REVIEW

Assay considerations for fluorescein isothiocyanate-dextran (FITC-d): an indicator of intestinal permeability in broiler chickens

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ABSTRACT Fluorescein isothiocyanate-dextran (**FITC-d**) is being used as an indicator of intestinal paracellular permeability in poultry research. Especially with the industry moving toward antibiotic-free production, intestinal function and integrity issues have been a research focus. An increasing number of scientific conference abstracts and peer-reviewed journal publications have shown that 4-kDa FITC-d is an efficient marker candidate for measurement of intestinal permeability and can be applied in broiler research. However, experimental protocols vary by personnel, instruments used, and research institution, and potential concerns related to this assay have yet to receive the same amount of attention. Understanding protocol consistency within and across laboratories is vital for obtaining accurate, consistent, and comparable experimental results. This review is aimed to 1) summarize different FITC-d assays in broiler research from peer-reviewed publications during the past 6 yr and 2) discuss factors that can potentially affect intestinal permeability results when conducting the FITC-d assay. In summary, it is essential to pay attention to details, including gavage dose, fasting period, sample handling and lab analysis details when conducting the assay in broiler research. Differences in birds (breed/strain, age, and gender) and experimental design (diet, health status/challenge model, and sampling age) need to be considered when comparing serum FITC-d concentration results between different in vivo animal trials.

Key words: FITC-d, marker, intestinal permeability, broiler

INTRODUCTION

The intestinal health of poultry has garnered much attention in the past few years, due to the industry moving towards removal of subtherapeutic antibiotics from the diet. Many intestinal disease issues previously controlled by antibiotics have increased prevalence (Cardoso Dal Pont et al., 2020). The gastrointestinal tract (**GIT**) is the first defense line and the largest immune organ of the chicken. The intestinal epithelium acts as a barrier protecting animals from intraluminal pathogens, toxins, and antigens, and selectively allows the passage of water, nutrients, and electrolytes (Groschwits and Hogan, 2009). The mucin layer is a transparent layer covered outside surface of the mucous membranes, which is permeable to gases, water, and nutrients while entrapping most microorganisms (Pelaseyed et al., 2014; Robert et al., 2017). Tight junctions are another crucial multi-protein complex structure that separates the apical and basolateral compartments by regulating passage through the intercellular space between adherent epithelial cells. The complex structure is involved in the regulation of paracellular permeability and membrane polarity (Condette et al., 2014; Robert et al., 2017). A disrupted intestinal barrier function is known to induce increased intestinal permeability and translocation of toxins, pathogenic bacteria, and parasites across the epithelium, which can further enhance systemic immune response or increase susceptibility to diseases (Grenier and Applegate, 2013). Thus, an effective bioassay to evaluate intestinal permeability is essential and meaningful for broiler research in different experimental models.

Markers used for assessment of intestinal permeability (in vitro, in vivo and ex vivo) include nondigestible sugars, polyethylene glycols, fluorescent-labeled dextran, horseradish peroxidase, ^{51}chromium-labeled ethylenediamine tetra-acetic acid (^{51}Cr-EDTA), ovalbumin, etc. (Chadwick et al., 1977; Elemer and Osborne-Pellegrin 1990; Zuckerman et al., 1993; Galipeau and Verdu, 2016; Volynets et al., 2016; González-González et al., 2019). In human research, the dual-sugar lactulose-mannitol test

assay (sometime using lactulose-L-rhamnose) is widely used to measure intestinal permeability instead of using one non-digestible marker alone (Wang et al., 2015; Galipeau and Verdu, 2016). Chen et al. (2016) used lactulose and L-rhamnose by measuring serum lactulose-to-rhamnose ratio to determine intestinal permeability in broiler chickens. The larger sized disaccharide lactulose (342 Da in molecular weight) can only pass through the intestine via the paracellular pathway if the barrier is disrupted. The smaller sized monosaccharide L-rhamnose (182 Da in molecular weight) is considered to pass through the mucosa via the transcellular as well as paracellular pathways (Galipeau and Verdu, 2016). Thus, if intestinal permeability is altered, oral co-administration of lactulose and rhamnose results in an increased lactulose-to-L-rhamnose ratio in blood or urine (Katouzian et al., 2005; Wang et al., 2015). Considering the advantages and disadvantages of each marker/probe and assay, the FITC-d assay has evolved as the "go-to" choice in recent broiler research due to its ease, timeliness, and cost of analytical measure.

Fluorescein isothiocyanate-dextran (**FITC-d**) has been known and used as an indicator for assessment of intestinal paracellular permeability (Napolitano et al., 1996). Dextrans are non-digestible polysaccharides with available molecular size ranging from 3-kDa to 2000-kDa (Wang et al., 2015; Woting and Blaut, 2018). FITC-d at 4-kDa molecular size is the one primarily used in poultry experiments currently, because it is large enough and does not cross the intestinal epithelial barrier in high quantities after oral administration unless the intestinal barrier is compromised (Gilani et al., 2017b). In contrast, smaller molecules (<300 Da) can passively cross the tight junction barriers (Sun et al., 1998). However, inflammation and injury of the intestinal epithelium can induce mucosal barrier dysfunction, resulting in barrier defects which increase the migration of 4-kDa FITC-d molecule to the serosal layer of the intestine, and subsequently can enter systemic circulation (Yan et al., 2009; Condette et al., 2014). In other words, after oral administration, the 4-kDa FITC-d molecules can cross the disrupted intestinal epithelium and be quantified in the blood. The serum FITC-d concentration can be measured and used as an indicator of paracellular permeability and extent and severity of intestinal mucosal barrier dysfunction.

The initial descriptions of intestinal permeability assay with 4-kDa FITC-d product are reported from human medical literature, using mice as an in vivo model or through ex vivo studies using Ussing chambers (Napolitano et al., 1996; Chatelais et al., 2011; Hamilton et al., 2015; González-González et al., 2019). When applying this assay to broiler research, experimental protocols for measuring serum FITC-d concentration to determine intestinal permeability vary from personnel, instruments used, and research institution. Potential concerns of this variation have yet to receive enough attention. Understanding such detailed information is vital for obtaining accurate and consistent experimental results, which can benefit researchers, allowing for the comparison of results among trials and minimize experimental error caused by the protocol. In this mini-review (Figures 1 and 2, created with Biorender.com), we summarize and compare different FITC-d permeability assays (mainly with 4-kDa molecular size product) in broiler research from peer-reviewed publications during the past 6 yr. In addition, factors that can potentially affect intestinal permeability results when conducting FITC-d assay are discussed.

