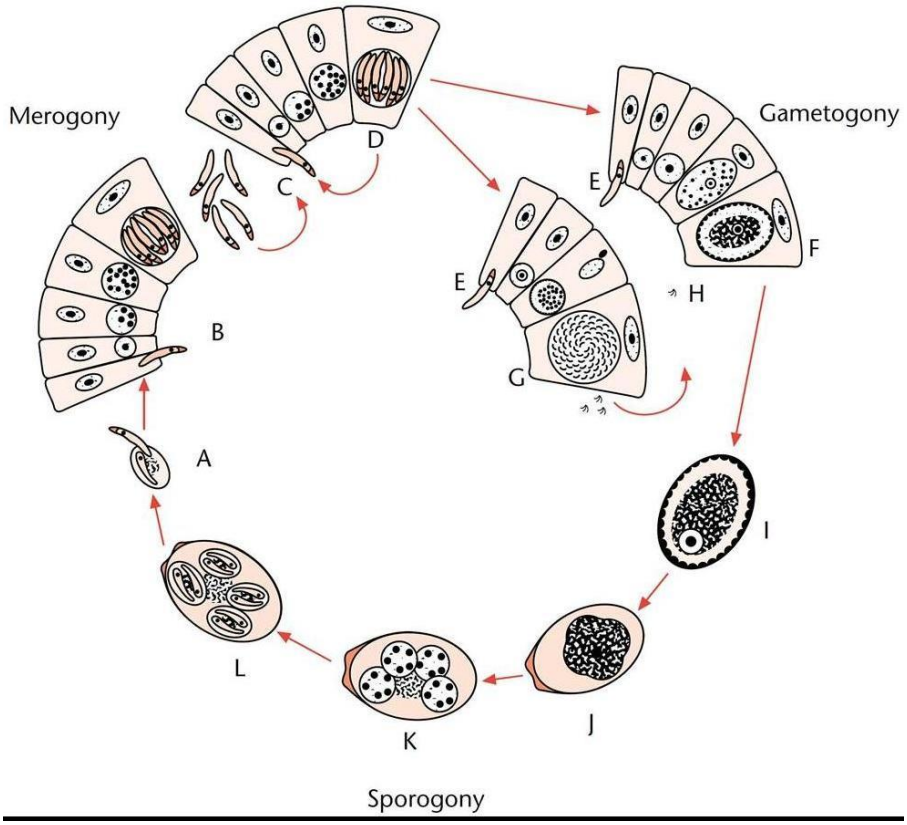


Figure 1.9. *Eimeria* life cycle. Sporozoites (A) excyst in the intestinal tract of the chicken and invade epithelial cells to for schizonts (B); these continue multiplying in the merogony stage (C, D), forming merozoits. After a genetically pre-determined number of replication cycles, merozoites undergo gametogony (E), producing macrogametocytes (G) and microgametocytes (F) inside the intestinal cells. Microgametes exit the cell and fertilise a macrogamete, forming a zygote (H). The oocyst formation happens within the host cell. Oocysts are released into the lumen and shed in the faeces (I). Sporogony occurs in the environment to produce a sporulated, infective oocyst (I-L) (Barta, 2001).

Life cycle and epidemiology

Eimeria is an obligate intracellular protozoan parasite, which follows a direct life cycle with faecal-oral transmission (*Figure 1.9*). Birds ingest sporulated oocysts from the environment, containing the parasite infective stages: sporozoites.

An oocyst has 4 sporocysts with 2 sporozoites each, making a total of 8 sporozoites. Once sporozoites are released by disruption in the intestinal track of the chicken through the still unclear mechanism of excystation (Belli *et al.,* 2006) they invade epithelial cells to start asexual replication, called schizogony and forming schizonts. The motile parasite stages are able to reach other cells and continue multiplying, stage called merogony forming merozoits (haploid cells) which undergo several generations of clone replication; this stage is self-limited (and usually stops before causing death of host), and genetically pre-determined depending on the *Eimeria* species (in most species is two to four generations). Afterwards, sexual replication starts as the merozoites transform into haploid non-motile macrogametocytes and motile (flagelated) microgametocytes inside the intestinal cells, stage called gametogony. The microgametes exit the cell and find a macrogamete to fertilise, forming a zygote. Zygots are diploid cells and will undergo meiosis and mitosis (to produce sporozoits, through the sporulation process). Fertilisation is a step necessary to form a viable oocyst, although little is known about the mechanism itself (Walker *et al.,* 2013). The oocyst formation happens within the host cell within a parasitophorous vacuole through the development of outer and inner oocyst walls. Oocysts are released into the lumen by rupturing the intestinal cells and are passed on faeces to the environment, where the exogenous phase of the life cycle starts.

Unsporulated oocysts become infective in the environment within 48 h, in the appropriate conditions of warmth, humidity and oxygenation. They can remain infective for a long time (from weeks to months) depending on the environmental conditions and are easily spread by animals, dust and through walking shoes, vehicles and other equipment. However, some conditions affect oocysts negatively, like high or freezing temperatures, desiccation and

ammonia, released by decomposition of litter and manure in chicken houses (Fanatico, 2006).

The oocyst wall is a bi-layered structure result of coalescence of veil- and wall-forming bodies (WFB) formed of 90% protein, dependant on carbohydrates for its construction and reinforced by protein-tyrosin crosslinks (these also implicated in the formation of the egg shell of trematodes and the cuticle of insects and nematodes, according to Belli *et al.,* 2006.). The same authors revise the nature of the oocyst wall, stating that it is a robust structure prepared to protect from mechanical and chemical damage; these would include many common disinfectants and some reagents used in laboratory such as sodium hypochlorite, potassium dichromate and sulphuric acid. Its components are produced and stored during macrogamete development, thus the macrogamete is considered the molecular storehouse of the next generation of parasites (Walker *et al.,* 2013).

Infection dynamics and immunology

The poultry production system has an influence in the infection dynamics of coccidiosis. In broiler chickens raised on floor and generally kept for five to six weeks, infection follows a pattern from low litter oocyst counts in their first weeks of life to peak excretion of oocysts around four to five weeks of life, decreasing afterwards towards the end of the raising period (Conway and McKenzie, 2007). Nevertheless, timing and magnitude of infection can be also influenced by a number of factors, including the use of a drug programme (Chapman, 1999).

Outbreaks of coccidiosis are rare in caged hens because the faecal-oral transmission of the parasite is stopped, as bird-to-bird contact and exposure to faecal material are reduced (McDougal and Reid, 1997). However, these production systems for layers were banned in Europe in 2012 and nowadays loose-housed layers are encouraged. Lunden *et al.* (2000) claimed that for hens housed on floor, the exposure to coccidia during the rearing period would condition their subsequent resistance to the infection. The time when

pullets are moved from rearing site to production site is particularly delicate, as the hens can potentially get in contact with residual contamination from the preceding flock (and can include different *Eimeria* species from the rearing site) and stress levels can rise because of transportation and diet change. In their study, clinical coccidiosis only involved birds younger than 24 weeks and oocyst excretion followed a pattern similar to broilers, with a peak between four and eight weeks after arrival at the production site (Conway and McKenzie, 2007).

In the case of *Eimeria*, natural immunity development will depend in the level of exposure to the parasites and the species. There is great antigen variability between species and immunity is species-specific (del Cacho *et al*., 2010). This means that the host develops immunity to homologous secondary infections, which does not prevent sporozoite invasion of intestinal cells but impede their development (Allen and Fetterer, 2002). This is particularly important for birds kept producing for longer periods (i.e. laying hens, slow growing- free range chickens), as *Eimeria* generally affects young birds but outbreaks can occur any time if birds have never before exposed to certain parasite species, combined with the fact that fluctuations in immunity can occur (Fanatico, 2006; Lunden *et al*., 2000).

The asexual replication stages are likely to be more important than the sexual stages in the induction of a protective immune response of the host (Rose and Hesketh, 1976). It is known that the immunogenicity varies between species, being considered *E. maxima* the most immunogenic and *E. necatrix* and *E. tenella* the least (Rose, 1976) (*Table 1.5*).

The humoral immune response based in antibody production is promoted from early stages of infection, and their longevity is around three months (Wallach, 2010). The contribution of antibodies against the parasite is not clear on its efficacy, while IgG and IgA are able to reduce parasite multiplication and IgG has proven to be important for the protection of intestinal mucosal surfaces (del Cacho *et al.,* 1998); this supports the idea of antibodies having some defensive role in the *Eimeria* infection.

The cell-mediated immune response plays a primary role in the protection against the infection, as there is a high cytotoxic activity of immunocompetent cells linked to the presence of *Eimeria* antibodies. Citotoxicity mediated by antibodies is a strategy for vaccine development. However, cytotoxic T-cells, which reside in the gut-associated lymphoid tissue, need continuous re-stimulation by parasitic antigens to keep immune memory, otherwise their population decreases in absence of stimulation (del Cacho *et al.,* 1998).

According to Chapman (1999), broilers acquire protective immunity gradually but it is not complete until birds are seven weeks of age, regardless of the drug programme used or house litter management. Their results support previous observations of Rose and Long (1962), who claimed that the development of immunity to *E. maxima* appeared to be quicker than to other *Eimeria* species.

1.3.4 METHODS FOR THE CONTROL OF AVIAN COCCIDIOSIS

Coccidiosis is most important parasitic disease and one of the main problems in conventional chicken farms. The highly resistant coccidia oocysts are likely to be present in the environment and animals' stocking density favours parasite transmission and re-infection (Witcombe and Smith, 2013). Farm management procedures are essential for the control of coccidiosis infection, although not sufficient if not supported by other measures, especially under intensive rearing conditions in commercial farms (Quiroz-Castañeda and Dantán-González, 2015). These procedures include good animal husbandry, cleaning and disinfection of housing and materials, carrying out an adequate sanitation period following the "all in-all out" principle in enclosed farm facilities (Fanatico, 2006).

Chemotherapy has been used as a strategy for coccidiosis control in the poultry industry. Anti-coccidial drugs have been widespread used in the poultry industry since 1950s as a method of prophylaxis to prevent coccidiosis

outbreaks, with many products being released to the market. These drugs are given at doses to allow parasite replication at a low level, in order to stimulate the host immune system (Lunden *et al*., 2000). They can be classified into synthetic compounds (or 'chemicals') and ionophorus antimicrobials (from bacterial fermentation), and can have a coccidiostatic or coccidiocidal effect. They work in a different way, being both able to arrest the development of *Eimeria* parasitic stages but they do not completely prevent parasite multiplication, allowing a gradual development of immunity (Chapman, 1999; Quiroz-Castañeda and Dantán-González, 2015). In the field, they are used following different strategies depending on the incidence of coccidiosis in farm and the characteristics of the production system.

As mentioned before, organic farming standards restrict the use of drugs and other synthetic chemicals in organic production, therefore the application of these products is not allowed. Only under professional veterinary advice, and if no other effective treatments are available, their application can be considered as part of a disease control programme. This must be agreed with the Soil Association in the animal health plan of the organic farm and the corresponding withdrawal period must be respected (Soil Association, 2014). From 2018, if an allopathic veterinary treatment has been applied, the producer must wait twice the legal withdrawal period as referred to in Article 11 of Directive 2001/82/EC, before selling the product as organic (Soil Association, 2018); this is fourteen days for eggs and fifty-six days for meat.

The identified limitation of anti-coccidial drugs is the emergence of drug-resistant strains after a drug has been used for prolonged periods, which are able to multiply or survive in the presence of drug concentrations that normally destroy or inhibit multiplication of the same parasite species (Chapman, 1997)

Shuttle and rotation programmes are employed to combat resistance, replacing single-drug use (McDougal, 2003). Briefly, a shuttle programme employs two different anti-coccidials during the raising period of the chickens: a synthetic anti-coccidial drug ('chemical') and an ionophorus antimicrobial, switching from one to the other at a mid point. Rotation programmes use one

drug at a time during the whole raising period, either a chemical or an ionophorus antimicrobial, and change to another one within the same type (rotate) after some time (Agrinews, 2015). Examples of chemicals are nicarbazin diclazuril and robenidin; some ionophorus antimicrobials are monensin, salinomicin and lasalocid. In order to select a programme for a poultry farm, it is advisable to consult with an experienced animal scientist who could assess the current situation looking at the type of farm and location, risk of *Eimeria* infection and parasite species usually found in the area (Barragán, 2006). The anti-coccidial drugs come as medicated feed and are offered to the animals by the farmer.

Chapman (1999) exposed that there has not been observed a decline in the use of anti-coccidial drugs in animal feed, even if most drugs are not longer as effective as they were. However, the development of new anti-coccidial drugs has been discouraged for the reasons of cost, legislative restrictions and consumer awareness of chemical residues in food products, combined with the fact that resistance to drugs can appear quickly (Blake and Tomley, 2014).

Vaccination has become an alternative approach for the control of coccidiosis. There has been a growing interest on developing vaccines because immunological control is recognized as the only practical alternative to anti-coccidial drugs in large-scale production (Chapman *et al.,* 2002).

Vaccines are composed of attenuated or non-attenuated live oocysts. They have proven to give similar level of protection for broilers as anti-coccidial programmes do, and, in addition, vaccinating birds with live vaccines allows re-seeding the chicken house with coccidia sensitive to drug use (Conway and McKenzie, 2007). This is possible because coccidia strains in vaccines are susceptible to drugs and so are able to transfer drug susceptibility to drug resistant strains present in the farms.

Producers have used the combination of chemotherapy and vaccination for years, as a strategy to restore drug sensitivity or replace the field strains with drug-sensitive strains. This may have helped to slow down the development of resistance to drugs (Witcombe and Smith, 2013).

Some factors have to be considered when using vaccines, like production and purchase costs, degree of pathogenicity and effectivity of the vaccine (ability to induce protective immunity). Vaccination provides the birds an initial exposure to sporulated oocysts but these must be recycled in the faeces to produce a “trickle” infection, able to induce protective immunity through parasite cycling within the host. Animal stocking density and type of housing are important factors contributing to this “trickle” infection (Price and Barta, 2010).

1.4 RESEARCH TECHNIQUES FOR DIAGNOSTICS IN PARASITOLOGY

1.4.1 METHODS FOR QUANTITATIVE MICROSCOPIC ANALYSIS: FAECAL EGG COUNTS (FEC)

Faecal egg counts (FEC) are laboratory tests performed to determine the type and number of internal parasites present in faecal samples. Their use has been extended from sheep to other farm species including poultry (Soil Association, 2010).

The presence of parasite eggs, larvae or worm adults in faeces is a positive evidence of infection. FEC give information about the parasite burden of the flock, being a valuable tool for monitoring both individuals and groups of animals. For instance, they can help to check whether or not an anti-parasitic treatment is required, verify its effectiveness and monitor the possible development of drug resistance (by testing before and after treatment).
While FEC inform about the level of infection, i.e. a high egg count would suggest high number of adult parasites, there are a number of factors affecting the correlation between the counts and the real parasite burden (Friedhoff, 1978).

FEC are commonly used at a farm level to monitor the health status of populations. The application of these methods in scientific research enables getting qualitative and quantitative results with regard to parasitism, and remains a reliable tool for diagnostics and comparison with any other technique applied (Velkers *et al*., 2010; Zajac and Conboy, 2012)

There are several FEC methods, commonly fairly cheap and easy to perform. Briefly, fresh faeces are taken from a group of animals in airtight containers and are kept cool. Examination takes place ideally within 48 hours. A measured amount of faeces is put together with measured volume of flotation solution that allows the parasite eggs to float to the surface, and then this is mixed well and strained to eliminate debris (Permin, 1998; Soil Association, 2010).

If the McMaster counting method is used, the filtered solution is stirred again and two samples are drawn off with a pipette to fill both sides of the McMaster chamber. The slide is allowed to stand and examined under the low power microscope. Other methods put the filtered solution in a tube with a cover slip on top, and examine the cover slip where the eggs have stuck after some waiting time. Parasite eggs are counted and a multiplication factor is applied to the egg count result to give a final ‘eggs per gram’ (epg) (Permin, 1998; Soil Association, 2010).

1.4.2 DNA EXTRACTION

Genomic DNA extraction from a biological sample involves the steps showed in Figure 1.10:

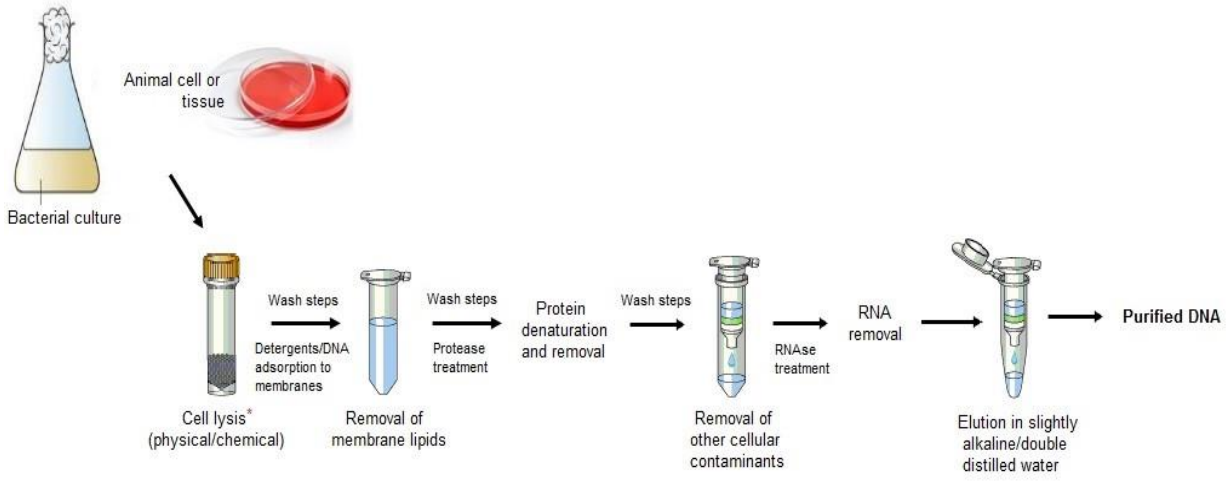


Figure 1.10. Basic steps involved in all DNA extraction methods. Adapted from Dhaliwal (2013) and Clontech Laboratories Inc. (2016).

Depending on the nature of the cell walls from the source, the method used should include an appropriate lysis step, i.e. vortexing or bead-beating for 1-2 minutes.

Selection of method for DNA extraction should consider the factors: sample origin as the source of DNA (e. g. soil, faeces, tissue, etc.), preparation or starting point for the sample to be processed (e. g. fresh, frozen, freeze-dried, fixed or preserved samples), moisture content, as water should be removed during the process and the percentage of water considered in the sample presentation, sample quantity and presentation (e. g. number of cells, mg of fresh/dried faeces, tissue, etc), intended use, as the yield, quality and purity of DNA should be suitable for the subsequent downstream application, automation and simplicity.

The basic criteria a method should meet are: efficient DNA extraction, sufficient amount of DNA obtained, removal of contaminants and quality and

purity of DNA assessed by UV absorbance (ratio A260/A280 > 1.8) (Dhaliwal, 2013).

The classic organic extraction method phenol-chloroform is a popular, very cheap and relatively easy to perform method. It gives very high DNA yields. However, some safety measures are required as it needs the use of a fume hood and generates hazardous substance waste. It may be relatively time consuming. In addition, the organic solvents used for extraction may damage the DNA and potential residues in the sample could affect downstream applications (Dhaliwal, 2013). Silica-based technology is widely used in current commercial kits. Silica is incorporated in spin columns and adsorbs DNA (dependant on pH and presence of certain salts). The sample is processed for cell lysis, protein digestion, DNA binding in the column, washing steps to remove contaminants and elution of pure DNA in a buffer. It is a simple, relatively fast method and suitable for automation, but more expensive (Dhaliwal, 2013).

1.4.3 DIAGNOSTIC BY POLYMERASE CHAIN REACTION (PCR) AND REAL-TIME QUANTITATIVE PCR (QPCR)

The research in molecular biology techniques for *Eimeria* parasites has been developed from the last decade, providing advanced tools for detection and diagnostic. These intend to overcome the limitations of traditional diagnostic techniques. Polymerase chain reaction and real-time quantitative PCR (qPCR) assays are examples of them.

PCR allows the selective amplification of DNA from complex genomes, being able to differentiate between species and strains depending on the target sequence contained in the DNA (Mullis, 1990). The technique principle is based on a denaturisation of double-strand genomic DNA by heating, primer annealing (hybridization) to complementary sequences in opposite strands of the DNA allowed by lower temperature, and finally an extension (synthesis) of the template from the primer sites with a thermostable DNA polymerase to

produce double-stranded products. This last step is usually repeated 20-40 times in an automated cycler, replicating the template by a factor of two in each cycle. As a result, millions of copies of the DNA target (amplicons) are available for subsequent analysis (Morris and Gasser, 2006). The DNA products can then be loaded on an agarose gel to allow migration of the amplicons depending on size (number of base pairs) by electrophoresis. The formation of bands of a desired size is considered proof of amplification and so detection of the target sequence in the original sample.

Quantitative PCR (qPCR) effects a selective amplification of DNA and real-time quantitation. It follows the PCR principle, with the extra addition of a fluorescent dye that binds to the synthesized DNA molecules and record of the fluorescence value during each cycle in the real-time qPCR cycler. The linear correlation between PCR product and fluorescence intensity is used to calculate the amount of template present at the beginning of the reaction. DNA-based probes used in the reaction are specific and sensitive but have high cost (Sigma-Aldrich Co., 2008).

A variety of methods and targets have been employed in the research of *Eimeria* parasites.

Random amplification of polymorphic DNA (RAPD fingerprint) is able to screen the whole genome and has been used to identify and study the variation between species. It is a simple and fast method, which does not require sequence specific primers, using arbitrary primers instead. However, low stringency conditions apply and hybridisation of nucleic acids with mismatched sequences can occur, affecting specificity and reproducibility (Morris and Gasser, 2006). In addition, it has been suggested that the technique may not be appropriate for the detection of mixed-species infections, as co-migrating bands on the agarose gel can contain multiple hidden sequences (Fernández *et al*., 2003).

Primers directed to conserved ribosomal DNA sequences (18S, 5.8S, 28S), flanking internal transcribed spacers of nuclear ribosomal DNA (either the ITS1 or ITS2 regions) have been used for species discrimination. These sequences represent a polymorphic region in the DNA and are present in

multiple copies per genome, fact that may affect sensitivity or risk false negatives as variation in the target sequence can impede primer binding. Morgan *et al.* (2009) used as target the ribosomal ITS2 sequence for qPCR, finding an unknown number of copies per genome, some degree of sequence heterogeneity and variability within the genome and between parasite strains and species. They concluded that the number of matching targets for each strain and species could not be predicted with this method.

Sequence-characterized amplified regions (SCAR markers), developed from RAPD, have been used for designing primers for species-specific amplification of *Eimeria* DNA. Produced bands are isolated, cloned and sequenced to get specific primers for which high stringency conditions apply. Highly conserved SCAR regions are strain or species-specific genetic markers. This method may be less sensitive because their lower abundance but more specific, as they are non-polymorphic markers present as a single-copy per genome (Blake *et al*., 2008). The proved null variability of the regions avoids false negative reactions and accurate relative quantitation, being a suited method when mixed strains or species are present because it allows discrimination between species and is able to characterize the strains present. In addition, other non-target pathogens do not affect the assay and it works regardless of the parasite lifecycle stage, covering the identification of both environmental and intracellular stages characterized by a different number of genomes (Blake *et al.*, 2008). Vrba *et al*. (2010) validated the limit of sensitivity to be below ten copies of the target sequence, what equals to a single oocyst (eight genome copies when fully sporulated); however, practical detection is also influenced by oocyst recovery, DNA extraction and its quality.

A multiplex PCR assay based on SCAR markers for the simultaneous detection of all seven *Eimeria* species was developed (Fernández *et al*., 2003) and a SCARdb database for researchers is available since then (http://www.coccidia.icb.usp.br/eimeriaScardb/index.jsp; previously http://puma.icb.usp.br/eimeriaScardb/; Fernández *et al.*, 2004).

It is known that genetic variability exists between strains of *E. maxima and E. acervulina* (to lesser extent). Quantitative PCR (qPCR) assays using the

selected SCAR markers were able to characterize all *Eimeria* species and strains present; *E. acervulina* marker was found to be polymorphic in this assay, therefore new candidate markers were designed (Vrba *et al*., 2010).

1.4.4 EVOLUTION IN DIAGNOSTICS OF COCCIDIOSIS

Diagnosis of *Eimeria spp*. infections still relies on the identification of oocysts in poultry faeces by optical microscopy and the examination of specific lesions found in different intestinal areas of the chicken. This is labour intensive, time consuming and need for trained personnel. In addition, there are some overlapping features observed between the different species that pose limitations of the traditional methods of diagnostic, a. e. the affected gut areas and the nature and extent of lesions, as well as the variability of oocyst size and shape (Morris and Gasser, 2006). In consequence, a species-specific diagnostic cannot be made based only in those criteria (Long and Joyner, 1984).

Real-time quantitative PCR is presented as the newest molecular technique and seems to be fast, robust and reliable, designed for getting results to make immediate decisions. Assays done were highly reproducible and correlated well with microscopic diagnosis (Blake *et al*., 2008). Compared to regular PCR, it does not need for gel electrophoresis but it requires technical input and this has higher cost due to the type of reagents needed. Some of its applications include coccidiosis research, live vaccine manufacture and veterinary practice, being possible to adapt to particular diagnostic needs and so reducing costs (Vrba *et al*., 2010).

An innovative method for the morphological identification of *Eimeria* species is COCCIMORPH, an online free-available software tool (www.coccidia.icb.usp.br/coccimorph; Castañón *et al*., 2007). It is a digital image processing system, which integrates oocyst features (size, shape, curvature, symmetry, internal structure characterization) from a large amount of sporulated oocyst images of all seven species of *Eimeria*. By uploading the

images to analyze, one can get an automatic classification (although accuracy is different depending on species). Despite the little information about the use of this tool in field samples and its limitations to compete with other diagnostic methods, it could be a useful, low cost screening tool for technicians in poultry farms (Chapman *et al*., 2013). In a study supplementing molecular identification of *Eimeria spp*. with COCCIMORPH, Kumar *et al.* (2014) found that some of the results agreed well with the PCR assays but the software failed to detect species in low occurrence. Thus, in practical field application it may need to be reinforced with other methods.

1.5 USE OF ADDITIVES AS SUPPLEMENTS IN POULTRY FEED

1.5.1 GENERAL PURPOSES OF ADDITIVES

The poultry production industry has nowadays become very technical, with the aim of increasing production rate at the smaller cost for the producer. Advances have happened thanks to the development of farming facilities and equipment, the improvement of management practices, health programmes and diet, and the advance on genetics for chicken breeds (Moura *et al*., 2006).

Diet formulation depends on the purpose of the production (meat or eggs) and it changes in composition and presentation according to the animal's age and physiological changes associated. With the objective of increasing weight gain in broilers and egg production and/or quality, diet has suffered reviews and modifications. Alteration of protein content, addition of organic acids and caloric restriction are examples of attempts to increase feed efficiency (Van der Klis, 2012). On the other hand, the use of antibiotics as growth promoters has been extensive but no longer possible in Europe after the EU ban in 1999 (Casewell *et al.,* 2003). These substances work controlling subclinical

disease, making nutrients more available and inducing metabolic effects in the animal by modifying the bacterial population of the intestine, both total number and type of bacteria present in the gut (Risley, 2005). It has been observed that feed additives used as growth promoters in poultry make lower improvement on performance compared with the use of antibiotics, and their effects can be inconstant and infrequently significant (Mallet *et al*., 2005). In any case, promoting animal growth is not the main goal of their application.

Thus, the modulation of the microbial population in the chicken gut has also been considered as a strategy. Products like prebiotics, based in non-digestible fibre compounds (carbohydrates), promote the growth of a selected group of bacteria by acting as a substrate for their feeding. Probiotics are used with the aim of increasing the intestinal population of beneficial bacteria (i.e. lactic acid producers *Bifidobacterium, Lactobacillus* and *Enterococcus*), which help to balance the presence of pathogenic bacteria by competing over nutrients and cell adhesion sites or producing toxic compounds (Fuller, 1989).

1.5.2 NEED FOR ALTERNATIVE APPROACHES TO CONTROL COCCIDIOSIS AND OTHER PARASITOSIS

The concern about the possibility of transferring to humans the resistance to antibiotics that bacteria affecting food-producing animals have developed, has increased throughout the last decades (Risley, 2005). With regard to coccidiosis, the extensive use of anti-coccidial drugs in the poultry industry has caused a generalized resistance to these drugs (Blake and Tomley, 2014). These facts together with and interest in food security issues have motivated legislative restrictions towards the use of antimicrobials and the implementation of alternative products and strategies.

New anti-coccidial alternatives are emerging and there are current research interests in natural products that could be used in the field to enhance poultry intestinal health and contribute to the control of pathogenic bacteria and

parasites. Many of these products are presented in the form of feed additives, which can potentially have a direct effect in commensal microflora or pathogen microorganisms and interact with the host physiology thanks to certain attributed properties, i.e. anti-inflammatory, anti-oxidant, immune stimulation, etc. (Quiroz-Castañeda and Dantán-González, 2015).

Additives like Chinese medicine herbal extracts (Wang *et al*., 1998), artemisinin (del Cacho *et al.,* 2010), other plant extracts (garlic, oregano, etc.) and essential oils such as artemisia, clove, tea tree and thyme (Remmal *et al.,* 2011) have been tested with positive results for the control of *Eimeria spp.* parasites. Moreover, some components present in these products have been identified and studied, finding qualities that make them more effective for certain *Eimeria spp*. infections. For instance, the anti-oxidant properties of γ-tocopherol (in corn, wheat and soybean) and curcumin (in turmeric) seem to work against infections by *E. acervulina* and *E. maxima* (Allen and Fetterer, 2002); artemisinin and n-3 fatty acids are effective against *E. tenella*, through mechanisms involving oxidative stress (Allen and Fetterer, 2002; Quiroz-Castañeda and Dantán-González, 2015).

1.5.3 DIATOMACEOUS EARTH (DE) AS A FEED ADDITIVE FOR POULTRY

CHARACTERISTICS AND APPLICATIONS OF DE

Diatomaceous earth (DE) is a natural soft siliceous sedimentary rock consisting in fossilized remains of diatoms, a type of microscopic single-celled and hard-shelled algae (*Figure 1.11*). There are different Diatom species with chemically the same silica shell (amorphous silicon dioxide, SiO_2) and physically an intricate perforated structure: hard, irregular and variable in shape, with holes that vary in number and size between the diatom species (typically sized from 10 to 200 µm) (Wang *et al*., 2012).

Diatoms were discovered in 1702 by Anton van Leeuwenhoek and in the nineteenth century were classified as plants because they perform photosynthesis. They are the most abundant type of phytoplankton on earth, able to grow with enough carbon dioxide, light, water and necessary nutrients, and mostly found in cold oceans all around the world, Diatomaceous earth deposits are made of fossilized exoskeletons of diatoms, which have sunk dead to the bottom of a river, lake or ocean bed and cumulated in layers over the years (Hoover, 1979) (*Figure 1.12*).

The micro structural characteristics of diatoms determine the quality of DE in terms of oil absorption, surface area and other properties. As a result, depending on the species predominance in diatomite deposits and also their age, the quality of the extracted DE will have some variation among suppliers depending on their source (NFF, 2015*a*).

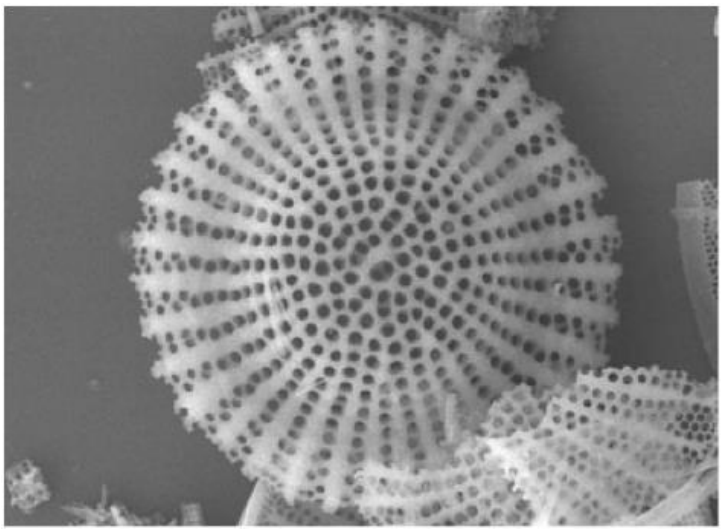


Figure 1.11. *Cyclostephanos spp*., the predominant diatom species in Diature™ DE. Source: Natural Feeds and Fertilisers (NFF) Ltd.

Presentation of DE is in fine powder with a chalky appearance. Food Chemical Codex Grade ('Food Grade') DE is considered non-toxic but must comply with regulations with regard to heavy metal content (arsenic and lead) and contain less than 1% crystalline silica, chemical compound that usually result from calcination (Absorbent Products, 2012).

Figure 1.12. Diatomite mine. They can be found in many places around the world, mainly in the United States, Europe and Africa. Source: Natural Feeds and Fertilisers (NFF) Ltd.

DE has a variety of uses in the industry including anti-caking agent, filter, and as a mild abrasive. More recently, it has been used in farming and agriculture for soil improvement and plant growth, as insecticide (for external parasites, crop pest control and grain storage), anti parasitic agent (wormer) and 'organic' feed additive for farm animal species including camelids to improve body condition and product quality. It has been claimed to help to maintain good hoof, skin and coat condition, fleece quality, improve weight gain and milk yield, reduce worm burdens in ruminants and prevent from scouring in young animals (NFF, 2015b).

In poultry, pigeons and caged birds it can be applied externally dusting the birds, in the dust baths or nest boxes for prevention of external parasites and feather quality. Sprinkled in the chicken house it helps to reduce moisture, odours and crawling insects such as darkling beetles and flies. The control of red mite (*Dermanyssus gallinae*) and northern fowl mite (*Ornithonyssus sylviarum)* is currently a big concern for laying hen farmers and bird keepers, with DE already commercialised and widely used for this purpose (Sparagano *et al.*, 2014).

POTENTIAL BENEFITS FROM THE USE OF DE IN FEED

Effect on bacteria and protozoa

Callus (2013) reported from an *in vitro* titration that DE showed an antimicrobial effect on *Campylobacter jejuni* colonies and also a dose-dependant protective effect for bacteria when dose of DE increased from 2% to 5%. There was a significant negative correlation between amount of DE used and the bacterial growth on plates (with the exception of DE at 5%). The commercial DE product provided by the company and used in both *in vitro* and *in vivo* trials contained different unknown microorganisms (bacteria and fungi).

In a study conducted by Wang *et al*. (2012), DE was used as a bacterial carrier for self-healing concrete, based on its properties for bacteria immobilization. Protective effect on bacteria was marked at a high pH and it improved with increased DE concentration. It was observed that DE particles had a strong capacity to adsorb bacterial cells on surface, providing a kind of microenvironment in which the local pH around the bacteria was not as high as that in the cement slurry; they could still decompose urea, activity that increased with higher DE concentrations.

DE used in physical filters have proven efficacy to remove protozoan oocysts from drinking water (enteric protozoa such as *Giardia*, *Entamoeba* and *Cryptosporidium*) (Schaefer *et al*., 2004). The efficiency of the process has been related to factors like the surface characteristics and size of the cyst, the amount of other particles in dissolution, the physical characteristics of the water, the DE pore grade and the filtration rate (Schaefer *et al*., 2004).

Silicon and micro-mineral absorption

Since Diature™ DE is composed of 91% amorphous silicon dioxide and contains several other micro minerals (as seen in Table 2.5), the absorption of these could have an impact on the animal's physiology.

