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Advances, Challenges and Future Applications of Avian Intestinal in Vitro Models

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Key words

Intestine, in vitro, model, epithelial cell line, tissue explant, organoid, enteroid, apical-out, infection, barrier, leukocyte

Abstract

There is a rapidly growing interest in how the avian intestine is affected by dietary components and probiotic microorganisms as well as its role in the spread of infectious diseases in both the developing and developed world. A paucity of physiologically relevant models has limited research in this essential field of poultry gut health and led to an over reliance on the use of live birds for experiments. The intestine is characterised by a complex cellular composition with numerous functions, unique dynamic locations and interdependencies making this organ challenging to recreate *in vitro*. This review illustrates the *in vitro* tools which aim to recapitulate this intestinal environment; from the simplest cell lines which mimic select features of the intestine but lack anatomical and physiological complexity, to the more recently developed complex 3D enteroids which recreate more of the intestine's intricate microanatomy, heterogeneous cell populations and signalling gradients. We highlight the benefits and limitations of *in vitro* intestinal models and describe their current applications and future prospective utilisations in intestinal biology and pathology research. We also describe the scope to improve on the current systems to include for example microbiota and a dynamic mechanical environment, vital components which enable the intestine to develop and maintain homeostasis *in vivo*. As this review explains, no one model is perfect but the key to choosing a model or combination of models is to carefully consider the purpose or scientific question.

Introduction

The global movement to reduce animal trials has resulted in an urgent need to develop alternative comprehensive non-animal lab models whose results closely resemble that of a living animal. Intestinal models are of particular interest for livestock studies since the gut is essential for nutrition and immunity as well as the target for many important pathogens, therapeutics and feed additives. However, models commonly used to study the livestock intestinal surface present major limitations. Avian gastrointestinal studies also have long been hampered by a lack of representative cell culture tools. This review describes the features of *in vitro* and *ex vivo* culture systems that can be employed as a model for the avian intestine and future prospective uses of *in vitro* intestinal models.

In vitro models of the intestine

Cell lines

Intestinal *in vitro* studies have historically used established cancer-derived or immortalised mammalian cell lines such as human Caco-2 and human HT29 colorectal adenocarcinoma cells. These cell lines have been in use for a comparatively long time, are cheap, easy to access and grow, and can be analysed using currently available technologies. However, immortalised intestinal epithelial cell (IEC) lines lack cellular heterogeneity, do not reproduce full tissue functionality, and typically evolve chromosomal aberrations and mutations during continuous passage which can affect growth, metabolism, physiology and reproducibility (Ben-David *et al.*, 2018). In addition, since they are not species-specific for livestock studies, they are not suitable for the study of species physiology or the interaction of species-specific intestinal pathogens. Several porcine and bovine small intestinal cell lines have been established (Chiba *et al.*, 2012; Miyazawa *et al.*, 2010; Schierack *et al.*, 2006; Zakrzewski *et al.*, 2013), but most avian epithelial cell lines are patented and not available for research purposes (patent WO/2020/157076). The only established easily-accessible epithelial cell line in the chicken is the lung epithelial cell line (CLEC)-213 (Esnault *et al.*, 2011).

Primary intestinal epithelial cell cultures

Primary isolated IECs are closer in phenotype and function to *in vivo* tissues than immortalised cell lines and are therefore more capable of recapitulating key features of animal physiology and host-pathogen interactions. A standard optimised isolation protocol for chicken primary small intestinal cultures does not exist but several groups have published methods to culture primary IECs. This includes an embryonic day (ED)18 chicken IEC culture which was used to investigate *E. tenella* infections and contained cells positive for epithelial-marker cytokeratin and epithelial adhesion

marker cadherin-1 (Dimier-Poisson *et al.*, 2004). Monolayers with improved morphology have since been obtained from embryonic and adult intestines, however, these degenerate after 7 - 10 days in culture (Bai *et al.*, 2019; Kaiser *et al.*, 2017). Although adult IEC chicken cultures were maintained for 6 - 7 passages by Rath *et al.* (2018), they were shown to have an atypical intercellular junction morphology which affects their applicability (Rath *et al.*, 2018). A more recent primary IEC culture showed survivability for 12 days when growing the monolayers on a Matrigel (an Engelbreth-Holm-Swarm mouse tumour extract)-coated surface and using medium supplemented with sodium butyrate (a histone deacetylase inhibitor which supports stem cell self-renewal and promotes differentiation), R-Spondin 1 (RSPO1), Noggin and CHIR99021 (all canonical β-catenin/Wnt pathway inducers), and epidermal growth factor (EGF, which maintains proliferation). The epithelial nature of the cells was confirmed by staining for villin and cytokeratin, and intercellular tight junctions were identified using zonula occludens 1 (ZO1) and occludin (OCLN) markers (Ghiselli *et al.*, 2021). IEC cultures derived from ED17 chicks, also seeded on Matrigel-coated wells but without the addition of exogenous recombinant growth factors, have been also been used in innate immunity studies to investigate inflammatory responses to bacterial products (Bar Shira & Friedman, 2018). More recently another IEC culture model was published derived from late embryonic chicks, that recapitulates all major differentiated cell lineages, including enterocytes, Paneth cells, Goblet cells, enteroendocrine cells and intra-epithelial leukocytes, and self-organises into an epithelial and mesenchymal sub-layer (Orr *et al.*, 2021). Functional studies using this model in transwell inserts demonstrated a robust epithelial integrity and inflammatory responses upon exposure to bacterial endotoxins.