PRODUCT

In most broiler research reported in the literature, the commercial FITC-d products from Sigma-Aldrich were mainly used, with an average molecular weight range from 3 to 5-kDa (solubility in water: 25 mg/mL) or 4-kDa (solubility in water: 50 mg/mL) (Table 1). Both products have been shown to be effective when conducting the FITC-d permeability assay in broilers. Even though no comparison studies have been done between these 2 molecular weight products, there should be no significant difference when comparing results among experimental treatments or having impact on the final conclusion if choosing either of the products. It is worth noting that the quantity of the FITC-d products includes 100, 250, 500, and 1,000 mg per bottle. In order to minimize the total purchase budget, shorten the storage time of an opened bottle, and reduce the chance of weighing error during solution preparation, it is recommended to calculate the total amount of FITC-d product that is needed at each sampling age (number of birds, amount of FITC-d for each bird), and to consider the interval time between different sample collection ages. In certain scenarios, the buffer solution (PBS or pure water) can be added directly into an amber glass bottle (adding slowly to avoid spilling due to the volume of inserted fused cone, especially for the 100 mg 4-kDa FITC-d product). On the other hand, when only small amount of solution is needed for an experiment, the FITC-d powder can be weighted meticulously with a scale. According to Sigma-Aldrich guideline, FITC-d powders should be stable at least for 2 yr at 4°C without the light exposure (Sigma-Aldrich, 1997).

SOLUTION PREPARATION

The 4-kDa FITC-d product is easily solubilized in water and is sensitive to light. Both ultrapure water (Baxter et al., 2017) and sterile 1× PBS (Teng et al., 2020) have been reported to dissolve the FITC-d powder. It should be noted that the FITC-d solution needs to be protected from light at all times after dissolving (such as covered with aluminum foil, using an amber microcentrifuge tubes, or storing in a closed dark-colored container) if a bottle of the product will be used for multiple sampling ages or projects.
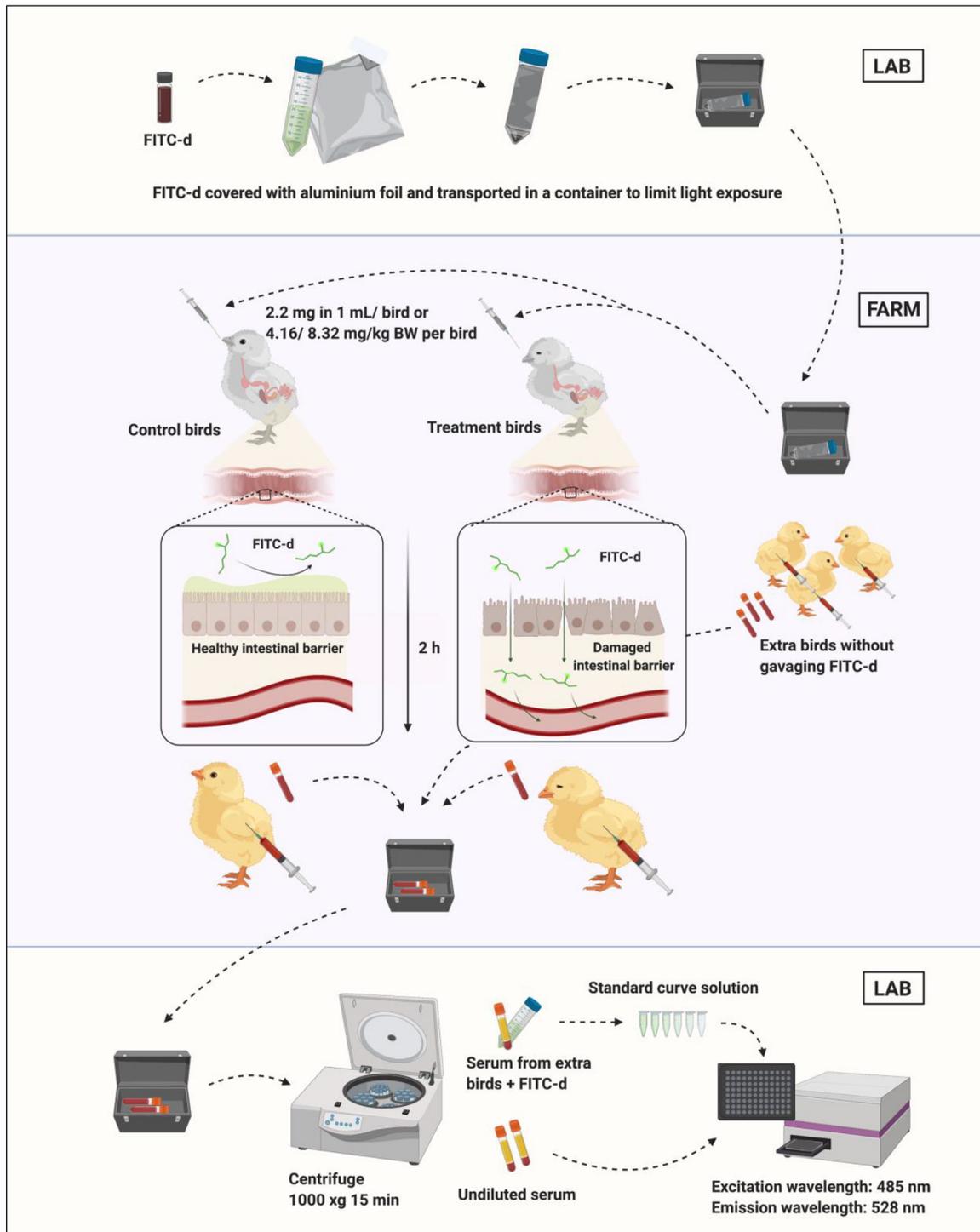
Figure 1. Overview of 4-kDa FITC-d intestinal permeability assay and workflow. Abbreviations: FITC-d, fluorescein isothiocyanate-dextran.