Silicon plays and essential role in bone formation and maintenance, as it improves the bone matrix quality and facilitates bone mineralization. Supplementing silicon to animals and humans has shown to improve bone mineral density and bone strength, although the exact mechanism for this action has not been identified.
It is argued that silicon in the form of silicon dioxide is poorly soluble in water and has limited intestinal absorption, therefore poor biological availability (Price *et al*., 2013). However, water-soluble forms of silicon are more biologically available so more adequate as dietary sources.

Silicon is important as an initiator of mineralization because it is highly concentrated in the immature osteoid but declines as calcium content rises in mature bone (Carlisle, 1970b). The same author also reported in other studies that supplementation with silicon accelerates the rate of bone mineralization and identified an effect on collagen that makes the bone matrix more calcifiable (Carlisle, 1970a).

In studies using an ovariectomized rat as an animal model of postmenopausal osteoporosis, silicon reduced the bone resorption and increased bone formation (Hott *et al*., 1993). In addition, Kim *et al.* (2009) proved that silicon at physiological levels improved calcium incorporation in bone and by adding calcium to silicon supplementation the bone mineral density increased in rat models. Besides, estrogens increase the intestinal absorption of calcium and this may also influence the absorption of silicon. Estrogenic status may be important for silicon metabolism as Macdonald *et al*. (2005) suggested a synergic interaction between them.

Silicon supplementation has proved beneficial effects for the increase of bone mineral density and reduction of bone fragility through direct measurements of bone mass and strength in different animal models, including broiler chickens (Merkley and Miller, 1983) and quail (Sahin *et al*., 2006).

Desiccant effect

DE is used for the control of insect infestations in grain stores with a presumed mode of action by desiccation, acting either as abrasive for the outer protective layer of insects (exoskeleton) or by absorption of the lipidic cuticular wax, important to water retention (*Figure 1.13*). The assumption of the effectivity of DE as parasiticide for internal parasites is based on these properties (Cockrell, 1993; Osweiler and Carson, 1997).

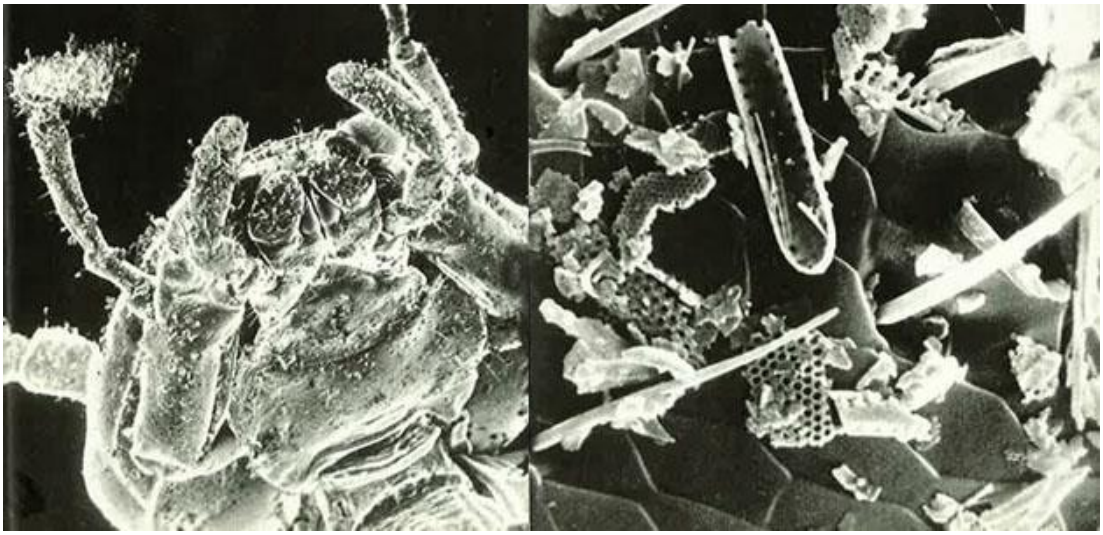


Figure 1.13. Food grade DE lacerating the rear part of a cockroach (a) and other insect (b), under high magnification. Source: Absorbent Products (2012), (pictures by Dean W. Blinn and J. Norman Grim).

The same mode of action seems to apply for chicken mites, which result in death by dehydration when DE is applied to the housing (Martin and Mullens, 2012). Efficacy appears to be greatly affected by the quality of the raw material used and reduced by high humidity levels (higher than 85%) in the poultry house (Sparagano *et al*., 2014). DE and kaolin applied in hen dust baths showed negative, immediate effects on chicken body lice (*Menacanthus stramineus*) and northern fowl mites (*Ornithonyssus sylviarum)* (Martin and Mullens, 2012).

RESEARCH FINDINGS

Dietary clay supplements in the form of phyllosilicates (such as bentonite, kaolinite and talc) can have a mechanical action on pathogens (binding) and effect on feed passage. They slow down the intestinal transit and adsorb nutritive elements, fact that could improve the absorption of nutrients in the intestinal tract and as a result achieving better chicken growth as reported by some authors (Quisenberry, 1968; Mallet *et al.,* 2005).

Dietary treatment with low-level addition of clay (2-5%) showed consistent significant results in increased hen body weight and egg size, and reduced percentage of moisture in droppings. Moreover, it was demonstrated an improved caloric efficiency resulting from their use although it remained unclear whether clay supplements made a nutritive contribution *per se* (Quisenberry, 1968). Mallet *et al.* (2005) studied the addition into broiler diet of talc, a hydrated magnesium silicate mineral. A beneficial effect of talc on body weight (BW) and food conversion rate (FCR) was found and results were similar to the obtained from administering avilamycin in feed. In addition, there was no difference in bacterial population counts between treatments. They concluded that clay products have proven no detrimental effects in broiler performance.

Within this context, diatomaceous earth has been considered as a product with a variety of potential applications in poultry farming.

Animal trials showed positive results regarding performance. For instance, Mathis and McDougald (1995) found improvement of feed conversion of broilers when adding DE to their diet. Bennet *et al.* (2011b) reported significantly higher egg production in hens fed DE for both different breeds, Lohmann Brown and Bovan Brown; the latter breed also showed an improvement in egg quality (larger eggs and heavier, thicker shells) under DE dietary treatment. In another study analyzing bone mineralization in hens under a 2% DE dietary treatment (birds aged from 18 weeks, lasting for 22

weeks), DE increased the density and mineralization of cortical structural bone (Bennett *et al*., 2011a).

Several *in vivo* trials involving the use of DE in grazing ruminants have been conducted in order to study its efficacy to control internal parasites, with unclear results (Bernard *et al*., 2009; Fernández *et al*., 1998; Osweiler and Carson, 1997).

Although abundant information about using DE for livestock and poultry is available from different sources such as press articles and specialised websites (McLean *et al.,* 2005), scientific studies involving the use of DE in poultry are limited and there is a lack of information about whether diatomaceous earth could prove efficacy to control protozoan and other internal parasites (Bennett *et al.,* 2011b). The same authors concluded in their study that the effect of DE on internal parasites of hens was not robust, because DE dietary treatment did not improve resistance in birds genetically more resistant to parasites, but it helped to lower parasite loads in Bovan Brown hens.

OBJECTIVES

The objectives of this study are summarized in the following points:

1. To perform a DNA extraction method comparison on chicken's freeze-dried and homogenised faecal samples.

2. To study the presence of the seven *Eimeria* species that infect the chicken in a flock of free-range organic laying hens of 54 weeks old, by using molecular identification methodology (PCR).

3. To investigate the effect of the inclusion of diatomaceous earth (DE) in the diet of free-range organic laying hens of 54 weeks on:

- ✓ The concentration of parasites commonly encountered in free-range hens, such as nematode species (*Ascaridia galli*, *Capillaria spp.* and *Heterakis gallinarum)*, and protozoa species (*Eimeria spp.)* in faecal samples.
- ✓ The concentration of *Eimeria acervulina* in faecal samples, by using quantitative PCR (qPCR) technique.
- ✓ The egg production.
- ✓ The internal and external egg quality and the eggshell quality.

CHAPTER TWO

MATERIALS AND METHODS

2.1 ANIMALS AND HOUSING

The animals used in the trial were 440 Novogen Brown layer hens of approximately 54 weeks of age, purchased from Tom Barron Hatchery (Preston, UK) and reared from day one in the farm under organic farming conditions. The location was a commercial organic farm based in Llanilar (Aberystwyth).

All the hens were vaccinated for Marek disease in the hatchery but they did not receive any other vaccine or given any anti-coccidial or worming drug during their entire life. Other prophylactic treatments including homeopathic remedies were commonly used and these were recorded in the farm book. No major health problems were encountered in the farm during the previous year. The housing was kept clean and disinfected as standard guidelines for egg production. The hens had access to the outdoors from week 6 of age, depending on weather conditions, and moved from the rearing shed to the laying shed at 16 weeks of age.

Flock cycle production in the farm usually lasted two years (2 laying cycles), with a maximum of 15 hours of lighting per day. Between cycles, the flocks were left to molt naturally in the autumn (September to November) before starting laying again with increasing photoperiod (day length).

The flock was split in two groups (n= 220) and housed in two different sheds with access to a shared open field to free range (2.5 hectares of surface), limited by an electrified fence to protect from external predators and a line of trees and bushes. Each shed was assigned to a dietary treatment at random, Control (CTR) or Intervention (INT) (*Figure 2.1*). There was not replication of the

treatments in this trial, meaning that there was only a single replicate. Field ranges were not partitioned so all the hens could use the entire field to move and forage, and eventually they could use any of the two sheds. (*Figure 2.2a).*

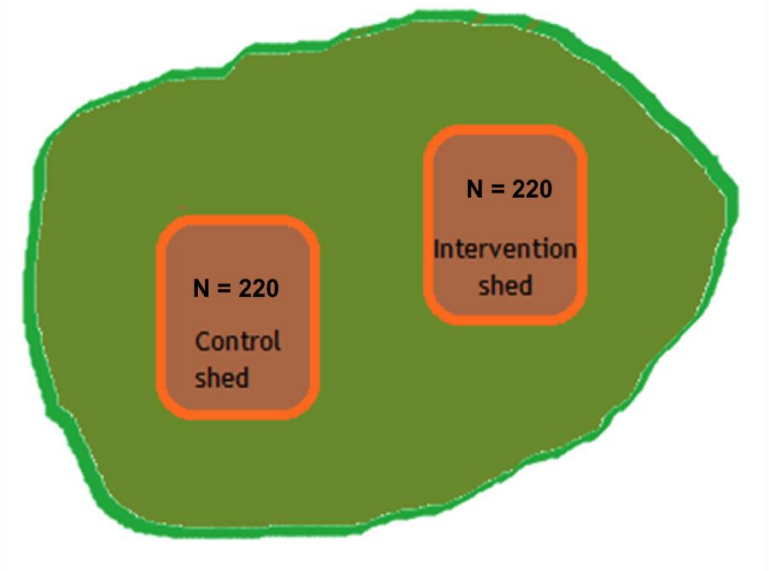


Figure 2.1. Scheme of the layout of housing sheds in the farm field.

Figures 2.2a and 2.2b. Images of the farm field in the summer months

Sheds were of the same dimensions and surface area: 24 ft x 14 ft (732 cm x 427 cm) and 336 square feet (31.25 m^2). They had slat-litter floor type with a central slat area of 2 ft (61 cm) above the floor and side floor corridors covered in litter, with a total of 50% floor and perch area. They were equipped with roost

perches, nest boxes (1 per 5 hens), 10 feeders per shed and bell drinkers. Hens could access the shed through a lateral ramp.

Before this flock moved into the laying sheds, these were cleaned by hot water pressure wash and underwent a sanitation period of 6 weeks during which they remained empty. Nest boxes and perches were regularly cleaned and disinfected with sanitizer products based on quaternary ammonium.

The field terrain was entirely composed of mixed grass, mostly available in the summer period but sparse over the winter season (experimental period of this trial). There was presence of mud and puddles scattered over the field (*Figures 2.2a and 2.2b*).

The farm fields followed part of a 5-6 year rotation of vegetables/arable/grass, to allow for recovery of the grass cover in the field as well as a natural way to control external parasites such as coccidia. Sheds were not moved within the field until the end of lay, when they were moved to a new field and the used field was then resting for at least 2 years.

2.2 DIETS AND FEEDING

2.2.1 DIETS AND FEED COMPOSITION

Two different diets were formulated and prepared in farm: a basal Control (CTR) and an Intervention diet (INT). Both diets were certified organic. The composition and ingredients of the basal diet was the same as the diet fed to this flock from the age of fourty weeks.

The ingredients of the basal control feed were: home grown naked oats (from Ceredigion-based fields), wheat/pea mix and whole wheat and oats (from Pembrokeshire-based fields), whole rapeseed, protein/mineral concentrate and limestone. Intervention feed contained additionally diatomaceous earth

(Diature™ DE, supplied by Natural Feeds and Fertilisers Ltd.) at a final concentration of approximately 1.33 % (w/w). Feed composition of both CTR and INT diets is presented in Table 2.1.

Poultry concentrate composition was based on: organic soya expeller, organic full fat soya, organic peas, prairie meal, organic rape expeller, organic sunflower expeller, brewer's yeast, di-calcium phosphate, calcium carbonate, sodium bi-carbonate and salt. Its chemical composition is presented in Table 2.2. From the cereal used to make the feed, the naked oats were home grown and subject to analysis (*Table 2.3*).

Table 2.1: Composition of Control (CTR) and Intervention (INT) feeds.

Ingredient (g/kg)	CTR	INT
Wheat/pea mix	296.3	292.4
Wheat/oat whole cereal mix	370.4	365.4
Organic naked oats	101.0	99.7
Poultry concentrate	168.4	166.1
Rapeseed (whole)	13.5	13.3
Limestone	50.5	49.8
Diature™ DE	-	13.3

Table 2.2. Chemical composition of 87.5 Organic poultry concentrate, Bowerings Animal Feeds (Somerset, UK).

Component	Amount
Main components (g/100g DM)	
Ether extract	8
Crude protein	36
Crude fibre	5.5
Ashes	9.5
Vitamins (IU/kg)	
Vitamin A (E672)	24000
Vitamin D3 (E671)	12000
Vitamin E (3a700)	50

Table 2.3. Chemical composition of organic naked oats (Bullion variety).

Component	Amount
Main components (g/100g DM)	
Ether extract	12.4
Ashes	1.9
Starch	55.5
Total oil (method B)	8.15
NCGD	93.2
Oven dry matter	82
Total sugars (as sucrose)	< 0.2
Energy (MJ/kg)	
ME (method poultry, Hartel)	14
ME (method ruminants, EqnE3)	15.5

Analysis performed by Eurofins Lab limited, Gogerdann (October 2011).
DM= dry matter; ME= metabolizable energy; NCGD= neutral cellulase gammanase digestibility.

COMMERCIAL DE EMPLOYED FOR THE PROJECT

Diature™ DE is commercialised by the company Natural Feeds and Fertilisers Ltd., based in Penrhyncoch, Aberystwyth. It is organically certified by Organic Farmers & Growers Ltd. (OF&G) and Quality Welsh Foods Certification Ltd. (QWFC), and is an approved feed additive (E551C).

Diature™ DE is composed of amorphous silicon dioxide (SiO_2) and has approximately 1195 ppm of soluble silica or PAS (Plant Available Silica), as extracted by a calcium chloride extraction method at an extraction ratio of 1:100; whereas synthetic calcium silicate has a PAS value of 268 ppm, and perlite and quartz sand, 43 ppm. Physical properties and chemical composition are presented in Tables 2.4 and 2.5.

Table 2.4. Physical properties of Diature™ DE (Sourced by NFF Ltd.).

Base Diatom species	*Cyclostephanos*
Surface area (m^2/g)	33
Oil absorption (by ASTM D281)	220
Particle size d10 microns	4.9
Particle size d50 microns	13.3
Particle size d90 microns	26.6
pH	8.3

2.2.2 FEED PREPARATION AND DELIVERY

An intervention feed batch of 1.5 tones was prepared using the farm mill within a working day, from ingredients stored in a sheltered storage barn at outdoor ambient temperature (range +0°C to +10°C in the months February-March). No thermal treatment was applied throughout the procedure. A batch of control feed to use in week 1 was also prepared in the day and the same process repeated once a week during the length of the trial. All feed was stored in 25 kg strong, labeled white plastic bags.

Shed feeders were refilled with a regularity of twice a week, in each occasion six intervention feed bags and six control feed bags were delivered to the appropriate sheds (150 kg feed per shed), thus allowing the feeding *ad libitum* of the hens during the length of the trial.

Late winter-early spring was the time of the year chosen for the trial, for the reasons of mild weather conditions and convenient age of this particular batch of hens. The first feed batch was put out on 18 February and the last batch on 22 March, to cover a length of five weeks of the trial. Feed was sampled in two occasions (week 3 and week 5) and preserved frozen at -80°C.

Table 2.5. Chemical composition of Diature™ DE (Sourced by NFF Ltd.).

SiO_2 91 %	Cl 328 ppm	Co 5 ppm	Ge 1 ppm	Sm 0.4 ppm
LOI 3.54 %	F 118 ppm	Ga 3 ppm	Mo 1 ppm	Dy 0.3 ppm
Al_2O_3 2.29 %	V 172 ppm	Pd 3 ppm	W 1 ppm	Er 0.21 ppm
CaO 0.77 %	Ba 106 ppm	La 2.3 ppm	Br <1 ppm	Ag 0.2 ppm
Fe_2O_3 0.66	Sr 83.8 ppm	Nd 2.3 ppm	Hf 0.7 ppm	Yb 0.2 ppm
MgO 0.42 %	Zr 19.5 ppm	Sc 2.2 ppm	U 0.69 ppm	Cd <0.2 ppm
Na_2O 0.33 %	B 15 ppm	As 2 ppm	Pr 0.57 ppm	Eu 0.13 ppm
C(total) 0.27 %	Zn 14.2 ppm	Nb 2 ppm	Be <0.5 ppm	Tl <0.1 ppm
K_2O 0.25 %	Cr 9 ppm	Pb 2 ppm	Bi <0.5 ppm	Ho 0.06 ppm
C(org) 0.14 %	Ni 9 ppm	Sn <2 ppm	In <0.5 ppm	Tb 0.06 ppm
TiO_2 0.12 %	Rb 8.4 ppm	Sb 1.7 ppm	Se <0.5 ppm	Lu <0.05 ppm
S 0.06 %	Cu 7 ppm	Cs 1.5 ppm	Ta <0.5 ppm	Tm <0.05 ppm
MnO 0.02 %	Li 6 ppm	Y 1.5 ppm	Te <0.5 ppm	Pt <10 ppb
P_2O5 <0.01 %	Ce 5.2 ppm	Th 1 ppm	Gd 0.42 ppm	Au <2 ppb

2.3 FEED ANALYSIS

Feed samples used for analysis of the dry and organic matter, ether extract and nitrogen (protein) were taken on week 3 and week 5 of the trial, for both CTR and INT treatments. They were preserved frozen at -80^{o}C until further analysis.

2.3.1 DRY MATTER AND ORGANIC MATTER DETERMINATION

Dry matter

Feed samples were milled to powder in an electric grinder (circa 1 mm diameter size). Approximately 2 g of sample was weighed by duplicate in digital scales (Sartorius analytical) and placed into ceramic crucibles. The ceramic crucibles were previously kept in the oven at +103°C to remove any possible moisture. Samples were placed in the oven, left at +103°C for 24 h. After 24 h crucibles were placed for 30 min into a desiccator to allow the samples cool down to room temperature and weighed after that time.

Percentage of dry matter was calculated following the formula:

$$DM = 100*(DW-TC)/(FW-TC)$$

Where:

DW is the weight of the dried sample with the crucible

FW is the weight of the initial sample with the crucible

TC is the weight of the crucible

Organic matter

Crucibles with dried samples were placed in an oven furnace at +550°C and were left for 3 h. After that time, crucibles were transferred into a dessicator for 1 h to cool down at room temperature and weighed.

Percentage of organic matter was calculated following the formula:

$$OM = 100*[1-(Ash-TC)/(DW-TC)]$$

Where:

DW is the weight of the dried sample with the crucible.

Ash is the weight of the sample with the crucible after 3h in the oven furnace.

TC is the weight of the crucible.

2.3.2 ETHER EXTRACT DETERMINATION

The equipment used for crude fat analysis was the XT15 Ankom Fat extractor (Ankom technology, USA), which applies the official method AOCS Am 5-04.

Approximately 0.2 g of milled feed sample was weighed in digital scales (TL-64, Denver Instrument Company), put into XT4 filter bags, previously weighed, and sealed. Samples were prepared in triplicate. The standard protocol for extraction was applied (60 min and 90°C) using as solvent petroleum ether 40-60°C (Fisher Scientific, UK); the solvent recovery was the same or greater than 97%.
Once the extraction was finished, bags were taken out and placed in the oven at +60°C for 2 h to remove all the extra solvent. They were then transferred to a glass desiccator and weighed.

Percentage of ether extract (EE) was calculated following the formula:

***EE* = 100*(ES-TB)/(IS-TB)**

Where:
ES is the weight of the bag with the sample after the extraction process.
IS is the weight of the bag with the sample before the extraction process.
TB is the weight of the bag without sample.

2.3.3 NITROGEN TO PROTEIN DETERMINATION

The analyzer Vario MAX cube (Elementar Analysensysteme, Germany) in CN mode was used for the determination of the nitrogen content of the feed.

An approximate amount of 200 mg of milled feed sample was added to each vial and loaded into the analyzer to be processed by the Dumas combustion method. The samples were analysed in a unique run with no replicates. Protein content was calculated from the resultant values applying the nitrogen to protein

conversion factor of 5.83, known as one of the “Jones factors” (Jones, 1941) and appropriate for feed based in wheat and oats.

2.4 SAMPLING AND PRODUCTION RECORD

2.4.1 EGG PRODUCTION RECORD

Egg production was recorded in the farm by the farm personnel on a daily basis.

2.4.2 FAECAL SAMPLING

Faeces were sampled on a weekly basis, taking 3 samples per shed in labeled zip bags. Floors and slat (area underneath the roost perches) were examined following a Z walking pattern, and fresh faeces were collected and distributed between the bags. Then, bags were closed with care to eliminate all air contained inside. Samples were stored at +4°C within 30 to 45 minutes after collection. These were subjected to faecal egg counts (FEC) (section 2.7.2), then frozen at -80°C and freeze-dried.
In addition, faeces were also tested at T=0, one week before starting the trial, with the goal of screening the type of parasites and amount of eggs present.

2.4.3 EGG SAMPLING

The sampling of eggs was performed twice, one at the beginning (week one) and a second one at the end of the trial (week five). On each sampling day, 15 eggs per shed were randomly chosen and kept in colored-id cardboard trays for three consecutive days, making a total number of 45 eggs per treatment. Samples were individually labeled with a marker pen and stored at +4°C within 60 to 90 min after collection. These were subjected to egg quality tests (section 2.5), then frozen at -80°C and freeze-dried. Egg component analysis (section 2.6) was subsequently performed on egg samples from week 5.

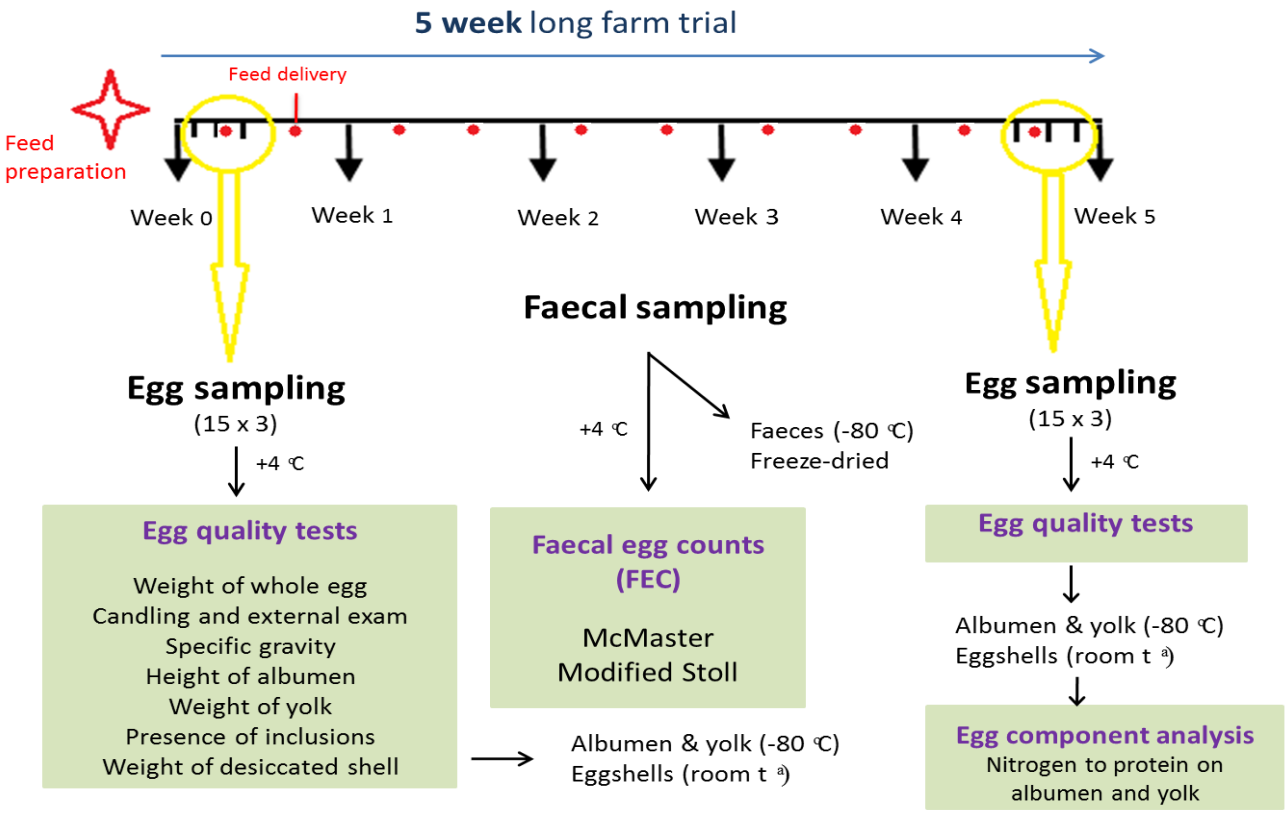


Figure 2.3. Timeline of the DE feeding trial (JT2) events.

Diet supplementation with diatomaceous earth (DE) was proposed in this trial in order to evaluate its efficacy for parasitic control and improved egg production and quality in layers. 440 Novogen Brown organic hens of approx. 54 weeks of age were split in two groups (n= 220) and housed in two different sheds with access to a shared field. Two diets were randomly assigned to the barns: control (basal organic diet) and intervention (basal organic diet with DE at 1.33%), feed was supplied twice a week; dietary treatment lasted for 5 weeks. Faeces were sampled and faecal egg counts (FEC) were performed once a week. Egg sampling for quality tests was performed at weeks one and five of the trial, collecting 15 eggs per barn for three consecutive days. After analysis, both faecal and egg samples were frozen at -80°C and freeze-dried. Egg production was recorded on a daily basis.

2.5 EGG QUALITY TESTS

All tests were performed on the egg samples collected twice, one at the beginning (week one) and a second one at the end of the trial (week five).

2.5.1 EGG EXTERNAL EXAMINATION AND CANDLING

Eggs were examined individually on the evening following collection.
The external examination enables a visual description of the eggshells. As described by Cutts *et al.* (2007), external flaws observed were:

- Dirtiness: eggshell stained with blood spots, faeces or both, covering 10% or more of the shell surface.
- Hairline cracks (fissures): very fine linear cracks.
- Star cracks: fine cracks radiating from a central point
- Repaired fractures: cracks that have re-calcified.
- Pinholes: very small holes (nail piercing holes).
- Pimples: small aggregates of calcified material on the eggshell.
- Body checks: groove and ridge marks found at the ends of the eggs,
- Rough texture/sandpaper shells: rough texture areas in the shell, often unevenly distributed.

Candling examination with an egg candler is a simple process that helps to identify shell defects, by passing the eggs over a bright light in a dark room (*Figures 2.4a and 2.4b*).

Figures 2.4a and 2.4b. Egg candler (a) and candling demonstration in a commercial setting (b). Sources: http://incubatorwarehouse.com (2.4a) and USDA Egg grading manual (2.4b).

For this purpose, a homemade egg candler was built using a clean 1 L milk box, wrapped with aluminum foil the inside of its surface. A hole of approximately 2 cm diameter was made on the distal end of one of the flat sides. A lamp was placed inside the pack and the open side of the box was sealed (*Figure 2.5*).

Figure 2.5. Homemade egg candler. Each egg was placed on the hole (left hand side of the box) and examined in a dark room with the cadler light switched on.

External defects inspected were all the types of fractured shell (hairline, star and pinhole), repaired fractures and shell-density aspects like:

- Weak shell areas: poor calcification areas in the shell. Graded as: 1 = < 0.5 cm^2 surface; 2 = in several scattered areas, < 2 cm^2 in total; 3 = many lighter shell areas or bigger surface cover.
- Very porous translucent shells: “mottled” or “glassy” egg appearance.

2.5.2 WEIGHT OF THE WHOLE EGG

Eggs were labeled with a marker pen and weighed individually on the evening of collection, using digital scales and a plastic support (weight expressed in grams).

2.5.3 WEIGHT OF THE EGGSHELLS, YOLK AND ALBUMEN

Broken shells were washed, distributed in foil trays and put to dry in a stove at 70°C for 24 h. Their dry weight (in grams) was taken using digital scales and a plastic support.

Yolk weights (in grams) were taken following egg breakage using digital scales and a plastic support.

Albumen weights were calculated by difference, with the formula:
Albumen weight (in grams) = whole egg - dry shell weight - yolk weight

2.5.4 EGGSHELL DENSITY

Eggshell density was calculated with the following formula (Mueller and Scott, 1940):
Density of the eggshell (in mg/cm^2) = weight of dried egg shell / surface of the shell

Where:

Surface of the shell is equal to 4.67 x (weight of whole egg) $^{2/3}$

2.5.5 YOLK COLOUR

The DSM Yolk Colour Fan was used for the evaluation of the colour of the yolk (*Figure 2.6a and 2.6b*).

Figures 2.6a and 2.6b. DSM Yolk Colour Fan and detail of its use. Source: http://www.dsm.com.

Broken eggs were laid on a laminated white card with homogeneous natural light conditions for inspection. The fan has fifteen coloured blades, numbered from one (pale yellow) to fifteen (dark orange/red). The blades are held above the egg yolk with the tips around its centre, and the yolk is viewed vertically from above. The number that corresponds better with the yolk colour is recorded. In the surveys, European customers have expressed a preference for the darker colours such as 12 and 14 in the Colour Fan scale (Beardsworth and Hernández, 2004).

2.5.6 ALBUMEN HEIGHT AND HAUGH UNIT CALCULATION

Eggs were taken out from the fridge to the laboratory bench, and were left there for approximately one hour to equilibrate their temperature. Then these eggs were carefully broken one by one and laid on a hard plastic flat piece sitting on the bench. A manual vernier caliper positioned vertically was used to take a measure of the height of the dense albumen (expressed in millimeters).

Haugh units (HU) were calculated with the formula:

$$HU = 100 \times \log(H + 7.57 - 1.7 \times W^{0.37})$$

Where:

H is observed height of the albumen (in millimeters).

W is weight of the whole egg (in grams).

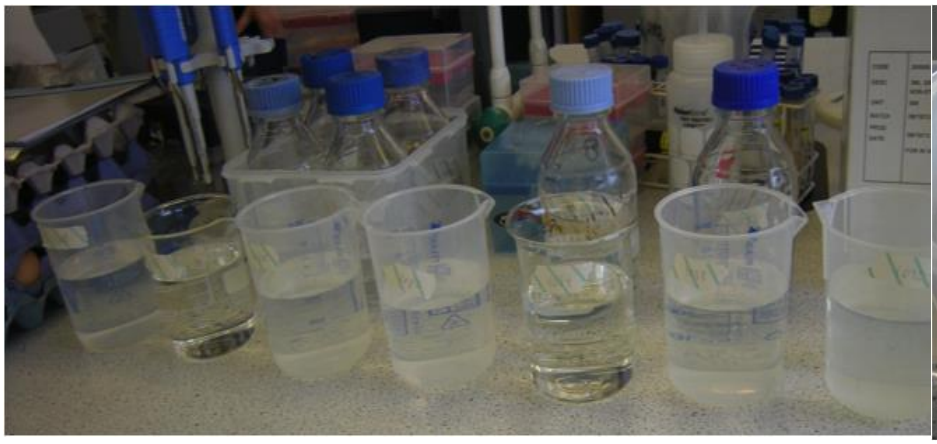
2.5.7 EGG INCLUSIONS AND OTHER CHARACTERISTICS

Inclusions such as meat (on chalazeae, albumen or yolk) and blood spots (on yolk) were identified and counted in the broken egg by visual examination. A minimum value of 3 was attributed if there was only one inclusion, but of big size. Notes were taken about other egg characteristics like watery whites and discoloured yolks, both considered internal defects.

2.5.8 EGG SPECIFIC GRAVITY

Salt solutions were made mixing distilled water with different quantities of laboratory grade sodium chloride, which yielded nine solutions of specific gravity, ranging from 1.068 to 1.098 mg/ml; a hydrometer was used to check the specific gravity (SG) of the solutions prior use. The solutions were preserved in 1 L glass bottles and reused for three days, then discarded. The temperature of eggs, materials and salt solutions were left to equilibrate overnight in the same laboratory bench at room temperature, and the test was performed the day after (completed within 48 h of egg collection).

In brief, the solutions were poured into glass or plastic medium-size beakers, and with the help of a tablespoon each egg was immersed in increasingly concentrated salt solutions until it floated on the surface of one of the solutions ("breaking" the surface of the liquid). The SG of the solution in which floated was assigned to that egg (*Figures 2.7a and 2.7b*).




Figures 2.7a and 2.7b. Salt solutions ordered by increasing specific gravity (from left to right) (a), and a labeled egg floating in one of the solutions (b).

2.6 EGG COMPONENT ANALYSIS

2.6.1 FREEZE DRYING EGGS

Fresh egg portions (albumen and yolk) from week 5 samples were individually stored in plastic containers and frozen at -80°C immediately after visual exam. These samples were freeze dried in batches using a freeze-drier, taking around three days to complete the process. Samples were stored at room temperature afterwards.

2.6.2 NITROGEN TO PROTEIN ON ALBUMEN AND YOLK

The analyzer Vario MAX cube (Elementar Analysensysteme, Germany) in CN mode was used for the determination of the nitrogen content on albumen and yolk.

An approximate amount of 150 mg of freeze-dried sample was added to each vial and loaded into the analyzer. 19 yolk samples (8 CTR, 11 INT) and 40

albumen samples (20 CTR, 20 INT) from week 5 were analyzed in a unique run.

Protein content was calculated from the resultant values applying the nitrogen to protein conversion factor of 6.25, known as one of the “Jones factors” (Jones, 1941) and appropriate for dairy products.

2.7 PARASITOLOGY TESTS

2.7.1 FLOTATION SOLUTION PREPARATION

Two different flotation solutions were made up in the laboratory and used for the faecal egg counts: salt saturated (specific gravity, SG= 1.18-1.20), and Shaether’s sugar solution (SG= 1.25).

Salt saturated solution was made according to the following procedure: 400 g of sodium chloride (NaCl laboratory reagent grade, Thermo Fisher Scientific, USA) were mixed in a glass beaker with 1 L of distilled water and dissolved over medium heat with agitation. Once the salt was dissolved and the fluid did not take any more salt (it means it was fully saturated), the specific gravity of the solution was checked with a hydrometer (Densimetre 1,000-1,200, Alla France), stored in 1L bottles and autoclaved.

