# Evidence-based consideration of dietary 'alternatives' to anticoccidial drugs to help control poultry coccidial infections

Leon J. Broom[ab*]

[a]Gut Health Consultancy, Exeter, Devon EX14 1QY, UK

[b]Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, UK

*guthealthconsultancy@gmail.com

Coccidiosis remains a major disease and economic challenge for the global poultry industry. Coccidiosis in chickens is caused by seven *Eimeria* species that target specific regions of the gastrointestinal tract and cause malabsorptive or haemorrhagic disease. These *Eimeria* species infect segment-specific epithelial cells and thus need to navigate the host's indigenous microbiome and intestinal defences to establish an infection and cause disease. Good husbandry practices, prophylactic use of anticoccidial drugs and/or live parasite vaccination have been the primary control measures employed but there are challenges with vaccination and growing constraints on anticoccidial drug use. This review, therefore, considers available information on the key steps of the infection process, notable microbiome- or host-related changes occurring, and the (potential) influence of dietary 'alternatives' to anticoccidial drugs. There is good available evidence to indicate that some phytogenics, prebiotics, probiotics, betaine, n-3 fatty acids, as well as carbohydrase enzymes and anti-IL-10 antibodies, can (beneficially) modulate at least some of these features in coccidiosis-specific challenge studies. As a minimum, these anticoccidial drug 'alternatives' could support the establishment of a desirable host microbiome and optimum immune system development. It is important to better understand the potential of these 'alternatives' in commercial production and how they can complement, or reduce, the use of anticoccidial drugs.

Keywords: chicken; coccidiosis; *Eimeria*; immunity; microbiome; phytogenics; probiotics; prebiotics

## Introduction

Coccidiosis remains a major impediment to, and cause of significant economic loss (globally >US$3 billion per annum) for, the poultry industry, as well as being a recognised predisposing factor for another prominent intestinal disease (necrotic enteritis) (Shivaramaiah *et al.*, 2014).

Coccidiosis in chickens is caused by host-specific *Eimeria* species that display tissue trophism and cause haemorrhagic (*E. brunetti*, *E. necatrix*, and *E. tenella*) or malabsorptive (*E. acervulina*, *E. maxima*, *E. mitis*, *E. praecox*) disease (Blake and Tomley, 2014). *E. tenella* infects the caeca and might be the easiest to recognise amongst poultry producers due to bloody faeces, but species infecting pre-caecal regions could be more relevant for nutrient digestion and absorption. Chickens become infected through ingestion of sporulated oocysts (resilient, thick-walled structure containing 4 sporocysts, each with 2 sporozoites), which are ubiquitous, particularly in areas of intensive production. Good husbandry and the prophylactic use of anticoccidial drugs and/or live parasite vaccination have been the primary control measures employed to date (Chapman, 2018). Resistance to anticoccidial drugs is widespread, while the availability of these drugs has become restricted due to legislation and consumer pressure on chicken production. Vaccination challenges include exposure to relevant *Eimeria* spp., appropriate oocyst cycling to induce adequate immunity without causing (sub)clinical disease, and cost (particularly live attenuated vaccines) (Blake and Tomley, 2014). Therefore, there is great interest in alternatives, such as next generation vaccines based on parasite genes and antigens that induce effective, less risky protection, or feed (or water) additives with coccidiosis control potential. There are various challenges in developing next generation vaccines (Blake *et al.*, 2017) and so this concise review considers the evidence-based potential of anticoccidial drug alternatives to support coccidiosis control strategies.

## Microbiome effects

Studies have reported various effects of *Eimeria* infection on the intestinal microbiota. While *Eimeria* infection has been reported to reduce gut microbial (alpha) diversity (Wu *et al.*, 2014), others have observed no such effect (Macdonald *et al.*, 2017). Notable intestinal microbiota changes following *Eimeria* infection (mixed species) include reduced abundance of *Ruminococcaceae* groups and increased abundance of three unknown *Clostridium* species (Wu *et al.*, 2014), and reduced *Candidatus Arthromitus* (Stanley *et al.*, 2014). However, these studies did not directly relate microbiota changes with the severity of intestinal pathology (lesion scores). Macdonald *et al.* (2017) reported no effect of *E. tenella* infection on taxa diversity in the caeca but it caused significant changes in the abundance of some taxa, particularly in birds with severe caecal pathology. These changes included increases in *Enterobacteriaceae* (*Escherichia coli*, *E. fergusonii*, *Shigella flexneri* or *S. sonnei*) and *Clostridium*, and reduced *Bacillales*, *Lactobacillales* and *Ruminococcus*. Of interest, asymptomatic infected birds (no lesions) had reduced *Bacteroides* and increased *Lactobacillus*. Although it remains largely unclear whether *Eimeria*-associated microbiota changes contribute to, or result from, intestinal (caecal) pathology, identified alterations could inform the appropriate application of interventions suitably modifying the gut microbiome. Based on current studies, such interventions could seek to reduce