DOSE

The dose selection for FITC-d product has varied among different researchers (Table 1). In mouse studies, researchers conducted luminal enteral or oral administration of FITC-d based on the body weight of mouse, for example, 60 mg/100 g body weight (Napolitano et al., 1996; Yan et al., 2009; Condette et al., 2014; Yang et al., 2018). This is similar to some of the poultry research by gavaging broiler chickens with 4.16 or 8.32 mg/kg BW per bird (Vicuña et al., 2015a; Baxter et al., 2017). However, in practice, a typical nutritional evaluation trial with broiler chickens normally has a larger number of animals and replicates per treatment than the mouse studies in order to obtain enough statistical power and gain acceptance from the poultry producers for the research results. Thus, the number of broiler chickens used for FITC-d permeability assay increases handling time and labor on the sample collection day. More importantly, multiple tasks of sample collections often carried out within the same day. It is difficult and time consuming to weigh each individual bird, calculate gavage dose, and conduct oral gavage accurately if labor is limited.
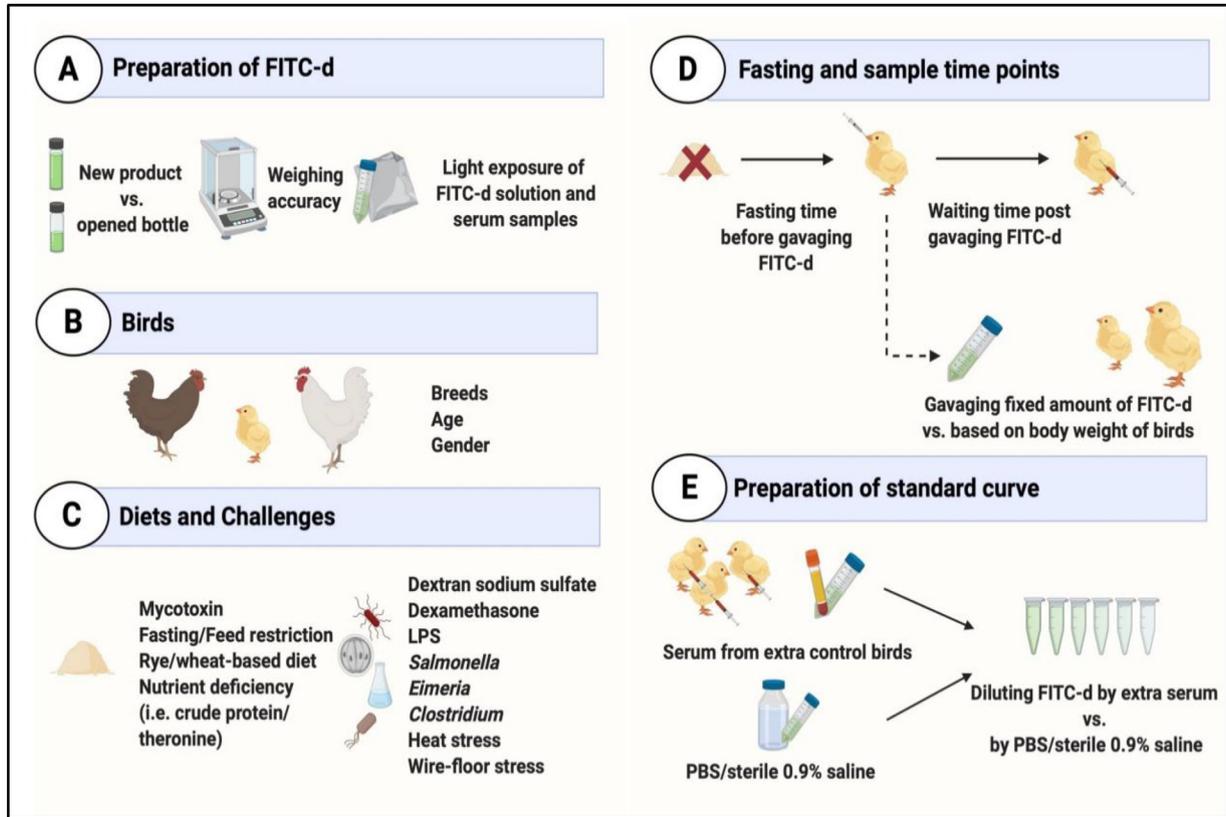
Figure 2. Potential factors that can affect serum FITC-d concentrations.

Alternatively, researchers can obtain the average body weight of a gavaged bird based on the average treatment body weight, or average weight of each cage (if possible) as long as the methodology is documented in future publications. Among previous studies, 2 main calculation methods have been applied for the FITC-d assay (Table 1): 1) based on the body weight of bird (i.e., mg/ kg FITC-d per kg broiler); 2) 2.2 mg/bird.

BEFORE GAVAGE

Feed in the GIT can affect passage-rate and absorption-rate of 4-kDa FITC-d, which makes the fasting period a consideration before the oral gavage. Fasting can result in reduced proliferation of intestinal epithelial cells, induced apoptosis, increased mucosal damage, decreased glutamine concentration and disrupted morphology, which can lead to increased intestinal permeability (van der Hulst et al., 1993, 1994; Chappell et al., 2003; Thompson and Applegate, 2006; Gilani et al., 2018a). In mouse studies, food and water were withdrawn for 4 h before administration of the FITC-d solution (Yan et al., 2009; Yang et al., 2018). In addition, results from other research with mice indicated that the duration of fasting may not be a major factor to affect the appearance of FITC-d in plasma, while fasting did affect the concentration of 4-kDa FITC-d in blood (Woting and Blaut, 2018). However, altered physiological and histological conditions of the GIT were found at 8, 12, or 24 h feed withdrawal in broilers

(Thompson and Applegate, 2006), as broiler chickens have a more rapid feed rate-of-passage compared to other animal species. In a study with specific age (21-day-old), Liu et al. (2017) tested the rate-of-passage of broiler chickens via the computed tomography assay and found the feed first appeared in colon 2.5 h post the feed ingestion, and the GIT is almost cleared of the iodinated contrast feed between 4 and 6 h. In broiler chicken, fasting or feed withdrawal has been shown to increase indicators of a stress response (e.g., increased corticosterone concentrations) (Najafi et al., 2016), affect intestinal morphology (increased jejunum villus height and decreased ileum crypt depth), reduce ileal mucin content (Thompson and Applegate, 2006), and increase the attachment of *Salmonella* Enteritidis to ileal tissues ex vivo (Burkholder et al., 2008). Ultimately, an increased intestinal permeability caused by fasting period before gavage can be a factor that confounds the serum FITC-d results. Multiple researchers have shown that fasting caused increased intestinal permeability in both 7-day-old (with 24 h fasting period) (Kuttappan et al., 2015b; Vicuña et al., 2015b) and 21-day-old broiler chickens (with a 19.5 h fasting period) (Gilani et al., 2017b). Further research has also reported that serum FITC-d concentration was significantly increased with increasing fasting time from 0, 4.5, 9.0 to 19.5 h in broiler chickens (Gilani et al., 2018a). However, no significant difference on serum FITC-d results was found between 9.0 and 19.5 h of fasting. Results from this study demonstrated that fasting time can cause increased permeability in as little as 4.5 h. Finally,

Table 1. Fluorescein isothiocyanate-dextran (FITC-d) assay on the measurement of intestinal permeability in broilers research.