Sheather’s sugar solution was made according to the following procedure: 454 g of white sugar were mixed in a glass beaker with 355 ml of distilled water; then the sugar was dissolved over low heat with agitation. After the sugar was dissolved and solution cooled down, 6 ml formaldehyde USP were added to prevent microbial growth. The specific gravity of the solution was checked with a hydrometer, stored in 1 L bottles and autoclaved.

2.7.2 METHODS FOR FAECAL EGG COUNTS (FEC)

FEC were performed within 24-48 h after faecal collection, following two different counting methods that are described below.
Protocols are included in Appendix.

2.7.2.1 PROTOCOL FOR METHOD: MCMASTER 25 EGGS PER GRAM (EPG) SENSITIVITY

The present procedure is an adapted version from Zajac and Conboy (2012) and Permin (1998) and is illustrated in Figure 2.9. It was performed with saturated salt solution (SG= 1.18-1.20) as follows:

Four grams of faeces were mixed with 26 ml of tap water in a beaker, stirred well with a spatula and left to soak at room temperature for 60-90 min. After soaking the suspension was filtered through a single layer of muslin, filling a 15 ml screw cap centrifuge tube. This was centrifuged at 600 x *g* for 5 min to pellet parasite eggs and oocysts. The supernatant was aspirated using a sterile serological pipette and electronic pipette, leaving 1-2 ml of supernatant and taking care not to disturb the pellet. The supernatant was then checked microscopically for unpelleted eggs. Two to three ml of saturated salt solution was used to carefully resuspend the pellet, and the volume then made up to 15 ml with saturated salt solution. The tube was inverted gently 8-10 times to mix the contents. An aliquot was then removed from the middle of the tube with a Pasteur pipette, and used to fill one of the McMaster slide chambers 1.5 mm depth (Weber Scientific International, England). The tube was then capped and inverted two times, before a second aliquot was removed using a Pasteur pipette to fill the second chamber. The filled McMaster slide was left at room temperature for a minimum of 5 min to allow eggs to float to the surface. The slide contents were examined with the 12.5 x objective lens of an Olympus BH-2-RFCA microscope (Olympus Corporation, Japan), and the number of eggs counted in all six fields of both slide chambers. All eggs were counted and recorded separately for each parasite species.

A McMaster counting slide has two chambers, each of 1.5 mm depth and 0.15 ml volume capacity, and marked off in 6 lanes (*Figure 2.8*). If both chambers are counted and the results are added, the total count represents the number of eggs in 0.3 ml. This represents 1/100 of the volume of the initial mixture.

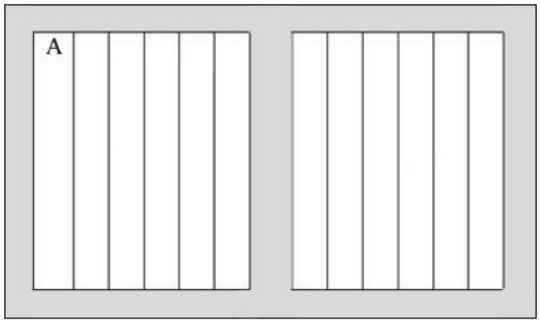


Figure 2.8. A two-chambered McMaster slide. *A* corresponds to one of the six lanes of a single chamber (adapted from Pereckiene *et al*., 2007).

The eggs per gram (epg) calculation is worked out as follows:
Epg = (count of eggs per parasite species x 100)/4 (g of faeces used)
Or multiplying the total count of eggs of each parasite species by 25.
The minimum sensitivity of the test is 25 epg.

As an alternative, it can also be applied the formula:

$$\textbf{Epg} = \frac{(N \times T)}{(V \times F)}$$

Where:
N is n^0 eggs counted.
T is total volume of faecal suspension (30 ml).
V is volume of aliquot examined in slide (2 x 0.15 ml = 0.3 ml).
F is grams of faeces used.

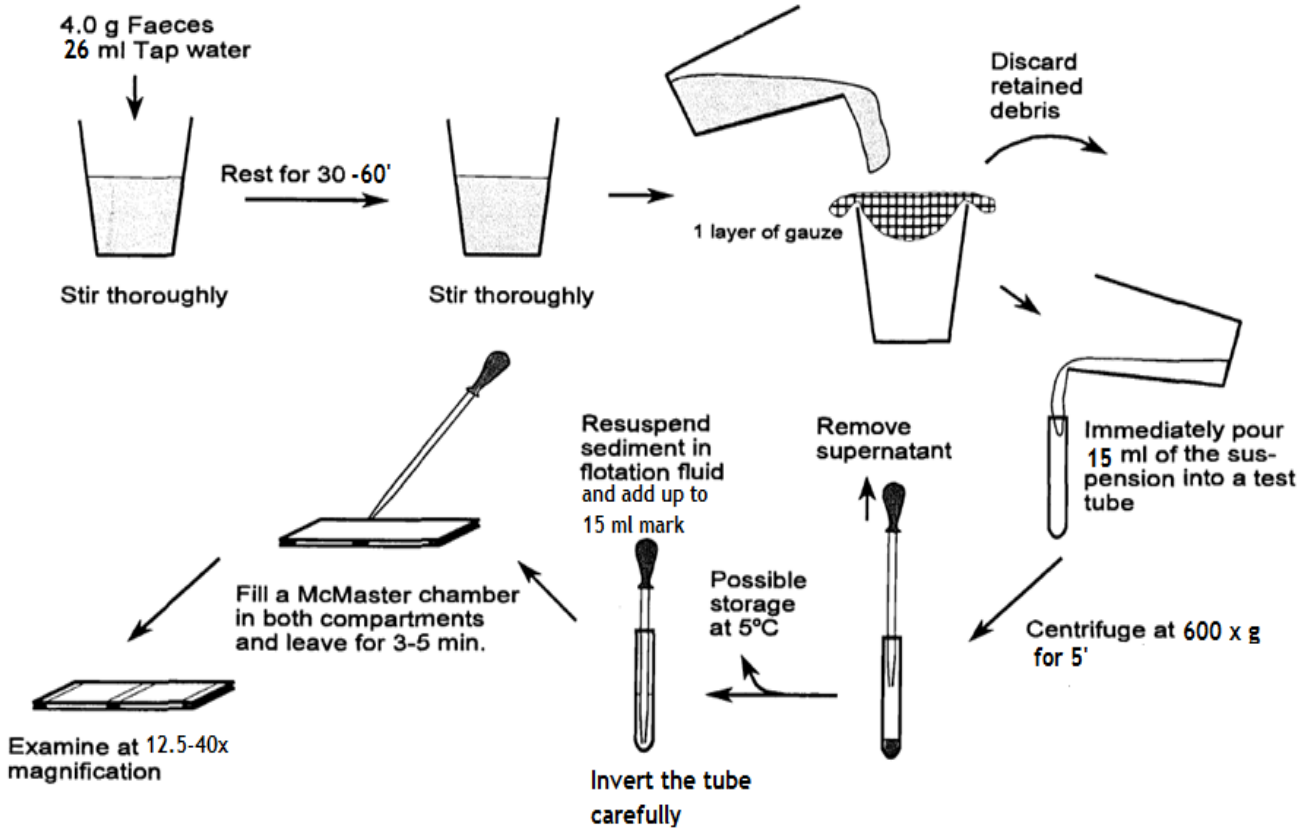


Figure 2.9. Procedure for the McMaster FEC method (adapted from Permin, 1998).

2.7.2.2 PROTOCOL FOR METHOD: MODIFIED STOLL TEST

The present procedure is an adapted version from Zajac and Conboy (2012) and Permin (1998) and is illustrated in Figure 2.10. It was performed with Shaether's sugar solution (SG= 1.25) as follows:

Five grams of faeces were mixed with 20 ml of tap water in a beaker, stirred well with a spatula and left to soak at room temperature for 60-90 min. After soaking the suspension was filtered through a single layer of muslin, 1 ml was transferred to a 15 ml centrifuge tube and filled up with flotation solution. This was centrifuged at 450 x *g* for 10 min. The tube was placed in a rack, flotation solution was added with a Pasteur pipette to make a meniscus and a coverslip was placed on top. The tube was left to stand for 15 min. After that, the

coverslip was removed, placed on a glass slide and examined with the 12.5 x objective lens of an Olympus BH-2-RFCA microscope (Olympus Corporation, Japan). All eggs found attached to the coverslip were counted and recorded separately for each parasite species.

The epg calculation was worked out as follows:
All the eggs present in 1 ml of faeces/flotation solution mixture were counted and this represents 1/25 of the volume of the initial mixture.

Epg = (count of eggs per parasite species x 25)/5 (g of faeces used)
Or multiplying the total count of eggs of each parasite species by 5.
The minimum sensitivity of the test is 5 epg.

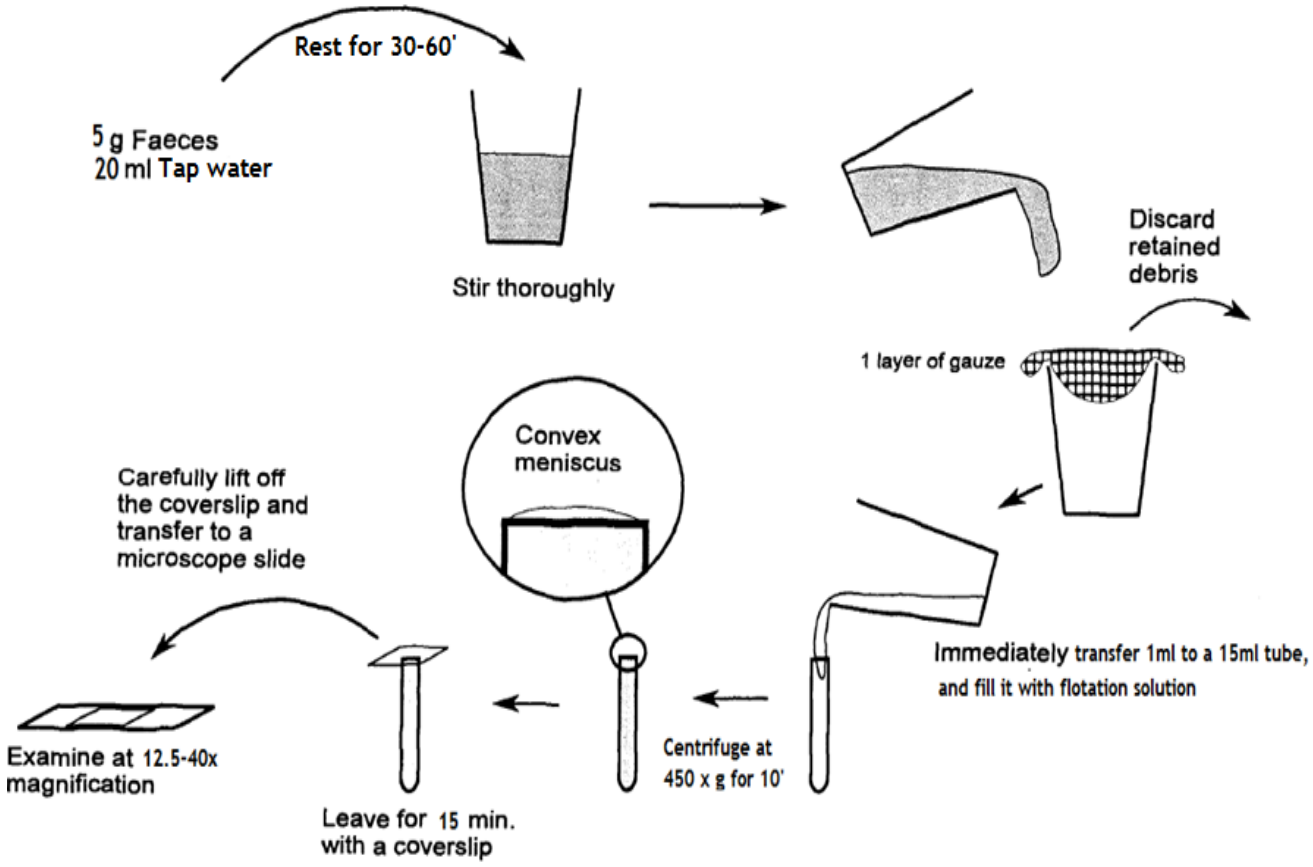


Figure 2.10. Procedure for the Modified Stoll FEC method (adapted from Permin, 1998).

2.7.3 PARASITE IDENTIFICATION

The parasite eggs present in the samples were identified using the keys of Foreyt (2001), Gibbons (2010), Kassai (1999), Kaufmann (1996), Urquhart *et al.* (1996), Wakelin (1965) and Zajac and Conboy (2012).

A customised key was designed for quick reference and is shown in the Appendix.

The parasite species *Capillaria* and *Eimeria* are not easily distinguishable by the egg, so the egg counts are referred to a number of eggs of a mix of species.

Samples tested by the Modified Stoll method (glass slides with cover slips) were taken to the Advanced microscopy and Bio-imaging laboratory to be examined with a Leica LMD 6000 microscope (Leica Microsystems, Germany). All the parasites present were identified, measured and photographed, as well as artefacts, “pseudoparasites” (pine and other pollen grains, hairs, fibrous material, free-living mites) and unidentified parasite eggs (i.e. from free-living mites, usually very large >100 µm) present in the samples.

2.7.4 RECOVERY OF EIMERIA OOCYSTS FROM FAECAL SAMPLES (FAECAL HARVEST) AND SPORULATION

2.7.4.1 FAECAL HARVEST AND SPORULATION

When the objective is to collect a large number of oocysts from poultry faeces, a faecal harvest can be done in a significant amount of faeces. The procedure involves soaking and sieving the faeces to get a homogenate. This is mixed with salt saturated solution and centrifuged, which forms a surface layer (containing oocysts), which is kept in a separate container. These steps are repeated several times. The recovered oocyst suspension is then washed in tap

water and centrifuged several times, and only the resultant pellet is kept for further use.

Eimeria parasites (oocysts) were harvested from faecal samples of the challenge broiler trial (AH6), being the experimental birds aged 30 days. Samples from 9 pens were taken and two batches of faeces (A and B, weighing 444 g and 461 g) were soaked in 800 ml and 900 ml tap water respectively, using 5 L beakers. They were kept overnight at + 4°C. The protocol is included in the Appendix.

Once a suspension of oocysts was obtained from the faecal samples, this was mixed with 2% potassium dichromate and distilled water and put in a shaker station at +28°C for four and a half days. The result was a culture of sporulated *Eimeria* oocysts, which was stored at +4°C until further use. The protocol is included in the Appendix.

2.7.4.2 COUNTING PARASITES

A. MCMASTER METHOD

Two samples of 1 ml are withdrawn and transferred to different 10 ml tubes. 9 ml of saturated salt solution are added to each sample, then mixed thoroughly and transferred to a McMaster slide. Both chambers are filled with the sample of one tube. The slide is left for 4-5 min to allow oocysts to float to the surface, so numbers can be accurately counted.

Chamber lines are counted as follows: if small numbers of oocysts are produced it is advisable to count all six lanes, including any oocysts in the tramlines. If oocyst numbers are large it is permissible to count just one lane and multiply by six, or the number of lanes x multiplication factor. Disrupted and irregular oocysts that look not viable and clumped oocysts were not counted.

Total parasite numbers are calculated with the formula:

X/0.15 x volume x dilution

Where:

X is the mean of the 2 counts.

Volume is total volume of mixture.

Dilution is the factor by which each 1 ml sample was diluted (10).

0.15 equals to the volume a McMaster chamber (ml).

B. MODIFIED FUCHS-ROSENTHAL METHOD

Modified Fuchs Rosenthal slide 0.2 mm cell depth (Weber Scientific International, England) has two chambers, each made up of 9 large squares and subdivided in 16 smaller squares (*Figure 2.11*).

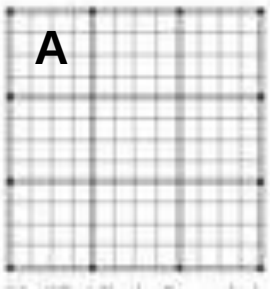


Figure 2.11. Single chamber of a Modified Fuchs Rosenthal slide. *A* occupies a volume of 1/5,000 ml.

A glass cover slip is placed on the slide, 20 µl of sample is added to fill the counting chamber by placing the pipette tip by the cover slip. The sample should be well mixed before sampling, and both chambers are filled with one sample (20 µl each). Parasites are allowed to settle for a few minutes. Briefly look over the whole slide to determine if there is an even spread of oocysts. Observe under a microscope (x 10 and x 40 magnification) and count according the following rules:

Number oocysts seen in all 9 big squares	Number of big squares to count
< 20	9 (all)
> 20	5 (corner and middle squares)
> 100	2 or 3

To calculate the number of parasites in 1 ml, the following rules apply:

Number of squares counted	Multiplication factor
5 large squares	1000
1 large square	5000
1 small square	80000

To obtain the total number of oocysts contained in the suspension the resulting number of parasites should be multiplied by the total volume of oocyst suspension (i.e. 200 ml).

C. DROP COUNT

An aliquot of the suspension is mixed well and 25 µl samples are withdrawn and transferred to clean glass slides with a cover slip. The number of parasites in each sample is counted under the microscope (drop count). As a rule 5-10 drop counts are done and an average is calculated. When counting a sporulated culture, a percentage of sporulated vs. unsporulated oocysts can be calculated to check for the quality of sporulation.

2.8 MOLECULAR BIOLOGY METHODS

The effect of the dietary inclusion of DE on the concentration of *Eimeria* parasites in free-range organic laying hens was studied in the DE feeding trial. The presence of the seven *Eimeria* species that infect the chicken and the concentration of *Eimeria acervulina* in faecal samples were studied by the means of FEC, PCR and qPCR in order to assess its significance for free-range organic laying hens.

2.8.1 TYPE OF MATERIAL USED FOR DNA EXTRACTION AND MOLECULAR ANALYSES

Refer to Figure 2.12 for more detail.

FAECES

Three sets of faecal samples were collected randomly from both the control and intervention sheds once a week for 5 weeks. Faecal samples from the DE feeding trial (JT2) were then freeze-dried and homogenised as described in section 2.8.2 (A).

Faecal samples from control and intervention pens from the challenge broiler trial (AH6) were collected when birds were aged 30 and 36 days. The trial used experimental broiler birds housed in pens with *Campylobacter* positive litter. These samples were used in fresh form (FEC) and preserved at +4°C.

OOCYSTS

Freshly purified oocysts were obtained from faecal samples of the experimental birds aged 36 days from the challenge broiler trial (AH6), as described in section 2.8.2 (B).

Oocysts were harvested from faecal samples of experimental birds aged 30 days from the challenge broiler trial (AH6) and incubated until sporulation, following the method described in section 2.7.4.1. They were preserved at +4°C and concentrated to use as described in section 2.8.2 (C).

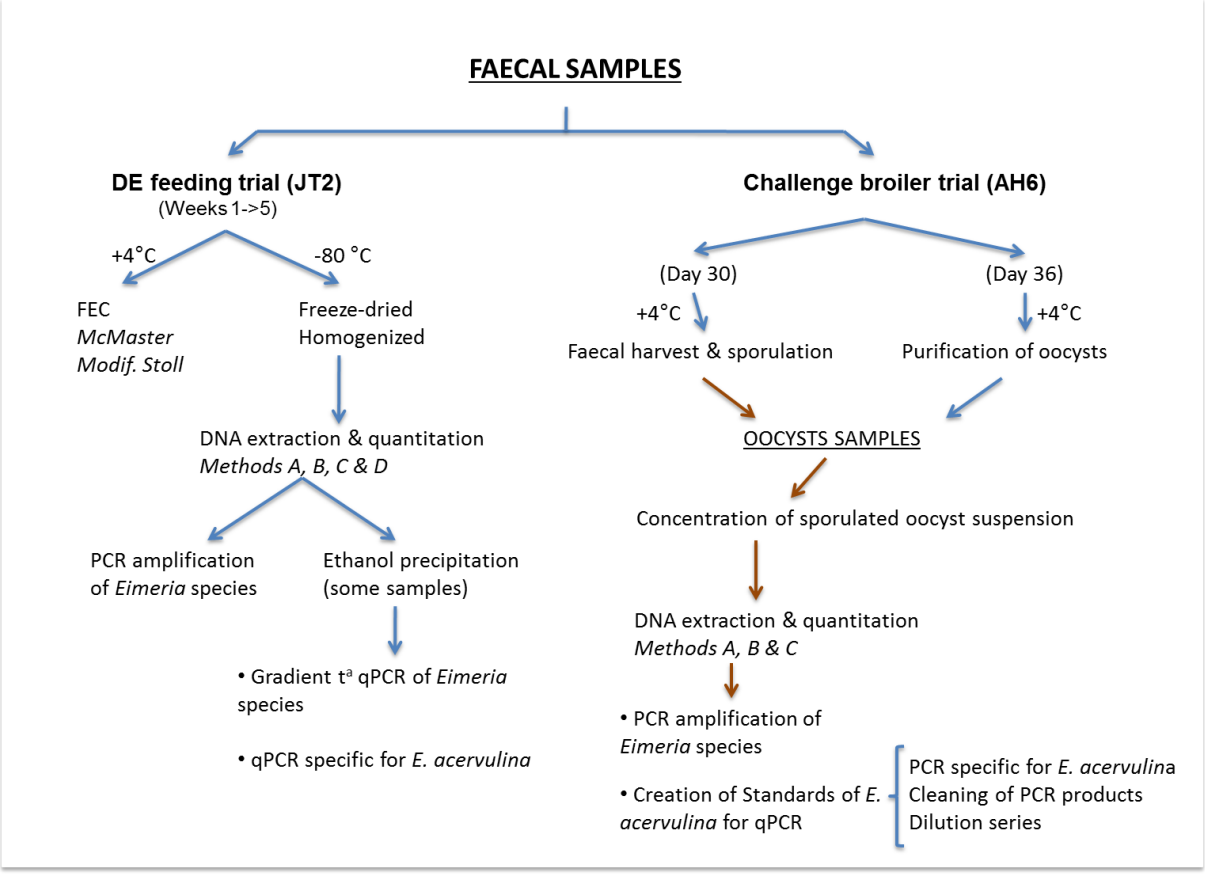


Figure 2.12. Type, preparation and use of samples for molecular methods.

2.8.2 PREPARATION STEPS OF MATERIAL BEFORE DNA EXTRACTION

Refer to Figure 2.12 for more detail.

A. FREEZE DRYING

Hen faecal samples were stored at +4°C for two days and frozen at -80°C afterwards. Plastic zip bags were placed open inside the freeze-drier for 48 h. Once freeze-dried, samples were individually mashed to powder with a mortar inside the bag to homogenize them.

B. PURIFICATION OF OOCYSTS FROM FAECAL SAMPLES

The present procedure is an adapted version from Kumar *et al.* (2014):

Four grams of faeces per pen were weighed and mixed with 5 ml of 2% potassium dichromate in a 15 ml tube. These samples underwent FEC to enumerate oocysts before further use. After that, tubes were pooled by treatment in 50 ml centrifuge tubes and filled up with saturated salt solution. On each faecal suspension, 2 ml of distilled water was gently overlaid and sample was left to stand for ten minutes and then centrifuged at 750 × *g* for 8 min. Using a disposable Pasteur pipette, the layer from the interface between the saturated salt and the water was transferred to a new 50 ml tube. This was repeated four more times. The new tube was filled up with distilled water and centrifuged at 750 × *g* for 8 min., then the supernatant was carefully removed without disturbing the pellet using an electronic pipette, leaving 3–5 ml fluid. Discarded supernatant was checked microscopically for unpelleted oocysts, and centrifuged again as some oocysts were seen. Sample was transferred into a 2.0 ml microcentrifuge tube (MCT), centrifuged at 6000 × *g* for 5 min and supernatant was discarded after microscopic screening for unpelleted oocysts. The pelleted oocysts were suspended in 1 ml distilled water and counted using the Modified Fuchs-Rosenthal method as in section 2.7.4.2 (B).

C. CONCENTRATION FROM SPORULATED OOCYST SUSPENSIONS

Two aliquots of 15 ml were taken from a 50 ml tube containing sporulated oocysts in 2% potassium dichromate and centrifuged at 750 × *g* for 10 min. The supernatant was discarded carefully without disturbing the pellet. The tubes were washed three times in distilled water by successive rounds of re-suspension and centrifugation (750 x *g*, 10 min). The pellet was re-suspended in few millilitres of water with a Pasteur pipette, filled up to the mark with water and centrifuged. Supernatant was discarded after centrifugation, with a previous microscopic check for unpelleted oocysts. After the last wash, supernatant was discarded carefully without disturbing the pellet. The oocyst pellet was then re-suspended in 1 ml distilled water and transferred to a MCT. Samples were ready for DNA extraction.

2.8.3 DNA EXTRACTION

Four methods were tested with the aim of extracting and isolating the optimal yield and quality of DNA. These were:

A. MpBIO FastDNA® Spin kit for soil

B. MpBIO FastDNA® Spin kit for faeces

C. Qiagen QiaAMP DNA Stool mini kit

D. Modified phenol-clorophorm (by Dr. Eric Pinloche)

Protocols are included in the Appendix.

All four methods were applied to extract the DNA of randomly selected DE feeding trial (JT2) freeze-dried and homogenised faecal samples, with four sample technical replicates. The aim was to perform a DNA extraction method

comparison. In addition, the method MpBIO FastDNA® Spin kit for soil (A) was also applied with some modifications.

The methods MpBIO FastDNA® Spin kit for soil (A), MpBIO FastDNA® Spin kit for faeces (B) and Qiagen QiaAMP DNA Stool mini kit (C) were applied to extract the DNA of a sample of sporulated oocysts, with four technical replicates. The initial volume of sample used was 100 µl of re-suspended oocysts.

The method Qiagen QiaAMP DNA Stool mini kit (C) was selected and employed to extract the DNA of a total of 36 DE feeding trial (JT2) freeze-dried and homogenised faecal samples. Full speed was applied on centrifugation steps (17,000 x *g*).

2.8.4 QUANTITATION AND PURIFICATION OF DNA

2.8.4.1 *DNA QUANTITATION*

The concentration and purity of nucleic acids can be determined using a full spectrum (220-750 nm) spectrophotometer. Absorbance of the sample is measured at different wavelengths, spectrum is represented graphically and ratios are calculated: A260/A280 assess the purity of the DNA, being a value higher than 1.8 generally accepted as 'pure' for DNA; lower values suggest the presence of contaminants such as protein or phenol, absorbing near 280 nm. The ratio A260/A230 is in the range of 1.8-2.2 for 'pure' DNA, lower values suggest co-purified contaminants. Sample concentration is expressed as ng/microliter based on the absorbance at 260 nm and the selected analysis constant (using the modified Beer-Lambert's equation) (Thermo Fisher Scientific Inc., 2008).

Equipment used was NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). All extracted DNA samples were quantitated in ng/µl.

An aliquot of 2 µl of elution buffer was used as blank for calibration. Then, to make a measurement, a DNA sample was briefly vortexed and 2 µl of sample

was aliquoted onto the lower measurement pedestal. The sampling arm was closed and the spectral measurement was initiated using the operating software on the PC.

2.8.4.2 *ETHANOL PRECIPITATION OF DNA*

This method was applied to selected DE feeding trial (JT2) DNA samples, in order to improve DNA concentration and quality. In six out of the 36 samples DNA extraction was repeated, as they did not present neither sufficient DNA yield nor adequate 260:280; 260:230 nm ratios as measured by spectrophotometry.

Ethanol precipitation was then applied to these samples, as follows:
Fifteen microlitres of 3M Sodium acetate pH 5.5 were added to each DNA sample tube, followed by 375 µl of 100% ethanol at room temperature and vortex was applied to mix for 20 sec. Samples were incubated 1 h at -20°C, then centrifuged at 16,000 x *g* and +4°C for 15 min and ethanol was discarded by pouring it out and applying one tap on tissue. A second addition of 500 µl of 70% ethanol at room temperature was performed, followed by centrifugation at 16,000 x *g* and +4°C for 5 min and discarding ethanol as before. These steps were repeated one more time. Afterwards, samples were centrifuged at 16,000 x *g* and +4°C for 1 min and remaining ethanol was aspirated using a pipette (fitted with a 200 µl tip). Samples were incubated for 15 min at room temperature with the tube caps opened to evaporate any ethanol. Finally, 50 µl of TE elution buffer was added to each sample, they were vortexed to mix and incubated on ice for 15 min.

DNA samples were quantitated and stored at +4 or -20°C until further analysis.

2.8.5 POLYMERASE CHAIN REACTION (PCR) AND AGAROSE GEL ELECTROPHORESIS

2.8.5.1 *PCR AMPLIFICATION OF EIMERIA SPECIES DNA IN FAECES*

DNA extracted from DE feeding trial (JT2) faecal samples by four different extraction methods was tested by PCR to check for the presence of DNA from five *Eimeria* species: *E. acervulina, E. brunetti, E. maxima, E. necatrix and E. tenella*.

For each species test, a single PCR reaction was performed including four DNA samples (one per extraction method), negative and positive controls. *E. acervulina* was tested in two PCR reactions. A general PCR protocol was applied, adapting in each case the species-specific primers used (*Table 2.6*) and the reaction annealing temperature (*Table 2.7*). All PCR reactions were set up in a PCR clean room, using PCR hoods, racks and filter tips pipettes that had been wiped with 10% ethanol, and then subjected to 10 min UV light sterilization prior to setting up PCR reactions.

General PCR method for amplification of Eimeria species DNA

PCR reactions were performed in 200 µl PCR tubes using MyTaq HS mix (Bioline, BIO-25045) which is a ready to use 2x mix for highly-specific, hot-start PCR. Primer pairs used to amplify *Eimeria* specific DNA were supplied by Eurofins Scientific (Luxembourg) and are listed in Table 2.6. The qPCR reaction mixture components were defrosted on ice, and reactions consisted of: 1x MyTaq HS mix, 0.4pMµl^{-1} forward primer, 0.4pMµl^{-1} reverse primer, 5 µl of chicken faecal DNA, and made up to 50 µl with PCR grade water (Roche Ltd). Note that the DNA template was added to the reaction mixture outside of the hood to prevent contamination of the hood and reagents. Each set of PCRs was always set up with a template negative control of 5 µl of PCR grade water. Reactions were thermo-cycled on a TProfessional basic (Biometra GmbH, Germany) as follows: 95°C for 1 min; 35 cycles of denaturing at 95°C for 1 min, annealing at primer specific temperature (*Table 2.7*) for 30 sec, extension at 72°C for 30 sec; followed by an 8°C final step.

Table 2.6. Primer and probe characteristics and sequences specific for the seven *Eimeria* species that infect the chicken (adapted from Vrba *et al*., 2010).

Species	Sequence source (SCARdb ID)	Primer and probe sequences	Amplicon size [bp]
E. acervulina	Ac-AD18-953	ACE-F: GCAGTCCGATGAAAGGTATTTG ACE-R: GAAGCGAAATGTTAGGCCATCT ACE-P: [6-FAM]ACAGTCCCCGCTGATGGTGTAACG[BHQ1]	103
E. brunetti	Br-J18-626	BRU-F: AGCGTGTAATCTGCTTTTGGAA BRU-R: TGGTCGCAGACGTATATTAGGG BRU-P: [6-FAM]CAACCGCAGCAAGCGAAGTTGA[BHQ1]	118
E. maxima	EmMIC1	MAX-F: TCGTTGCATTCGACAGATTC MAX-R: TAGCGACTGCTCAAGGGTTT MAX-P: [6-FAM]ATTGTCCAGCCAAGGTTCCCTTCG[BHQ1]	138
E. mitis	Mt-A09-716	MIT-F: CAAGGGGATGCATGGAATATAA MIT-R: CAAGACGAATGGAATCAATCTG MIT-P: [6-FAM]CCCGCGAGGGTTTCAGTTGATG[BHQ1]	115
E. necatrix	Nc-AD10-702	NEC-F: AACGCCGGTATGCCTCGTCG NEC-R: GTACTGGTGCCAACGGAGA NEC-P: [6-FAM]CCGTAGCATAGCTCAGGCAGCCAC[BHQ1]	134
E. praecox	Pr-A09-1108	PRA-F: CACATCCAATGCGATATAGGG PRA-R: ACAGAAAAACGCAAAGAGCAA PRA-P: [6-FAM]AGCAGCAGCTGCCTCTCATTGACC[BHQ1]	117
E. tenella	Tn-E03-1161	TEN-F: TCGTCTTTGGCTGGCTATTC TEN-R: CAGAGAGTCGCCGTCACAGT TEN-P: [6-FAM]CTGGAAAGCGTCTCCTTCAATGCG[BHQ1]	100

F= forward primer; R= reverse primer; P= probe.

Table 2.7. PCR annealing temperatures for primers of *Eimeria* species.

	DNA from faecal samples (a)	DNA from sporulated oocyst suspension (b)
E. acervulina	58˚C/55˚C	56˚C
E. brunetti	57˚C	55˚C
E. maxima	56˚C	54˚C
E. mitis	-	55˚C
E. necatrix	64˚C	58˚C
E. praecox	-	54˚C
E. tenella	58˚C	56˚C

Annealing temperatures applied were obtained: (a) using the online tool from: http://www.operon.com/tools/oligo-analysis-tool.aspx, and (b) according to the primer manufacturer's information sheet (Eurofins Scientific, Luxembourg).

PCR products were resolved by agarose-TAE gel electrophoresis as described in section 2.8.5.3.

2.8.5.2 *APPLICATION OF PCR FOR EIMERIA SPECIES DETECTION FROM A SPORULATED OOCYST SUSPENSION*

DNA from a sporulated oocyst suspension extracted by the three DNA extraction methods MpBIO FastDNA® Spin kit for soil (A), MpBIO FastDNA® Spin kit for faeces (B) and Qiagen QiaAMP DNA Stool mini kit (C) was tested by PCR to check for the presence of all seven *Eimeria* species: *E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E.praecox and E. tenella*.

For each species test, a single PCR reaction was performed including six DNA samples (two per extraction method), negative and positive controls. A general PCR protocol was applied as described in the previous section 2.8.5.1, adapting

in each case the species-specific primers used (*Table 2.6*) and the reaction annealing temperature (*Table 2.7*).

2.8.5.3 *AGAROSE GEL ELECTROPHORESIS*

The appropriate amount of molecular biology grade agarose (Melford Laboratories Ltd., UK) was weighed into a conical flask. The required volume of 0.5 x TAE buffer (Bio-Rad Laboratories Inc., USA) was added. The solution was heated in a microwave at low power for 3 minutes, swirled and left to cool down at room temperature. SYBR safe DNA gel stain (Invitrogen™, Thermo Fisher Scientific, USA) was added and mixed gently swirling the flask.

The solution was poured onto a clear plastic tray with a teeth comb, sitting on a balanced horizontal surface. It was left to cool down for one hour. The tray was transferred to an electrophoresis buffer tank, with the top of the gel by the cathode side (negative electrode), and the comb was removed. 0.5 x TAE buffer was used to refill the tank, covering the surface of the gel.

The gel was loaded using a pipette, filling the wells with: 1:10 dilution of Standard ladder (Hyperladder™ IV, Bioline, UK), made with 10 µl of Standard ladder, 20 µl of 5x DNA loading and 70 µl of distilled water. To get a side ladder reference, 5 µl were added to one or several empty wells (first and last column).

For genomic DNA using a 1% w/w agarose gel, a mix of 2.5 µl of 5x DNA loading (Bioline, UK) with 10 µl of DNA sample was prepared on parafilm. After mixing, 10 µl of this mix was added to an empty well. The process was repeated for each DNA sample. For PCR products resolved using a 2% w/w agarose gel, amounts were: 2 µl of 5x DNA loading mixed with 8 µl of DNA sample and 8 µl added to each well. Gel was run for 45-55 min at a voltage of 70-80 V for small to medium gels, and 130 V for larger gels.

The agarose gel was removed from the tank and gently slid onto the transilluminator drawer (Gel Doc XR, Bio-Rad Laboratories Inc., USA), avoiding bubbles underneath the gel. The drawer was closed and UV light switched on.