*Enterobacteriaceae*, *Clostridium* and *Bacteroides*, and increase *Bacillales*, *Lactobacillales* (*Lactobacillus*) and *Ruminococcus* to help buffer identified microbiota changes. It is also important to recognise that *Eimeria* infection is associated with increases in the intestinal prevalence of (including foodborne) pathogens such as *C. perfringens*, *Campylobacter jejuni* and *Salmonella* (Collier *et al.*, 2008; Macdonald *et al.*, 2019; Baba *et al.*, 1982), aspects of which will be considered in a section below.

There are limited data outlining the impact of *Eimeria* infection on intestinal metabolites. Such metabolites are a crucial form of 'communication' and interaction between the microbiota and the host (Maslowski, 2019). Wu *et al.* (2014) reported that mixed *Eimeria* infection did not affect caecal digesta short-chain fatty acid (SCFA) concentrations (or pH), while Stanley *et al.* (2014) found that infection reduced caecal acetic acid but increased butyric, isobutyric, and isovaleric acid concentrations. Any changes in SCFA concentrations will reflect shifts in, and interplay between, microbial populations, as well as absorption dynamics.

Generally, these studies investigate the (luminal) caeca (even if using *Eimeria* targeting other intestinal regions). The caeca are regarded as having high microbial density and diversity, and sample both descending and ascending microbes (Wu *et al.*, 2014). However, changes occurring in other gut regions, particularly relevant to the infecting *Eimeria* species, would be informative. Further studies should also seek to establish the relationship between *Eimeria* infection, the mucosa-associated microbiota, metabolites and intestinal pathology. Some of the traditional 'alternatives to antibiotics' have been documented to appropriately influence at least some of the caecal microbiome changes outlined, which are associated with *Eimeria* infection, and thus could be suitable candidates to counteract or modulate these effects and have some benefit for challenged birds (Table 1).

Table 1. Important steps in the *Eimeria* infection process, potentially important host-microbiome modifications, identified anticoccidial drug alternatives and notable supporting references.

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|---|---|---|---|
| **Component** | **Potentially important/desired influence** | **Anticoccidial drug alternatives** | **Notable references** |
| Microbiome effects | ↑ *Bacillales*, *Lactobacillales* & *Ruminococcus*<br><br>↓ *Enterobacteriaceae*, *Clostridium* and *Bacteroides*<br><br>?Short-chain fatty acids | Enzymes<br><br>Phytogenics<br><br>Probiotic<br><br>Prebiotic | ?Craig *et al.*, 2020<br><br>Oviedo-Rondón *et al.*, 2006<br><br>Giannenas *et al.*, 2012<br><br>Shanmugasundaram *et al.*, 2013<br>Markazi *et al.*, 2017 |

| | | | |
|---|---|---|---|
| Intestinal secretions | ↑ IgA<br>? Mucus<br>↑ LEAP2<br>? Tight junction proteins | Probiotics | Dalloul *et al.*, 2003<br>Park *et al.*, 2020 |
| Epithelial cell invasion & integrity | ↓ parasite viability<br><br>↓ invasiveness<br><br>↑ cell refractoriness/integrity<br><br>Influence cell redox state | Betaine<br><br>n-3 fatty acids<br><br>Phytogenics<br><br><br><br>Probiotics | Allen *et al.*, 1998<br><br>Allen *et al.*, 1998<br><br>Khalafalla *et al.*, 2011<br>Burt *et al.*, 2013<br>Jitviriyanon *et al.*, 2016<br>Chikara *et al.*, 2018<br><br>Tierney *et al.*, 2004<br>Hessenberger *et al.*, 2016 |
| Immune responses | Cell-mediated immunity<br>Cytotoxic T-cells<br>Heterophils<br>Macrophages<br><br>Proinflammatory IFN-γ:IL-10 | Anti-IL-10 antibodies<br><br>n-3 fatty acids<br><br>Phytogenics<br><br>Prebiotics<br><br><br>Probiotic | Sand *et al.*, 2016<br><br><br>Allen *et al.*, 1998<br><br>Kim *et al.*, 2019.<br><br>Shanmugasundaram *et al.*, 2013<br>Markazi *et al.*, 2017<br><br>Dalloul *et al.*, 2005<br>Stringfellow *et al.*, 2011 |