Reference	Molecular weights	FITC-d dose	Fasting	Blood collection post gavage	Breed/strain	Sampling age	Challenge type	Diet	Experimental model	Results (compared with control)
Zanu et al., 2020	4 kDa	4.17 mg/kg BW	No fasting	3 h from jugular vein	Ross 308 male broiler	d 16	5,000 oocysts of field strains of E. acervulina and E. maxima and 2,500 oocytes of E. brunetti on d 9 and 10E8 *Clostridium perfringens* on d 14, 15	Wheat-soybean meal	Necrotic enteritis disease model	Challenge increase the serum FITC-d (0.03 vs 0.34 ug/mL)
Teng et al., 2020	4 kDa	2.2 mg/mL/bird	No fasting	2 h by cardiac puncture	Cobb 500 male broiler	d 16, 18, 19, 20 and 22	Orally gavage graded levels of mixed *E. maxima, E. acervulina,* and *E. tenella*	Corn-soybean meal	*Eimeria* induced coccidiosis model	Graded challenge linearly increased serum FITC-d on 5,6 and 7 d postinfection
Barekatain et al., 2020	4 kDa	2.2 mg/mL/bird	No fasting	2.5 h from jugular vein	Ross 308, male broiler	d 21	0.5 mg/kg BW dexamethasone breast muscle injection on d 14, 16, 18 and 20	Rye-soybean meal or wheat-soybean meal	Rye-based diet plus dexamethasone induced leaky gut model	Only dexamethasone increase serum FITC-d; no significant effect with inclusion of rye
Schneiders et al., 2019	4 kDa	2.2 mg/mL/bird	No fasting	2 h from jugular vein	Ross 708, broiler	d 14 to 24	Orally gavage with 2 × 10E5 *E. maxima*	Corn-soybean meal	*Eimeria* induced coccidiosis model	Increase serum FITC-d on 5 and 6 d postinfection
Barekatain et al., 2019ab	4 kDa	2.2 mg/mL/bird	No fasting	2.5 h from jugular vein	Ross 308, male broiler	d 21	0.5 mg/kg BW dexamethasone breast muscle injection on d 14, 16, 18 and 20	Wheat-sorghum-soybean meal	Reduced protein plus dexamethasone induced leaky gut model	Dexamethasone increase serum FITC-d
Baxter et al., 2019	3−5 kDa	8.32 mg/kg BW	No fasting	1 h from femoral vein	2015 genetic line broiler; 1995 genetic line broiler; Jungle fowl chicken (mixed-sex)	d 10 and 21	Switch feed diet (rye-corn; rye-rye or corn-rye) during each growing phase	Corn-soybean meal or rye-soy-bean meal	Rye-based diet induced leaky gut model	On d 10, the 2015 and 1995 genetic line broiler from rye-based diet increased serum FITC-d, but jungle fowl was unaffected; On d 20, the rye involved diet increased serum FITC-d in 2015 and 1995 genetic line broilers
Hernandez-Patlan et al., 2019	3−5 kDa	8.32 mg/kg BW	No fasting	1 h from femoral vein	Cobb 500, male broiler	d 21	Orally gavage with 1 × 10E8 cfu *S.* Typhimurium on d 1; 2 × 10E4 *E. maxima* on d 13; 1 × 10E9 cfu mixed *Clostridium perfringens* on d 18, 19	Corn-soybean meal	Necrotic enteritis disease model	Challenge increase serum FITC-d (0.69 vs 0.31 ug/mL)
Bortoluzzi et al., 2019a	4 kDa	2.2 mg/mL/bird	No fasting	2 h by cardiac puncture	Cobb 500, male broiler	d 21	Orally gavage with ~5,000 *E. maxima* on d 14, with/without 1 × 10E8 *Clostridium perfringens* on d 19, 20 and 21	Corn-soybean meal	Necrotic enteritis disease model	The inoculation of *Clostridium perfringens* on top of *E. maxima* challenge did not increase serum FITC-d
Bortoluzzi et al., 2019b	4 kDa	2.2 mg/mL/bird	No fasting	2 h by cardiac puncture	Cobb 500, male broiler	d 21	Orally gavage with ~5000 *E. maxima* on d 14, following challenge with 1 × 10E8 *Clostridium perfringens* on d 19, 20 and 21	Corn-soybean meal	Necrotic enteritis disease model	Serum FITC-d was two times higher in challenged treatment than the non-challenged treatment
Gilani et al., 2018a	4 kDa	2.2 mg/mL/bird	0, 4.5, 9 or 19.5 h	2.5 h from jugular vein (meanwhile birds received lactulose, mannitol and rhamnose solution 60 min post the FITC-d gavage)	Ross 308, male broiler	d 38	Feed restriction for 4.5, 9 or 19.5 h	Wheat-soybean meal- sorghum-canola meal expeller	Feed restriction model	Serum FITC-d increased with increasing fasting time, no difference between 9.0 and 19.5 h fasting treatments (0.94. 1.25, 1.69 and 1.61 ug/ml for 0, 4.5, 9.0 and 19.5 h feed withdraw respectively)
Gilani et al., 2018b	4 kDa	2.2 mg/mL/bird	24 h post hatch for delay-feeding treatment	2.5 h from jugular vein	Ross 308, male broiler	d 2, 4 and 7	24 h delay-feeding on day of hatch	Local commercial starter crumble diet	Delayed feeding model	No difference for serum FITC-d between the first 24 h delay feeding treatment compared with control on d 2, 4 and 7. But serum FITC-d increased on d 7 compared to d 4
Latorre et al., 2018	3−5 kDa	8.32 mg/kg BW	No fasting	1 h from femoral vein	Cobb 500, male broiler	d 25				

Table 1 (*Continued*)