By using the Gel Doc Bio-Rad software bands could be visualized and pictures saved or printed.

2.8.6 QUANTITATIVE REAL-TIME PCR (QPCR)

2.8.6.1 *CREATION OF STANDARDS OF E. ACERVULINA FOR QPCR*

A PCR reaction was performed including 8 DNA samples from a sporulated oocyst culture, negative and positive controls. The same method as described in 2.8.5.1 was applied, using *E. acervulina* primer pair and adapting the thermocycling conditions (annealing temperature at +56°C and 40 cycles). Following PCR, a 2% w/w agarose gel electrophoresis was performed and pictures of the gel with UV trans illuminator were taken.

PCR products from 4 wells were cleaned using the Isolate II PCR and gel kit (Bioline, UK) to generate standards for the qPCR. Samples were adjusted in volume to 50 µl with ultrapure water, then 1 volume of sample was mixed with 2 volumes of Binding buffer CB (100 µl). An Isolate II PCR column was placed in a 2 ml collection tube and sample was loaded, it was centrifuged for 30 sec at 11,000 *x g* discarding flow. Then 700 µl Wash buffer CW was added to the column and was centrifuged for 30 sec. at 11,000 *x g* discarding flow. This step was repeated one more time. Tube was centrifuged at 11,000 *x g* for 1 min to remove residual ethanol and column was placed into a 1.5 ml MCT. 20 µl Elution buffer C was added directly to silica membrane, followed by incubation at room temperature for 1 min and centrifugation for 1 min at 11,000 *x g.* A pool of clean PCR products (mixing 10 µl/sample) was made and DNA quantitated.

Nine standards (Std) were made, performing a 1:10 dilution series of the previous pool; i.e. Std 1 (10^{-1}) was made of 10 µl of the pooled DNA in 90 µl ultrapure water; Std 2 (10^{-2}) was 10 µl Std 1 added to 90 µl ultrapure water, and so on (up to 10^{-9}).

2.8.6.2 *APPLYING GRADIENT TEMPERATURE QPCR TO SCREEN FOR ALL EIMERIA SPECIES*

Real-time qPCR was used to test for the amplification of all seven *Eimeria* species in a pool of DNA from the DE feeding trial (JT2)

As an attempt to optimise the qPCR reaction, a gradient of temperature was applied, selecting a range that covered all the primer's appropriate working temperatures. The number of cycles applied was higher than the usual for qPCR, and it was decided based on the expectancy of low target DNA concentration. No negative controls or standards were used in the gradient PCR and tests for each species were run in duplicate (using two plate rows). A unique DNA sample was used made by pooling 2 µl from each of the 36 DE feeding trial (JT2) DNA samples, and then diluting it 1:5. From this sample, 5 µl/well was used as template. All qPCR reactions were set up in a PCR clean room, using PCR hoods, racks and filter tips pipettes that had been wiped with 10% ethanol, and then subjected to 10 min UV light sterilization prior to setting up qPCR reactions.

Real-time qPCR reactions were performed in a 96-well PCR plate using SensiFAST Probe No-ROX mix (2x) (Bioline, UK). Primer pairs and probe used to amplify *Eimeria* specific DNA were supplied by Eurofins Scientific (Luxembourg) and are listed in Table 2.6. The PCR reaction mixture components were defrosted on ice, and reactions consisted of: 1x SensiFAST Probe No-ROX mix, 0.4 $pM\mu l^{-1}$ forward primer, 0.4 $pM\mu l^{-1}$ reverse primer, 0.1 $pM\mu l^{-1}$ probe, 5 µl of chicken faecal DNA, and made up to 20 µl with PCR grade water (Roche Ltd). Note that the DNA template was added to the reaction mixture outside of the hood to prevent contamination of the hood and reagents. Plate was sealed with Microseal 'B' adhesive seals (Bio-Rad Laboratories Inc., USA) and pulse centrifuged at 1500 rpm, to be finally thermo-cycled on a Thermo cycler DNA engine Opticon (MJ Research, Bio-Rad Laboratories Inc., USA) as follows: 95°C for 5 min; 55 cycles of denaturing at 95°C for 30 sec, annealing at gradient temperature (between 53°C and 64°C, with 1 degree difference between plate columns) for 30 sec, extension at 72°C for 15 sec; followed by an 8°C final step.

For each pair of samples, an average Ct value was calculated. The four higher Ct values were discarded; among the other values, the two in the higher and lower extremes were also left out. The temperature corresponding to the lowest Ct value was then chosen as best amplification temperature for that particular *Eimeria* species.

2.8.6.3 *APPLYING QPCR SPECIFIC FOR E.ACERVULINA*

Real-time qPCR was used to test for the presence and quantitation of *E. acervulina* in all 36 DNA samples corresponding to control and intervention treatments from week 0 to week 5 from the DE feeding trial (JT2). Three qPCR reactions were performed.

On the first attempt, each week's DNA samples were run in duplicate, six *E. acervulina* standards Std 1 (10^{-1}) to Std 6 (10^{-6}) were added in triplicate and one negative control with water was added. All qPCR reactions were set up in a PCR clean room, using PCR hoods, racks and filter tips pipettes that had been wiped with 10% ethanol, and then subjected to 10 min UV light sterilization prior to setting up qPCR reactions.

Real-time qPCR reaction was performed in a 96-well PCR plate using SensiFAST Probe No-ROX mix (2x) (Bioline, UK). Primer pairs and probe used to amplify *E. acervulina* DNA were supplied by Eurofins Scientific (Luxembourg) and are listed in Table 2.6. The qPCR reaction mixture components were defrosted on ice, and reactions consisted of: 1x SensiFAST Probe No-ROX mix, 0.4 $pM\mu l^{-1}$ forward primer, 0.4 $pM\mu l^{-1}$ reverse primer, 0.1 $pM\mu l^{-1}$ probe, 5 µl of chicken faecal DNA, and made up to 20 µl with PCR grade water (Roche Ltd). Note that the DNA template was added to the reaction mixture outside of the hood to prevent contamination of the hood and reagents. Plate was sealed with Microseal 'B' adhesive seals (Bio-Rad Laboratories Inc., USA) and pulse centrifuged at 1500 rpm, to be finally thermo-cycled on a CFX96 Touch Real-Time PCR Detection System including C1000 thermal cycler chassis & CFX96 optical reaction module in software-controlled mode (Bio-Rad Laboratories Inc., USA), as follows: 95°C for 5 min; 55 cycles of denaturing at 95°C for 30 sec,

annealing at 62°C for 30 sec, extension at 72°C for 15 sec; followed by an 8°C final step.

On the second attempt, the same method was followed with some modifications: the standards used were less concentrated, Std 4 (10^{-4}) to Std 9 (10^{-9}) and were added in triplicate, as well as three negative controls with water. The number of cycles was reduced to 40 instead of 55.

The same procedure as explained before was applied on a third attempt, but using a fresh probe aliquot.

2.9 STATISTICAL ANALYSIS

Data reported were analysed using SPSS software package version 22 (SPSS, Chicago, IL, USA). Two-way and one-way ANOVA were carried out. FEC data were transformed by $\log_{10}$ before statistical analysis. Means between groups were tested by a Tukey-test and differences were considered significant at $P<0.05$. P between 0.1 and 0.05 were considered trends. Multivariate analyses, including Permutational Analysis of variance (Adonis) and Principal Component Analysis (PCA), were performed using the package “vegan” from the R statistical program (R Core Team, 2014).

CHAPTER THREE

RESULTS

3.1 PARASITOLOGY

3.1.1 PARASITE IDENTIFICATION

Three sets of chicken faecal samples were collected randomly from both the control and intervention sheds of the DE feeding trial, once a week for 5 weeks. Faecal samples were subjected to faecal egg counts (FEC) using McMaster and Modified Stoll methods.

Eight parasite species eggs were identified by optical microscopy in the faecal samples of the DE feeding trial (JT2). These were classified according to their taxa as follows: nematodes (roundworms): *Ascaridia galli*, *Heterakis gallinarum*, *Capillaria spp.* and *Trichostrongylus tenius*; cestodes (tapeworms): *Railletina spp.* and *Choanotaenia infundibulum*; trematodes: *Echinostoma spp.* and finally, protozoa (coccidia): *Eimeria spp.* A complete list of pictures is shown in the Appendix.

The most commonly encountered parasite species eggs were *Ascaridia galli*, *Capillaria spp., Eimeria spp.* and *Heterakis gallinarum*. From the genera *Capillaria and Eimeria,* several species were present in the samples. Presumably the species of *Capillaria* responsible for caecal (*C. anata*) and intestinal capillariosis (*C. bursata, C. caudinflata and C. obsignata)* were detected in the faecal samples by optical microscopy.

3.1.2 FAECAL EGG COUNTS (FEC) USING THE METHODS Mc MASTER AND MODIFIED STOLL

3.1.2.1 *EFFECT OF THE FEC METHOD ON THE CONCENTRATION OF PARASITES*

The two counting methods used had different sensitivities (*Figure 3.2*), which affected the estimation of parasite counts. A highly significant difference (p<0.01) in egg counts between the two counting methods was identified for the parasite species *Ascaridia galli*, *Eimeria spp*. and *Heterakis gallinarum*; in contrast, results were not significant for *Capillaria spp.* (p>0.05) (*Table 3.1* and *Figure 3.1*).

Table 3.1. Effect of the FEC method on the concentration of parasites in free-range organic laying hens. Hens were fed a basal organic diet for five weeks. McMaster and Modified Stoll FEC methods were performed once a week (N= 30). Results expressed in log_{10} eggs/g faeces. SEM: standard error of the means.

	Mc Master	Mod. Stoll	SEM	P
Ascaridia galli	1.27^{a}	1.71^{b}	0.090	0.001
Heterakis gallinarum	1.39^{a}	1.87^{b}	0.090	0.0004
Capillaria spp.	1.07	1.20	0.101	0.342
Eimeria spp.	2.41^{a}	1.84^{b}	0.065	<0.0001

Superscripts indicate differences between means.

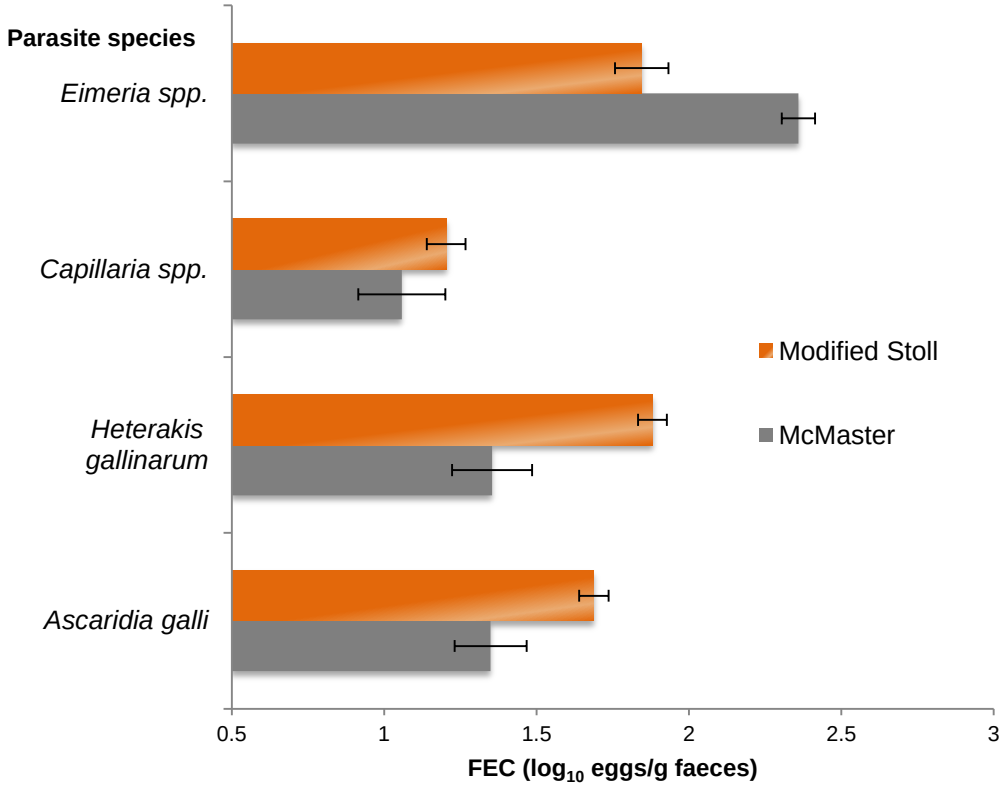


Figure 3.1. Effect of the FEC method on the concentration of parasites in free-range organic laying hens. Hens were fed a basal organic diet for five weeks. McMaster and Modified Stoll FEC methods were performed once a week (N= 30). Error bars indicate the standard error of the means (SEM).

Sensitivity differed between the two counting methods (*Figure 3.2*). In the case of *Eimeria spp.,* all the samples tested (N= 30) were positive to both FEC methods. Modified Stoll method detected Ascarids (*A. galli* and *H. gallinarum*) and *Capillaria spp.* in a higher number of samples than McMaster method.

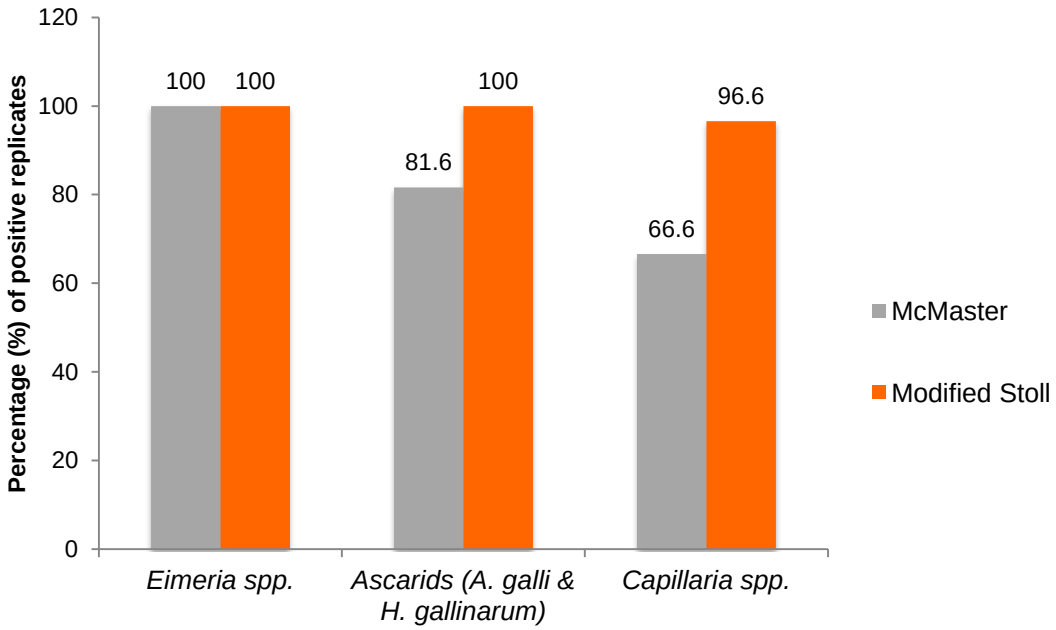


Figure 3.2. FEC method sensitivity. Faecal samples from free-range organic laying hens fed a basal organic diet for five weeks were subjected to faecal egg counts (FEC) once a week, using McMaster and Modified Stoll methods (N= 30). Data is expressed as percentage (%) of positive replicates.

3.1.2.2 *EFFECT OF THE DIET ON THE CONCENTRATION OF PARASITES*

Dietary treatment did not have a significant effect in parasite counts (p>0.05), no matter which FEC method was used (*Tables 3.2 and 3.3*). The intervention group had slightly higher overall mean counts for all parasite species with the exception of *Eimeria spp.* by McMaster method, with lower FEC than CTR FEC (*Table 3.2)*. Nevertheless, these results were not statistically significant.

Table 3.2. Effect of the dietary inclusion of DE on the concentration of parasites in free-range organic laying hens. Hens were fed a basal organic diet with (INT) and without (CTR) supplementation of diatomaceous earth (DE) for five weeks. FEC were performed in faecal samples once a week using the McMaster method. Results expressed in log_{10} eggs/g faeces. SEM: Standard error of the means.

	CTR	INT	SEM	P
Ascaridia galli	1.13	1.41	0.161	0.228
Heterakis gallinarum	1.23	1.55	0.172	0.205
Capillaria spp.	1.02	1.11	0.190	0.743
Eimeria spp.	2.45	2.38	0.071	0.454

Table 3.3. Effect of the dietary inclusion of DE on the concentration of parasites in free-range organic laying hens. Hens were fed a basal organic diet with and without supplementation of diatomaceous earth (DE) for five weeks. FEC were performed in faecal samples once a week for five weeks using the Modified Stoll method. Results expressed in log_{10} eggs/g faeces. SEM: Standard error of the means.

	CTR	INT	SEM	P
Ascaridia galli	1.68	1.74	0.055	0.405
Heterakis gallinarum	1.83	1.90	0.055	0.400
Capillaria spp.	1.16	1.24	0.078	0.446
Eimeria spp.	1.82	1.87	0.093	0.713

3.1.2.3 *EFFECT OF THE DIET ON THE CONCENTRATION OF EIMERIA SPP. USING MCMASTER METHOD*

No significant effect of the diet was identified with respect to FEC counts of *Eimeria spp.* oocysts in collected faecal samples over the five-week trial period.

The parasite dynamics were different compared to the ones for *A. galli* and *H. gallinarum* (*Figures 3.4 and 3.5*). The lowest FEC values of *Eimeria spp.* were reached by week 4, and then there was a rebound on week 5 (*Figure 3.3*).

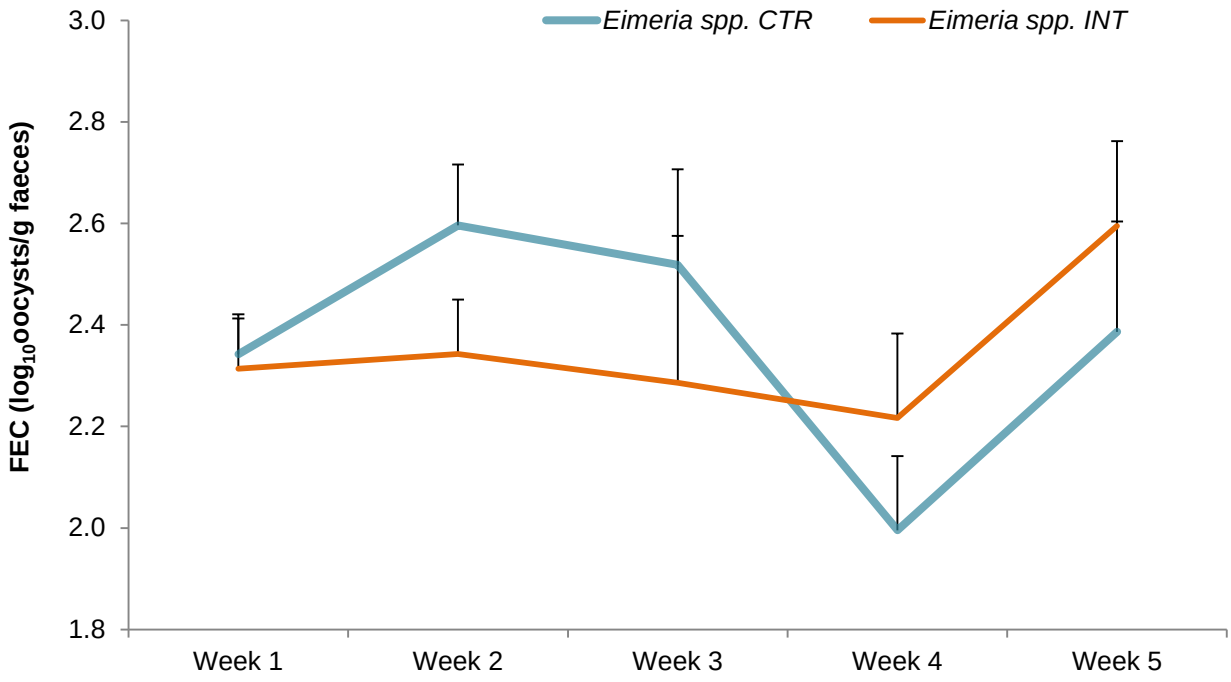


Figure 3.3. Effect of the dietary inclusion of DE on the *Eimeria spp.* FEC in free-range organic laying hens. Hens were fed a basal organic diet with (INT) and without (CTR) supplementation of diatomaceous earth (DE) for five weeks. FEC were performed in faecal samples once a week using the McMaster method (N= 3). Error bars indicate the standard error of the means (SEM).

The FEC of Eimeria oocysts in intervention samples remained stable up to week 4 at between 2.2 and 2.4 $\log_{10}$ oocysts per gram of faeces, and then it reached its highest value by week 5. CTR FEC followed a decreasing trend, with a marked descent by week 4 and a rebound by week 5 (*Figure 3.3*). These results were not significant.

3.1.2.4 *EFFECT OF THE DIET ON THE CONCENTRATION OF ASCARIDS USING M. STOLL METHOD*

The interaction week*diet was significant ($p<0.05$) for both *A. galli* and *H. gallinarum* parasites FEC.

Intervention group reduced in 27% the initial FEC for both *A. galli* and *H. gallinarum* parasites (comparison of FEC at week 1 and at week 5), but this

difference was not significant. The FEC for the intervention group were higher than those for the control group at the beginning of the trial, but they remained lower from week 3 until the end of the trial with no statistical significance (*Figures 3.4 and 3.5*).

Despite the decreasing trend in Ascarids FEC observed for the Intervention group, overall FEC mean values of each parasite species were higher but not significant for the Intervention group (*Table 3.3*).

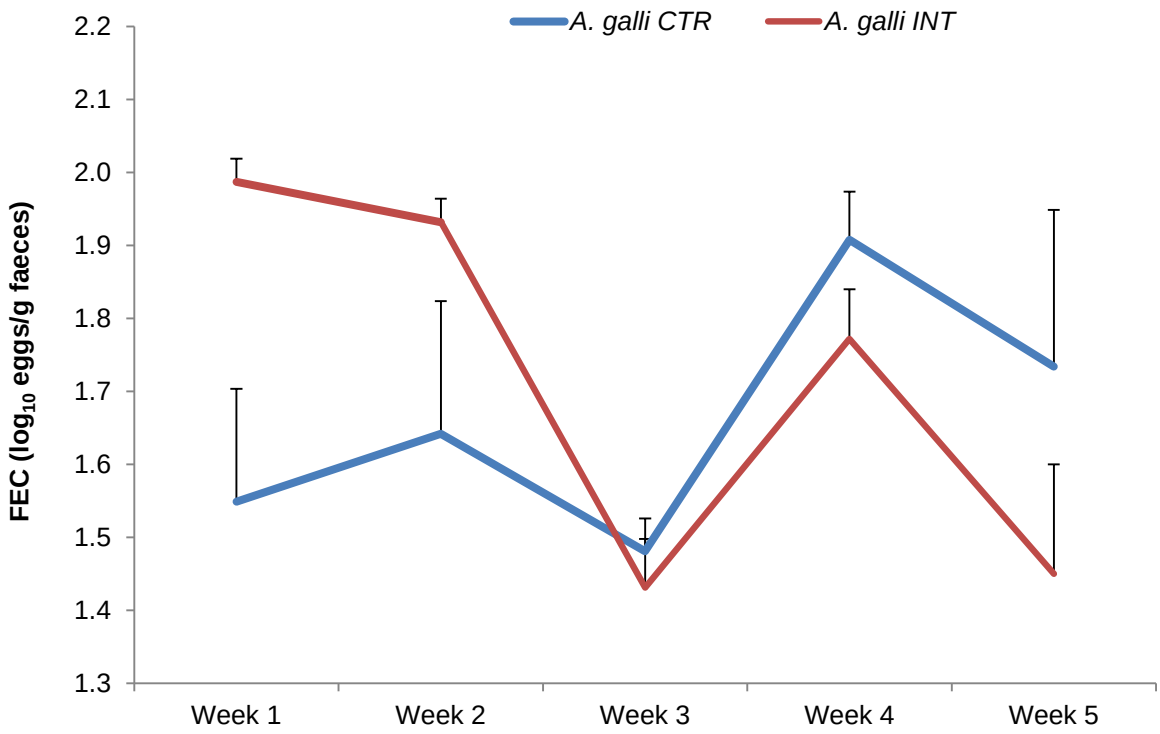


Figure 3.4. Effect of the dietary inclusion of DE on the *A. galli* FEC in free-range organic laying hens. Hens were fed a basal organic diet with (INT) and without (CTR) supplementation of diatomaceous earth (DE) for five weeks. FEC were performed in faecal samples once a week using the Modified Stoll method (N= 3). Error bars indicate the standard error of the means (SEM).

For *A. galli* parasites, INT FEC followed a decreasing trend overall, with a marked descent by week 3, although there was a rebound by week 4. These FEC reduced in 27% from week 1 (1.99 $\log_{10}$) eggs per gram of faeces) to week 5 (1.45 $\log_{10}$ eggs per gram of faeces). CTR FEC followed an increasing

trend from week 3 onwards, with the highest value by week 4 (*Figure 3.4*). These results were not significant.

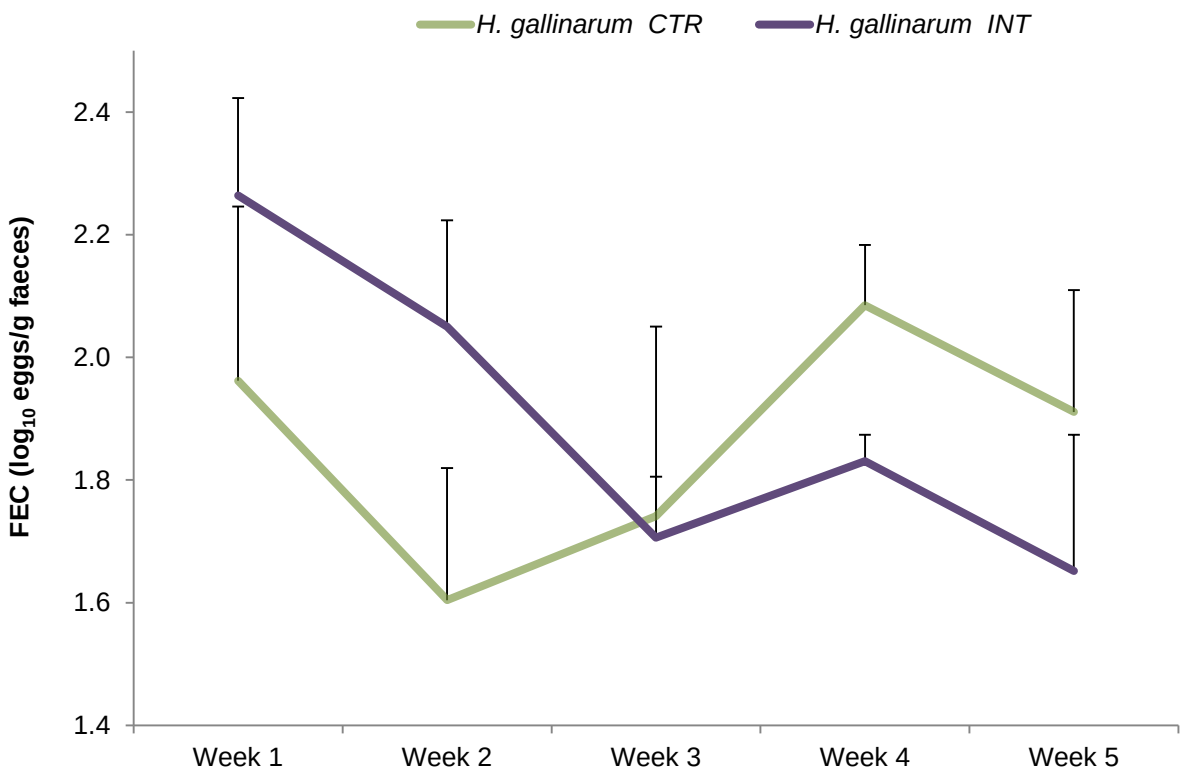


Figure 3.5. Effect of the dietary inclusion of DE on the *H. gallinarum* FEC in free-range organic laying hens. Hens were fed a basal organic diet with (INT) and without (CTR) supplementation of diatomaceous earth (DE) for five weeks. FEC were performed in faecal samples once a week using the Modified Stoll method (N= 3). Error bars indicate the standard error of the means (SEM).

For *H. gallinarum* parasites, INT FEC followed a decreasing trend, with a marked descent by week 3 and a lowest value by week 5. These FEC reduced in 27% from week 1 (2.26 $\log_{10}$) eggs per gram of faeces) to week 5 (1.65 $\log_{10}$ eggs per gram of faeces). CTR FEC followed an increasing trend, with the highest value by week 4 (*Figure 3.5*). These results were not significant.

3.1.2.5 *EFFECT OF THE DIET ON THE CONCENTRATION OF CAPILLARIA SPP. USING M. STOLL METHOD*

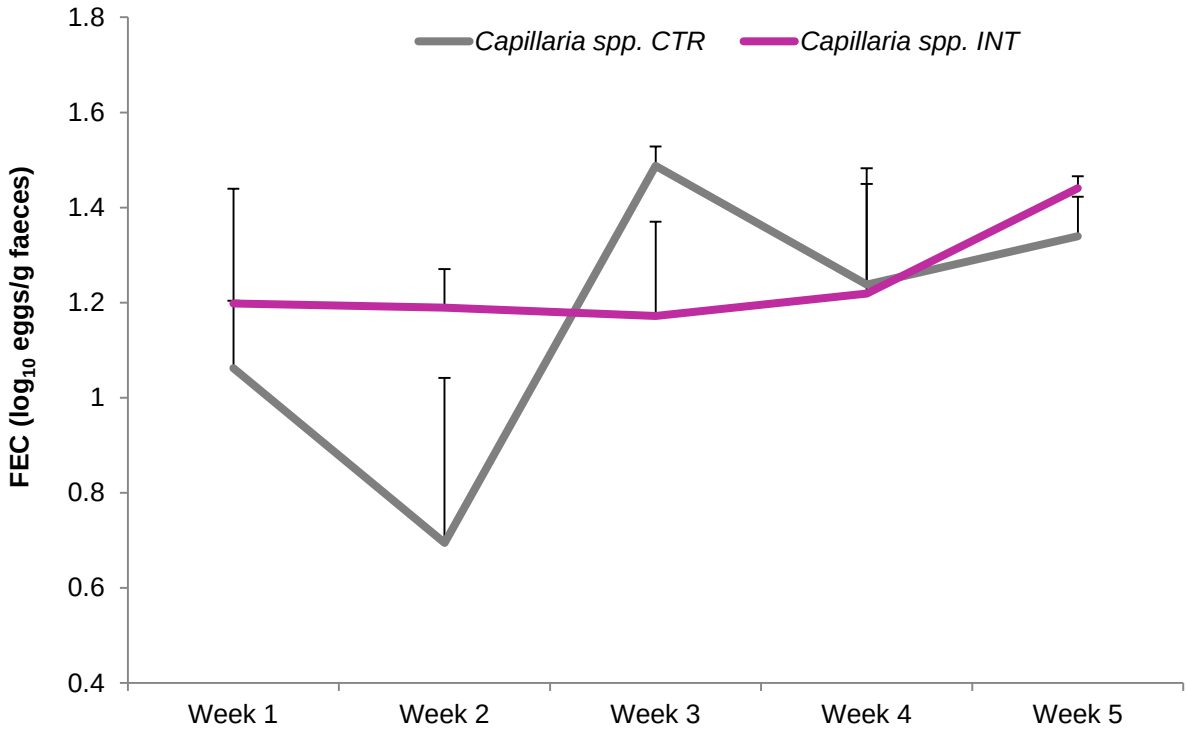


Figure 3.6. Effect of the dietary inclusion of DE on the *Capillaria spp.* FEC in free-range organic laying hens. Hens were fed a basal organic diet with (INT) and without (CTR) supplementation of diatomaceous earth (DE) for five weeks. FEC were performed in faecal samples once a week using the Modified Stoll method (N= 3). Error bars indicate the standard error of the means (SEM).

For *Capillaria spp.* parasites, INT FEC remained stable up to week 4, and then reached its highest value by week 5, similarly to *Eimeria spp*. INT FEC. A considerable increase in CTR FEC was detected between weeks 2 and 3, from where the counts remained higher than the previous weeks (*Figure 3.6*). These results were not significant.

3.2 EFFECT OF DE ON EGG PRODUCTION

3.2.1 DAILY EGG PRODUCTION

The dietary intervention group produced numerically more eggs per day (195) than the control group (191) (*Figure 3.7*).

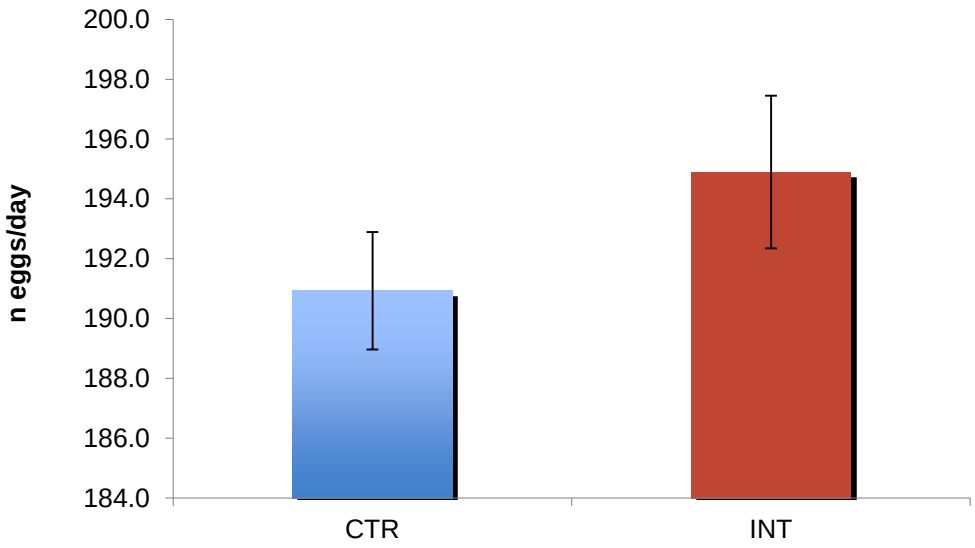


Figure 3.7. Average daily egg production in the experimental flock of free-range organic laying hens (220 per treatment). Hens were fed a basal organic diet with (INT) and without (CTR) supplementation of diatomaceous earth (DE) for five weeks. Eggs were counted approximately once a day for five weeks (N= 28). Error bars indicate the standard error of the means (SEM).

3.2.2 DAILY EGG PRODUCTION PER WEEK

The average eggs per day every week from 1 to 5 was slightly higher for INT group, with a larger difference on week 2 (*Figure 3.8*). However, due to lack of replicates, these data were not statistically analysed.

The dietary intervention group produced an average of 22.2 eggs more per week, so meaning a positive difference of 1% of egg production overall. Considering the size of the studied flock, the minimal differences found between flocks do not allow to infer a relevant effect due to the applied

experimental treatment and its productive consequences on the poultry industry.