## Intestinal secretions

Beyond (antagonistic) interactions with the microbiome, *Eimeria* must also overcome various intestinal secretions to infect epithelial cells. IgA is an important secretion helping protect mucosal surfaces from pathogens (Macpherson *et al.*, 2018). Whilst cell-mediated immunity is regarded as the cornerstone of protection from *Eimeria* (re)infection (outlined later), both non-specific and specific antibodies (e.g. IgA) are likely to contribute to parasite-host dynamics and protective mechanisms (Shivaramaiah *et al.*, 2014). Evidence for the contribution of antibodies to host protection from *Eimeria* infection include significant production of parasite-specific (including intestinal) antibodies post-infection (Trees *et al.*, 1989) and passive immunity transfer to offspring or naïve chicks providing good or partial protection against primary infection with homologous or heterogenous *Eimeria* species, respectively (Rose, 1974; Smith *et al.*, 1994). Work conducted in the development of the first commercial subunit vaccine for *Eimeria* control

(CoxAbic®) supports a role for antibodies in protection against coccidiosis (Sharman *et al.*, 2010).

Mucus is another dominant secretion into the gut environment, which seeks to trap less desirable microbes and facilitate their removal from the intestine via digesta flow. *Eimeria* infection increases mucus production, associated with the host's inflammatory response, to help eliminate coccidia from the gut (Collier *et al.*, 2008). Ileal mucin RNA expression was increased 6 days post-infection (p.i.) (with *E. acervulina* and *E. maxima*) and, at peak inflammation (8 days p.i.), ileal goblet cell theca (distended apical portion of cell containing membrane bound mucins) area was greatest in infected birds compared to non-infected controls. Chicken intestinal mucus has been reported to reduce invasion of chicken epithelial cells by enteropathogens (Byrne *et al.*, 2007) and thus may decrease bacterial translocation (Macdonald *et al.*, 2019). A challenge with enhancing mucus production as a defence strategy against *Eimeria* is that a thicker mucus layer may impede the effective diffusion of digestive enzymes and nutrients (Bontempo *et al.*, 2006), enhance endogenous losses (Cowieson *et al.*, 2016), and/or encourage the proliferation of microorganisms that use mucus as a nutrient source, including potential pathogens such as *Clostridium perfringens* and *Campylobacter spp.*, and their expression of virulence factors (Macdonald *et al.*, 2019). In support of this, attempts to thin the intestinal mucus through the addition of N-acetylcysteine to the feed of *E. tenella*-infected chickens reduced the associated increase in caecal *C. jejuni* (Macdonald *et al.*, 2019).

Specific intestinal epithelial cell (IEC) subsets, such as Paneth cells and enterocytes, secrete low molecular weight (mw) peptides and higher mw proteins (e.g. lysozyme) that provide broad-spectrum antimicrobial activity. In addition, low mw peptides are reported to have immunomodulatory activity, including chemokine receptor binding to promote a proinflammatory response (Su *et al.*, 2018). Lysozyme (muramidase) is considered to be most active against the peptidoglycan structure of gram-positive bacteria, while lower mw peptides are active against various microbial pathogens (Wang, 2014). Of the lower mw peptides/proteins, there are no α-defensins encoded by the chicken genome, but 14 β-defensins, 4 cathelicidins and liver expressed antimicrobial peptide-2 (LEAP2) are (Robinson *et al.*, 2015). Information regarding antimicrobial protein activity against *Eimeria* is scarce. Several β-defensins show somewhat variable intestinal expression in *Eimeria*-challenged chickens, dependant on sampled location and breed or line, while LEAP2 shows some consistency in down-regulation following challenge (Su *et al.*, 2018). LEAP2 down-regulation was not considered to result purely from villous shortening and the resultant effect on general gene expression but could not be related to disease susceptibility. Moreover, basal or homeostatic antimicrobial peptide/protein expression may be important in influencing the course of infection (Su *et al.*, 2018).