Mammalian intestinal organoids and enteroids

Organoids are self-organising 3D tissue-like structures with biological functions similar to the living organ. These long-term self-renewing culture systems are proving to be more physiologically relevant than traditional monolayer culture techniques lines and are reducing the number of *in vivo* studies. Over the past decade methods for generating organoid culture systems have been published for most human and mouse organs including intestine, lung, liver, kidney, and brain (reviewed in Hofer & Lutolf, 2021). Intestinal organoids and enteroids, also known as ‘mini-guts’, mirror *in vivo* 3D intestinal epithelial morphology and are already facilitating research into basic biology, disease modelling, and drug development. Enteroids are typically epithelial only and are developed from intestinal tissue biopsies containing adult intestinal stem cells (ISC) (Stelzner *et al.*, 2012). These adult ISCs can originate from the tissue of juveniles and late stage embryos and are intrinsically programmed with their location specific functions (Lancaster & Knoblich, 2014; Middendorp *et al.*, 2014), reviewed in (Fatehullah *et al.*, 2016). In contrast, intestinal organoids often contain epithelial and mesenchymal cells and are typically developed from induced pluripotent or early embryonic stem cells. Embryonic

stem cells are obtained from the inner mass of pre-implantation embryos whereas induced pluripotent stem cells are generated by reprogramming differentiated somatic cells e.g. skin fibroblasts, to regain pluripotency (Takahashi & Yamanaka, 2006).

Propagation of primary adult intestinal crypt cultures was initially achieved in 1992 when Evans *et al.* (1992) cultivated murine crypt epithelial cells on collagen in the presence of contaminating mesenchymal fibroblasts (Evans *et al.*, 1992). The expertise to grow self-organising continuously expanding enteroids in a mesenchymal-free environment was first developed in the mouse in 2009 (Sato *et al*., 2009). By isolating intestinal crypts or single multipotent adult ISC, embedding the cells in a Matrigel matrix and adding external growth factors, 3D epithelial enteroids and organoids can be generated with organised crypt and villus domains, a polarised epithelium, a functional lumen and comprising most differentiated cell types at normal ratios (Figure 1). Since then enteroids have been developed in various livestock species using the same concepts as the murine culture system (reviewed in Beaumont *et al*., 2021).

Avian enterospheres

Although most advances in 3D enteroids are limited to mammalian species, the development of chicken enteroid models has been ongoing for almost 10 years. However, attempting to grow chicken enteroids in the microenvironments successful for most mammals has so far yielded limited results. As with other livestock species, the induction cues needed to differentiate avian intestinal organoids from pluripotent stem cells have not yet been determined so currently their 3D cultures are all derived from adult ISC. Pierzchalska *et al.* (2012) first published evidence of chicken *in vitro* 3D intestinal cultures grown using the classical murine method from ED18 – 20 small intestine (Pierzchalska *et al.,* 2012). They were cultured in Matrigel domes with comparable morphology obtained when using prostaglandin E2 (PGE2) as a cost-effective alternative to RSPO1 and Noggin. The isolated epithelial fragments were observed to close into spheres with an expanding lumen enclosed by a thin intact layer of cells. These structures remained viable for several weeks but did not form crypt-like buds. In current organoid nomenclature these non-budding thin-walled spherical structures are closest in description to enterospheres so shall be described as such going forwards (Stelzner *et al.*, 2012). This group has subsequently published variations on this model including a hanging drop culture system where enterospheres were suspended in media containing 5% Matrigel (Panek *et al.,* 2018).

Although the majority of chicken 3D cultures are small intestinal in origin, Powell & Behnke (2017) demonstrated the ability to passage Matrigel-embedded caecal enterospheres, created from adult bird caecal tissue, over 30 times (Powell & Behnke, 2017). Despite the chicken's body temperature being 41°C, all cultures are incubated at 37 - 38°C. This is similar finding in porcine enteroid cultures,

which express higher levels of *LGR5* (an intestinal stem cell marker) at 37°C despite the pigs body temperature being 39°C (Khalil *et al.*, 2016). All Matrigel-based culture methods for poultry have so far resulted in thin-walled cystic structures with few if any defined crypt- and villus-like domains. These therefore do not resemble the 3D architecture of the *in vivo* intestine nor the classical enteroid morphology of other species. Typically these enterospheres have only been evidenced to contain enterocytes, by identifying villin and sucrase isomaltase markers and proliferating cells (Li *et al.*, 2018; Panek *et al*., 2018; Pierzchalska *et al.*, 2012; Powell & Behnke, 2017). However, a more recent publication reported the identification of stem cells, Paneth cells, enteroendocrine cells, epithelial cells, and goblet cells in their enterosphere cultures, although this was predominantly by using RT-PCR on a limited gene set (Zhao *et al.*, 2021). Pierzchalska *et al.* (2012) also identified myofibroblasts strongly attached to the enterosphere surfaces when they were seeded in Matrigel domes (Pierzchalska *et al.*, 2012). These were assumed to originate from epithelial-mesenchymal transition however low expression of both the myofibroblast marker α-smooth muscle actin (αSMA) in the hanging drop cultures, and mesenchymal marker vimentin in Matrigel dome cultures from other groups indicates the presence of this cell type is variable and could be caused by isolation or other culture related factors (Powell & Behnke, 2017; Zhao *et al.*, 2021).