Reference	Molecular weights	FITC-d dose	Fasting	Blood collection post gavage	Breed/strain	Sampling age	Challenge type	Diet	Experimental model	Results (compared with control)
							Oral gaveve 1 × 10E7 cfu *S.* Typhimurium at d 1; 2.5 × 10E4 *E. maxima* at d 18; 1 × 10E8 *Clostridium perfringens* at d 23, 24	Corn-soybean meal	Necrotic enteritis disease model	Challenge increase serum FITC-d (203.23 vs 15.05 ng/mL)
Baxter et al., 2017	3–5 kDa	4.16 or 8.32 mg/kg BW	24 h fasting on d10 only for feed restriction treatment	1 or 2.5 h from femoral vein	Cobb, broiler	d 11	None	Not mention	24 h feed restriction model	Increase dose of FITC-d from 8.32 to 4.16 mg/kg; shorten the blood collection time from 2.5 to 1 h showed more evident reading results
Gilani et al., 2017a, Exp 3	4 kDa	2.2 mg/mL/bird	No fasting	2.5 h from brachial vein (meanwhile birds received lactulose, mannitol and rhamnose solution 60 min post the FITC-d gavage)	Ross 308, male broiler	d 21	Intraperitoneally administered 1 mg/kg BW of lipopolysaccharide or sterile saline	Commercial broiler diet	Lipopolysaccharide challenge model	No difference on serum FITC-d between the LPS and control group (1.81 vs 1.91 ug/mL)
Gilani et al., 2017b	4 kDa	2.2 mg/mL/bird	19.5 h fasting on d 20 only for feed restriction treatment	2.5 h from brachial vein (meanwhile birds received lactulose, mannitol and rhamnose solution 60 min post the FITC-d gavage)	Ross 308, male broiler	d 21	Dextran sodium sulphate group were given 0.75% dextran sodium sulphate from d 16 to 20	Commercial broiler diet	Dextran sodium sulphate plus feed restriction model	Serum FITC-d increased after fasting compared with control (3.6 vs 1.91 ug/mL); no difference between dextran sodium sulphate and control group
Prado-Rebolledo et al., 2017, Exp 2	3–5 kDa	4.16 mg/kg BW	No fasting	2.5 h from femoral vein	Male broiler	d 4	Orally gavage with 4 × 10E4 cfu/0.25 mL/bird *S.* Enteritidis on d 1	Unmedicated broiler starter diet	*Salmonella* enteritidis challenge model	*Salmonella* Enteritidis increased serum FITC-d (1.59 vs 0.34 ug/mL)
Zhang et al., 2016	3–5 kDa	2.2 mg/mL/bird	No fasting	2 h from jugular vein	Ross 708, male broiler	d 14 and 21	Birds from challenge group were receive 24 h fasting on d 13 combined with the gavaged with a coccidial vaccine at 25 × dose, which contained live oocysts of *E. acervulina, E. mivati, E. maxima* and *E. tenella*	Corn-soybean meal	Threonine deficiency with feed withdrawal combined with coccidial vaccine challenge model	only feed withdrawal combined with overdose vaccine (0.32 vs 0.22 ug/mL) increase serum FITC-d on d 14; Threonine deficiency (0.84 vs 0.71 ug/mL) or feed withdrawal combined with overdose vaccine (0.93 vs 0.62 ug/mL) increase serum FITC-d on d 21
Kuttappan et al., 2015a	3–5 kDa	2.2 mg/mL/bird	34 or 29 h fasting only for feed restriction treatment	2.5 h from femoral vein	Cobb, broiler	d 4	Orally gavage with 1 mL dextran sodium sulfate 0.45 g/bird/d on d 3 and 4; or withdrawn feed 5 h before the first dextran sodium sulfate gavage and continued until blood collection (total 34 h/29 h feed restriction)	Broiler starter diet	Dextran sodium sulfate and feed restriction model	Dextran sodium sulfate and feed restriction increased the serum FITC-d than the control (0.44 vs 0.28 uL/mL in Exp 1; 0.50, 0.49, 0.28 ul/mL in Exp 2; 0.44, 0.49, 0.27 uL/mL in Exp 3)
Kuttappan et al., 2015b	3–5 kDa	2.2 mg/mL/bird	24 h fasting only for feed restriction treatment	2.5 h from femoral vein	Broiler and Leghorns	d 14 or 7	Dextran sodium sulfate 0.75% in drinking water for 3 d; or 24 h feed restriction	Corn-soybean meal or Rye-soybean meal	Dextran sodium sulfate, feed restriction, rye-based diet model	Feed restriction and rye-based diet showed higher serum FITC-d
Vicuña et al., 2015a	3–5 kDa	4.16 mg/kg BW	No fasting	2.5 h from femoral vein	Cobb, broiler	d 10	Dexamethasone in feed (0.57, 1.7 or 5.1 mg/kg) from d 4 to 10; or injection (1 mg/kg) on d 3, 5 and 9	Corn-soybean meal	Dexamethasone induced leaky gut model	All dexamethasone treatments showed increased serum FITC-d
Vicuña et al., 2015b	3–5 kDa	0.55, 1.1 or 2.2 mg/bird BW	24 h fasting only for feed restriction treatment	2.5 h from femoral vein	Cobb, broiler	d 7 or 14	Dextran sodium sulfate 0.75% in drinking water for 5 d; or 24 h feed restriction	Corn-soybean meal or Rye-soybean meal	Dextran sodium sulfate, feed restriction, rye-based diet with different FITC-d dose model	Feed restriction, rye-based diet and dextran sodium sulfate increase the serum FITC-d
Tellez et al., 2014	3–5 kDa	2.2 mg/mL/bird	No fasting	2.5 h from femoral vein	Cobb, broiler	d 10	None	Corn-soybean meal or Rye-soybean meal	Rye-based diet induced leaky gut model	Rye increased the serum FITC-d in both trials (0.42 vs 0.20; 0.52 vs 0.31 ug/mL)

within certain challenge models or experimental designs, broilers chickens are euthanized for necropsy to determine intestinal lesion scores or collect intestinal digesta for measuring nutrient digestibility. Having birds in a fed-state, rather than fasting, may be a better option concerning its potential effect on intestinal lesion score development (such as in trials with coccidiosis challenge model and need lesion score evaluation) as well as the need of collecting intestinal digesta (such as in trials with endogenous nutrient digestibility evaluation). In short, as fasting affects the outcomes of the FITC-d permeability assay, it is not advised to do so unless this is part of a specific experimental need. If fasting is utilized, it is critical to maintain similar feeding behavior and feed withdrawal times to ensure similar amounts of feed left in the GIT between treatments.

ORAL GAVAGE

A reusable metal oral gavage needle together with the regular disposable syringe (3−5 mL in volume size) works effectively in broiler research (Bortoluzzi et al., 2019a and 2019b). As the gavage needle/tube could fall off because of bird movement, it is essential to use a syringe with a luer-lock. Another option is to gavage the broilers via a repeater pipette (Teng et al., 2020), which doses the FITC-d solution more accurately. With both oral gavage equipment, it is crucial to ensure the exact amount of 4-kDa FITC-d solution is gavaged into the crop without spilling.