Although the precise role of intestinal secretions in the susceptibility of chickens to coccidiosis is somewhat unclear, their oral provision, or rather interventions that influence their secretion, have been reported to attenuate the impact of *Eimeria* infection (Table 1).

## Epithelial cell invasion and integrity

While the components mentioned so far provide barriers that can prevent *Eimeria* contact with IEC, enhancing the resistance of IEC to parasite invasion could also be beneficial. *Eimeria* invade and multiply in intestinal cells, with different species targeting specific regions of the gut. Although the segment-specific features that promote this regionalisation are not clearly understood, the mode of invasion is considered to be largely conserved across Apicomplexa (Besteiro *et al.*, 2011). To invade the cell, *Eimeria* spp. secrete proteins and create a structure called the moving junction (MJ) to firmly adhere to the host cell. The MJ moves distally along the parasite to facilitate its entry into the cell. Apical membrane antigen 1 (AMA1) seems to be a key element of the MJ structure and cell invasion, certainly for *E. tenella*, as expression is highest during the sporozoite stage (Jiang *et al.*, 2012).

Reducing parasite viability, blocking/interfering with invasion mechanisms and/or promoting cell refractoriness to infection could reduce IEC invasion by *Eimeria*. These effects could be mediated through direct inhibitory effects on the parasite, reducing the availability of IEC sites for adherence, and/or increasing the hostility of IEC to invasion and parasite survival. Different compounds (e.g. phytogenics) have been reported to reduce the viability of *E. tenella* sporozoites (Khalafalla *et al.*, 2011) or their invasion of an epithelial cell line *in-vitro* (Burt *et al.*, 2013; Jitviriyanon *et al.*, 2016 ), although these effects are likely to be time and/or concentration dependent. By occupying potential attachment sites, secreting antimicrobial compounds or signalling molecules, and/or stimulating epithelial defences (e.g. via toll-like receptor signalling), probiotics could interfere with the ability of *Eimeria* to access and invade IEC (Tierney *et al.*, 2004; Hessenberger *et al.*, 2016). There was, however, evidence that the probiotic bacteria had a negative impact on the epithelial line used in these studies, likely through production of antagonistic compounds (e.g. organic acids), but such effects would need verifying *in-vivo*. It is not surprising that *Eimeria* infection disrupts cell physiology and energy homeostasis, which may be explained by increased levels of reactive oxygen species (ROS) and lipid peroxidation (Galli *et al.*, 2019). Moreover, Allen *et al.* (1998) suggested that different *Eimeria* species could differ in their susceptibility to oxidative stress according to the intestinal segment they thrive in and infect (e.g. relatively anaerobic caeca vs. small intestine regions). A recognised property of numerous phytogenic compounds is their potential to alter redox states (Chikara *et al.*, 2018) and so any derived benefit could include modifying cellular oxidative stress and helping to stabilise the physiology and integrity of infected cells. Clearly, optimal availability of vitamins (e.g. A and

E) and trace minerals (e.g. selenium and zinc) that contribute to host antioxidant capability, as well as immune function, could also be advantageous (Wunderlich *et al.*, 2014). Betaine and n-3 fatty acids have also shown benefit in coccidiosis studies, which is proposed to be through modulation of epithelial cell oxidative and osmotic stress, and/or immune responses (Allen *et al.*, 1998). Epithelial cells express various receptors that can sense microbial metabolites, notably SCFA such as butyric acid, which influence various cellular pathways and thus their metabolism, function and integrity (Maslowski, 2019). For example, with intestinal epithelial cell line models, butyrate is reported to increase epithelial cell integrity (assessed by maintaining or increasing trans-epithelial electrical resistance) following challenge (e.g. lipopolysaccharide exposure) (Yan and Ajuwon, 2017). The effects of SCFA may, however, be bimodal and thus beneficial effects are likely to be dependent on appropriate concentrations (Kurita-Ochiai *et al.*, 2006). Recent developments have also included inducing antibodies against important components of the MJ structure (e.g. AMA-1), including through recombinant bacteria administration (Jian *et al.*, 2018), or the application of specific binding peptides to inhibit cell invasion by *Eimeria* (Ma *et al.*, 2019).