Chicken enterospheres grown in microenvironments successful for other species are a positive advancement in species-specific 3D cultures, but as yet fail to recapitulate the morphology of their organ of origin and are as such limited *in vitro* models. On a molecular level there is a complex interplay of growth factors and signalling pathways that positively regulate self-renewal and differentiation of LGR5+ ISCs (de Lau *et al*., 2011; Lahar *et al.*, 2011; Sato *et al.*, 2011). For example, basolateral cell receptors need to interact with specific combinations of extracellular matrix (ECM) proteins for the development of enteroids from isolated crypts (Gjorevski *et al*., 2016; Vogel *et al*., 2006). Ongoing investigations of chicken enteroid growth within different ECM analogues with variable mechanical (elasticity or stiffness) and chemical compositions are required to determine the physical niche that is beneficial for chicken enteroid ISCs. In addition, trialling a greater variety of small molecules at differing concentrations and/or producing chicken-specific recombinant growth factors could contribute to successful differentiation and longevity of chicken enteroids.

Avian enteroids

In 2020, Acharya *et al.* demonstrated the growth of compact spheroids from isolated day old chick villi when they were floated in suspension instead of embedded in extracellular matrix. The resultant floating enteroid structures displayed an external epithelial layer and a solid core of tissue. They shed large numbers of cells from the surface, as would be expected along the villus in intestinal

homeostasis, but only displayed very limited budding from the main spheroid structure after stimulation with EGF and human insulin-like growth factor (IGF)-1 (Acharya *et al.,* 2020; Figure 3).

More recently Nash *et al.* (2021) developed a floating enteroid model but, in contrast to other chicken enterosphere and enteroid cultures, without the requirement for most commonly used exogenous growth factors (Nash *et al.*, 2021). This resulted in an enteroid model with multiple villus-like budding structures that lasted in culture for 1 – 2 weeks. The enteroid structures were demonstrated to contain representative gut epithelial populations of ISCs, Paneth cells, enteroendocrine cells, goblet cells, and enterocytes using a combination of transcriptional analysis, transmission electron microscopy and immunohistochemistry. Interestingly the floating chicken enteroid morphology developed rapidly with multiple small buds present by 24 hours and mature villus-like structures evident by 2 - 3 days (Figure 3). In contrast, newly established mouse enteroids take 7 - 10 days to become fully mature structures (Sato *et al*., 2009). This ED18 model was also shown to mature to post-hatch functions in culture, showing transcriptional changes related to development of digestive function and organ development. To expand the potential applications of the floating avian 3D enteroid culture system, Nash *et al.* (2021) developed cultures from different regions of the small and large intestine and from several poultry species (Nash *et al.*, 2021).

Typically, gel-embedded enteroid crypts feed into a central functional lumen and are lined by highly polarised epithelial cells whose apical brush borders face internally and basolateral surfaces lie in contact with the ECM scaffold (Sato *et al*., 2009). A practical limitation of the 'basal-out' 3D geometry and internal lumen of these classical enteroids is that they prevent easy access to the apical epithelium. The most striking discovery with the floating cultures was the 'apical-out' orientation of the floating poultry enteroids, with the apical brush border of the epithelial cells facing the media, and the basal epithelial cell surface abutting a dense central cellular core (Nash *et al.*, 2021). Transcriptional and histological analyses by Nash *et al.* (2021) showed this enteroid core is representative of the lamina propria and contains functional leukocytes, glial and mesenchymal cell populations (publication in prep) making this a distinctive model from their mammalian counterparts (Nash *et al.*, 2021). The externally accessible epithelial surface of the floating chicken enteroids allowed for the uncomplicated replication of the natural infection process by simply adding viral, bacterial and protozoal pathogens to the media (Nash *et al.*, 2021). The enteroids immune-epithelial component was also demonstrated to trigger appropriate immune responses to each pathogen, as described *in vivo*, using a custom highly multiplexed qPCR array (Borowska *et al.*, 2019). However, the complexity of this 3D model does not allow for the characterisation of immune responses originating solely from the epithelium only, in contrast to the 2D model (Orr *et al.*, 2021), nor the full array of *in vivo* adaptive immune responses.