BLOOD COLLECTION

In mouse research, blood samples have been collected by cardiac puncture, retro-orbital bleeding or tail vein punctures with/without anesthesia at 4 h (more common) or 6 h after the FITC-d administration (Napolitano et al., 1996; Yan et al., 2009; Condette et al., 2014; Yang et al., 2018; Woting and Blaut, 2018). Mice fasted for 6 h showed higher 4-kDa FITC-d concentration in plasma 1 h after gavage than 4 h after gavage (Cani et al., 2009). Other research demonstrated the 4-kDa FITC-d reached its maximal concentration in plasma less than 4 or even 2 h post oral gavage depends on the stain of mouse (Woting and Blaut, 2018). The difference may due to factors that can affect FITC-d intestinal uptake and renal excretion (Woting and Blaut, 2018). In broiler research, bleeding from the jugular (neck) vein, brachial (wing) vein, femoral (leg) vein or cardiac puncture are four common methods (Table 1). Taking animal welfare into consideration, blood collection is conducted after CO_2 inhalation or cervical dislocation euthanasia procedures (longer than normal waiting time after the euthanasia may lead to less blood amount to be collected). Researchers conducted blood collection 2.0 or 2.5 h after oral gavage broiler with 2.2 mg FITC-d/bird (Tellez et al., 2014; Zhang et al., 2016). Other researchers collected blood

1.0 h after the oral gavage, but with higher gavage dose at 8.32 mg FITC-d/bird (Baxter et al., 2017).

Application of lower dose FITC-d and shorter periods between the gavaging of FITC-d and blood collection would reduce the ability to determine a treatment-related difference of gut permeability. In contrast, if higher dose and longer periods of time were conducted in the experiment, FITC-d background may increase because the birds without receiving pathogenic challenge or dietary treatments would still exhibit limited intestinal leakage. A 2 h gap has shown consistent and little background of FITC-d in non-*Eimeria*-challenged birds among several experiments or different sampling ages within the same study (Castro et al., 2020; Teng et al., 2020; Yadav et al., 2020). However, future permeability assay studies should be designed to find the optimal point in time with corresponding FITC-d doses (time-point for serum FITC-d to reach the peak concentration with concomitant low concentrations of background fluorescence) for blood sampling in broiler chickens.

Meanwhile, the rate-of-passage of FITC-d along the GIT in broiler chickens should also be considered when selecting blood collection time post the gavage. The speed and amount of blood collection and final collected amount may vary depending on the proficiency of the personnel. It is recommended to store the blood collection tube immediately in a dark-colored container and keep at room temperature for 2 to 3 h until all samples are well clotted (Bortoluzzi et al., 2019a and 2019b; Teng et al., 2020). Centrifuging blood samples from broiler chickens can be set at 500 to 2,000 g for 10 to 15 min at 4°C (Tellez et al., 2014; Vicuña et al., 2015a; Zhang et al., 2016; Gilani et al., 2018a; Baxter et al., 2019).

FITC-D ANALYSIS

Enough additional FITC-d solution should be reserved for making a standard curve. Triplicate wells are recommended for running each standard curve concentration, while duplicate wells are good for serum sample measurement (Bortoluzzi et al., 2019a and 2019b). The flat bottom black opaque plate (minimize back-scattered light, background and crosstalk of fluorescence when reading the plate) is used for measuring FITC-d product. If multiple plates are used for FITC-d reading (large number of birds when sampling), a new standard curve is needed for each plate. The results from each plate are calculated based upon its correlative standard curve. In mouse studies, some researchers directly used PBS to dilute FITC-d for calculating the standard curve (Yang et al., 2018). Other researchers demonstrated that the ideal dilutions of FITC-d are performed with non-hemolytic serum from healthy, non-FITC-d gavaged mice (Wang et al., 2015). In broiler chickens, researchers have measured FITC-d fluorescence concentration after diluting serum: non-diluted, 1:1, 1:5 or 1:10 of PBS or sterile 0.9% saline (Tellez et al., 2014; Kuttappan et al., 2015a; Vicuña et al., 2015a;

Baxter et al., 2017). The aim of the higher dilution for serum samples is to eliminate the background color and fluorescence in the serum. However, Baxter et al. (2017) also reported a 1:5 dilution may be better than 1:10 dilution, with a significantly higher amount of serum FITC-d concentration, indicating dilution might result in reducing the reading value of FITC-d. In the same paper, the authors optimized the FITC-d permeability assay by using blank chicken sera for preparing FITC-d standard curve. However, the final dilution ratio of serum samples may need to be optimized by the end user (Gilani et al., 2017b; Gilani et al., 2018b). The ideal standard curve is able to cover a range that most serum FITC-d reading values are distributed towards the midpoint of the standard curve. For example, we use 0 to 2,000 ng/mL as the range to prepare the standard curve of 4-kDa FITC-d permeability assay for serum samples of broiler chickens. Based on our experience, using sera from extra unchallenged broilers that were not gavaged with FITC-d to prepare the FITC-d standard curve is recommended. Enough additional broiler chickens fed a control diet are needed for the recommended protocol. Since the extra serum is used for correcting possible background color in the serum, it is optional to measure the treatment samples (with a total volume of 100 uL/well) directly without dilution if enough serum was collected from the birds. For 4-kDa FITC-d, the fluorescence measurement is conducted with a microplate reader at an excitation wavelength of 485 nm and the emission wavelength of 528 nm (Kuttappan et al., 2015a'b). A previous study reported that FITC-d were stable for more than three days at 37°C in biological media (Kurtzhals et al., 1989). In poultry studies, some researchers conducted the FITC-d measurements on the same day of the sampling (Bortoluzzi et al., 2019a; Teng et al., 2020), whereas other researchers have kept serum samples at −20 C until further analysis (Gilani et al., 2017b). However, no specific experiment has been done to compare the difference between "analysis on the same-day" versus "analysis after storage at −20°C".

OTHER RELATED FACTORS

Serum FITC-d concentration results vary considerably across different studies, with obvious magnitude of difference even being observed between control birds (Figures 3 and 4). Multiple factors can affect the serum FITC-d concentration, including the breed/strain, age and gender of the birds, as well as the experimental design (diet, health status/challenge model, and sampling age).

Breed/Strain, Age, and Gender

The serum FITC-d concentrations from broiler research vary slightly between different genetic lines (Gilani et al., 2017a; 2018a). Researchers have evaluated the intestinal permeability among modern commercial broiler, 1995 genetic line broiler and unselected Giant Jungle Fowl (birds within each treatment were mixed genders) using a rye-based diet experimental model (Baxter et al., 2019). The modern commercial broiler has been reported to have higher serum FITC-d concentration than the jungle fowl on d 10 when fed the corn-control dietary treatment, with 1995 genetic line birds as intermediate. However, there was no different on serum FITC-d concentration fed a rye-based diet treatment on d 10. On d 20, contrary results were found that jungle fowl had the highest serum FITC-d concentration when the dietary treatments were switched. The authors speculated that the jungle fowl takes longer time to develop a fully functional GIT; thus, higher passage of FITC-d in younger jungle fowl may be contributed to the immaturity of the intestinal tract.