Tight junction complexes, formed by cytoplasmic and transmembrane proteins to regulate the paracellular space between IEC, may not be of primary importance as *Eimeria* infect the epithelial cells themselves. However, loss of IEC and gut barrier integrity would be detrimental as it would further compromise host-microbiome homeostasis and increase the likelihood of antigen passage from the luminal side of the epithelium into the lamina propria and beyond.

Notable studies reporting seemingly positive effects of anticoccidal drug alternatives influencing IEC invasion and integrity, in the context of *Eimeria* infection, are provided in Table 1.

## Subepithelial mechanisms

Some immune cells, notably natural killer (NK) and γδ T cells, reside among the epithelial cells (intraepithelial lymphocytes; IEL), but the majority are located within the lamina propria below the epithelium. Cells in this compartment include both innate (e.g. dendritic cells (DCs), heterophils (avian equivalent of mammalian neutrophil), macrophages, and NK cells) and adaptive (e.g. T and B cells) immune cells in more, or less, organised lymphoid follicles. Bursectomisation of chickens does not interfere with the development of protective immunity against disease, indicating a prominent role for cell-mediated immunity (Lillehoj, 1987). The early response to *Eimeria* infection in immunised chickens includes migration of heterophils (and lymphocytes), causing initial leukopenia (Rose *et al.*, 1984), and, subsequently, a primary role for cytotoxic T-cells as *Eimeria* spp. are intercellular parasites (Shivaramaiah *et al.*, 2014). *E. acervulina* sporozoites were found in, or associated with, cytotoxic T-cells and macrophages

during primary infection (Lillehoj and Trout, 1996). The contribution of cytotoxic T-cells has been demonstrated through their selective depletion with monoclonal antibodies, which increased infection and oocyst shedding following *E. acervulina* and *E. tenella* challenge (Lillehoj *et al.*, 2004). The location of IEL suggests that these cells contribute to the initial response to infection, particularly as they do not require priming (Smith and Hayday, 2000). In mice, $T_H$ cells are activated during primary infection and IFN-γ may play a key role during both the initiation of an immune response and effector responses (Shivaramaiah *et al.*, 2014). Studies have also suggested the importance of various other pro-inflammatory, regulatory and anti-inflammatory cytokines during *Eimeria* infection (Shivaramaiah et al., 2014; Broom and Kogut, 2019). However, immune responses to *Eimeria* challenge are complex and are largely species, and probably strain, specific, and influenced by parasite developmental stage. This, along with various uncertainties regarding the importance of specific immune components for protection from *Eimeria* infection and/or disease progression, make it challenging to confidently seek to influence the immune response appropriately. Work has, however, shown that birds of a higher proinflammatory mediator, including interleukin (IL)-6, CXCLi2, and CCLi2, phenotype are more resistant to the pathological consequences of *E. tenella* compared to lower expression birds (Swaggerty *et al.*, 2015). Higher or lower relative expression of IFN-γ or IL-10, respectively, or oral supplementation of anti-IL-10 antibodies have also been associated with greater resistance to *Eimeria* infection (Broom and Kogut, 2019, Sand *et al., 2016*) This does suggest the possibility to appropriately influence the immune response of birds to both primary and subsequent *Eimeria* infection through exogenous means, while immune cells are also able to sense microbial metabolites, which can influence their phenotype and function (Maslowski, 2019). Table 1 outlines poultry coccidiosis studies where exogenous additives have been associated with beneficially modifying immune responses to *Eimeria* spp.