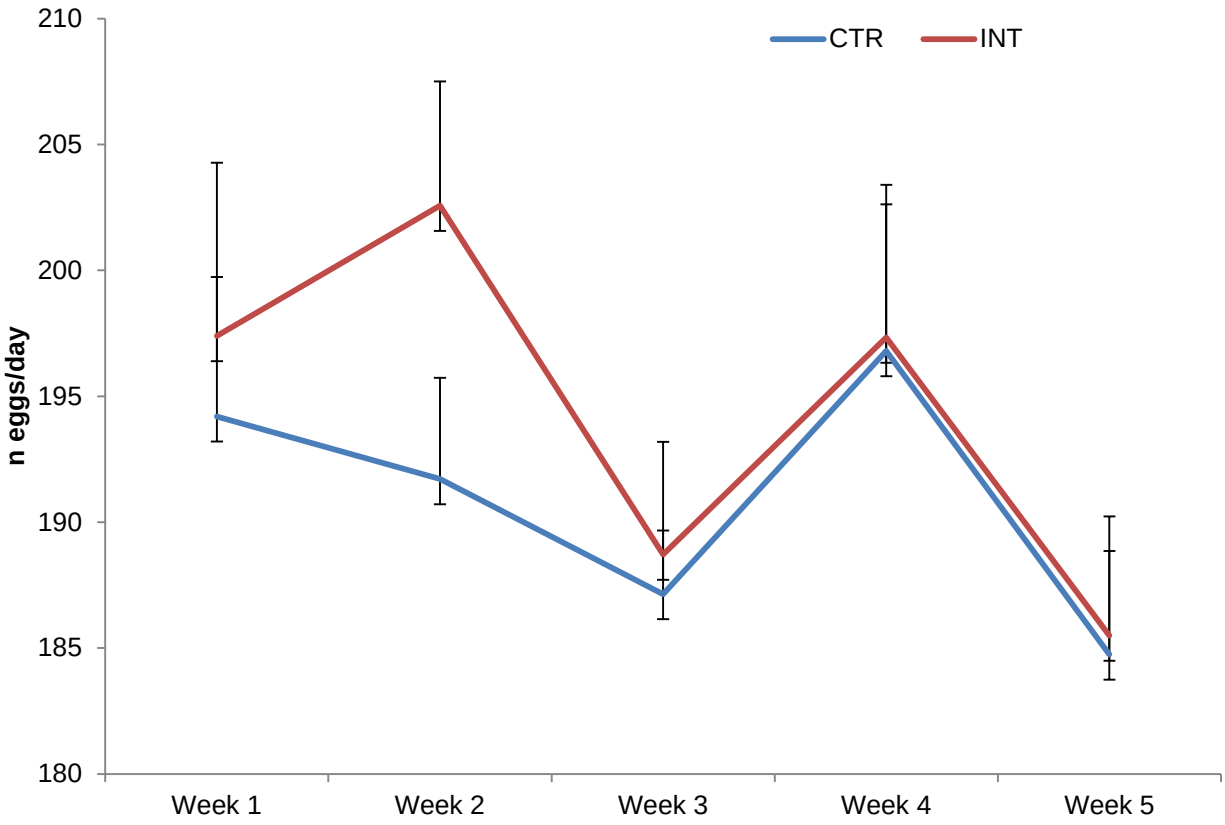


Figure 3.8. Average of daily egg production by dietary treatment in the experimental flock of free-range organic laying hens (220 per treatment). Hens were fed a basal organic diet with (INT) and without (CTR) supplementation of diatomaceous earth (DE) for five weeks. Eggs were counted approximately once a day for five weeks. Error bars indicate the standard error of the means (SEM).

3.2.3 LAYING INDEX

Laying index or egg production per animal and day was calculated following the formula:

Laying index = Q/(N*k)

Where:

Q is number of eggs laid in the period.

N is number of hens.

k is number of days (observations).

Hens belonging to INT group were more productive than CTR hens, as the egg production per animal and day in the former (0.88) was higher than in the latter (0.86) (*Figure 3.9*). However, due to lack of replicates, these data were not statistically analysed.

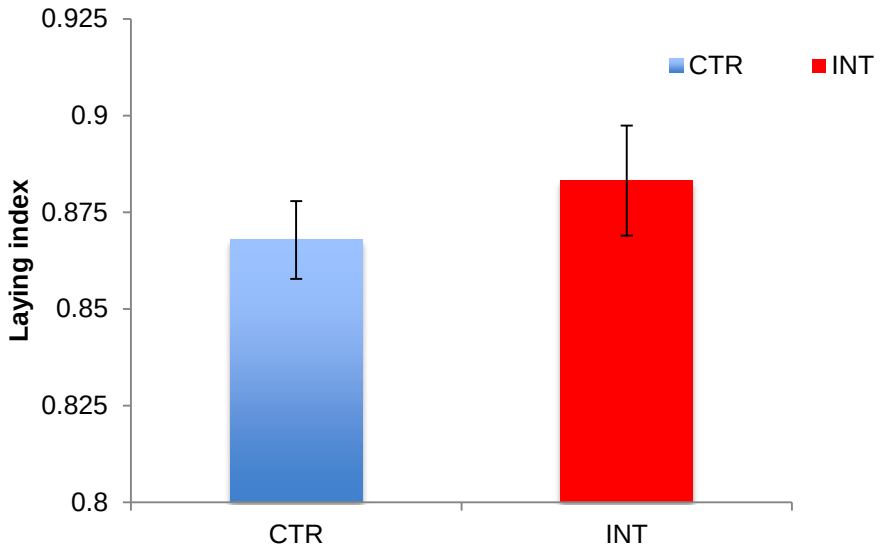


Figure 3.9. Laying index in the experimental flock of free-range organic laying hens (220 per treatment). Hens were fed a basal organic diet with (INT) and without (CTR) supplementation of diatomaceous earth (DE) for five weeks. Eggs were counted approximately once a day for five weeks (N= 28). Error bars indicate the standard error of the means (SEM).

3.3 EGG QUALITY

3.3.1 EXTERNAL EGG QUALITY

External egg quality results are shown in Table 3.4a and 3.4b. Pictures corresponding to external egg quality are shown in the Appendix.

Table 3.4a. External egg quality features and component weights of free-range organic eggs. Hens were fed a basal organic diet with (INT) and without (CTR) supplementation of diatomaceous earth (DE) for five weeks. Egg sampling was performed at weeks one and five of the trial, collecting 15 eggs per treatment for three consecutive days (N= 45). SEM: Standard error of the means.

	Week 1		Week 5			Significance		
	CTR	INT	CTR	INT	SEM	Diet	Week	Diet*Week
Whole egg weight (g)	66.77	66.0	66.8	66.3	0.70	NS	NS	NS
Dried shell weight (g)	6.59	6.81	6.60	6.55	0.102	NS	NS	NS
Albumen weight (g)	41.5	40.9	41.7	41.1	0.55	NS	NS	NS
Yolk weight (g)	18.7	18.7	18.5	18.8	0.21	NS	NS	NS
Eggshell density (mg/cm^2)	85.6	89.3	85.9	85.7	1.08	NS	NS	T
Specific gravity (g/L)	1086.9^b	1090^a	1086.9^b	1086.2^b	0.92	NS	*	*

***T means 0.1>P>0.05; * means P<0.05; NS means P>0.1*. *Superscripts indicate differences between means*.**

Over the 5-week period, hens fed the DE intervention (INT) diet laid eggs that showed an increase in the whole egg weight, and albumen and yolk weight by week 5, but these results were not significant (*Table 3.4a*). Nevertheless when compared to eggs laid by hens fed the control (CTR) diet, only the yolk weight in INT was higher than that from CTR eggs. The weight of the eggshell, eggshell density and specific gravity decreased in INT by week 5. All these values remained lower than in eggs collected from CTR fed hens. The interaction diet*week and the differences between weeks were significant for egg specific gravity, although the differences between diets were not. A trend in the interaction diet*week was identified for eggshell density.

Table 3.4b. External egg quality features of free-range organic eggs. Hens were fed a basal organic diet with (INT) and without (CTR) supplementation of diatomaceous earth (DE) for five weeks. Egg sampling was performed at weeks one and five of the trial, collecting 15 eggs per treatment for three consecutive days (N= 45). Results expressed in percentage (%).

	Week 1		Week 5	
	CTR	INT	CTR	INT
Egg features				
Dirty	26.7	24.4	20	24.4
Cracked	6.7	4.4	6.7	4.4
Pinholes	2.2	0	2.2	4.4
Pimples	6.7	15.5	24.4	33.3
Body checks	4.4	11.1	8.9	11.1
Rough texture	20	8.9	11.1	8.9
Weak shell areas				
1	8.9	6.7	15.5	4.4
2	6.7	4.4	2.2	4.4
3	2.2	8.9	0	0
Mottled/glassy	4.4	0	3.3	13.3

"Weak shell areas" are referred to poor calcification areas in the shell. Graded as: 1 = < 0.5 cm^2 surface; 2 = in several scattered areas, < 2 cm^2 in total; 3 = many lighter shell areas or bigger surface cover

The number of dirty and cracked eggs (with star or hairline cracks) was the same between weeks for both treatments (*Table 3.4b*). Eggs collected from INT fed hens accounted a higher number of eggs with pinholes (double) and mottled/glassy eggs (four times more) compared to CTR fed hens at week 5. Due to lack of replicates, these data were not statistically analysed.

The number of eggs with pimples was higher for both treatments at week 5. For CTR fed hen eggs, this increased by four times and INT fed hen eggs doubled the amount of eggs affected, still INT was higher than CTR at week 5. The number of eggs with body checks or rough texture did not change between weeks for INT. By week 5, weak shell areas were smaller and fewer

eggs were affected for INT. As mentioned before, these data were not statistically analysed.

3.3.2 INTERNAL EGG QUALITY

Internal egg quality results are shown in Table 3.5a and 3.5b. Pictures corresponding to internal egg quality are shown in the Appendix.

Table 3.5a. Internal egg quality features of free-range organic eggs. Hens were fed a basal organic diet with (INT) and without (CTR) supplementation of diatomaceous earth (DE) for five weeks. Egg sampling was performed at weeks one and five of the trial, collecting 15 eggs per treatment for three consecutive days (N= 45). SEM: Standard error of the means.

	Week 1		Week 5			Significance		
	CTR	INT	CTR	INT	SEM	Diet	Week	Diet*Week
Albumen features								
Height (mm)	5.6	5.8	6	6.2	0.13	NS	*	NS
Haugh units	69.9	72.5	74.2	75.6	1.17	NS	*	NS
Yolk features								
DSM colour	8.6	9.1	10.3	10.5	0.181	T	*	NS
Blood spots per egg	0.53^{b}	0.36^{b}	0.4^{b}	1^{a}	0.161	NS	NS	*
Meat spots per egg	0.822	0.756	0.467	0.556	0.174	NS	NS	NS

***T means 0.1>P>0.05; * means P<0.05; NS means P>0.05*. *Superscripts indicate differences between means*.**

Albumen height, Haugh units and DSM yolk colour increased their values for INT by week 5. These values were higher than CTR (*Table 3.5a*). The differences between weeks were significant but the interaction diet*week was not. An increase of approx. 1.5 points in the mean values of yolk colour, measured with the DSM Yolk Colour Fan scale, was identified for both treatments. INT accounted higher scores than CTR In both weeks 1 and 5, meaning darker yolks. For this feature, differences between weeks were significant, diet was a trend and the interaction diet*week was nor significant.

INT increased the number of blood spots and decreased meat spots by week 5. Both values were higher than CTR but not significant. A trend in the interaction diet*week was identified for blood spots.

Table 3.5b. Internal egg quality features of free-range organic eggs. Hens were fed a basal organic diet with (INT) and without (CTR) supplementation of diatomaceous earth (DE) for five weeks. Egg sampling was performed at weeks one and five of the trial, collecting 15 eggs per treatment for three consecutive days (N= 45). Results expressed in percentage (%).

	Week 1		Week 5	
	CTR	INT	CTR	INT
Watery white	8.90	6.70	4.40	6.70
Blood spots	22.2	22.2	22.2	44.4
Meat spots	35.6	37.8	24.4	33.3
Discoloured yolk	-	-	-	4.4

INT accounted higher number of eggs affected by watery whites or discoloured yolks compared to CTR by week 5 (*Table 3.5b*). Up to one third of the eggs examined presented inclusions (blood or meat spots); the number of meat spots decreased for both treatments but blood spots increased for INT by week 5. Due to lack of replicates, these data were not statistically analysed.

3.3.3 MULTIVARIATE ANALYSIS

The most relevant egg quality features (weights of: whole egg, dried shells, albumen and yolk; eggshell density, specific gravity, Haugh units, DSM colour, blood and meat spots) were analyzed together following a principal component analysis (PCA), and a permutational analysis of variance (PERMANOVA) to study the effect of the use of DE in the egg quality in overall.

Results from the PCA analysis are shown in Figures 3.10 and 3.11.

Despite the differences observed in the PCA plots, PERMANOVA analysis didn't show any statistical difference due to the interaction treatment x time, and hence the differences observed were not high enough to be considered significant.

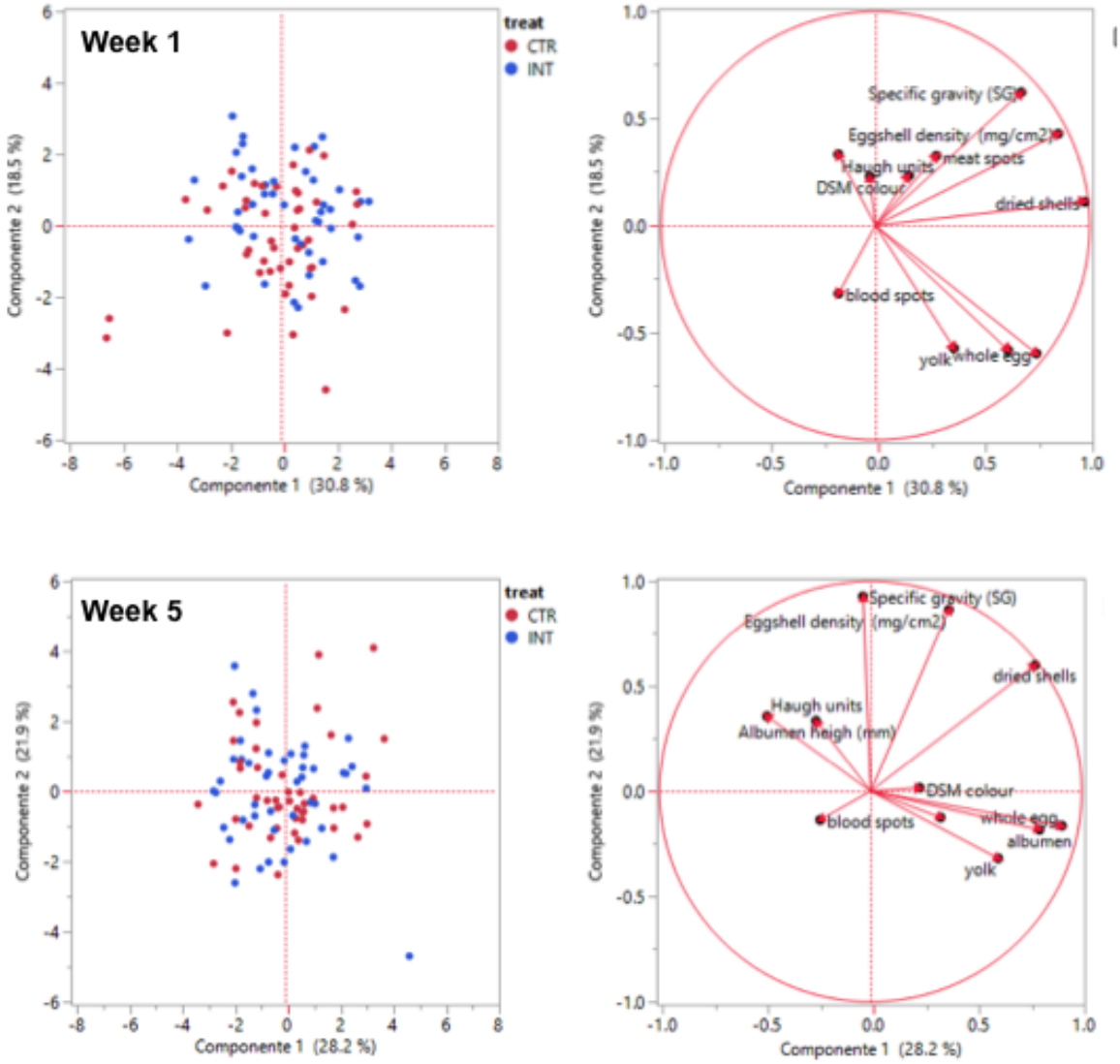


Figure 3.10. PCA from egg quality features data in week 1 (upper figures) and in week 5 (lower figures). Left figures show the PCA biplots and right figures show the egg quality variables vectors. Red dots indicate CTR samples and blue dots indicate INT samples.

As shown in Figure 3.10, there is not an evident treatment effect on the external quality of the egg in any of the studied time points, that is no overall differences were found between CTR and INT neither at week 1 nor at week 5. However, when same analysis was performed within treatment, we could see a grouping effect in INT that wasn't observed in CTR (*Figure 3.11*). Thus, INT eggs from week 5 showed an overall egg quality different to that observed at week 1. This variation was partially explained by the changes in eggshell density, specific gravity and dried shell mass, which seemed to decrease throughout the experimental period in INT samples.

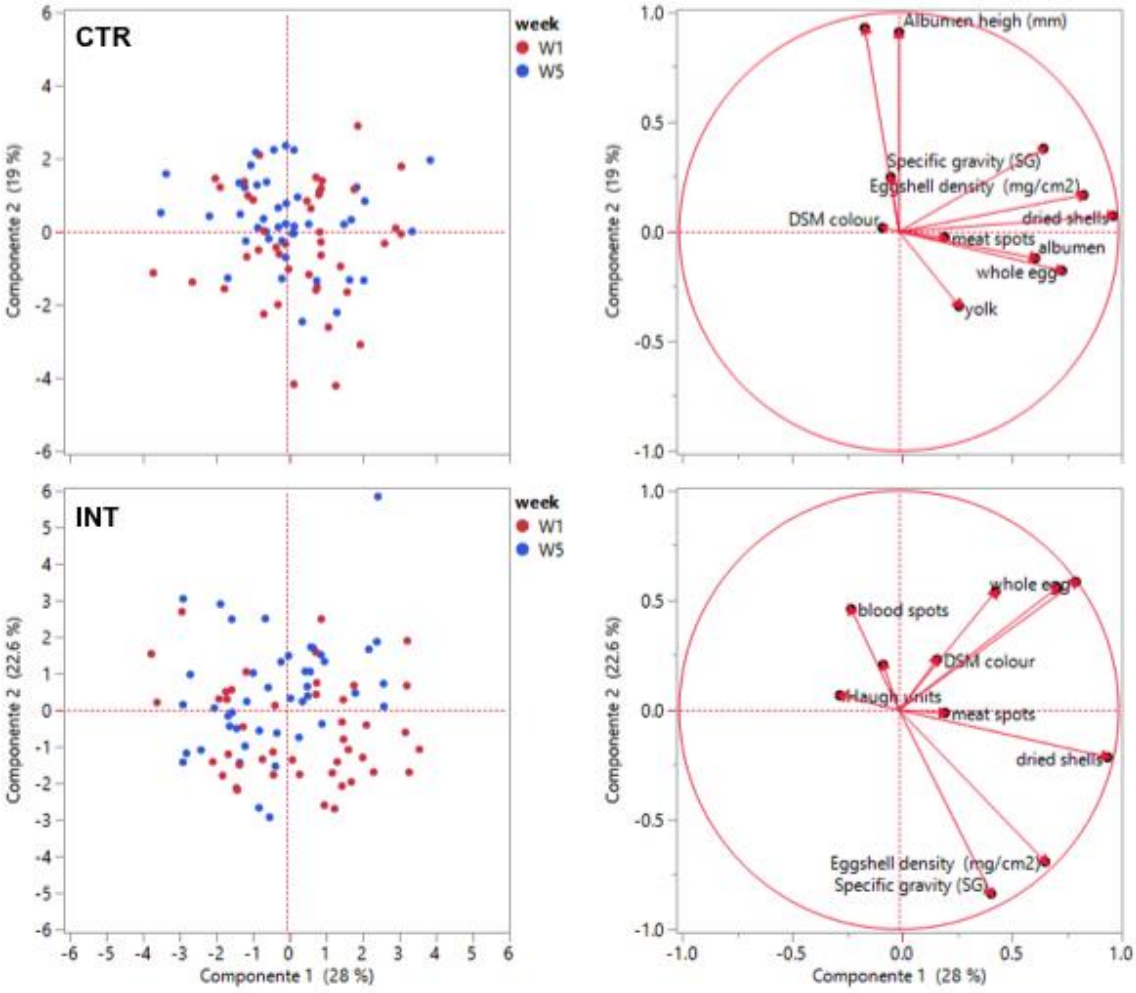


Figure 3.11. PCA from egg quality features data for CTR (upper figures) and for INT (lower figures). Left figures show the PCA biplots and right figures show the egg quality variables vectors. Red dots indicate Week 1 samples and blue dots indicate Week 5 samples.

3.4 EGG COMPONENT ANALYSES: NITROGEN TO PROTEIN IN EGG ALB & YOLK

Protein content of yolk and albumen from week 5 samples showed small variation between CTR and INT (*Table 3.6*), with not statistically significance.

Table 3.6. Protein content in free-range organic eggs. Hens were fed a basal organic diet with (INT) and without (CTR) supplementation of diatomaceous earth (DE) for five weeks. Freeze-dried albumen samples from CTR (N= 20) and INT (N= 20), and yolk samples from CTR (N= 8) and INT (N= 11) from eggs collected at week 5 were analyzed. Results expressed in g/100 g MS. SEM: Standard error of the means.

	CTR	INT	SEM	Significance
Albumen	82	81	0.55	NS
Yolk	31.2	31.4	0.42	NS

3.5 FEED ANALYSES

Analyses of feed samples taken on week 3 and week 5 of the trial showed that INT feed had less protein and was richer in organic matter and fat compared to CTR feed (*Table 3.7*). These data were not statistically analysed.

Table 3.7. Feed component analysis of feed applied to an experimental flock of free-range organic laying hens. Hens were fed a basal organic diet with (INT) and without (CTR) supplementation of diatomaceous earth (DE) for five weeks. Feed sampling was performed at weeks one and five of the trial.

Feed component	CTR	INT
DM (g/100 g FM)	86.9	86.3
OM (g/100 g DM)	87.3	87.9
Crude fat (g/100 g DM)	17.3	18.2
Protein (g/100 g DM)	12.9	12.3

3.6 MOLECULAR BIOLOGY APPLIED TO CHICKEN FAECAL SAMPLES

3.6.1 DNA EXTRACTION AND QUANTITATION

3.6.1.1 *FREEZE DRIED CHICKEN FAECES*

A DNA extraction method comparison for chicken faeces was performed, using the methods: MpBIO FastDNA® Spin kit for soil (A), MpBIO FastDNA® Spin kit for faeces (B), Qiagen QiaAMP DNA Stool mini kit (C) and Modified phenol-clorophorm (D). Results are shown below (*Table 3.8 and Figure 3.12*).

Table 3.8. DNA quantitation using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). DNA from a randomly selected freeze-dried faecal sample from the DE feeding trial (JT2) with four technical replicates was extracted by four DNA extraction methods: MpBIO FastDNA® Spin kit for soil (A), MpBIO FastDNA® Spin kit for faeces (B), Qiagen QiaAMP DNA Stool mini kit (C) and Modified phenol-clorophorm (D).

Method	ng/µl	260/280	260/230
A. MpBIO FastDNA® Spin kit for soil	264.48	1.52	0.91
B. MpBIO FastDNA® Spin kit for faeces	31.59	1.80	0.19
C. Qiagen QiaAMP DNA Stool mini kit	121.52	1.99	1.80
D. Modified phenol-clorophorm	1000.22	1.88	1.68

DNA extracted by the modified phenol-clorophorm showed the higher DNA yield, whereas the lower DNA yield was obtained with the MpBIO FastDNA® Spin kit for faeces. In terms of quality scores (260/280 and 260/230 ratios) both kits from MpBIO showed poor 260/230 ratios (below 1). Taking into account all the studied parameters (DNA yield and purity ratios), The Qiagen QiaAMP DNA Stool mini kit method was selected as most appropriate, due to

the good 260/280 and 260/230 ratios and reasonably good DNA yield and purity of DNA extracted using this technique.

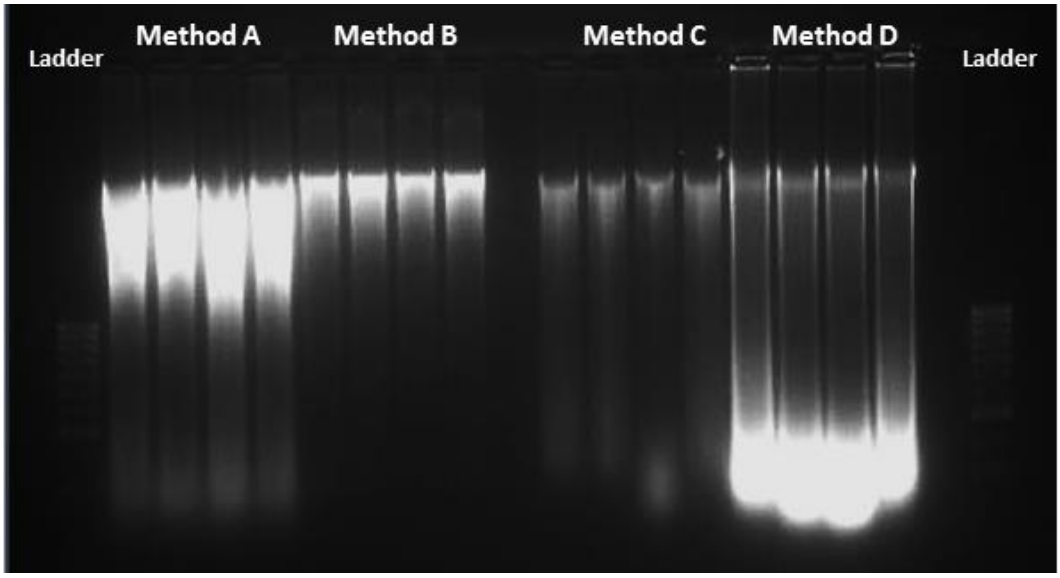


Figure 3.12. Agarose gel (1%) of DNA from a randomly selected freeze-dried faecal sample from the DE feeding trial (JT2) with four technical replicates, extracted by four DNA extraction methods: MpBIO FastDNA® Spin kit for soil (A), MpBIO FastDNA® Spin kit for faeces (B), Qiagen QiaAMP DNA Stool mini kit (C) and Modified phenol-clorophorm (D).

The effect of the dietary inclusion of DE on the concentration of *Eimeria* parasites in free-range organic laying hens was studied in the DE feeding trial. Faecal samples were collected once a week for five weeks from hens fed a basal organic diet with (INT) and without (CTR) supplementation of diatomaceous earth (DE). DNA extraction was performed using the Qiagen QiaAMP DNA Stool mini kit method (*Table 3.9).*

Table 3.9. DNA quantitation using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). DNA from a total of 36 freeze-dried and homogenised faecal samples from the DE feeding trial (JT2) were extracted by the Qiagen QiaAMP DNA Stool mini kit method.

Trial week	Treatment	Sample	ng/µl	A260	A280	260/280	260/230
Week 0	CTR	W0 CTR1	154.31	3.086	1.466	2.1	2.05
		W0 CTR2	95.55	1.911	0.974	1.96	1.73
		W0 CTR3	71.4	1.428	0.711	2.01	1.74
	INT	W0 INT1	103.66	2.073	1.015	2.04	1.95
		W0 INT2	62.84	1.257	0.637	1.97	1.58
		W0 INT3	98.02	1.96	1.009	1.94	1.64
Week 1	CTR	W1 CTR1	71.73	1.435	0.719	1.99	2.75
		W1 CTR2	124.24	2.485	1.209	2.06	1.93
		W1 CTR3	82.75	1.655	0.804	2.06	1.92
	INT	W1 INT1	155.87	3.117	1.5	2.08	2.05
		W1 INT2	94.35	1.887	1.019	1.85	1.75
		W1 INT3	121.515	2.43	1.221	1.99	1.79
Week 2	CTR	W2 CTR1	63.69	1.274	0.701	1.82	1.38
		W2 CTR2	77.97	1.559	0.818	1.91	1.43
		W2 CTR3	63.65	1.273	0.577	2.21	1.7
	INT	W2 INT1	69.95	1.399	0.751	1.86	1.42
		W2 INT2	97.19	1.944	1.047	1.86	1.45
		W2 INT3	74.49	1.49	0.799	1.86	1.3
Week 3	CTR	W3 CTR1	137.44	2.749	1.388	1.98	1.71
		W3 CTR2	65.16	1.303	0.68	1.92	1.66
		W3 CTR3	91.86	1.837	0.991	1.85	1.45
	INT	W3 INT1	248.85	4.977	2.468	2.02	1.81
		W3 INT2	233.53	4.671	2.277	2.05	1.97
		W3 INT3	174.43	3.489	1.73	2.02	1.84
Week 4	CTR	W4 CTR1	67.69	1.354	0.725	1.87	1.61
		W4 CTR2	39.17	0.783	0.375	2.09	1.37
		W4 CTR3	49.87	0.997	0.54	1.85	1.52
	INT	W4 INT1	30.07	0.601	0.342	1.76	1.31
		W4 INT2	135.68	2.714	1.347	2.01	2.07
		W4 INT3	50.36	1.007	0.557	1.81	1.37
Week 5	CTR	W5 CTR1	49.39	0.988	0.584	1.69	1.35
		W5 CTR2	105.06	2.101	1.187	1.77	2.11
		W5 CTR3	129.79	2.596	1.287	2.02	1.95
	INT	W5 INT1	58.77	1.175	0.587	2	1.79
		W5 INT2	105.71	2.114	1.05	2.01	1.85
		W5 INT3	163.11	3.262	1.57	2.08	2.16

Sample coding: W= week; CTR= Control; INT= Intervention.

Six of the samples from the DE feeding trial (JT2) (*Table 3.9*) were selected for a repeat DNA extraction and ethanol precipitation (*Table 3.10*) in order to improve DNA concentration and quality, as they did not present neither

sufficient DNA yield nor adequate 260:280; 260:230 nm ratios as measured by spectrophotometry.

Ethanol precipitation helped to concentrate the DNA and to wash buffer and other ethanol soluble residues in these samples. In addition, ratios were also improved (*Table 3.10*).

Table 3.10. DNA quantitation using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). Ethanol precipitation was applied to selected DE feeding trial (JT2) DNA samples, previously extracted by the Qiagen QiaAMP DNA Stool mini kit method.

Sample	ng/µl	A260	A280	260/280	260/230
W2 CTR1	229.88	4.598	2.504	1.84	1.26
W3 CTR2	180.67	3.613	1.907	1.89	1.55
W4 CTR2	109.53	2.191	1.288	1.7	1.07
W5 CTR1	142.5	2.85	1.709	1.67	1.13
W4 INT1	89.21	1.784	1.046	1.71	1.08
W4 INT3	176.78	3.536	1.986	1.78	1.21

Sample coding: W= week; CTR= Control; INT= Intervention.

3.6.1.2 *SPORULATED OOCYSTS*

The DNA of sporulated *Eimeria spp.* oocysts was used for the creation of standards of *E. acervulina* for qPCR. A large number of oocysts from poultry faeces were harvested from faecal samples from the challenge broiler trial (AH6), then underwent sporulation, DNA extraction and a PCR reaction with *E. acervulina* primer pair.

None of the three DNA extraction methods tested was found to be appropriate for extracting the DNA from the *Eimeria* oocysts, as the purity ratios (260/280 and 260/230) did not reach the optimal quality (*Table 3.11*). The method MpBIO FastDNA® Spin kit for soil (A) was close to be adequate because it gave better DNA yield and an adequate 260/280 ratio, but the low 260/230 ratio suggest that samples were not purified enough.

Table 3.11. DNA quantitation using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). DNA from a sample of sporulated oocysts harvested from faecal samples from the challenge broiler trial (AH6) with four technical replicates was extracted by three DNA extraction methods: MpBIO FastDNA® Spin kit for soil (A), MpBIO FastDNA® Spin kit for faeces (B) and Qiagen QiaAMP DNA Stool mini kit (C).

Method	ng/µl	260/280	260/230
A. MpBIO FastDNA® Spin kit for soil	20.24	1.91	0.04
B. MpBIO FastDNA® Spin kit for faeces	2.39	0.89	0.03
C. Qiagen QiaAMP DNA Stool mini kit	2.97	1.11	0.43

Four DNA samples extracted by the method Qiagen QiaAMP DNA Stool mini kit (C) were selected for a PCR reaction with *E. acervulina* primer pair. PCR products were then cleaned in order to obtain purified samples for the creation of standards of *E. acervulina* for qPCR (*Table 3.12*). The sample "pool" of clean PCR products was used for creating nine dilution standards of *E. acervulina* for qPCR.

Table 3.12. DNA quantitation of clean PCR products using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). PCR products (samples 9, 10, 11 and 12) were obtained from a PCR reaction with E. acervulina primer pair on four samples of DNA of sporulated oocysts harvested from faecal samples from the challenge broiler trial (AH6). PCR products were cleaned using the Isolate II PCR and gel kit (Bioline, UK).

Sample	ng/µl	260/280	260/230
9	58.6	1.89	1.68
10	54.9	1.85	1.71
11	64.3	1.86	1.55
12	61.5	1.86	1.38
Pool	57.3	1.88	1.58

"Pool" was made adding 10 µl from each of the samples: 9, 10, 11 and 12.

3.6.2 PCR AMPLIFICATION OF EIMERIA SPECIES IN FREEZE DRIED CHICKEN FAECAL SAMPLES

The *E. acervulina* PCR reactions gave just light amplification bands for some of the samples tested (gel pictures not shown). The expected amplicon size of *E. acervulina* was 103 base pairs (Vrba *et al.*, 2010). Nevertheless, a sample made by a pool of isoconcentrated DNA samples from the DE feeding trial (JT2) was found positive in another *E. acervulina* PCR reaction (*Figure 3.13 and 3.14 -positive control bands*), with bands of an amplicon size of 103 base pairs (Vrba *et al.*, 2010). This was the only one *Eimeria* species detected by PCR in the DNA samples from the DE feeding trial (JT2).

PCR reactions with no amplification products were obtained for the other *Eimeria* species tested: *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix* and *E. praecox.*

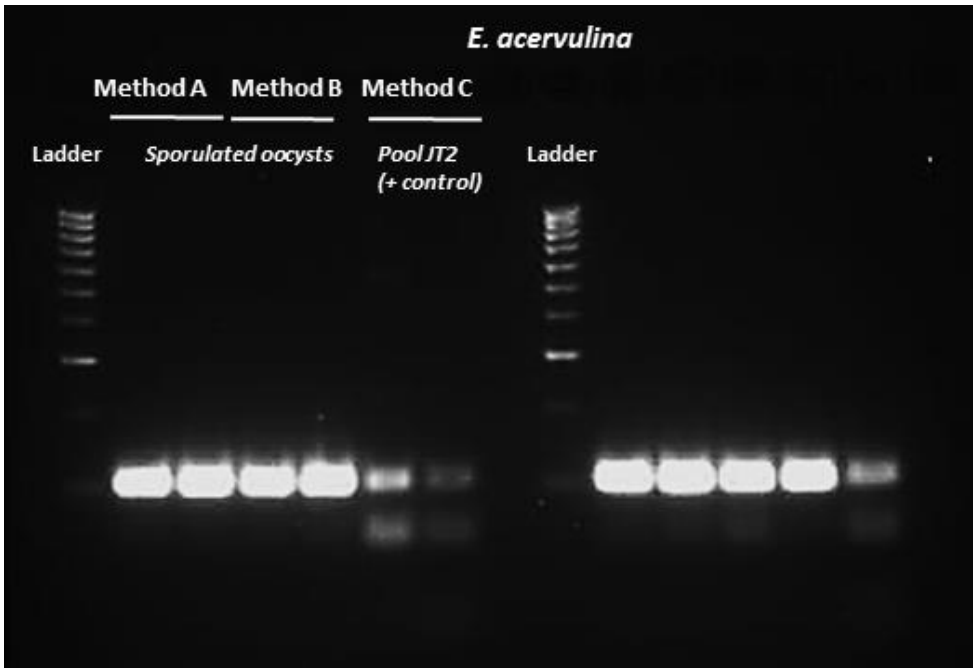


Figure 3.13. Agarose gel (2.5%) of PCR products from the amplification of *E. acervulina* in a DNA sample of sporulated oocysts, harvested from faecal samples from the challenge broiler trial (AH6) with two technical replicates. DNA was extracted by two DNA extraction methods: MpBIO FastDNA® Spin kit for soil (A) and MpBIO FastDNA® Spin kit for faeces (B), As positive control, the PCR products from the amplification of *E. acervulina* in a sample made by a pool of isoconcentrated DNA samples from the DE feeding trial (JT2) was added to wells 5 and 6 of strip one. DNA was extracted from faecal samples by the method Qiagen QiaAMP DNA Stool mini kit (C) with two technical replicates. Negative control was ultrapure water.