A 24-h delay-feeding program did not increase intestinal permeability on d 2, 4, and 7 in broiler chickens compared to control birds (Gilani et al., 2018b). The authors mentioned young birds may still absorb yok at the first few days post-hatch. This can reduce the adverse effect of fasting on intestinal permeability. Another reason may be due to feeding after 24 h can restore the permeability to a normal status on d 4. However, the FITC-d concentration was significantly higher on d 7 compared to the concentration on d 4. This result suggested that intestinal permeability can change by age, even in healthy broiler chickens.

In mice, female mice had a reduced gut permeability compared with the male mice. This can be explained by the effects of estrogen, which can maintain the intestinal barrier function by promoting bicarbonate secretion into the gut as well as enhancing the expression of tight junction proteins (Woting and Blaut, 2018). Thus, when conducting the FITC-d assay in broiler research, gender may also need to be considered if using straight-run (mixed gender) birds in the trial.

Experimental Design (Diet, Health Status, and Sampling Age)

Studies have shown the epithelial barrier function can be affected by feed composition (Chatelais et al., 2011; Tellez et al., 2014; Hamilton et al., 2015). An ex vivo research model found an enhanced ileal permeability when piglets were fed with a high-protein formula milk (Chatelais et al., 2011). The increased permeability may be related to altered immune responses and microbiota changes (Takiishi et al., 2017). Another mouse study reported high-fat diet increased paracellular permeability (increased FITC-d flux) in both small and large intestine using ex vivo Ussing chambers (Hamilton et al., 2015). Similar results with increased intestinal permeability were also found in broilers (Kuttappan et al., 2015b), which may due to the high-fat diet causing altered expression of tight junction. A review article has concluded that the high-fat diet would alter gut permeability by stimulating barrier-disrupting cytokines and enriching the gut microbiome with barrier-damaging
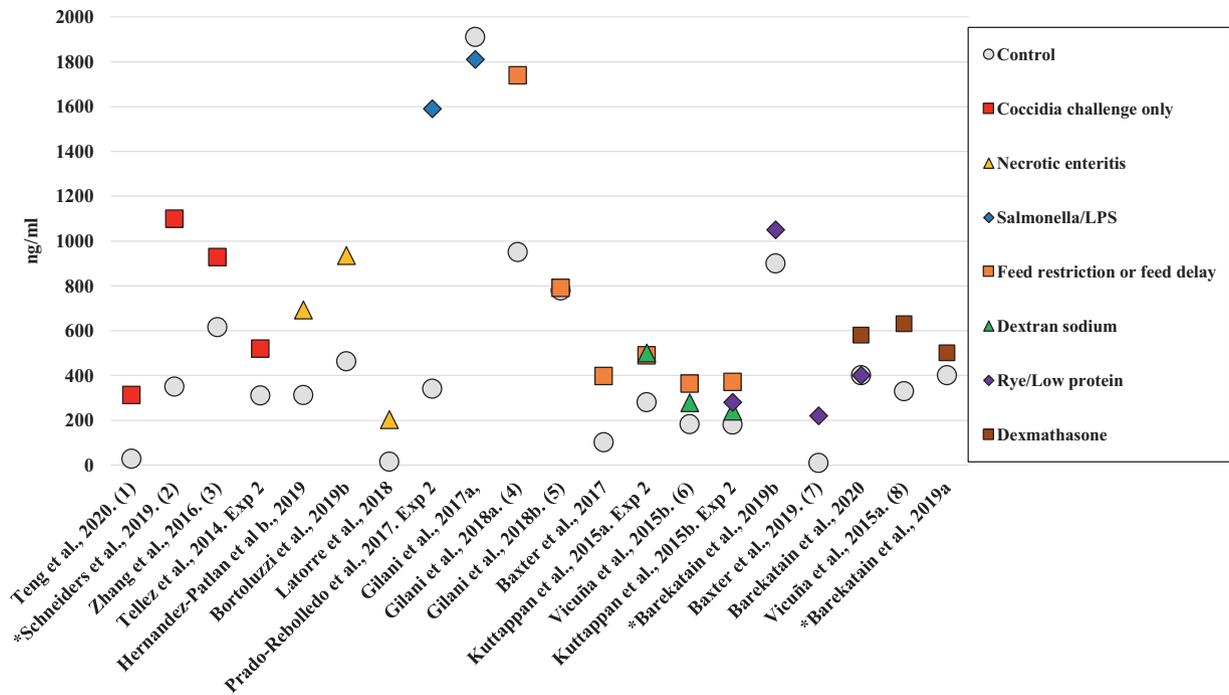
Figure 3. Comparing serum FITC-d concentration among previous studies (Scatter plot). * FITC-d value is estimated from the figure of original article. (1) Data selected from days post-inoculation (DPI 5); (2) Data selected from DPI 6; (3) Coccidia challenge and feed restriction; (4) Data selected from 19.5 h; (5) Data selected from d 7; (6) Data selected from 2.2 mg/bird; (7) Data selected from Rye-Rye diet of MB2015; (8) Data selected from Table 3 of original article.

species (Rohr et al., 2020). In addition, a poorly digested diet (such as rye-based diet) can affect increase intestinal viscosity, which may influence the movement of FITC-d along the GIT (Tellez et al., 2014). The rye in the diet disrupted intact intestinal barrier, resulting in increased serum FITC-d concentration in broiler chickens (Kuttappan et al., 2015b). Other research has demonstrated a reduced protein diet affected intestinal