Another drawback of floating enteroids is that they do not allow for long-term self-renewing propagation. Since human and murine apical-out enteroids also do not demonstrate passage capabilities, this is could be an issue of floating culture conditions on ISC survival (Co *et al.,* 2019). Either through prohibiting contact of media-supplemented growth factors ligands with the reverse polarised epithelial basolateral epithelial receptors, and/or through the change in complex two-way interactions between ISCs and ECM scaffold (Playford *et al*., 1996). This reduced longevity has been partly addressed with the development of methods to cryopreserve enteroids and villi at the point of isolation (Nash *et al.*, 2021). Biobanking of frozen culture material means the enteroids can be reproducibly used across many studies and material can be shared between labs, opening up research into indigenous breeds from other countries. This also removes the need to use fresh tissue, predominantly that of embryos rather than post-hatch birds, for each experiment.

Tissue explants

Ex vivo cultures of intestinal tissue explants are closer in phenotype and function of *in vivo* tissues than immortalised cell lines and are therefore more capable of recapitulating key features of animal physiology and host-pathogen interactions. However, explants in contrast to enteroids maintain and undergo senescence, cell death and necrosis over relatively short timespans (some after only 24 – 48 h) introducing concerns over the accuracy and reproducibility of biological experiments. Therefore, intestinal tissue explant models are generally used to investigate short term inflammatory and secretory responses of the entire gut mucosa to external challenge. These tissue culture systems have been described for the pig (Girard *et al.*, 2005; Kolf-Clauw *et al*., 2009) and bovine (Girard *et al.*, 2007) small intestine, mainly for studies exploring enteropathogen infections. Few groups have published explants of gastrointestinal tissue for poultry. Chicken ileal explant models have been used to measure nitric oxide (NO) production, epithelial inflammatory markers (*TLR4, IL-1β, IL-8*) and epithelial secretory responses (mucin (MUC)2, IgA and polymeric immunoglobulin receptor) after stimulation with LPS (Kallapura *et al*., 2015; Zhang *et al.*, 2017). However these cultures were reported to only be viable for 2 h which is not sufficient to evaluate tight junction responsiveness (Zhang *et al*., 2017).

Ligated intestinal loop models

Although not an *in vitro* model *sensu stricto*, ligated intestinal loop models are tools in which gut loop segments in a single anaesthetised animal are ligated to form independent and hermetic compartments whilst the animal is under terminal general anaesthesia. Experiments are performed within each loop e.g. injection of pathogens or toxins into the lumen, and samples for further analysis are collected a few hours of continuous general anaesthesia later. These models allow the use of multiple treatment and control groups in the same animal thereby reducing the number of hosts

required to obtain significant results. In addition, any pain is controlled by general anaesthesia thus reducing ethical concerns when conducting pathogenesis experiments on live animals. These techniques are not commonly employed, but they have been described in both calves and chickens (Menge *et al*., 2004; Parent *et al.*, 2018).

Applications and limitations of avian enteroids

The remainder of the review will focus on chicken 3D enteroid models since this relatively new and rapidly developing field has more promising future applications for intestinal studies than the other described *in vitro* systems. As demonstrated by the mammalian organoid field, there is marked potential to improve on these current cultures creating more physiologically accurate and/or more high-throughput applicable systems which will further enhance their utility in the basic research and industry setting.

Host-pathogen studies

Early mechanisms of host-pathogen interactions at the mucosal interface can be difficult to elucidate and manipulations of experimental conditions difficult to orchestrate in live animal studies therefore complementary *in vitro* studies are needed. Current 2D models of the chicken intestine have limited cell-types and/or intestinal architecture so cannot reproduce certain hallmarks of natural infections. Since 3D enteroids more closely mimic the morphology and physiology of the intestine they are emerging as viable alternatives for many established infection models. However, with basal-out gel-embedded enteroids, in order to replicate the physiological route of entry of enteric pathogens, microinjection (Heo *et al.*, 2018), transition to 2D enteroid cultures (Luu *et al.*, 2019) or enteroid fragmentation (Derricott *et al.*, 2019) are required to gain access to the apical epithelial surface. These techniques are costly and time consuming and, for the mechanical disruption technique, both apical and basolateral sides of the enteroid cells are exposed. In addition, investigating intestinal immunity *in vitro* remains challenging since most currently available intestinal organoid cultures are epithelial-only models. Access to species-specific intestinal epithelial-immune cell models are a necessary to study coordinated enteric immune responses in health and disease.

Features demonstrated in the floating avian enteroids, which make them highly applicable for host-pathogen studies, are not only their apical-out conformation with an easily accessible brush border, but also an intact epithelial barrier, the innate presence of lamina propria and intraepithelial leukocytes, and the development of poultry species- and digestive site-specific models (Nash *et al.*, 2021). These cultures have already provided a useful platform for interrogating the invasion and

replication efficiency and innate immune interactions of different species and strains of enteric bacteria, virus and protozoa in the post-hatch chick intestine (Nash *et al.* 2021). The assessment of floating chicken enteroid infections with wild type and Type 3 Secretion System 1 mutant *Salmonella* Typhimurium strains showed both were highly immunogenic, but the mutant bacteria showed limited invasion and replication and a differential innate immune gene expression (publication in prep). These studies indicate the model should be useful to explore the questions relating to intestinal colonisation, host genetics and innate immunity in laying hen breeds, as well as measuring the level of attenuation of live vaccine candidates. In addition, since floating enteroids can be developed from various bird species, they could be used alongside mammalian and avian respiratory and enteroid models to model Avian Influenza Virus species crossover events *in vitro,* including the viruses potential for mutation to virulence (Bryson *et al*., 2020).