3.6.3 PCR AMPLIFICATION OF EIMERIA SPECIES IN SPORULATED OOCYSTS SAMPLES

The *E. acervulina* PCR reaction was positive in samples extracted by methods MpBIO FastDNA® Spin kit for faeces (B) and Qiagen QiaAMP DNA Stool mini kit (C) (*Figure 3.14)*. However, the *E. tenella* PCR reaction showed unclear results, because several bands appeared in each sample column in addition to faint bands matching the expected amplicon size of 100 base pairs (Vrba *et al.*, 2010). It seemed that different size DNA fragments had been amplified in that PCR reaction, meaning either that primers bind to multiple sites on the *E. tenella* genomic DNA or that another source of DNA was involved.

PCR reactions with no amplified products were obtained for the other *Eimeria* species tested: *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix* and *E. praecox* (some gel pictures not shown).

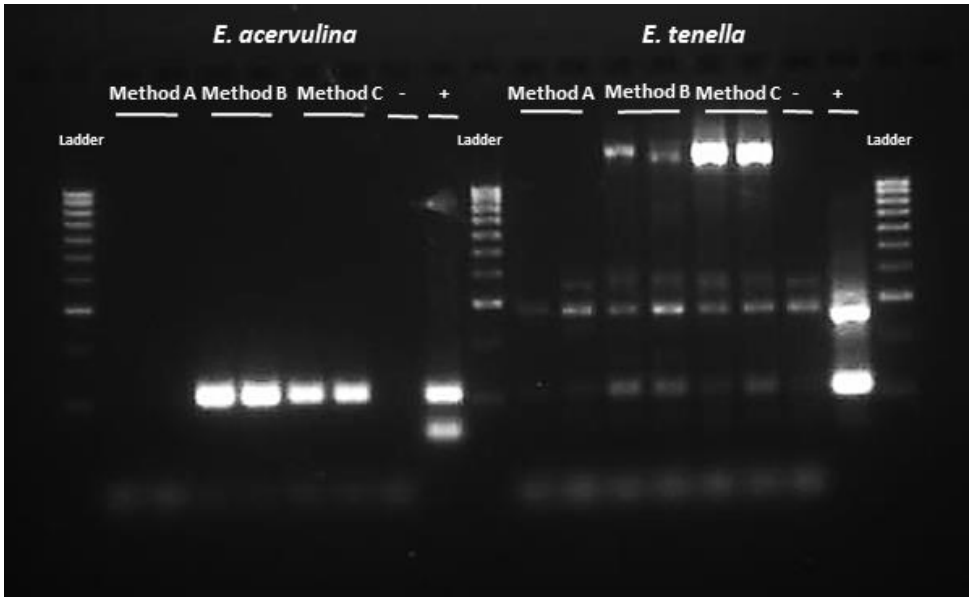


Figure 3.14. Agarose gel (2.5%) of PCR products from the amplification of *E. acervulina* (left*)* and *E. tenella* (right) in a DNA sample of sporulated oocysts harvested from faecal samples from the challenge broiler trial (AH6) with two technical replicates. DNA was extracted by three DNA extraction methods: MpBIO FastDNA® Spin kit for soil (A), MpBIO FastDNA® Spin kit for faeces (B) and Qiagen QiaAMP DNA Stool mini kit (C). As positive control, the PCR products from the amplification of *E. acervulina* in a sample made by a pool of isoconcentrated DNA samples from the DE feeding trial (JT2) was added to well 8 of the named strip; the same procedure was followed for *E.tenella*. DNA was extracted from faecal samples by the method Qiagen QiaAMP DNA Stool mini kit (C) with two technical replicates. Negative control was ultrapure water.

The *E. brunetti* PCR reaction *(Figure 3.15),* showed bands of lower size than the expected amplicon size of 118 base pairs for this species (Vrba *et al.*, 2010).

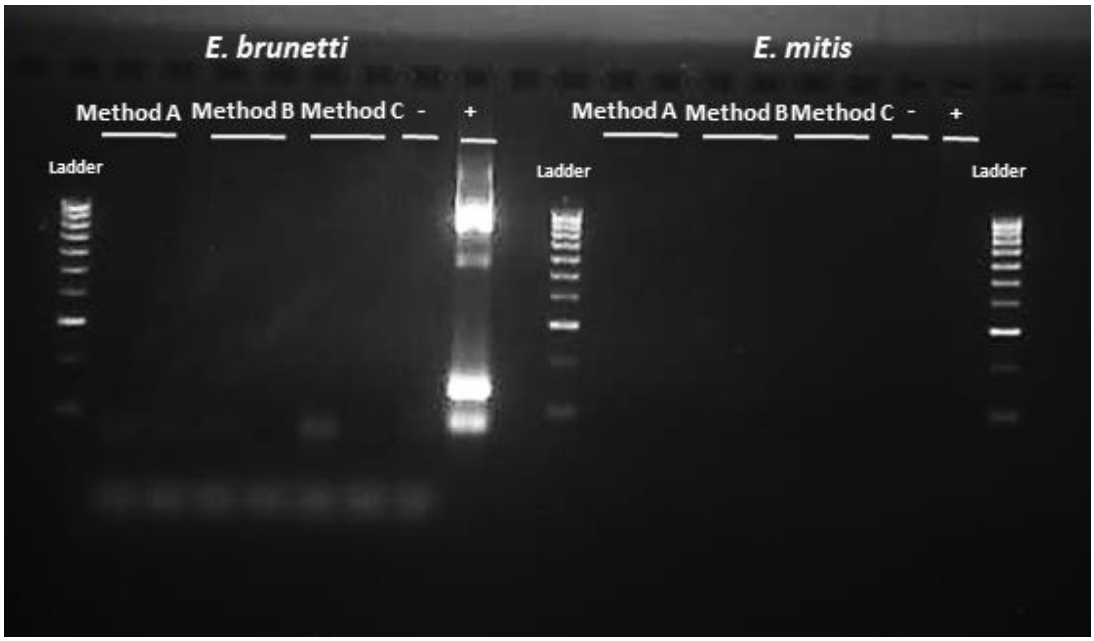


Figure 3.15. Agarose gel (2.5%) of PCR products from the amplification of *E. brunetti* (left) and *E.mitis* (right) in a DNA sample of sporulated oocysts harvested from faecal samples from the challenge broiler trial (AH6) with two technical replicates. DNA was extracted by three DNA extraction methods: MpBIO FastDNA® Spin kit for soil (A), MpBIO FastDNA® Spin kit for faeces (B) and Qiagen QiaAMP DNA Stool mini kit (C).

3.6.4 GRADIENT TEMPERATURE QUANTITATIVE PCR (QPCR) TO SCREEN FOR ALL EIMERIA SPECIES

Only the samples tested for *E. acervulina* amplified in the qPCR reaction, being the results for the rest of the *Eimeria* species negative. The best temperature for amplification of the *E. acervulina* DNA was 62°C, as we obtained the lowest Ct value in the qPCR; the range of temperature tested was from 53°C to 64°C. The temperature 62°C was applied on the annealing step of the qPCR reactions performed, looking for the presence and quantitation of *E. acervulina* in DNA samples from the DE feeding trial (JT2).

3.6.5 QUANTITATIVE PCR (QPCR) SPECIFIC FOR E.ACERVULINA

The quantitation of *E. acervulina* in DNA samples from the DE feeding trial (JT2) showed that diet did not have a significant effect in the parasite load (p>0.05) (*Table 3.13*).

Table 3.13. Effect of the dietary inclusion of DE on the concentration of *E. acervulina* parasites in faecal samples from free-range organic laying hens. Hens were fed a basal organic diet with (INT) and without (CTR) supplementation of diatomaceous earth (DE) for five weeks. Faecal samples were collected once a week then freeze-dried and homogenised. DNA was extracted by the Qiagen QiaAMP DNA Stool mini kit method and tested by qPCR for the presence and quantitation of *E. acervulina.*

	CTR	INT	SEM	P
pg DNA/ml	8.11	7.71	0.374	0.462
log num copies/ul	4.86	4.83	0.020	0.457

CHAPTER FOUR
DISCUSSION

The trial consisted in the inclusion of diatomaceous earth (DE) in the diet of free-range organic laying hens of 54 weeks of age, with two groups of 220 hens each fed a basal diet (control group) and diet supplemented with 1.33% DE (intervention group) for five weeks.

The objectives of the trial were to study the effect of the dietary inclusion of DE on the concentration of parasites measured by means of faecal egg counts (FEC) and molecular identification of *Eimeria spp.* in faecal samples by means of PCR and qPCR, the egg production and egg quality by means of external and internal egg quality tests and indirect methods to test eggshell quality.

The trial was conducted in a commercial organic farm located in the Aberystwyth area, which offered optimal conditions in terms of farm field area, flock size, age of the animals and general management. A matter of discussion could be the fact that the field was not split into two physically separated groups, laying sheds were in one single open range for use between all birds. Splitting the groups was not possible because the farmer was not allowed to introduce modifications in her setting, according to organic farming legislation. Therefore we had to assume that 95% of the hens were feeding and laying in their correspondent shed, a fact that naturally occurred since the hens moved into the laying sheds at sixteen weeks of age. Hens have persistent laying habits, reason why pullets are moved into the laying shed before starting egg production, as they get used to lay in the provided nest boxes rather than on the floor or outdoors (Webster, 2007). Moreover, since it was not possible to conduct a compartmentalization in the shed, some of studied parameters were performed in a single experimental replicate. Thus, there are some factors related with the environmental conditions and

housing that potentially may affect the results and it was not possible to rule them out from the study, such as the direction the shed was facing, seasonal variations and weather conditions during the trial. Besides, the age of the animals is a factor that highly influences the parasitic load in both animals and floor, although faecal samples were gathered and tested at the beginning of the trial and no differences were found between sheds. Moreover, all external conditions were affecting both treated and control animals, and it shouldn´t influence the direct comparison between them at a given time. A potential interaction between treatment and environmental conditions can't be totally discarded and it could be considered a flaw in the current study. A possible solution to amend this issue it would involve the repetition of the actual trial over time, in order to allow for an increase in the experimental replicates without compromising major changes in the environmental conditions, but this option it was not available at the time due to the length of the study.

All the feed used for the trial was prepared on site using the farm mill, which enabled monitoring the quality of the procedure. The addition of DE to the basal feed resulted in a powdery mix; the final concentration of DE in the intervention feed was no higher than 1.5 % (w/w) to avoid feed rejection by the hens. On the initial week of adaptation there was more feed waste in the INT shed than in CTR shed, but it decreased as the trial advanced. It has been observed in studies that chickens fed DE have a higher feed intake with either better feed conversion rate (FCR) in broilers (Modirsanei *et al.*, 2008) or feed efficiency (grams of feed per gram of egg) in organic Bovan brown hens, with the DE composition in essential trace elements a possible reason for these effects (Benett *et al*., 2011b). In this feeding trial there was no option to measure the hen's body weight or feed intake per bird, but it can be confirmed that the intervention diet was well accepted by the hens. The possibility of taking these two measurements would be of interest for future research, because that data allow the assessment of the hen's body condition and the calculation of feed conversion rate (ratio between feed consumed and egg mass produced).

Egg production, egg quality and feed

Egg production was increased in the intervention group, seen in better daily egg production and laying index, and that effect was consistent throughout the length of the trial. Even so, the number of eggs laid by the flock decreased as the trial advanced, as expected in hens getting through their last third of the first year production cycle. In a typical egg production curve (as seen in Figure 1.4), hens of 54 weeks old would be in their depletion period, where laying index (egg production per animal and day, expressed as percentage) undergoes a weekly descent of 0.5 to 0.7% (García-Trujillo *et al.*, 2009).

Nevertheless, production numbers suffered some fluctuation between weeks. An increased number of eggs produced on week 4 were registered for both groups, which contrasted with the decreasing general trend in egg production for CTR group and a depressed production that INT group accounted on week 3. According to García-Trujillo *et al.* (2009), organic egg production systems are more likely to have atypical production curves, due to the amount of factors that can influence egg production.

Bad weather conditions registered along the second to third week of trial could have affected negatively both feed intake and egg production. Because hens remain longer time indoors in rainy and windy days, they have reduced physical exercise and lower natural light intake. These modifications of the daily routine could potentially alter their performance (DEFRA, 2001).

After five weeks of dietary intervention, the DE diet group laid larger eggs with higher albumen and yolk weights compared to week one, but no improvement was identified in the eggshell quality parameters (weight, density and egg specific gravity) or number of broken eggs compared to control group. The eggshell quality results obtained by the intervention group hens were slightly lower but very similar to those obtained by the control group hens, which experienced little modification throughout the trial. The intervention group eggs had more pinholes and glassy eggs, although their eggshells showed a lower number of weak shell areas and of smaller size. The intervention egg specific gravity value was superior to 1.082, meaning that the egg breakage

index was lower than 5%, a value considered optimal for an egg producer (Wineland, 1992).

According to Barroeta *et al.* (2010), egg size and quality vary as the laying period advances; while eggs increase in size there is a higher risk of breakage due to thinner and more fragile eggshells. Many physiological factors influence the eggshell thickness, not only inherent to the animals but also related to their management and environment. For instance, the ability to absorb dietary calcium and mobilize calcium from bone declines with age; so older hens produce larger eggs with thinner shells (Gupta, 2008). Therefore, considering the flock age and time point in the egg production cycle, an increase on egg weight and a decrease of eggshell densities were expected.

Our positive results of egg production and egg component weights in the intervention group were comparable to those of Bennett *et al*. (2011b), who conducted a study applying a DE dietary treatment to Bovan Brown and Lohmann Brown hens. Similarly to us, their results for eggshell quality were not improved for the intervention group. They reported higher eggshell weight and thickness between weeks 20 and 28, but these parameters declined from week 28 until week 38 when the trial finished.

Additional tests to complete the external quality egg exam would be by direct measurement of shell strength by analysing its resistance to deformity, compression and puncture, requiring some technical skill. We lacked the equipment necessary to perform these tests, so indirect methods to evaluate eggshell quality were chosen, like egg specific gravity and density. These have the advantage of reducing the error caused by the operator due to their simplicity.

Candling examination was selected for being a classic method for the evaluation of eggs. It was performed with a homemade egg candler, a tool easy to build that gave reliable results for identifying shell defects. In order to get a better assessment of the internal egg quality, candling was complemented with other broken-egg tests such as measurement of the albumen height and Haugh units, yolk DSM colour, blood and meat spots, as well as weight of the whole egg, dried shell, albumen and yolk. It could be

argued that using the DSM Yolk Colour Fan for measuring the yolk colour is a subjective method, however it is considered reliable despite the limitations of being operator-dependant and the illumination of the room. It has the advantage of using the same criteria as consumers apply to judge the colour of the yolk, and can result be as precise as more sophisticated techniques like reflectancy colorimetry (Overfield, 1996).

The albumen quality parameters were better for the intervention group eggs, with the Haugh unit mean value reaching USDA quality grade AA, described as “clear and firm (thick)” albumen texture (*Table 1.3*). In addition, DSM yolk colour mean value was higher than 10, not far from the darker colours that European customers have expressed as preference (Beardsworth and Hernández, 2004).

In contrast, internal defects such as watery whites and discoloured yolks were identified in few eggs, these being related with storage time and conditions. These features could affect consumer acceptance of the product. The presence of egg inclusions is a normal finding and it is influenced by hen genetics, flock age, stress and diet (Soler *et al.*, 2011). Eggs laid by the intervention group hens had a higher incidence of blood spots, maybe related to modified conditions of the hens’ diet and some additional stress, besides the fact that the ageing of the hens may favour their presence, as observed in other studies (Buttow Roll *et al*., 2009). There were no differences in protein content of albumen and yolk between treatments.

For the purpose of evaluating the effect of DE in egg production and particularly in eggshell quality, increasing the length of time of the trial, repeating the trial using the same flock in their second laying period (after moult) or doing a feed swap between groups would be of interest for future research. An additional implementation to be considered would be the introduction of a physical barrier between flocks.

In this trial only non-invasive procedures were allowed by reason of organic farming legislation. Performing additional tests like blood sampling would have been good to assess the bird’s general health condition (haematology and biochemistry panels, including blood calcium levels). In addition, analysis of

plasma carotenoid levels would inform about coccidiosis because it is a sensitive parameter for measuring the pathogenic effect of *Eimeria spp.*, and variation of levels indicate damage of the intestinal integrity (Conway *et al*., 1993; Zhu *et al*., 2000). The technique of dual x-ray absorptiometry (DXA) is a mean of measuring bone mineral density and has been applied to birds *in vivo* (Hester *et al*., 2004; Schreiweis *et al*., 2003, 2004 and 2005). As it involves manipulation of live birds, there was no option to apply this technique in this trial but it could be considered an interesting way to study the relationship between egg production and bone quality.

The base ingredients for the feed were the same for CTR and INT feeds, but when these were analyzed the intervention feed contained less protein but was richer in organic matter and fat; this could mean higher energetic content, although this was a parameter not measured.

Both feeds had a protein content ranging from 12 to 13 g/100 g DM, amount slightly below the recommendations for laying hens in final production stage (14-15.5 g per 100 g of dry matter) (Benedí *et al.,* 1995; García-Trujillo *et al.*, 2009). The choice of the grain used to formulate the feed can make a difference with regard protein and energy content of the final product. Organic wheat, oats and peas were used to make the feed, which combined gave a medium protein level. Whole soya or soya meal could be a good choice to increase protein level in the feed, however soya can be an expensive ingredient (García-Trujillo *et al.*, 2009). Good quality pastures could also be an alternative (Blair, 2008).

Parasitology methods

Two faecal egg count (FEC) methods were selected for a weekly examination of hen's faeces. Both were performed with an initial step of mixing faeces with tap water and straining through muslin, which should help to release the parasite eggs into the water solution and to reduce faecal debris of the sample (grass, fibrous material, feathers, etc.). This was also improved by a centrifugation step in the case of the McMaster method. However, these modifications to the methods increased the time required to perform the tests.

Many flotation solutions can be used, each one more or less suited to the parasite species studied. As the specific gravity (SG) of the flotation solution increases, a greater variety of parasite worm eggs and protozoan oocysts float but excessive faecal debris can also float, which decreases the efficiency of the test. Most parasite stages float efficiently at SG between 1.18-1.30 (Ballweber *et al*., 2014).

For the McMaster method, the solution of choice was salt saturated solution (SG= 1.18-1.20), which floats common helminth and protozoa cysts, but can damage the parasite eggs rapidly. For the Modified Stoll method, the Shaether's sugar solution (SG= 1.25) was chosen, which floats common helminth and protozoa cysts (it is preferred for *Cryptosporidium spp.*), can float tapeworm and nematode eggs of higher density and does not distort parasite eggs as much as other solutions (Foreyt, 2001). The use of this solution for faecal flotation tests requires centrifugation because of its high viscosity.

As seen in Zajac and Conboy (2012), the recommended centrifugation speed ranged from 300 to 650 *x g* for both methods. Prior adaptation of protocol for centrifugation speed, several speeds were tested; a speed on the higher range (600 x *g*) was chosen for the McMaster method, because it pelleted all the parasite eggs (the supernatant was checked for unpelleted eggs), without being too firm to resuspend afterwards. A medium speed (450 x *g)* was chosen for the Modified Stoll method, as it proved to be both effective and innocuous for the integrity of the eggs.

In order to avoid egg distortion, a fact that would affect negatively the sensitivity of the tests, only a few samples were prepared simultaneously for microscopy screening. The waiting time indicated for the samples to stand in the final step was strictly applied to both tests, because that allows all the parasite eggs to float. Overall, following these rules contributed to achieve the expected level of sensitivity and accuracy of the tests.

The FEC results of samples prepared by each method were significantly different for the species *A. galli*, *H. gallinarum* and *Eimeria spp.* In addition, it was observed that the achieved sensitivity of the method varied depending on

the species tested. As a result, it seemed that Modified Stoll was an adequate method for nematode species (including *Capillaria spp*.), whereas McMaster was more suited for isolating and counting coccidian parasites (*Eimeria spp.*).

This outcome agrees with what was expected for each of the applied methods: the minimum analytic sensitivity for parasite detection is 5 eggs per gram of faeces (epg) for Modified Stoll and 25 epg for McMaster. Accordingly, while the first was able to detect the less numerous parasite species in the samples, the second may have failed to detect those or could underestimate the count, as it has demonstrated poor specificity with low egg concentrations (Bosco *et al.*, 2014; Haug *et al.*, 2006).

Nevertheless, the widely-used McMaster is the preferred method for counting purposes when faecal samples have some degree of parasite contamination: the slide is designed for counting under the microscope and the protocol can be adapted depending on the concentration of eggs in the faeces, in order to get an easy but accurate count (Haug *et al.*, 2006; Pereckiene *et al.*, 2007). For our trial, this method was selected and its protocol was adapted accordingly with the aim of increasing the sensitivity and minimizing under or over estimation of the counts. The proportion of faecal material vs. fluid was adjusted in order to get a moderate oocyst density, easy to read; the whole area under the grid of both chambers was counted (a whole slide per sample), as it seems to give more accurate results (Bosco *et al.*, 2014; Cringoli *et al*., 2004).

In order to further investigate the potential effects of DE in the *Eimeria spp.* oocysts, *in vitro* assays could be performed. For instance, an *in vitro* sporulation inhibition assay exposing unsporulated oocysts to different concentrations of DE under controlled sporulation conditions, and identifying the amount of sporulated oocysts by optical microscopy to measure the sporulation rate. Futhermore, an *in vitro* viability assay exposing sporulated oocysts to different concentrations of DE under controlled conditions, and identifying intact and damaged oocysts by fluorescence microscopy. Similar assays have been performed exposing *Eimeria* oocysts to artemisinin (del Cacho *et al*., 2010) and extracts from pine bark (*Pinus radiata*) (Molan *et al*.,

2009) to test for sporulation, and also to ten plant essential oils to test for oocyst viability (Remmal *et al*., 2011).

Parasitology results

Free-range systems are expected to have some degree of parasite presence in the pastures. The parasite species identified in this farm trial matched the most common encountered for chicken, according to Permin (1998) and Permin *et al.* (1999), and the quantitation results were overall low. For instance, the counts of *Eimeria spp.* in a commercial broiler farm can be thousands of times higher than the counts found in this trial, indicating higher degree of infection; in that case, clinical coccidiosis is more likely to occur, seeing clinical signs like diarrhoea, dehydration and poor growth. The fact that the majority of these parasite species have direct cycles, with a faecal-oral transmission, make very likely to find them in faecal exams of foraging areas with certain degree of contamination.

Likewise the study conducted by Permin *et al.* (1999) in Denmark, the parasite prevalence in our trial was, in a decreasing order: *Heterakis gallinarum*, *Ascaridia galli* and *Capillaria spp*. Moreover we detected *Eimeria spp.* (counts fluctuating between 100 and 500 oocysts per gram of faeces). Anecdotally, a few cestode parasites were detected.

Laying hens are animals kept in production for a long time, and generally they develop an immune response and resistance to parasites. Changes in the immune response can affect ovulation and fecundity of parasites, leading to a variation on the prepatent period, an intermittent egg production, etc., which could be reflected in false negative results. In addition, diurnal fluctuation of egg output together with the volume and consistency of faeces are factors that influence the concentration of eggs found in faecal material.

Depending on the parasite species, the FEC and parasite burden may not be well correlated, and it is also difficult to estimate the clinical signification of a known burden; i.e. *A. galli* induces a strong immunogenic response; the parasite egg output is higher when few adult females are present and it

decreases as the adult burden increases (Permin, 1998). Besides, a positive but low estimation of a parasite with high pathogenicity may be more clinically relevant that higher counts of lesser pathogenic species.

Some farming practices like pasture rotation can limit the contamination of the pastures. This allows regeneration of the field and helps to break up the parasite life cycle, but involves moving the flock or the hen house to a new pasture every 2-3 months (Fanatico, 2007); this may be implemented turning over the field ground (exposing eggs to the drying effect of sunlight) and planting crops in the fields that were emptied (García, 2006). The farmer followed these practices according to an integrated organic farming plan.

Dietary treatment with DE did not have a significant effect in parasite counts of free-range organic laying hens. In this farm trial, the parasite's FEC have fluctuated as the trial advanced, in a different way depending on species and treatment group.

Environmental conditions during the trial were particularly harsh along the second to third week with storms, increased rainfall, strong winds and lower temperatures. These factors may have influenced the presence and detection of parasites in faeces; therefore they may have contributed to explain the changes observed on the FEC of weeks 4 and 5.

Comparing FEC from week 3 to week 5, *Eimeria spp.* parasites decreased and then rebounded, contrary to what happened to *A. galli* and *H. gallinarum* in the same period of time, observed for both CTR and INT groups. Dietary treatment seemed to reduce the FEC after 5 weeks for *A. galli* and *H. gallinarum* parasite species, but failed to do so for *Eimeria spp.* and *Capillaria spp.*

In the study conducted by Bennett *et al*. (2011b), a DE dietary treatment (DE inclusion at 2%) helped to lower parasite loads in Bovan Brown hens. McMaster FEC were performed every two weeks for a period of 12 weeks, with birds initially aged 16 weeks. They had as results significantly lower *Capillaria spp*. FEC and slightly lower *Eimeria spp.* and *Ascaridia spp.* FEC. However, in our trial both INT *Capillaria spp*. and *Eimeria spp.* FEC remained

stable and then reached their highest value by week 5, resulting higher than CTR. In addition, different trends for CTR and INT groups were observed for *Ascaridia galli* FEC; in our case INT achieved lower FEC for the last 3 weeks of trial, but Bennett *et al*. (2011b) only got a lower INT FEC values in the middle and at the end of their trial, after an increasing trend on the INT FEC. In brief, we obtained lower *A. galli* and *H. gallinarum* FEC in the last weeks of trial in the groups with DE dietary treatment, but did not match their results for *Capillaria spp*. and *Eimeria spp.*

DE dietary treatment seemed to have some effect in the egg output of parasite species with longer prepatent periods (minimum of four weeks) such as *A. galli* and *H. gallinarum*. Plausible reasons to explain these could be that as long as the hens consumed the diet, they either passed a smaller amount and/or less viable, infective eggs, or that the adult parasite population inside the gut was somehow affected, and that made them less able to produce eggs; as a result, we obtained lower FEC in the last 3 weeks of trial.

Nevertheless, faster changes in egg output would be expected for parasite species with shorter prepatent periods such as *Eimeria spp.* (less than one week time) and *Capillaria spp* (three to four weeks time). In our case, INT group egg output did not fluctuate much over the time, and even if the FEC rose on week 5, it is possible that the DE dietary treatment helped to keep a steady egg output throughout the trial.

Osweiler and Carson (1997) evaluated the efficacy of dietary DE in organic sheep, obtaining lower nematode FEC in animals fed a 5% DE diet compared with control group, but results were no significant due to a large variability between groups. Similarly to Fernández *et al.* (1998), who did their research in beef steers, they realised that parasite counts of group fed a 2% DE diet reduced over the time, up to similar levels of control and anthelminthic drench groups. This was attributed to either the development of immune response to parasites or the death of old worms.

Young birds of less than ten weeks old are much more sensitive to *Eimeria* parasites, but as immunity is species-specific, outbreaks can occur at any

time if birds have never been exposed before to certain species (Fanatico, 2006). Relocation of pullets from rearing site to production site is considered a challenge for their immune system, as that involves some stressful factors like new environment and housing, different sanitary conditions, change of diet, and exposure of immunologically naïve birds to new *Eimera* species (Lunden *et al.*, 2000). For this reason, it would be of interest doing this study in a flock of younger hens (15 to 25 weeks old). In addition, increasing the length of time of the trial, repeating the trial using the same flock in another season of the year (i.e. late spring) or doing a feed swap between groups would give more and useful information for interpretation the parasitological part of the study.

There was an interest in screening for *Cryptosporidium spp.* presence in the hen's faeces in order to complete a general idea of the parasite's prevalence. Cryptosporidiosis is a severe disease in avian species such as turkey and quail, but chickens seem to develop an age-related resistance to disease, thus affecting birds younger than eleven weeks old. Nevertheless, its prevalence has proved high in chickens with immune-suppression or concurrent disease, being therefore considered a first order disease (de Graaf *et al.*, 1999). Similarly to *Eimeria spp.*, identification of *Cryptosporidium spp.* microscopically is a difficult task due to small oocyst size. As a result, it was considered more appropriate to look for this parasite species by molecular biology methods in future work.

Molecular biology applied to chicken faecal samples for the study of Eimeria parasites

Possible sources of *Eimeria spp*. oocysts for DNA extraction are chicken faeces and poultry litter, although the latter is less optimal as it contains PCR inhibitor substances, which are often difficult to remove (Chapman *et al*., 2013). In addition, oocysts are likely to degrade in litter by bacterial action and ammonia from droppings (Morris and Gasser, 2006).

Eimeria spp. oocysts can be recovered, concentrated and purified from chicken faeces for further use; by doing this, the amount of faecal material in the sample is reduced and parasites are more concentrated, a fact that may be of importance for detection when shedding is low and samples contain low number of oocysts. The advantages of using purified oocysts instead of faeces for DNA extraction are increased PCR assay sensitivity due to improved DNA extraction and detection of parasite sub-populations, although it entails a time consuming procedure and poses the risk of uneven oocyst purification (Kumar *et al*., 2014; Vrba *et al*., 2010).

The faecal samples collected every week were processed for FEC with priority, because faecal material degrades with time and so do parasite eggs. Once these tests were completed, faeces were frozen, freeze-dried and stored until further use. As the number of *Eimeria spp.* oocysts in the samples was detected low, the method for purification of oocysts from faecal samples described in section 2.8.2 (B) could have been applied to the faecal samples in order to concentrate the oocysts. By doing this step, the DNA extraction could have been improved and so the PCR results; however it would have been considerably more time consuming.

Due to the resistant nature of the oocyst wall, it has to undergo mechanical disruption prior DNA extraction, this step being the most important limiting factor for good sensitivity (Chapman *et al*., 2013). Depending on the DNA extraction method used, bead-beating with glass beads (Qiagen and modified phenol-clorophorm methods) or homogenization with the FastPrep® Instrument (MpBio methods) were applied to ensure oocyst disruption.

No suitable DNA extraction method was found for extracting DNA from *Eimeria* oocysts samples. The low 260/230 ratios obtained suggested that samples were not purified enough. Some substances present in the samples can increase the absorbance at 230 nm, such as carbohydrates, sodium chloride, peptides and phenol (or aromatic compounds), but it can also be due to contamination with guanidine hydrochloride, substance present in the components of the DNA extraction kits (QIAGEN, 2017). Ethanol precipitation or a repeat extraction with fresh samples could be applied to improve results,

but this was not considered as we obtained an optimal amplification of *E. acervulina* in these samples.

E. acervulina was detected by PCR in the oocyst samples resulting from the faecal samples of the challenge broiler trial (AH6). A gram of fresh faeces from the experimental birds aged 30 weeks contained on average 13,900 *Eimeria* oocysts, and the volume of suspension used for DNA extraction was 100 µl, which contained approximately 2,000 oocysts.

Weak positive amplification bands were obtained in the *E. tenella* PCR reactions, however other bands of size 300-350 base pairs were amplified in the sample columns and negative control. This could be explained by either a failure of the primers, as they bound to multiple sites on the *E. tenella* genomic DNA (lacked specificity), or that another source of DNA was involved within the DNA templates, the primers or the water used for negative control. Performing a qPCR using the specific primers and probe for *E. tenella* could have complemented the PCR test.

Eimeria spp. oocysts were present in the faecal samples, evidenced by the examination of preparations for FEC by optical microscopy. Morphological differences between *Eimeria* species oocysts make the discrimination by microscopy difficult, task that was not attempted in this study. Nevertheless, several species were likely to be present, judging by the differences in size and shape observed between oocysts. One of the objectives of the present study was to develop and adapt novel techniques to the early diagnosis and identification of parasitic load in organic poultry; hence, although the number of oocysts detected were low, otherwise expected considering the type of farming and the age of the hens, molecular biology methods were applied to assess the significance of *Eimeria spp*. in free-range organic laying hens. Thus, a series of protocols have been developed (available in the Appendix section) for their use in practical conditions.

E. acervulina was the only one *Eimeria* species detected by PCR in the DNA samples from hen's faeces of the DE feeding trial (JT2), yet identified just in a sample made by a pool of isoconcentrated DNA samples. A number of possible reasons are that oocysts were in low number, the degree of

sporulation was low (and so the number of DNA copies), PCR reactions were not well performed or failed, PCR inhibition by faecal material or that only that *Eimeria* species was present in the samples.

A gram of fresh hen faeces contained on average between 270 and 290 *Eimeria* oocysts, and the amount of dried faecal material used for a single DNA extraction was 50 milligrams, meaning that the expected number of oocysts in the samples for DNA extraction was very low (approximately between ten and twenty). Another element to consider is the degree of sporulation of the oocysts present in the samples, as this would determine the amount of DNA on them. This is because unsporulated oocysts from fresh faeces are diploid and contain two copies of the *Eimeria* genome, whereas there are eight haploid copies in fully sporulated oocysts and between two and eight copies in intermediate stages (Morgan *et al*., 2009; Vrba *et al*., 2010). Despite that, the limit of sensitivity for detection of *Eimeria spp.* seems to be below ten copies of the target sequence, what equals to a single oocyst (eight genome copies when fully sporulated); this was confirmed by Vrba *et al*. (2010) in qPCR assays using the primers and probes of the publication (same as those used in this trial).

DNA extraction was carefully performed, with the additional application of mechanical techniques to ensure oocyst disruption. PCR reactions were performed using DNA samples extracted by the four DNA extraction methods employed and repeated several times, in order to test whether that factor affected DNA amplification, obtaining negative results. PCR assay sensitivity could have been reduced by the presence of faecal debris in the samples, which seem to cause PCR inhibition by reducing the efficiency of the purification steps of the DNA extraction of chicken faeces (Kumar *et al.*, 2014).

The Qiagen QiaAMP DNA Stool mini kit method was found to be the most appropriate for DNA extraction from chicken faecal samples, due to the good 260/280 and 260/230 ratios and reasonably good DNA yield and purity of DNA extracted using this technique. Previous studies have reported the

selection of this method for its suitability (Kumar *et al.*, 2014; Morgan *et al.,* 2009).

E. acervulina has been recognized as an ubiquitous species (Williams *et al.,* 1996). In some prevalence studies, it was found to be the most commonly encountered species in Swedish layer flocks (Lunden *et al.,* 2010) and in French organic and standard broilers, broiler breeders and laying pullets (Williams *et al.,* 1996). *E. tenella* and *E. maxima* seemed to be species frequently detected in a variety of productions, including free range broilers, pullets and layers in Tunisia (Kaboidi *et al.,* 2016) and alternative organic and "label" chickens across northern France (Williams *et al.,* 1996). These three *Eimeria* species are considered the most concerning for the U.S. chicken production (Fanatico, 2006), being *E. acervulina* the one with more reproductive potential although less pathogenic than species like *E. maxima* (Lunden *et al.,* 2010).