barrier function and led to higher intestinal permeability in broiler chickens (Barekatain et al., 2019a, 2019b). A diet deficient in specific amino acids, such as threonine, was reported to affect intestinal permeability as threonine is particularly important for the production of mucin. Mucin was reported to act as the first defense line in the GIT against pathogen invasion (Zhang et al., 2016). In addition, threonine plays important roles in
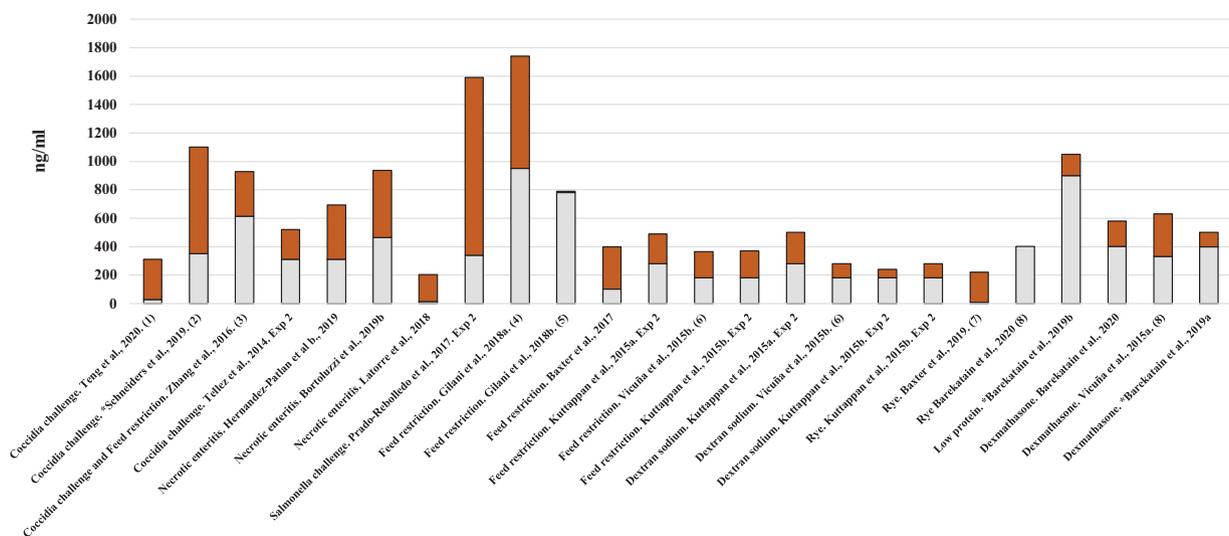
Figure 4. Comparing serum FITC-d concentration among previous studies (Stacked bar plot). * FITC-d value is estimated from the figure of original article. (1) Data selected from days post-inoculation (DPI 5); (2) Data selected from DPI 6; (3) Coccidia challenge and feed restriction; (4) Data selected from 19.5 h; (5) Data selected from d 7; (6) Data selected from 2.2 mg/bird; (7) Data selected from Rye-Rye diet of MB2015; (8) Data selected from Table 3 of original article.

producing immune cytokines and superoxide molecules (Chen et al., 2017). Altered dietary threonine concentrations can influence the gene expression of mucin, IFN- γ, and interlukin-1 in the intestine of birds (Chen et al., 2017). Mycotoxins (such as deoxynivalenol), even at subclinical dose, have also been reported to induce increased permeability in broilers by altering intestinal tight junction function and inflammatory cytokine secretion (Grenier and Applegate, 2013).

Intestinal permeability can be affected by different research models. These models have included fasting, chemical perturbation (e.g., dextran sodium sulfate and dexamethasone), as well as pathogen challenge. More than 24 h of feed withdrawal significantly induced intestinal physiological disruption (Kuttappan et al., 2015a). The impact of the fasting period on intestinal permeability in broilers has been described in the previous section. Using dextran sodium sulfate, a heparin-like polysaccharide, can induce inflammation, disrupt epithelial lining, and increase mucosal permeability in broiler chickens. This could decrease the villus height, reduce epithelial cell height, increase goblet cell density, and disrupt tight junction of the intestine (Kuttappan et al., 2015a,b). Dexamethasone is also found to increase serum FITC-d concentration and intestinal permeability, which can be explained by the induced stress-like inflammatory response in GIT (Vicuña et al., 2015a). Other research models, including *Eimeria* challenge (Schneiders et al., 2019; Teng et al., 2020), *Eimeria* together with *Clostridium perfringens* challenge (Bortoluzzi et al., 2019a,b), *Salmonella* challenge (Prado-Rebolledo et al., 2017; Köhler et al., 2007) and wire-floor stress model (Wideman et al., 2012), can also induce inflammation and increase intestinal permeability.

The effects of sampling age on serum FITC-d concentration were discussed in two recent publications, with *Eimeria* challenge models (Schneiders et al., 2019; Teng et al., 2020). Coccidiosis is a common poultry infectious disease caused by *Eimeria* spp. The FITC-d assay has been used to evaluate effects of *Eimeria* infection on broiler intestinal permeability. Schneiders et al. (2019) orally gavaged 14-day-old broilers with 2×10^5 *E. maxima* sporulated oocysts, and conducted FITC-d permeability assay daily for 10 consecutive days after inoculation. They reported that intestinal permeability was significantly higher in challenged birds at 5 and 6 d postinoculation, with the serum FITC-d concentration peaking at 6 d postinfection (22% increase of serum FITC-d concentration than in the unchallenged birds). However, no significant difference was noted in serum FITC-d concentrations from 7 to 10 d postinoculation. Similar results were found in another broiler trial. Teng et al. (2020) evaluated intestinal permeability on 3, 5, 6, 7, and 9 d postinoculation with 13-day-old broiler chickens. Birds from the challenge treatment were orally gavaged with four different dosages of mixed species of *E. maxima, E. tenella, and E. acervulina.* There was a significant linear increase of serum FITC-d concentration on 5, 6, 7 d postinoculation. However, by 9 d, the difference between challenged and control birds were not different. It was worth noting that, the authors mentioned the peak of serum FITC-d concentration was not only related to the timing of sampling, but also was affected by the dose of *Eimeria* used in the challenge model. It was reported that the high challenge dose caused the peak of intestinal permeability of birds earlier than the low challenge dose. Since a crowding effect occurs when an enormous number of parasites compete, limited enterocytes for schizonts development and results in self-inhibition, the high dosage challenged treatment reached the peak of gut permeability at 5 d postinfection, instead of 6 d postinfection. However, the leaky gut did not recover until 9 d postinfection in those birds challenged with the highest dosage of mixed *Eimeria* spp. Thus, the optimal sampling day for *Eimeria* challenge model is to conduct FITC-d assay on 5 d postinfection with dosage higher than mixed 25,000 *E. maxima,* 25,000 *E. tenella,* and 125,000 *E. acervulina* per milliliter, while on 6 d postinfection when given a challenge dosage less than mixed 12,500 *E. maxima,* 12,500 *E. tenella,* and 62,500 *E. acervulina* per milliliter.

CONCLUSION

Serum 4-kDa FITC-d concentration is a useful and effective bioassay to measure intestinal permeability in broiler chicken research. It can be widely applied to different gut inflammation or disease challenge research models. Serum FITC-d concentration results vary across previous studies with a huge magnitude of difference. Understanding and optimizing the details and factors of protocol that may affect FITC-d concentration results is crucial for poultry researchers. Difference in oral gavage dose, fasting period, sample handling, and lab analysis details should be taken into consideration when conducting the 4-kDa FITC-d intestinal permeability assay in broiler research. Multiple factors can affect the serum FITC-d concentration, including the breed/strain, age and gender of the birds, as well as the experimental design (diet, health status/challenge model, and sampling age). A preliminary trial with small number of birds is recommended in order to optimize the protocol that fit certain research animals, experimental design, and lab analytic instruments. Future research also needs to consider the respective contribution of both paracellular and transcellular intestinal permeability within different animal research models.

DISCLOSURES

All the authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations,

knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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