Growth of micro-organisms that do not grow in cell lines

Cultivation of previously unculturable viruses e.g. human norovirus, is now possible in enteroid models (Ettayebi *et al.*, 2016). This indicates that the differentiated cellular environment present in organoids can provide essential factors for the infection and expansion of microorganisms. This attribute could make the floating chicken enteroid system particularly useful for the culture of certain important chicken enteric viruses. These include astroviruses, rotaviruses and adenoviruses which are currently difficult to study as they do not grow in available cell lines but their effective control would have significant economic implications to the global poultry industry (Adebiyi *et al.*, 2019; Mettifogo *et al.*, 2014) (reviewed in Day & Zsak, 2013).

Studying host-protozoal interactions *in vitro* is challenging due to the universal difficulties culturing these parasites. Enteroid cultures in other species have recently been exploited to model protozoal infection such as *Cryptosporidium* and *Toxoplasma*, showing these parasites are able to replicate *in vitro* and induce enteroid innate immune responses (Cardenas *et al*., 2020; Derricott *et al.*, 2019; Heo *et al*., 2018; Luu *et al*., 2019; Zhang *et al.*, 2016). In poultry, assessment of disease manifestations and vaccine efficacy caused by the economically important protozoa *Eimeria* is predominantly achieved using live chicken challenge experiments with highly variable study designs (Soutter *et al.*, 2020). This is in part because immortalised mammalian cell lines can only support the early part of the *Eimeria* life-cycle (Tierney & Mulcahy, 2003). So far the full life-cycle of *E. tenella* (one of the most globally prevalent *Eimeria* species) has only been replicated *in vitro* in primary embryonic chick kidney cells. Unfortunately this infection produced very small numbers of unsporulated oocysts which sporulated very poorly (Hofmann & Raether, 1990). The chicken lung cell line (CLEC-213) also allows sporozoite infection but only replicates up to the gamete stage (Bussière *et al.*, 2018). Since these chicken models

are not intestinal in origin, they cannot fully recapitulate *in vivo* interactions of *E. tenella* with its host. Multivalent vaccines that do not require the use of chickens in their production and will induce cross protection against a combination of *Eimeria* spp. are required. In addition, although natural *Eimeria* infections induce strong cell mediated immune responses, there are no assays which predict how protective a coccidiosis vaccine might be. The ability to study interactions of different *Eimeria* spp. with the host intestinal mucosa *in vitro* could help to identify solutions to these problems and improve the safety and success of vaccines (Blake *et al.*, 2011; Liu *et al.*, 2018). Early work with digestive-site specific floating chicken enteroids is proving promising for the study of these protozoal pathogens in poultry (Nash *et al.*, 2021).

Genetic lines

Organoids derived from biopsies from individual humans or animals can be used for personalised medicine applications including patient-specific drug testing and autologous-grafting to repair damaged tissues (Kondo & Inoue, 2019; Sugimoto *et al.*, 2021). Although these applications may be relevant for pets they are not indicated for livestock species, however development of chicken-breed specific enteroids would allow the study of genetic diversity between breeds *in vitro*. Traits of interest that could be analysed include how phenotypes influence disease resistance, resilience, microbiota compositions and differences in nutrient absorption (Clark *et al*., 2020; Giuffra *et al.*, 2019). Genome editing has been used in human and mouse intestinal organoids to introduce specific genetic mutations to study intestinal disease phenotypes (reviewed in Roper & Yilmaz, 2019)). CRISPR Cas9 technology has not yet been utilised in livestock species enteroids, however enteroid cultures developed from pigs genetically edited to express a mutated myosin 5B gene displayed physiologically appropriate consequences of the gene mutation (Engevik *et al.*, 2020). Creating enteroids from individuals already harbouring or prone to disease can also allow modelling of disease processes without prior knowledge of the causative genes or conditions (Chandra *et al.*, 2019; Dekkers *et al.*, 2013).

Nutrition

The intestinal epithelial layer has many essential functions in protection and digestion therefore a representative *in vitro* model of the gut must also demonstrate the full cellular repertoire, polarity and barrier functions present *in vivo* (reviewed in Odenwald & Turner, 2017)). Differentiated cell types with prominent roles in digestion, e.g. absorptive enterocytes and digestive hormone secreting enteroendocrine cells, have been identified in both chicken enteroids and enterospheres (Nash *et al.*, 2021; Zhao *et al.*, 2021). In addition, the identification of surface microvilli, and upregulation of digestive enzyme and ion/nutrient transporter genes e.g. *SLC13A1, FABP2, SCP2, LCT, ARG2* indicates

floating enteroids could be used to assess food digestion, nutrient absorption and gut hormone regulation. Studies in pig enteroids have already demonstrated functional apical to basolateral transport of nutrients and increased epithelial proliferation after supplementation with dietary glutamine (van der Hee *et al.*, 2020; Zhu *et al.*, 2020). In addition these models have been used to show the impact of feed-associated vitamin A, L-glutamine and mycotoxins on ISC homeostasis, as well as insights into host-pathogen interactions in relation to feed efficiency in pigs (Li *et al.*, 2019; Wang *et al.*, 2020; Zhu *et al.*, 2020).