Finally, the quantitation of *E. acervulina* by qPCR showed that dietary treatment with DE did not have a significant effect in *E. acervulina* parasite load of free-range organic laying hens. This result matches what was obtained for the *Eimeria spp.* parasite load measured by FEC, where no dietary effect was identified. Thus, the present results suggest that *Eimeria spp.* presence did not affect significantly the flock; in other words, it did not cause any major detrimental effect for the health of the birds or their productivity.

CONCLUSIONS

- Dietary treatment with DE applied to free-range organic hens did not have a significant effect on the concentration of parasites commonly encountered in free-range hens, such as nematode species (*Ascaridia galli*, *Capillaria spp.* and *Heterakis gallinarum)*, and protozoa species (*Eimeria spp.* and *E. acervulina* - analized using qPCR technique). However, diet seemed to have some effect in the egg output of *A. galli* and *H. gallinarum* parasite species as lower FEC were obtained in the last weeks of the trial.

- The FEC methods more suitable to apply to chicken faeces were the Modified Stoll method for isolating and counting nematode species and the McMaster method for coccidian parasites (*Eimeria spp.*).

- The Qiagen QiaAMP DNA Stool mini kit method was the most appropriate method for DNA extraction from chicken faecal samples, but no suitable DNA extraction method was found for extracting DNA from *Eimeria* oocysts samples.

- Dietary treatment with DE applied to free-range organic hens showed increased daily egg production and laying index compared to control. The eggs were larger and had higher albumen and yolk weights after five weeks of dietary treatment.

- No detrimental effects were observed as a result of adding DE to the hen's diet, neither an improvement on eggshell quality or lower number of broken eggs. Nevertheless, the eggs obtained were considered good quality eggs, because they reached the higher USDA quality grade (AA), according to their Haugh unit and specific gravity values.

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APPENDIX

AP1. PROTOCOLS FOR FAECAL EGG COUNTS (FEC)

MCMASTER METHOD 25 EGGS PER GRAM (EPG) SENSITIVITY

1. Mix 4 g of faeces in 26 ml of tap water in a beaker, stirring well with a spatula. Leave it to soak for 60-90 min.
2. Mix again stirring and filter the suspension through a single layer of muslin, squeezing the contents in the gauze.
3. Immediately after the filtering procedure, fill the 15 ml tube with the filtrate. Centrifuge at 600 x *g* for 5 min.
4. Discard the supernatant with an electronic pipette, leaving 1-2 ml supernatant and being careful not to disturb the pellet. The supernatant is then checked microscopically for unpelleted eggs.
5. Add 2-3 ml of saturated salt solution and resuspend the pellet very carefully, using a Pasteur pipette to suck up and down. Avoid making bubbles in the solution.
6. More salt solution is added to the original 15 ml volume and the tube is inverted carefully 8-10 times.
7. Remove a sample with a Pasteur pipette from the middle of the tube and fill one of the McMaster slide chambers 1.5 mm depth (Weber Scientific International, England). Cap and invert the tube two times, then repeat the procedure to fill the second chamber.
8. Leave the filled slide to stand for a minimum of 5 min to allow eggs to float.
9. Examine with the 12.5 x objective lens of the optical microscope and count the number of eggs in all six fields of both slide chambers. All eggs are counted and recorded separately for each parasite species.

MODIFIED STOLL METHOD

1. Mix 5 g of faeces in 20 ml of tap water, stirring well with a spatula. Leave it to soak for 60-90 min.
2. Mix again stirring and filter the suspension through a single layer of muslin, squeezing the contents in the gauze.
3. Immediately after the filtering procedure, transfer 1 ml to a 15 ml centrifuge tube and fill it up with flotation solution. Centrifuge at 450 x *g* for 10 min. Take tubes out carefully.
4. Place the tubes in a rack and add extra flotation solution with a Pasteur pipette to make a meniscus. Place a cover slip on top without causing overflow of the liquid. Leave it stand for 15 min.
5. Remove the coverslip carefully and place it on a glass slide. Examine with the 12.5 x objective lens of the optical microscope, systematically scanning the whole coverslip. All eggs are counted and recorded separately for each parasite species.

AP2. KEY FOR POULTRY PARASITE IDENTIFICATION

A customised key was designed for quick reference, following Foreyt (2001), Gibbons (2010), Kassai (1999), Kaufmann (1996), Urquhart *et al.* (1996), Wakelin (1965) and Zajac and Conboy (2012).

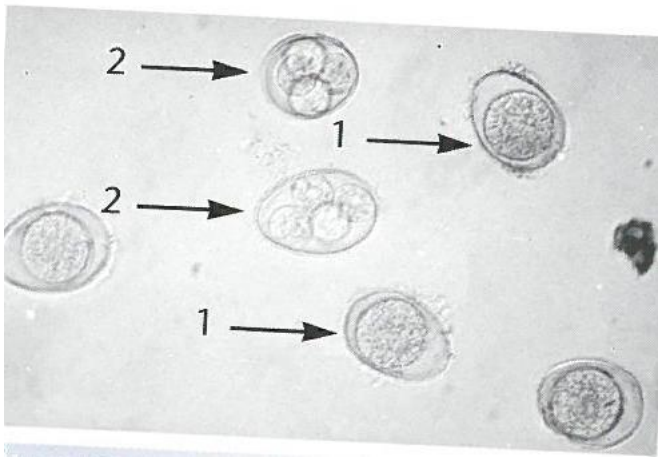


Figure A.1. *Eimeria spp. oocysts: (1)* unsporulated oocyst, (2) sporulated oocyst.

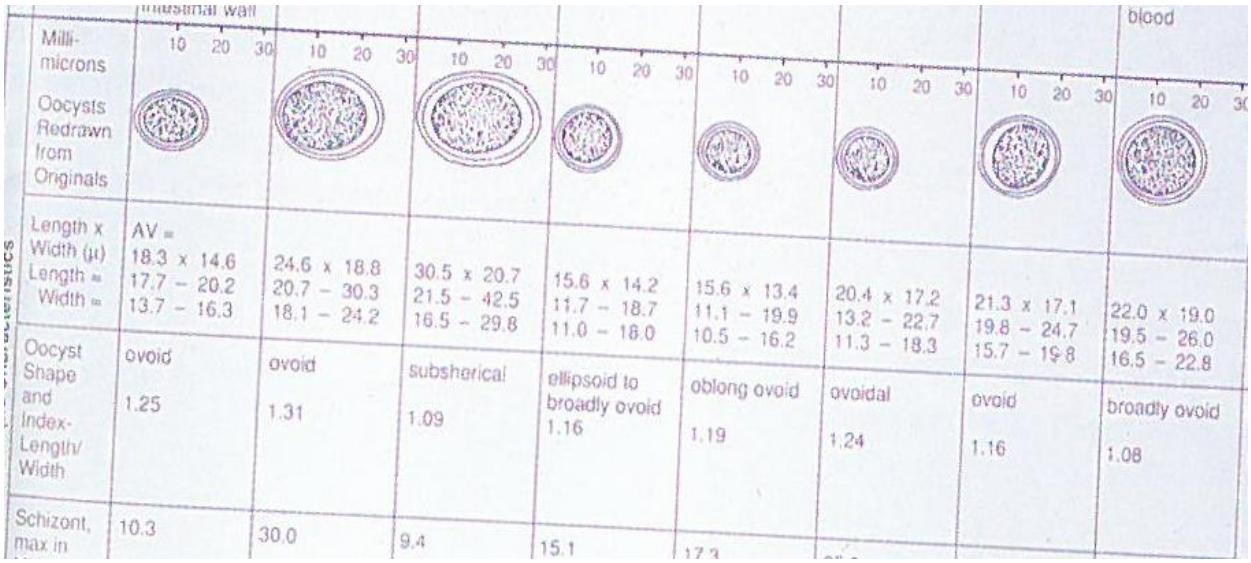
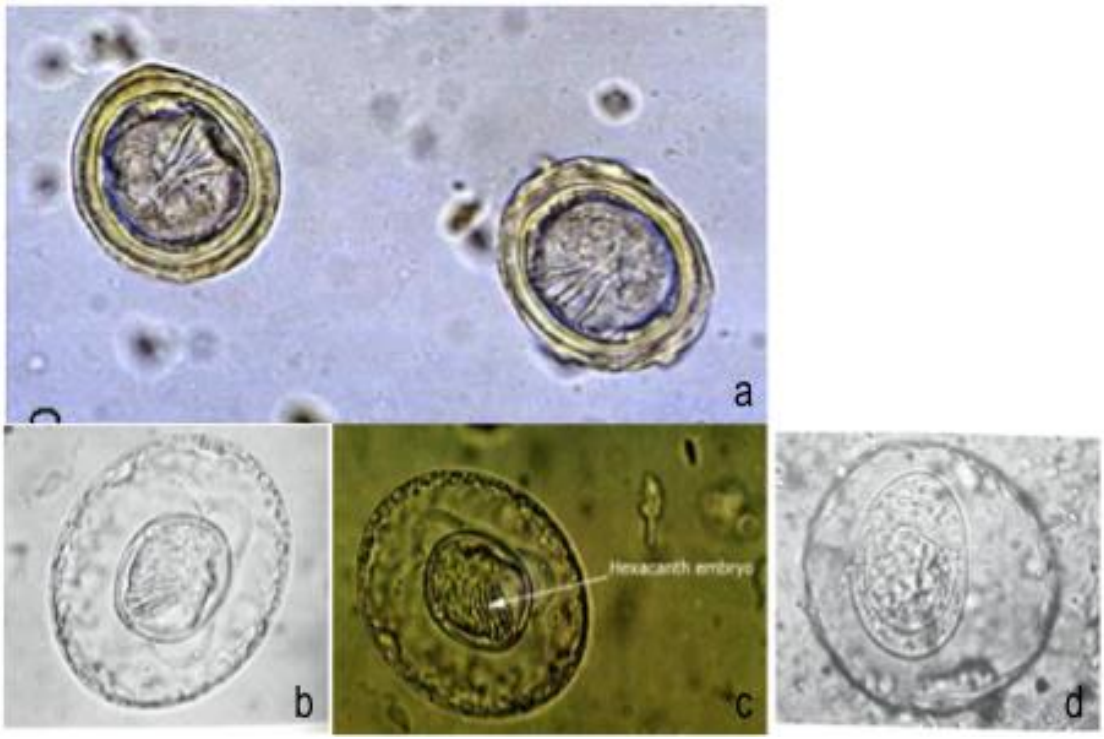
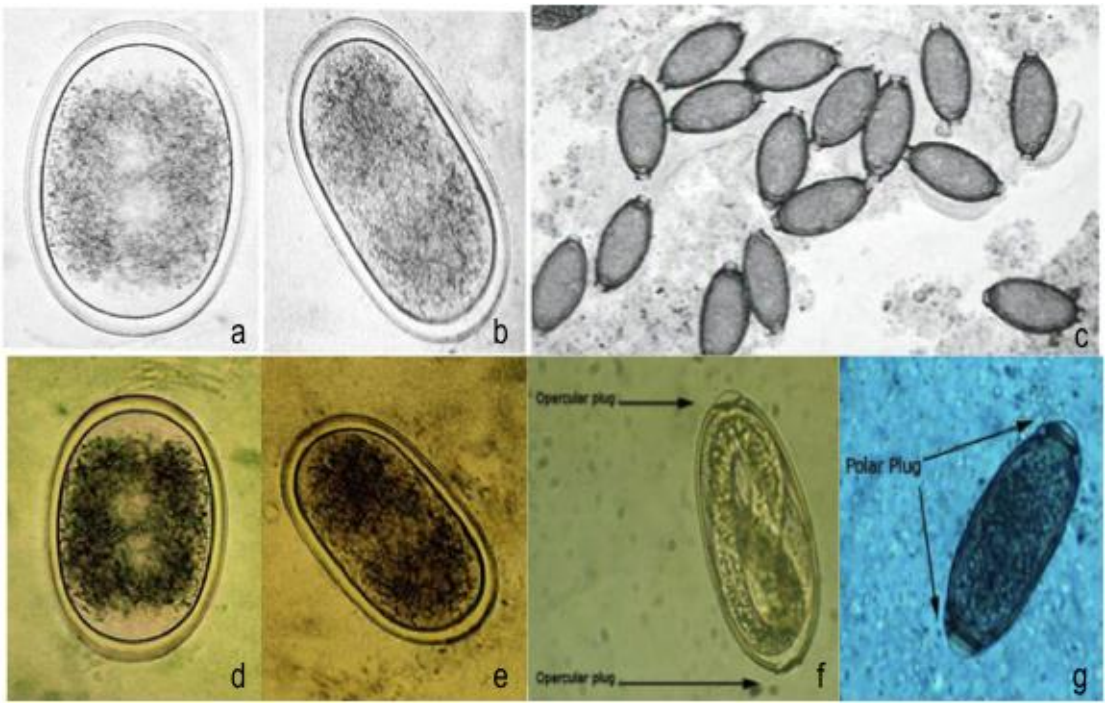


Figure A.2. Description of the *Eimeria* species infecting chicken: *E. acervulina, E. brunetti, E. maxima, E.mivati, E. mitis, E. necatrix, E. praecox and E. tenella* (from left to right).

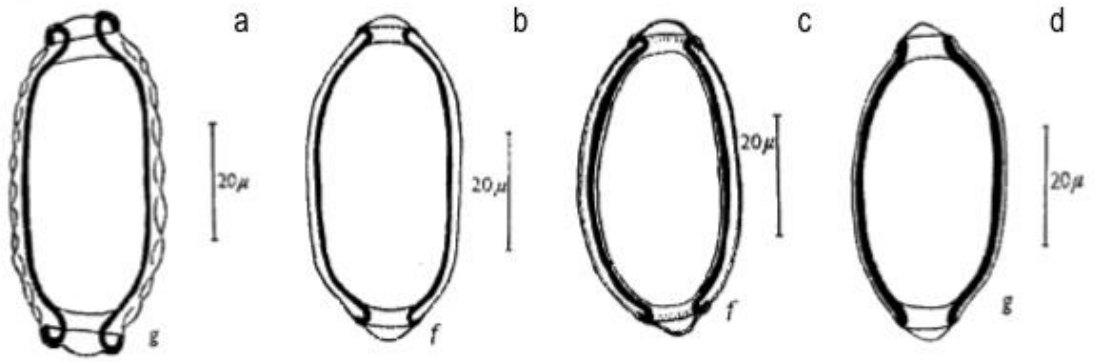


Figures A.3a, A.3b, A.3c and A.3d. *Choanotaenia infundibulum* (a). No uterine capsules, eggs loose in parenchyma. Egg 47 µm x 54 µm, outer shell delicate; processes present at poles, appear to recede when embryophore fully developed***;*** ***Raillietina spp.*** **(b, c).** Egg 25 µm – 50 µm, with thick and smooth shell. Hexacanth embryo present. Eggs shed only when mature segment and egg capsules disintegrate; ***Hymenolepis spp.*** **(d).**

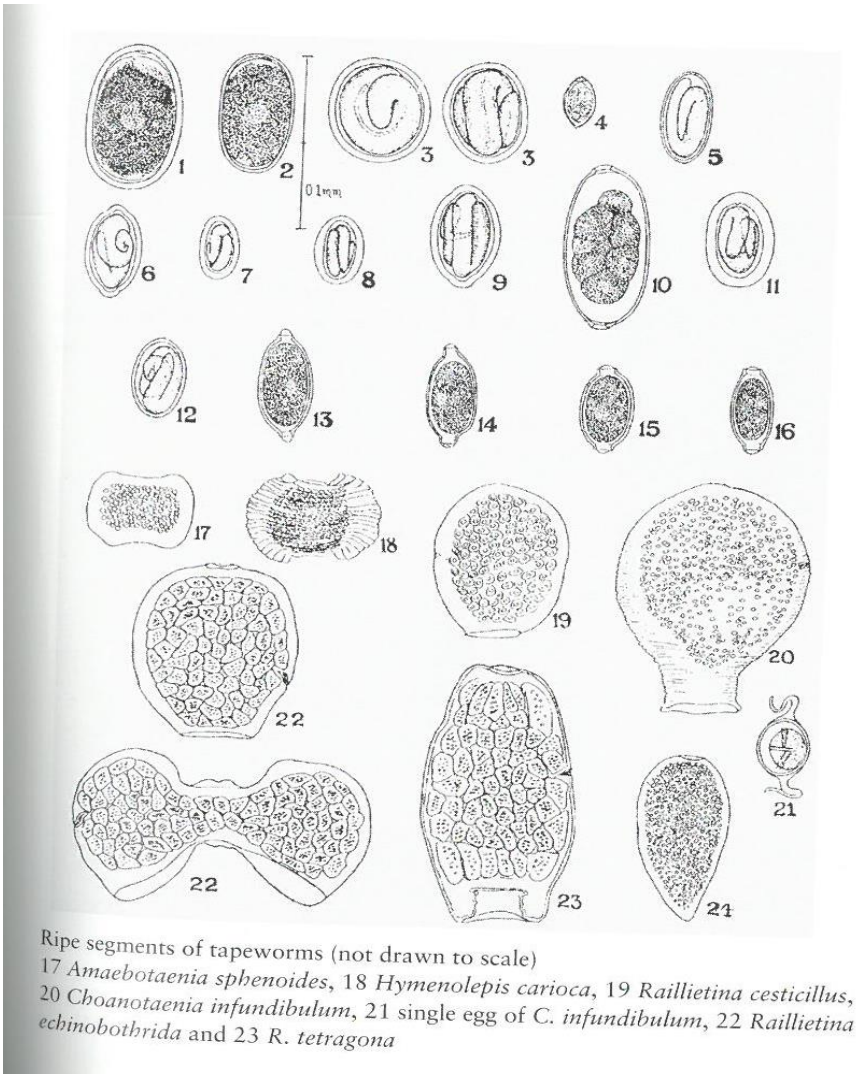


Figures A.4a, A.4b, A.4c, A.4d, A.4e and A.4f. Parasite eggs found in chicken: *Ascaridia spp.* (a, d). Egg length 68 µm - 90 µm and width 40 µm – 50 µm. Thick smooth shell made of three layers, with slight barrel-shaped sidewalls. Contents unsegmented; ***Heterakis spp.*** **(b, e)** Egg length 59 µm –75 µm and width 31 µm– 48 µm. Shell thick, smooth sidewalls. Contents unsegmented; ***Capillaria spp.*** **(c, g)** Egg length 43 µm – 65 µm and width 20 µm – 35 µm. Shell thick, smooth and brown. Slightly barrel-shaped with asymmetrical sidewalls. Two protruding polar plugs present. Contents granular and unsegmented; ***Syngamus trachea*** **(f)** Egg length 78 µm – 100 µm and width 43 µm – 60 µm. Shell smooth, side-walls Slightly barrel-shaped with opercular plugs at both poles. Morula of 8 – 16 blastomeres present.

Figures A.5a, A.5b and A.5c. Parasite eggs found in chicken: *Echinostoma spp.* (a) Egg length 97 µm – 126 µm and width 60 µm – 70 µm; ***Trichostrongylus tenuis*** **(b, c)** Egg length 65 µm – 75 µm and width 35 µm – 42 µm. Shell thin and smooth. Oval parallel sides with dissimilar poles.



Figures A.6a, A.6b, A.6c and A.6d. Parasite eggs found in chicken: Capillaria anatis (a); Capillaria bursata (b); Capillaria caudinflata (c) and Capillaria obsignata (d).



Ripe segments of tapeworms (not drawn to scale)
17 *Amaebotaenia sphenoides*, 18 *Hymenolepis carioca*, 19 *Raillietina cesticillus*, 20 *Choanotaenia infundibulum*, 21 single egg of *C. infundibulum*, 22 *Raillietina echinobothrida* and 23 *R. tetragona*

Fig. 630
Helminth eggs and segments of cestodes found in chicken [3]

1 *Ascaridia galli*
2 *Heterakis gallinarum*
3 *Subulura brumpti*
4 *Prosthogonimus* sp.
5 *Strongyloides avium*
6 *Tetrameres americana*
7 *Acuaria spiralis*
8 *Acuaria hamulosa*
9 *Gongylonema ingluvicola*
10 *Syngamus trachea*
11 *Hartertia gallinarum*
12 *Oxyspirura mansoni*
13 *Capillaria annulata*
14 *Capillaria retusa*
15 *Capillaria columbae*
16 *Capillaria longicollis*

Figure A.7. Helminth eggs, segments of cestodes and ripe segments of tapeworms found in chicken.

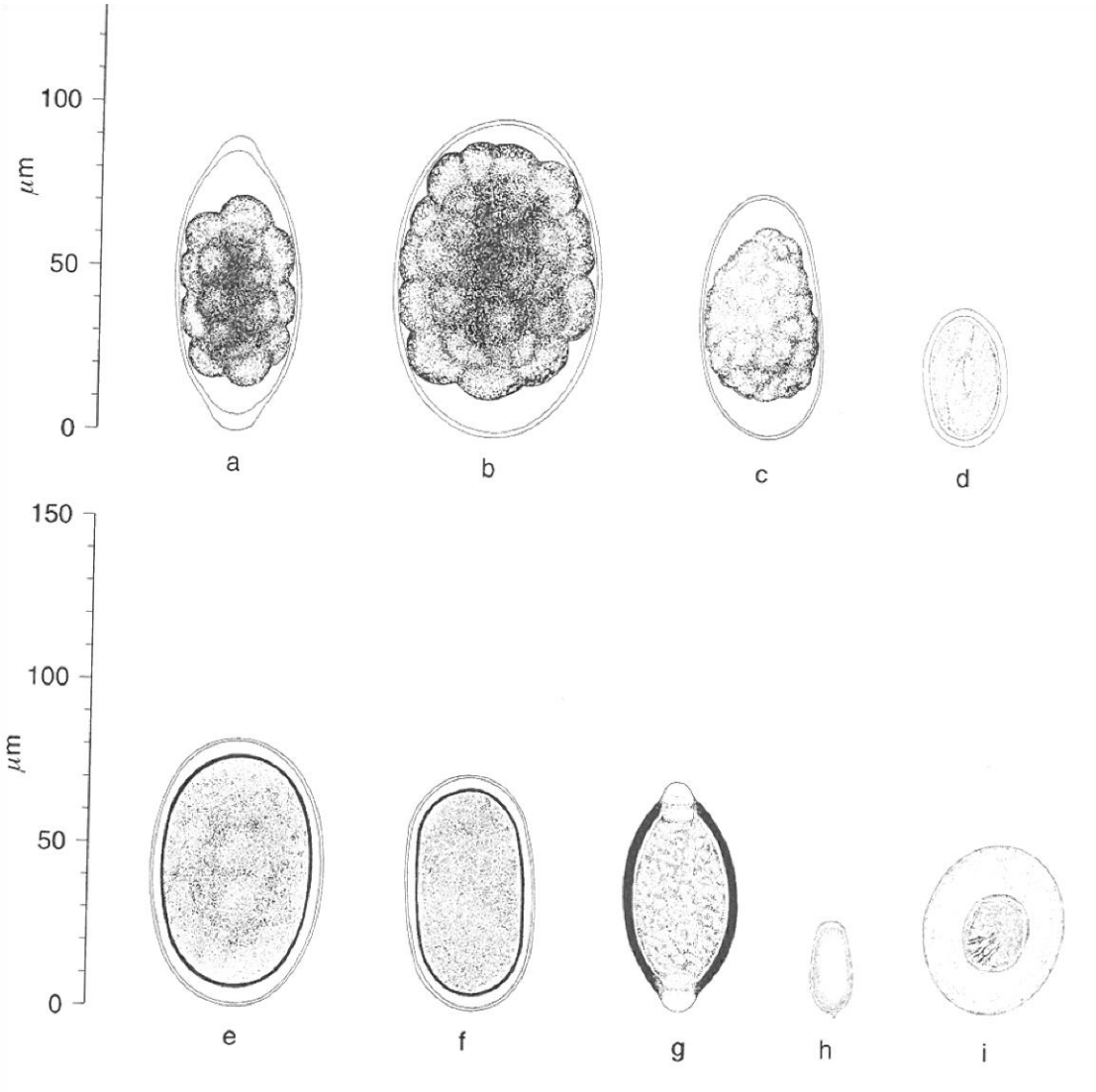


Figure A.8. Parasite eggs found in chicken: *Syngamus trachea* (a); *Amidostomum spp.* (b); *Trichostrongylus tenuis* (c); *spiruroid-type egg* (d); *Ascaridia spp.* (e); *Heterakis spp.* (f); *Capillaria spp.* (g); *Prosthgonimus spp.* (h); *tapeworm egg* (i).

AP3. PROTOCOL FOR *EIMERIA SPP.* FAECAL HARVEST

1. A countertop laboratory blender was used to homogenise the soaked faecal suspensions (A and B). Medium speed was applied for 15 minutes, or longer if required, until there were no solid lumps left.

2. Assessment of number of parasites present: 2 x 1 ml samples of the homogenate were collected using a Pasteur pipette diluted 1:10 with saturated saline, mixed well and counted in a McMaster slide ["McMaster method" in section 2.7.4.2 (A)].

3. A sieve (mesh size of approx. 300 µm) was placed on top of a fresh beaker. The homogenate was poured onto the sieve in small amounts, agitated and pressed by hand. Any remaining of faecal material in the sieve was flushed with an equal amount of water to help flush out remaining parasites (done twice). Finally, any remaining water was carefully squeezed out by hand. This step was repeated until all homogenate was strained through the sieve. Pressed faeces were discarded.

4. Assessment of number of parasites lost during sieving: done as in step number 2.

5. The homogenate was stirred with a palette knife and poured into 0.5 L sterile centrifuge bottles; these were capped and weighed on scales to balance. Bottles were centrifuged at 750 x *g* for 10 min.

6. After centrifuging, approx. 2/3 of supernatant was poured carefully into a beaker and a drop on a glass slide was checked microscopically for any oocysts that may have failed to pellet. Supernatant was discarded if no oocysts were seen.

7. Centrifuge bottles were half filled with saturated salt solution and shaken vigorously to homogenise the pelletted material. Then, bottles were topped up to the shoulder with saline, balanced and centrifuged as before (step 5).

8. Post-centrifugation the oocysts should form a layer at the surface of the salt, giving the appearance of a layer of “scum”. The floating layer was removed using an electronic pipette (removing liquid from sides to centre until approx. all scum was taken), then poured into a sterile beaker. Centrifuge bottles were shaken and re-suspended again with saline up to the shoulder.

9. Bottles were centrifuged at 750 x *g* for 10 min. to pellet the oocysts.

10. Flotation washes with saline (steps 8-9) were repeated 3 times, by re-mixing the remaining saline solution with the pellet in the centrifuge bottle. On the second wash, mixed salt supernatant was checked for unpelleted oocysts on a microscope slide to make sure it could be discarded (very few oocysts were seen).

11. After that, the oocyst suspension collected in a separate beaker was washed four times in fresh tap water by successive rounds of re-suspension and centrifugation (750 x *g*, 10 min) to remove any residual salt that could inhibit sporulation.

    a. The spared oocyst suspension was poured into fresh 0.5 L centrifuge bottles to wash. These were filled 1:4 in tap water to centrifuge (the more water, the easier is to wash the salt off).

    b. The steps of centrifugation-discard 2/3 supernatant pouring into beaker and refilling with water were done 3 times. This time oocysts should have pelleted, so centrifuge bottles were not shaken to not disturb it. Discarded supernatant was checked for unpelleted oocysts between each wash on a microscope slide.

    c. Before a last wash, sample pellets were pooled to one bottle, shaking pellet and rinsing from one to another plus a final rinse with water. From this bottle the content was split to 50 ml tubes, topped all up with water to do a fourth wash.

12. The oocyst harvest was enumerated microscopically using a McMaster slide [“McMaster method” in section 2.7.4.2 (A)], as it was

too dirty to use a modified Fuchs Rosenthal slide, and was diluted as required with 2% potassium dichromate in distilled water to a final concentration of ~ 15,000 oocysts/ml in conical flasks.

13. Total number of oocysts in the final suspension was calculated using a McMaster slide.

14. As final concentration should be of approx. 15,000 oocysts/ml, final volume (ml) was calculated doing: total n∘ oocysts/15,000.

15. Final volume (V) included: suspension of oocysts + potassium dichromate + distilled water.

    - V of 6% potassium dichromate to add = (total V- oocyst suspension V)/3
    - V distilled water to add = total V - oocyst suspension V - potassium dichromate V

Preparation for sporulation

16. Four sterile 250 ml glass conical flasks were filled to half with the final mix and sealed with perforated parafilm on the neck.

17. Flasks were placed in a rack fitted on an automated shaker station, set to a low speed (150 rpm) and +28°C. No additional aeration was required.

18. They were left for 4.5 days. After that time, the mix was checked that sporulation had occurred by microscopic examination for defined sporozoites/sporocysts within the oocysts [“Drop count” in section 2.7.4.2 (C)].

19. Given a suitable level of sporulation, the oocyst culture was transferred to 50 ml tubes and stored in 2% potassium dichromate solution at +4°C.

AP4. PROTOCOLS FOR DNA EXTRACTION METHODS

A. *MpBIO FastDNA® Spin kit for soil – modified version from manufacturers' handbook.*

Kit contents

Sodium phosphate buffer, MT buffer, PPS solution, Binding matrix, Concentrated SEWS-M and DES buffer.

Protocol

1. Add approx. 50 mg of freeze-dried faecal sample to a Lysing Matrix E tube.
2. Add 978 µl Sodium Phosphate Buffer to sample in Lysing Matrix E tube.
3. Add 122 µl MT Buffer. Mix well to avoid clogging.
4. Homogenize in the FastPrep® Instrument for 40 sec at a speed setting of 6.0 and rest in ice for 1 min. Repeat step.
5. Centrifuge at 14,000 *x g* for 8 min to pellet debris.
6. Transfer supernatant to a clean 2.0 ml microcentrifuge tube (MCT). Add 250 µl PPS (Protein Precipitation Solution) and mix by shaking the tube by hand 10 times.
7. Centrifuge at 14,000 *x g* for 5 min to pellet precipitate. Transfer supernatant to a 2.0 ml MCT.
8. Re-suspend Binding Matrix suspension and add 1.0 ml to supernatant in MCT tube.
9. Place on rotator for 2 min to allow binding of DNA. Place tube in a rack for 3 minutes to allow settling of silica matrix.
10. Remove and discard 500 µl of supernatant being careful to avoid settled Binding Matrix.
11. Re-suspend Binding Matrix in the remaining amount of supernatant. Transfer approx. 700 µl of the mixture to a SPIN™ Filter and centrifuge at 14,000 *x g* for 1 min. Empty the catch tube and add the remaining

mixture to the SPIN™ Filter and centrifuge at 14,000 *x g* for 2 min. Empty the catch tube again.

12. Add 500 µl prepared SEWS-M and gently re-suspend the pellet using the force of the liquid from the pipette tip. NOTE: Ensure that ethanol has been added to the Concentrated SEWS-M.
13. Centrifuge at 14,000 *x g* for 1 min. Empty the catch tube and replace. Repeat SEWS-M wash (step 12) and centrifuge for 2 min. Empty the catch tube and replace.
14. Without any addition of liquid, centrifuge again at 14,000 *x g* for 2 min to “dry” the matrix of residual wash solution. Discard the catch tube and replace with a new, clean catch tube.
15. Air dry the SPIN™ Filter for 5 min at room temperature.
16. Gently resuspend Binding Matrix (above the SPIN filter) in 100 µl of DES (DNase/ Pyrogen-Free Water).
17. Centrifuge at 14,000 *x g* for 3 min to bring eluted DNA into the clean catch tube. Extracted DNA should be stored at +4 or -20°C until further analysis.

B. MpBIO FastDNA® Spin kit for faeces – modified version from manufacturers’ handbook.

Kit contents

Sodium phosphate buffer, PLS solution, MT buffer, PPS solution, Binding matrix, Wash buffer #1, Wash buffer #2 and TES buffer (C6H14NO6SNa).

Protocol

1. Add approx. 50 mg of freeze-dried faecal sample to a Lysing Matrix E tube.
2. Add 600 µl Sodium Phosphate Buffer to sample in Lysing Matrix E tube.
1. Add 200 µl PLS solution. Mix well to avoid clogging.
2. Centrifuge at 14,000 *x g* for 5 min and discard supernatant using pipette.

3. Add 978 µl Sodium Phosphate Buffer to sample in Lysing Matrix E tube.
4. Add 122 µl MT Buffer and vortex to mix so that no sample is stuck to the lysis tube walls.
5. Homogenize in the FastPrep® Instrument for 30 sec at a speed setting of 6.0 and rest in ice for 1 min. Repeat step two more times.
6. Centrifuge at 14,000 *x g* for 10 min to pellet debris.
7. Add 250 µl PPS (Protein Precipitation Solution) to a clean 2.0 ml MTC. Transfer supernatant to MCT, shake vigorously to mix and incubate on ice for 10 min.
8. Centrifuge at 14,000 *x g* for 5 min to pellet precipitate.
9. Transfer supernatant to a MCT.
10. Re-suspend Binding Matrix suspension and add 900 µl to supernatant in MCT tube.
11. Shake gently by hand to mix and allow binding of DNA, then place on rotator for 4 min.
12. Centrifuge at 14,000 *x g* for 2 min and discard supernatant.
13. Wash the binding mixture pellet by gently resuspending with 650 µl Wash buffer #1. Transfer all mixture a SPIN™ Filter and centrifuge at 14,000 *x g* for 1 min.
14. Add 500 µl prepared Wash buffer #2 to the SPIN™ Filter and gently re-suspend the pellet using the force of the liquid from the pipette tip. NOTE: Ensure that ethanol has been added to the Wash buffer #2.
15. Centrifuge at 14,000 *x g* for 2 min. Empty the catch tube and replace. Repeat Wash buffer #2 (step 12) and centrifuge for 2 min. Empty the catch tube and replace.
16. Without any addition of liquid, centrifuge again at 14,000 *x g* for 2 min to “dry” the matrix of residual wash solution.
17. Discard the catch tube and replace with a new, clean catch tube.
18. Add 100 µl of TES above the SPIN™ Filter and gently re-suspend the pellet.
19. Centrifuge at 14,000 *x g* for 3 min to bring eluted DNA into the clean catch tube. Extracted DNA should be stored at +4 or -20°C until further analysis.

*C. Qiagen QiaAMP DNA Stool mini kit – modified version from manufacturers' handbook***.**

Kit contents

Buffer ASL, InhibitEX® tablets, Proteinase K, Buffer AL, Buffer AW1 (concentrate), Buffer AW2 (concentrate) and Buffer AE.

Protocol

1. Weigh 50 mg of freeze-dried faecal sample in a 2 ml screw-cap tube and place the tube on ice.
2. Add 1.4 ml Buffer ASL to each sample. Then add 1 big glass bead + 0.3 g of powdered beads. Bead-beat for 2 min.
3. Heat the suspension for 5 min at 70 °C using a heat block.
4. Vortex for 15 sec and centrifuge sample at 14,000 x *g* for 1 min to pellet stool particles.
5. Add 1.2 ml of the supernatant into a new 2 ml MTC and discard the pellet.
6. Add 1 InhibitEX Tablet (broken in 2 halves) to each sample and vortex immediately and continuously for 1 min or until the tablet is completely suspended. Incubate suspension for 1 min at room temperature to allow inhibitors to adsorb to the InhibitEX matrix.
7. Centrifuge sample at 14,000 x *g* for 4 min to pellet inhibitors bound to InhibitEX matrix.
8. Transfer all the supernatant into a new 1.5 ml MCT and discard the pellet. Centrifuge the sample at 14,000 x *g* for 3 min. *Transfer of small quantities of pelleted material from step 7 will not affect the procedure.*
9. Add 15 µl proteinase K into a new 1.5 ml MCT.
10. Add 200 µl supernatant from step 8 into the MCT containing proteinase K.
11. Add 200 µl Buffer AL and vortex for 15 sec, ensuring that they are thoroughly mixed to form a homogeneous solution. Note: Do not add proteinase K directly to Buffer AL.
12. Incubate at 70°C for 10 min.