## Infection dynamics

Nolan *et al.* (2015) reported a dose-dependent relationship between inoculum size and parasite genome copy number in the caeca of *E. tenella* infected chickens at 5 dpi, which was not apparent following extensive oocyst shedding at 8 dpi, highlighting the importance of sampling time. There were also strong positive relationships between inoculum dose, lesion scores and oocyst output, even though the 'crowding affect' likely reduced parasite fecundity (Williams, 2001), but statistical differences between treatment groups were generally lacking. The cyclical nature of oocyst excretion and development of immunity has been reported (Williams, 1995). As birds attain solid immunity, oocyst excretion is strongly inhibited, leading to reduced immunity, thus increased oocyst excretion, followed by enhanced immunity and reduced oocyst excretion, etc. Thus, it is important to appreciate the balance needed between interventions that reduce the viability of the parasite and/or interaction with the host, and necessary interaction to generate

immunity. However, what seems to be less clear cut is the relationship between pathology (lesion scores) and growth performance. Numerous studies have indicated complex relationships between lesion score severity, immunity and bird growth (Long *et al.*, 1980; Boulton *et al.*, 2018a; Broom, 2019). This makes understanding differences in how birds 'manage' *Eimeria* infection (e.g. resilient, resistant and/or tolerant) very pertinent (Boulton *et al.*, 2018b).

## Conclusion and possible interventions

This review has considered important steps in the *Eimeria* infection process, notable modifications at the host-microbiome interface and the potential for alternatives to anticoccidial drugs to beneficially interact with this dynamic. Establishing an appropriate intestinal microbiota is considered key to optimal gut health. Facultative anaerobes of the *Enterobacteriaceae* family are the initial colonisers of the chicken intestine (Ijaz *et al.*, 2018) until available oxygen becomes scarce. Therefore, early provision, perhaps including *in-ovo* administration, of chicken relevant, non-pathogenic *Enterobacteriaceae* strains could help establish the early microbiome, utilise oxygen to facilitate natural succession by stricter anaerobes, and aid immune system development. This could be followed by supplementation with bacteria, for example, *Bacillales*, *Lactobacillales* (*Lactobacillus*) and *Ruminococcus*, whose reduction is associated with coccidiosis, greater pathology and/or compromised immune system maturation. Prebiotics shown to support the growth of administered probiotic strains or populations already residing in the intestine could also be considered. Alternatively, if the proliferation of specific bacterial taxa (e.g. *Enterobacteriaceae*, *Clostridium* and *Bacteroides*) can be firmly connected to coccidiosis susceptibility and/or severity then there could be interest in the development of appropriate bacteriophages to directly target these bacteria. Phytogenics and enzymes (i.e. carbohydrases) have also indicated desirable shifts in the composition or metabolic activity of the gut microbiota during coccidiosis challenge and may thus be worthy of further investigation and consideration. However, phytogenics have been reported to have broad spectrum antimicrobial activity and so any potentially unwanted microbiome shifts should be avoided. There are limited published data with SCFA supplementation and coccidiosis challenge but their influence on the gut microbiome and host physiology could be interesting in this context.

Specific phytogenics may be important at the time of *Eimeria* infection if their *in-vitro* anti-*Eimeria* and/or inhibitory effects on IEC invasion are truly replicated *in-vivo*. In addition, the degree of *Eimeria* oocyst depletion and any implications for their cycling and the development of host immunity need to be considered. Moreover, phytogenics (and betaine) have the potential to modulate cellular oxidative stress and help support the integrity of infected IEC, while keeping in mind the importance of ROS for immune responses. Similarly, probiotics have also been

reported to influence intestinal secretions, that can help protect the gut mucosa, or invasion of IEC.

Beyond the apparent importance of cell-mediated immunity in protection against *Eimeria* infection, the crucial elements of immune responses have not been clearly delineated, which makes exogenous influence of appropriate immune responses through additives challenging. Various strands of evidence do point towards an (early) inflammatory bias increasing resistance to infection and could thus be indicative. Phytogenics, prebiotics, probiotics, n-3 fatty acids and, perhaps more novel, anti-IL-10 antibodies have been reported to modify the immune response landscape to *Eimeria* infection.

There is clear evidence indicating that the appropriate use of suitable alternatives to anticoccidial drugs do have the potential to play an important part in supporting the resilience of poultry to coccidiosis. At the very least, these alternatives could play a part in the establishment of a desirable host microbiome and optimum immune system development. Once refined, faecal microbiota transplant and postbiotics could also become important tools. However, it is imperative to substantiate any benefits seen from controlled experiments in commercial-type production and to fully understand their potential and limitations, and to integrate them with more specific and established coccidial control measures as appropriate.

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