Screening feed additives and heat stress

Feed costs make up to 70% of the costs of broiler production and feed efficiency is a major variable to determine the cost of meat. Therefore the availability of enteroid-based tools to study and screen food compounds would prove useful for the poultry industry. The innermost layer of the intestinal luminal surface consists of a single cell thick epithelial lining which acts as a barrier, preventing the entry of microbiota and harmful pathogens while still allowing the selective passage of dietary nutrients, ions, and water (Russell *et al.*, 2015). Increased intestinal permeability is a sign of perturbed intestinal barrier function so assessing the integrity of the epithelial barrier *in vitro* can be useful in the study of intestinal inflammation following pathogen infection as well as the effect of feed additives or probiotics. TEER measurements have been used to measure epithelial integrity in 2D chicken enteroid monolayers, however these methods are not applicable to the 3D structure of enteroids (Orr *et al.*, 2021). Analytic dye analysis has been used to measure the passage of permeability solutes over the epithelial layer via paracellular routes for both 2D and 3D enteroids. For example in porcine 2D intestinal cell-line studies it has been used to shown changes in gut barrier integrity on exposure to heat-stress (Zhou *et al.*, 2020). However for gel-embedded basal-out intestinal organoids/enteroids these intestinal permeability studies are either technically challenging e.g. requiring micro-injection of dye in the enteroid lumen, or only allow analysis of stimulants applied to the basal epithelial surface (Bardenbacher *et al.*, 2019; Hill *et al.*, 2017; Pearce *et al.*, 2018). Due to the apical-out nature of floating chicken enteroids, a much more simplified protocol of immersing them in FITC-dextran has enabled the quantitative measurement of epithelial permeability after various external challenges (Nash *et al.*, 2021).

Defined consortia – microbiome

In order that enteroids mimic the differentiated form and function of parental tissues, the introduction of monocultures or more complex microbiota cultures is being investigated. So far, cell lines in anaerobic transwells can only sustain microbiota co-cultures for under 24 h (Ulluwishewa *et al.*, 2015). However more complex anaerobic microfluidic-based systems such as Gut-Chips allow 5 day co-

cultures, and microbial communities have persisted in 3D basal-out human colon organoids for over 4 days after a high-throughput microinjection technique (Tottey *et al.*, 2013; Williamson *et al.*, 2018). Murine enteroid-microbiota studies have demonstrated a positive correlation between microbial stimulation of the gut and epithelial regeneration, and identified how host lipid metabolism genes are modulated by commensal gut bacteria (Lukovac *et al*., 2014; Nigro *et al.*, 2014; Park *et al*., 2016). In ED18 chicken enterosphere cultures, the probiotic bacteria *Lactobacillus acidophilus,* a TLR4 ligand*,* and Pam3CSK4, a TLR2 ligand, promoted enterosphere growth and increased PGE_2 production (Pierzchalska *et al.*, 2017). Developing a host-microbiota system for apical-out enteroid cultures with an aerobe-anaerobe interface seems highly feasible and would allow for the controlled exploration of the microbiotas role in e.g. epithelial barrier integrity, nutrient absorption and ISC function, as well as how IECs regulate the composition of the microbiota and distinguish between tolerance of and defense.

Bioprinting and upscaling

The advent of 3D organoid bioprinting, which has already been demonstrated for the bovine, may eventually enable co-culturing of livestock enteroids with defined spatial positioning of different cell populations (Töpfer *et al.*, 2019). In addition, the application of microfluidic organ-on-chip technology, where mechanical complexity such as oxygen gradients and peristalsis motions are introduced, could further improve the physiological relevance of organoid models and even introduce inter-organ communication (Nikolaev *et al.*, 2020; Sidar *et al.*, 2019; Skardal *et al.*, 2020).

Bioprinting techniques could also overcome many of the scalability limitations of standard enteroid cultures (Töpfer *et al*., 2019; Velasco *et al.*, 2020). High throughput screening is indicated for many commercial applications in poultry enteroids therefore ongoing work is required to decrease variability in the cultures and characterise reproducible responses to challenge. Organoids are a relatively new technology and they belong to a rapidly developing field. Therefore the correct concentration and combination of growth factors for each species, digestive site and age are still being optimised. Individual reports describe a variety of methods to generate and maintain enteroid cultures, possibly resulting in variability in the enteroid phenotypes. This is particularly evident in the chicken enteroid field, where no two ISC isolation techniques are the same and an array of growth factors are in use (Acharya *et al.*, 2020; Pierzchalska *et al.*, 2012; Powell & Behnke, 2017). The floating cultures do not use Matrigel or supplemental growth factors which significantly reduces potential effects of raw culture material variability on culture reproducibility as well as the financial impact of this model.