13. Add 200 µl of ethanol (96–100 %) to the lysate, and mix by vortexing.
14. Label the lid of a new QIAamp spin column placed in a 2 ml collection tube. Carefully apply the complete lysate from step 13 to the QIAamp spin column without moistening the rim. Close the cap and centrifuge at 14,000 x *g* for 1 min. Empty the collection tube containing the filtrate and replace.
15. Carefully open the QIAamp spin column and add 500 µl Buffer AW1. Close the cap and centrifuge at 14,000 x *g* for 1 min. Empty the collection tube and replace.
16. Carefully open the QIAamp spin column and add 500 µl Buffer AW2. Close the cap and centrifuge at 14,000 x *g* for 3 min.
17. Place the QIAamp spin column in a new 2 ml collection tube and discard the old collection tube with the filtrate. Centrifuge at 14,000 x *g* for 1 min.
18. Transfer the QIAamp spin column into a new, labeled 1.5 ml MCT. Carefully open the QIAamp spin column and pipet 150 µl Buffer AE directly onto the QIAamp membrane. Close the cap and incubate for 1 min at room temperature, then centrifuge at 14,000 x *g* for 1 min to elute DNA. Extracted DNA should be stored at +4 or -20°C until further analysis.

D. Modified phenol-chloroform (by Dr. Eric Pinloche)

Reagents needed

Lysis buffer, 3M Potassium acetate pH 5.5, 5M NaCl, CATB/NaCl, chloroform, isopropanol, 70% ethanol and TE buffer.

Protocol

1. Weight 50 mg of freeze-dried faecal sample in a 2 ml MCT (with flat cap) or in a screw-top tube, but not conical eppendorfs. Add 1 big bead.
2. Add 800 µl of Lysis buffer (warm 1L bottle + agitate with magnet).

3. Bead-beat for 2 min (for *Eimeria* oocysts). For bacteria 30 sec, yeasts 1 min (if using dried material).
4. Incubate in heat block at 80 °C for 5 min (put weight on top as caps can pop-up). If samples are foamy afterwards, centrifuge at 0.5 *x g* for 1 min.
5. Add 50 µl of 3M Potassium acetate pH 5.5, invert by hand 5 times (so you hear the bead shaking), and check that they are well mixed.
6. Incubate in ice for 2-3 min, just to get them cool and until you see the precipitate.
7. Centrifuge at 17,000 *x g* for 5 min.
8. Transfer 600 µl of supernatant to a new 2 ml MCT.
9. Add 100 µl of 5M NaCl.
10. Add 100 µl of CATB/NaCl, making sure that is heated first (it jellifies) and you pipette fast. Vortex for 5-10 sec.
11. Incubate in heat block at 60°C for 5 min.
12. Add 400 µl of chloroform under the fume hood (wet the tip of the pipette on the liquid first, that avoids it to leak so fast). Vortex well to get a strong emulsion.
13. Centrifuge at 17,000 *x g* for 5 min. The supernatant should be rather clear in colour, and the pigments should remain trapped with the chloroform in the bottom layer; that means you have a good quality DNA.
14. Recover 600 µl of supernatant* to a new 1.5 ml eppendorf tube under the fume hood, empty and discard the original tubes in the special waste. *Use the technique: incline the tube towards the side of the centrifuged waist tail, and place the pipette tip in the opposite side; suck liquid moving the pipette slowly from top to bottom, to avoid absorbing the waist of the solid layer.
15. Add 600 µl of isopropanol (at room temperature) and invert by hand.
16. Centrifuge at 17,000 *x g* for 5 min (at room temperature). Discard supernatant in bin and tapping into tissue.
17. Add 500 µl of 70% ethanol, centrifuge at 17,000 *x g* for 1 min and discard supernatant in bin and tapping into tissue. Do this step twice.

To remove the ethanol completely, you can pulse the samples and take out the last drop using the pipette.

18. Leave the samples to stand for 15 min with the caps opened at room temperature.
19. Add 150 µl of TE buffer incubate in heat block at 50°C for 1 min and leave samples to stand for 5 min at room temperature. Then vortex as much as needed to re-suspend the DNA or leave them at +4°C overnight. Extracted DNA should be stored at +4 or -20°C until further analysis.

AP5. PARASITE EGG AND OOCYST PICTURES

NEMATODES (ROUNDWORMS)

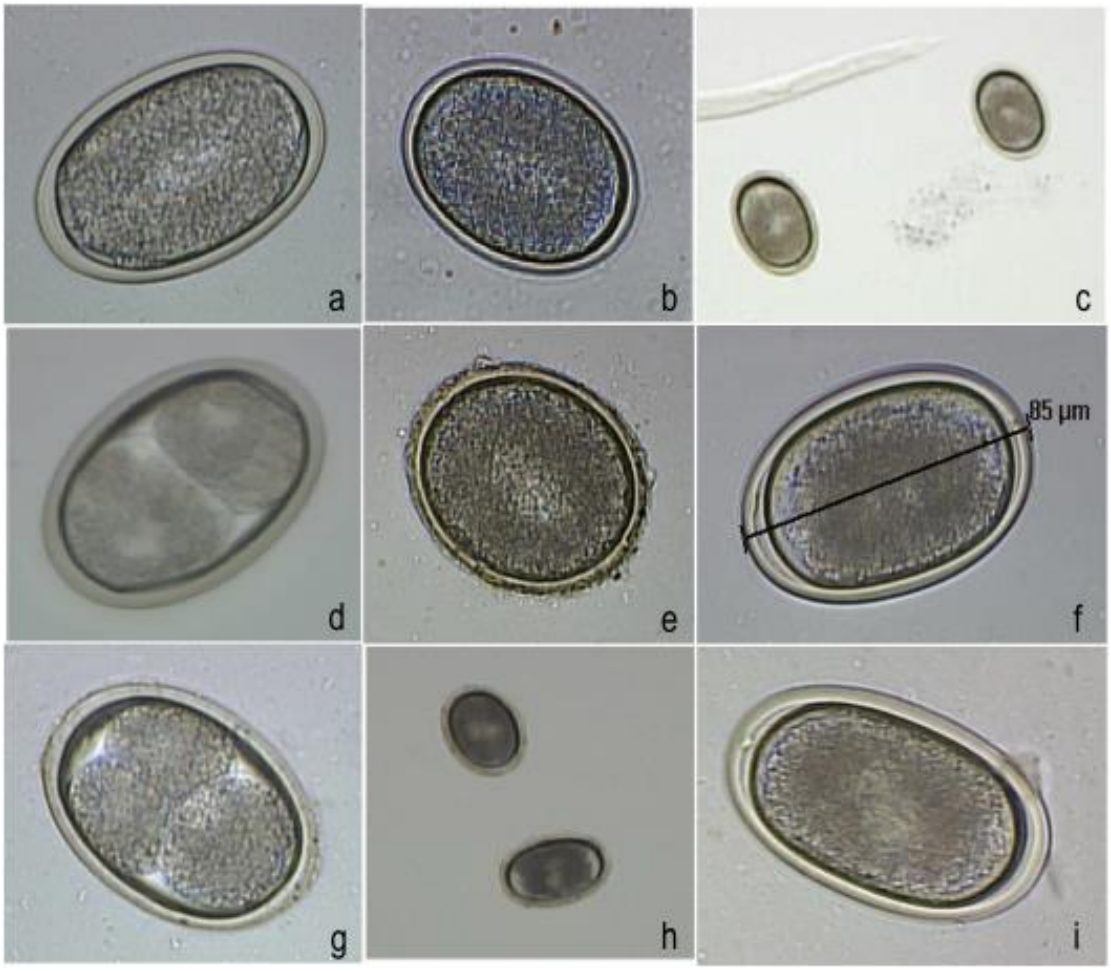


Figure A.9. *Ascaridia galli* eggs; immature eggs (a, b, e, f, i) and eggs in embryonation stage (c, d, g, h).

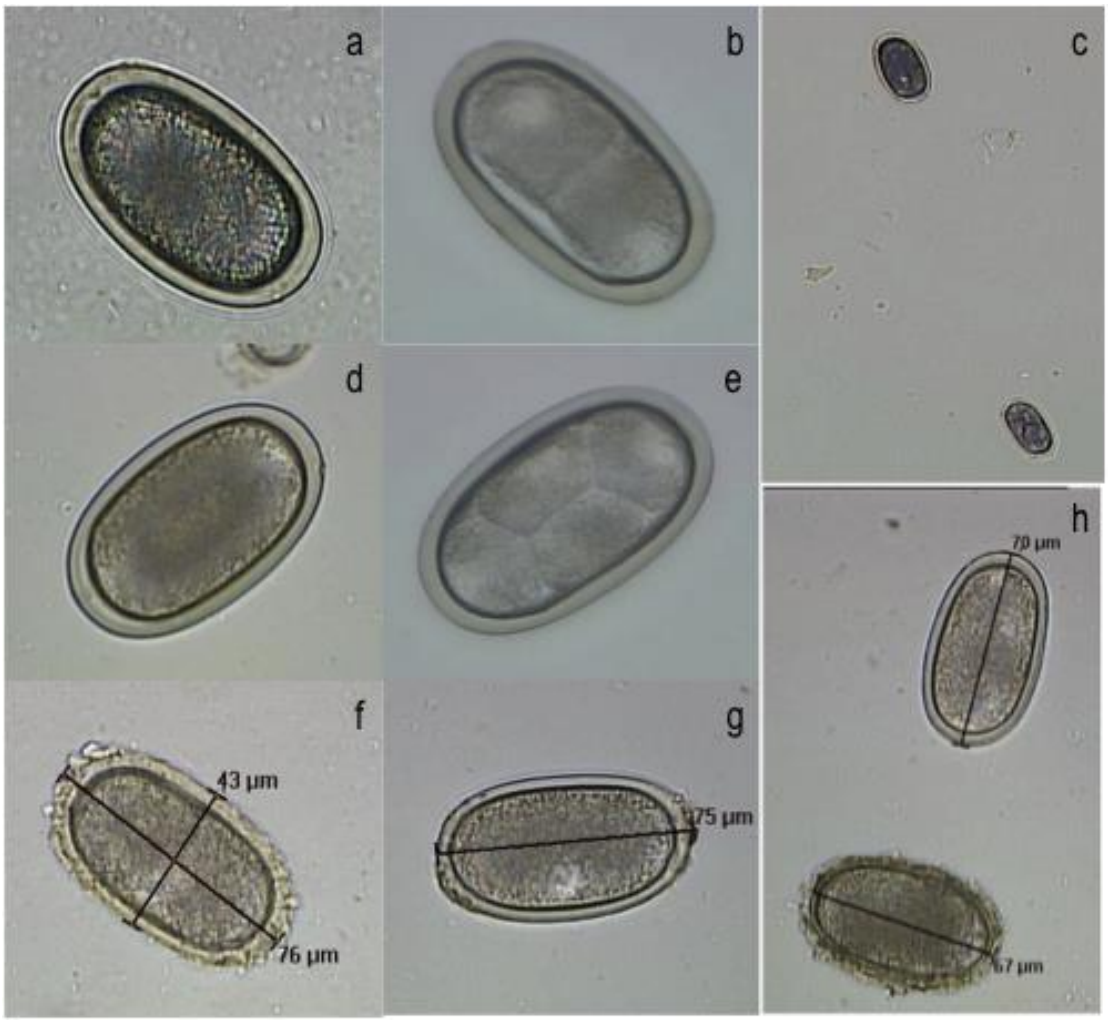


Figure A.10. ***Heterakis gallinarum*** **eggs; immature eggs (a, b, c, d, f, g, h) and eggs in embryonation stage (b, e).**

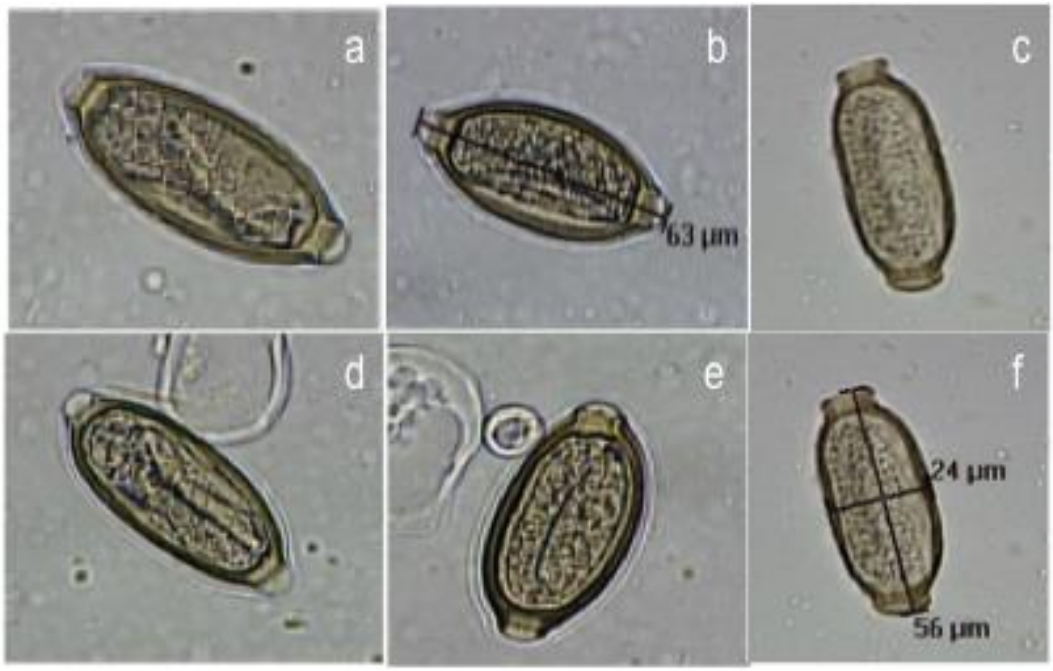


Figure A.11. ***Capillaria spp.*** **eggs*: Capillaria anatis (c, f) and Capillaria caudinflata (a, b, d, e).***

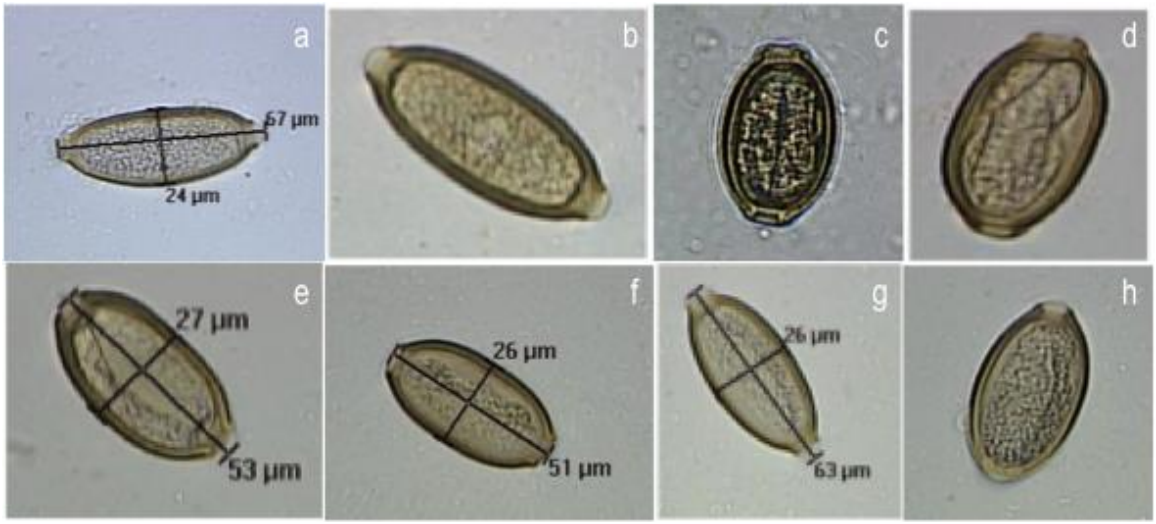


Figure A.12. ***Capillaria spp.*** **eggs*: Capillaria bursata (a, b, g) and Capillaria obsignata (c, d, e, f, h).***

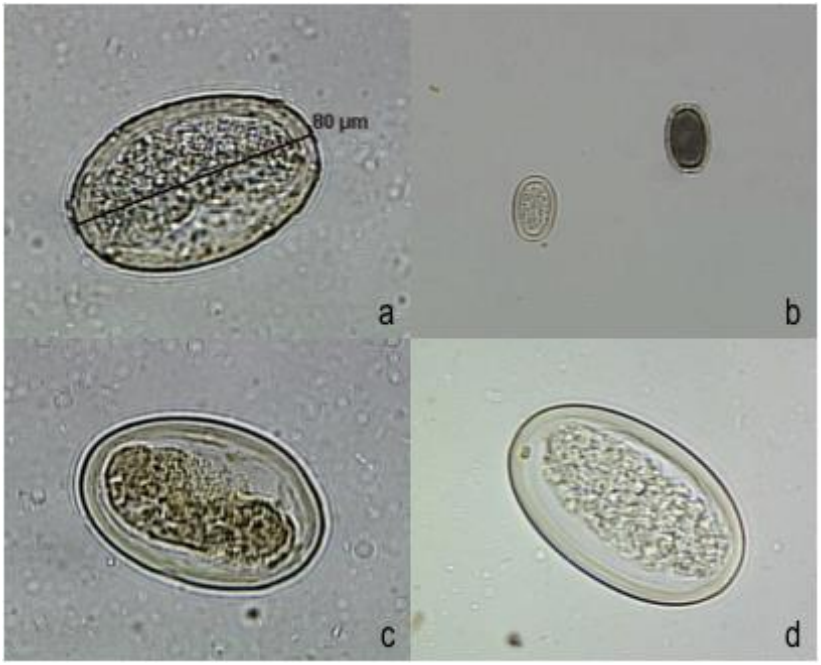


Figure A.13. Unidentified species of Strongyle-type parasite eggs (a, c, d). Unidentified egg (left) with an *Ascaridia galli* egg (right) (b).

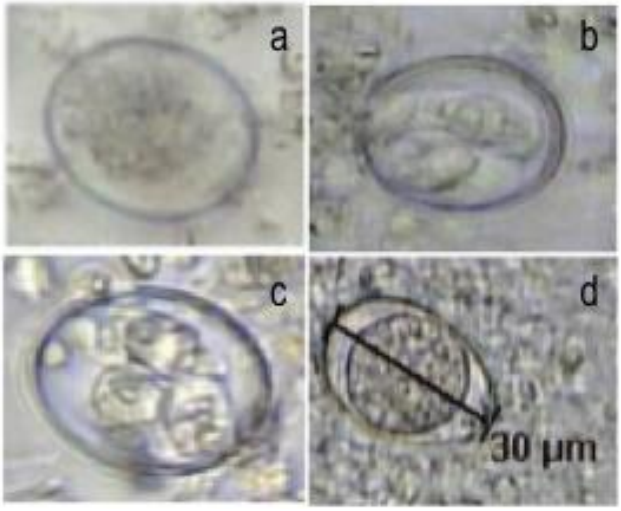


Figure A.14. *Eimeria spp.* oocysts. Unsporulated (a, d) and sporulated oocysts (b, c), containing four sporocysts each.

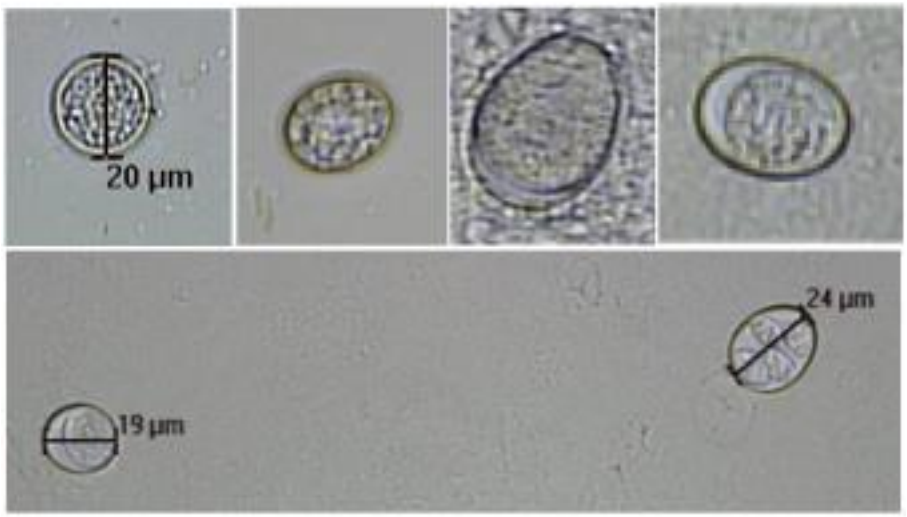


Figure A.15. *Eimeria spp.* oocysts.

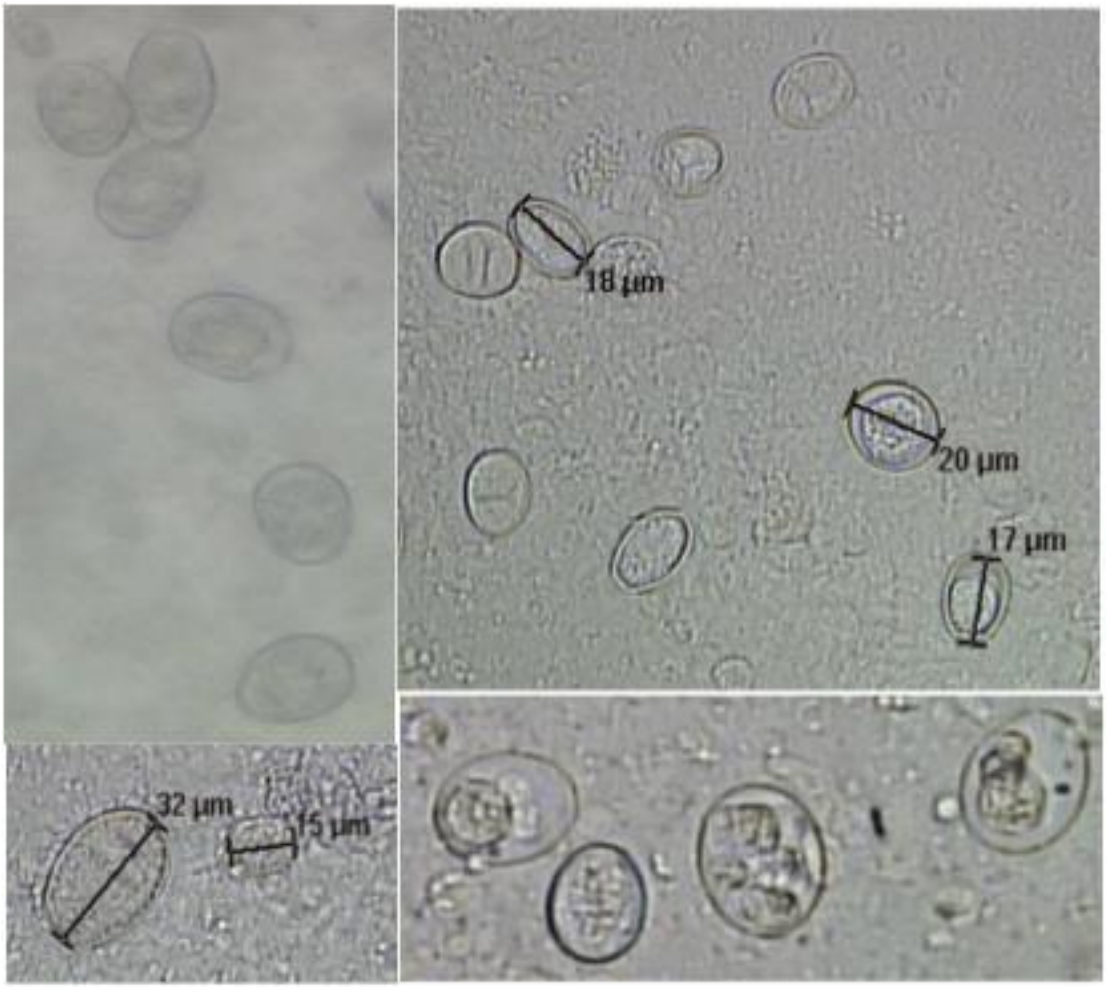


Figure A.16. *Eimeria spp.* oocysts of a variety sizes and shapes, probably corresponding to different species.

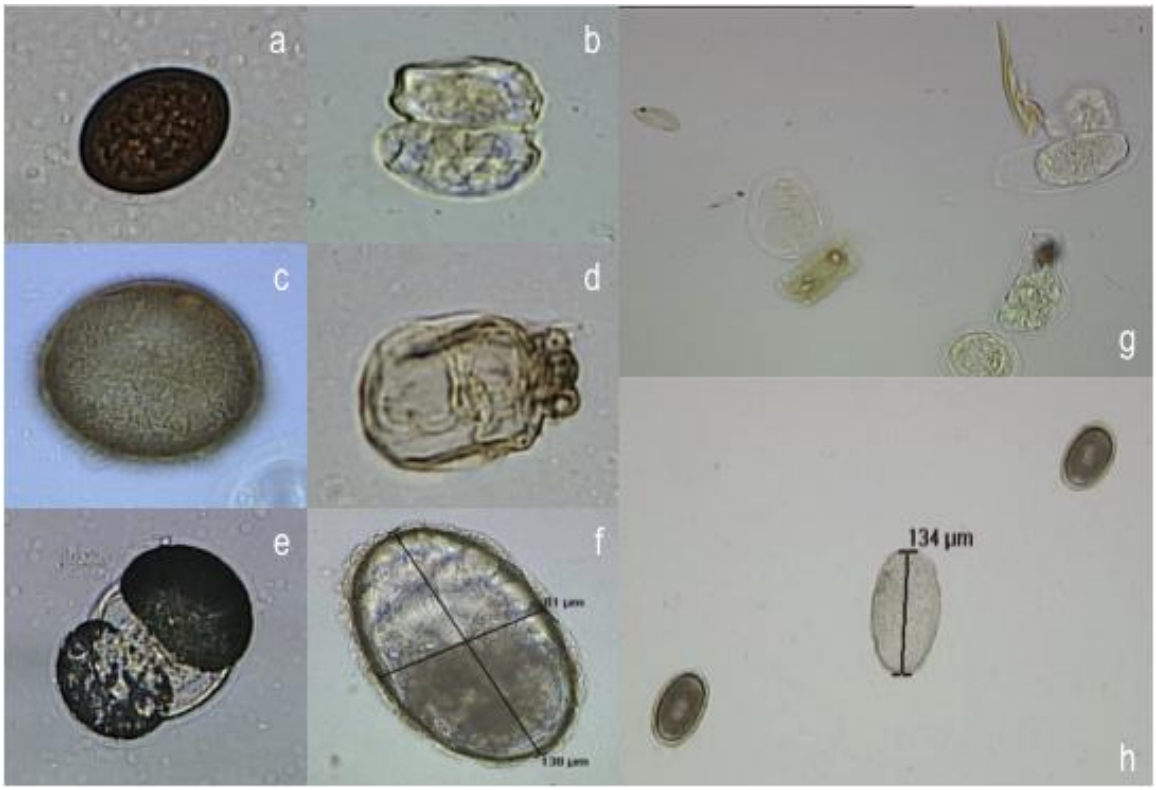


Figure A.17. Artefacts found in faecal tests: pollen (a, c) and pine pollen grain (e), fibrous material (b, g), free-living mite (d), mite egg (f) and a mite egg between two *Ascaridia galli* eggs (h).

AP6. EGG QUALITY PICTURES

EXTERNAL EGG QUALITY

Pictures were taken at the time of quality analysis.

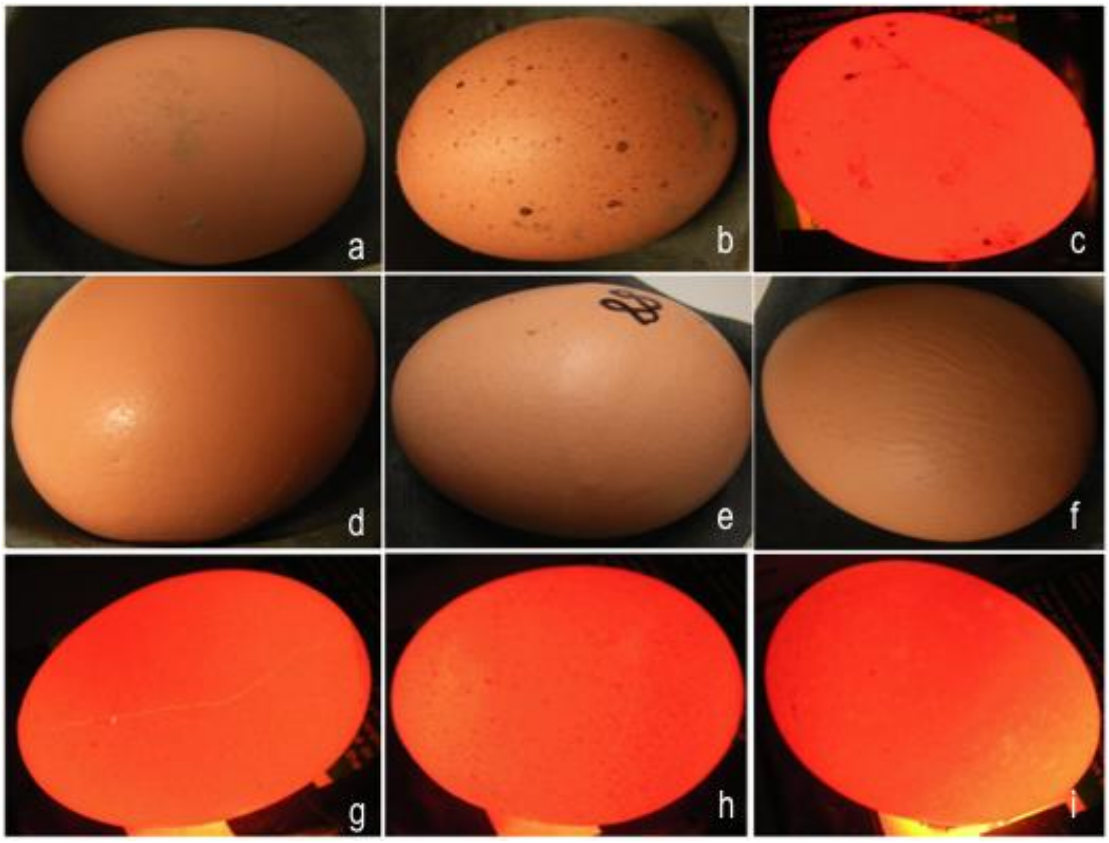


Figure A.18. Dirty eggs (a, b) and a dirty egg seen in the candler (c); pinhole (d); pimple (e); body checks (f); hairline cracked egg (g); egg with weak shell areas (h); mottled egg (i).

Pictures were taken at the time of quality analysis.

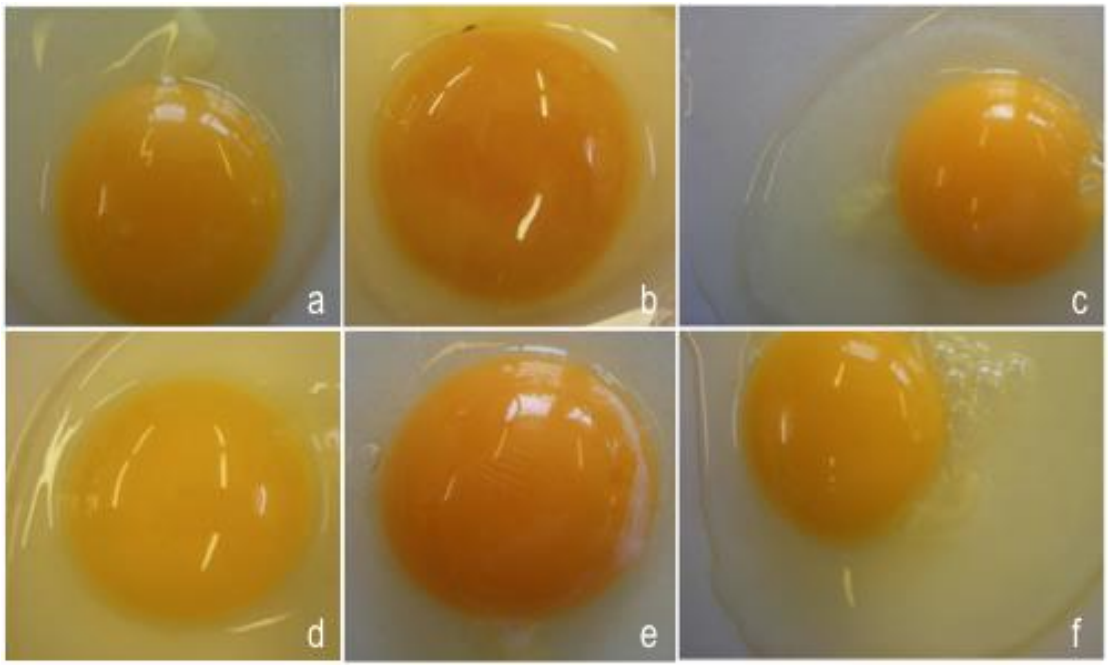


Figure A.19. Eggs with discoloured yolks (a, b); eggs with watery whites (c, f); pale yolk (d); moderate coloured yolk (e).

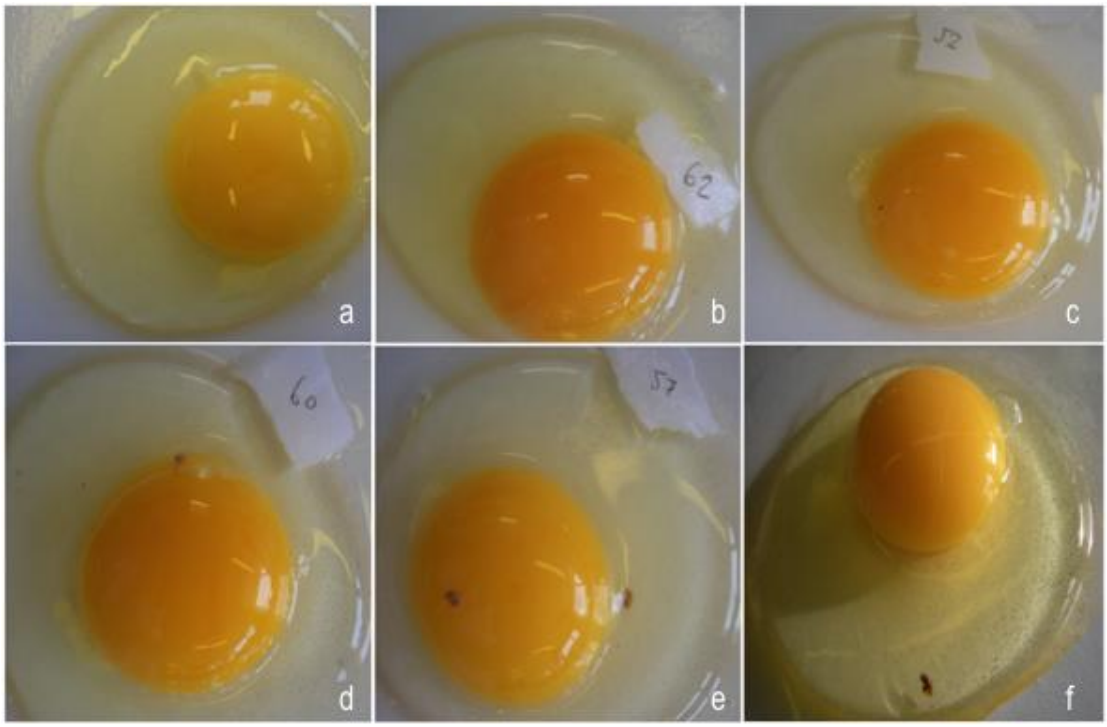


Figure A.20. Egg with no inclusions (a); eggs with blood spots (b, c); eggs with meat spots (d, e); a single large meat spot (f).