Large-scale analytic techniques for apical-out enteroids have not been established, with most technologies aimed at 2D cultures e.g. TEER measurements for epithelial barrier integrity studies (Roodsant *et al.*, 2020). Therefore, alongside the development of novel *in vitro* models, the creation or adaption of analytical tools addressing the model's particular idiosyncrasies also needs to be considered. One such tool already in use are customised qPCR arrays for assessment of chicken enteroid immune gene expression (Borowska *et al.*, 2019). These have already shown great potential to measure enteroid responses to pathogen infection, but could also be utilised in host-microbiota and other enteroid challenge screening studies (Nash *et al.* 2021).

Future prospectives of *in vitro* intestinal models

Research in the essential field of poultry gut health has been compromised for a long time by a shortage of physiologically relevant *in vitro* models, leading to an over reliance on the use of live birds for experiments. No one model system is perfect, but the key to choosing a model is to carefully consider the purpose or scientific question. Indeed, the extrapolation of results from unsuitable cell culture systems is a major bottleneck in the drug discovery process across the species. Transformed cell lines are useful for high throughput screening, for example to measure the toxicity of compounds. However they may be less translatable if used in host-pathogen interaction studies, especially if the pathogen replicates in that particular cell type *in vitro* but not *in vivo.* Models with increased cellular heterogeneity are more representative of the *in vivo* organ, however, typically these more complex culture systems are more labour intensive and costly. Although of utmost importance, the reliability of many models is unknown and to ensure translatability of the system, direct comparisons of compounds and pathogens tested *in vivo* and *in vitro* is needed. On the other hand, *in vivo* experiments are not the model system Holy Grail as trials using SPF chickens or commercial birds under experimental conditions do not encompass the full environmental effect the birds experience in a commercial setting.

Prior to the development of organoids, the fundamental analysis of certain species-specific biological processes such as host-pathogen interactions and metabolism were severely hampered by the lack of appropriate *in vitro* model options. The introduction of enteroids has provided species- and organ-specific models which create more reliable intestinal responses than current *in vitro* options whilst also contributing to the 3Rs (reviewed in Beaumont *et al*., 2021; Kar *et al.*, 2021). Experiments using 2D and 3D enteroids or enterospheres can allow the study of early infection events e.g. pathogen adhesion and entry, in far more detail and synchrony than the oral inoculation of birds. Major advantages of the newly developed floating chicken enteroids is that they combine a complex cell

system with an easy accessible apical-out orientation and grow in a simple culture medium without an ECM (Nash *et al.* 2021). The future hopefully holds even more complex microphysiological models of the poultry gut with gut-on-chip technologies starting to emerge in the human field which allow the study of biology beyond conventional cell models, including shear stress, nutrient circulation, and peristalsis (reviewed in Marrero *et al*., 2021). We hope that this review of the current and potential types and applications of chicken *in vitro* models will fuel interest in those associated with the field and help to drive new approaches in these promising tools. In doing so we can continue to create models which better mimic tissue- and organ-level physiology and function, addressing the 3Rs and facilitating much needed advancements in poultry gastrointestinal research and product development.

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751 **Table 1 Differences between avian and murine enteroid cultures**

Publication	Species	Animal age	Crypt/villi isolation	ECM	Culture media
Sato *et al.,* 2009	Mouse	mature	EDTA (Gentle Cell Dissociation Reagent)	Matrigel (Engelbreth-Holm-Swarm mouse sarcoma extract)	Intesticult (EGF, Noggin, RSPO1)
Pierzchalska *et al.,* 2012; Li *et al.,* 2018; Zhao *et al.,* 2021	Chicken	ED18 – 20 week birds	EDTA, EGTA or collagenase	Matrigel	DMEM/F12 or DMEM +/- HEPES, Glutamine, antibiotic & antimycotic, 10% FBS, ITS, chicken serum, RSPO1, Noggin, EGF, L-WRN media (Wnt3a, RSPO3, Noggin), Wnt3a, PGE_2, CHIR99021
Co *et al.,* 2019	Mouse	mature	EDTA	Floating (after establishment in Cultrex Reduced Growth Factor Basement Membrane Matrix, Type II)	DMEM/F12, HEPES, Glutamine, B27, N-acetylcysteine, gastrin, EGF, nicotinamide, TGF-βi, p38MAPKi, FGF10, L-WRN media
Acharya *et al.,* 2020; Nash *et al.,* 2021	Chicken	ED18 – 1 day old chick	Collagenase or mechanical trituration	Floating	DMEM/F12, HEPES, Glutamine, antibiotic +/- B27, sodium bicarbonate, sodium pyruvate, antimycotic, 10% FBS, ITS, polyamine, bovine pituitary extract

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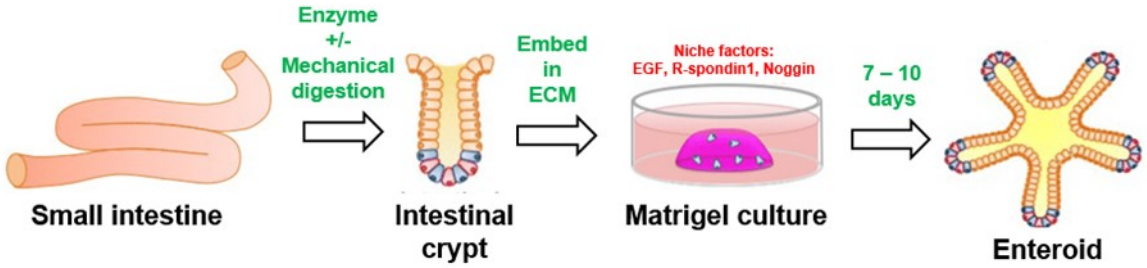
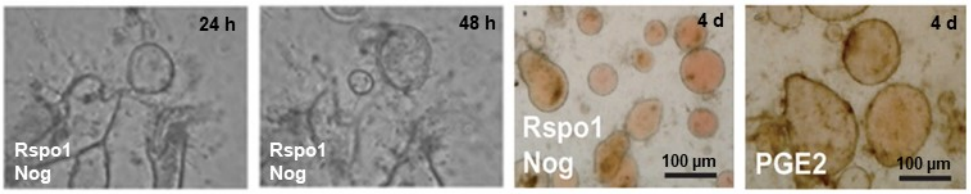
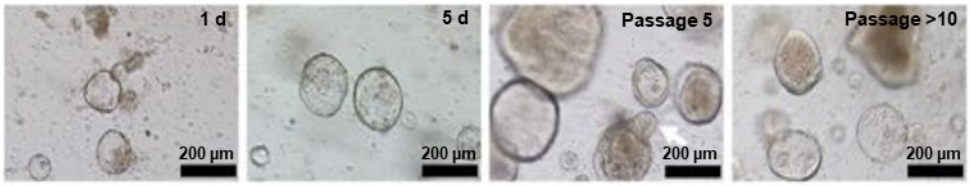
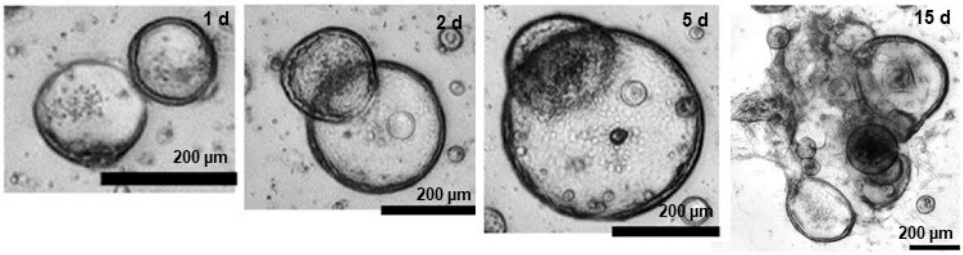


Figure 1. Method for culturing enteroids in Matrigel. Intestinal crypts are isolated from the small intestine using a combination of enzymatic and mechanical digestion. The crypts are embedded in an extracellular matrix (ECM), a dome of Matrigel, and surrounded by a media containing essential growth factors, enabling them to develop into enteroids over 7 - 10 days. Adapted from Chusilp *et al.* (2020) under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/) (Chusilp *et al.*, 2020).

Ref	Culture Images
(Pierzchalska *et al.*, 2012)	
(Powell & Behnke, 2017)	
(Li *et al.*, 2018)	

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761 **Figure 2: Chicken enterosphere culture morphology**. Brightfield images of enterospheres developed
762 from chicken intestinal tissue. Images adapted from publications under the terms of the Creative
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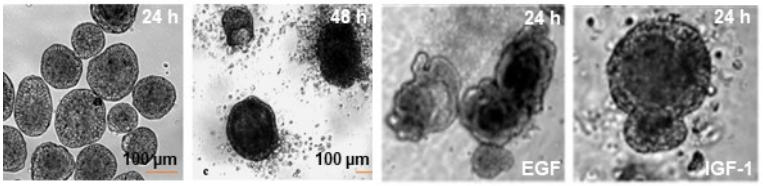
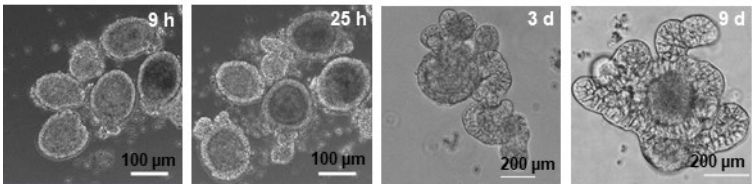
764 (http://creativecommons.org/licenses/by/4.0/).

Ref	Culture Images
(Acharya *et al.*, 2020)	
(Nash *et al.*, 2021)	

765

766 **Figure 3: Floating chicken enteroid culture morphology**. Brightfield images of enteroids developed
767 from chicken intestinal tissue. Images adapted from publications under the terms of the Creative
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