

# Probiotics and gut health: linking gut homeostasis and poultry productivity

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**Abstract.** The use of probiotics in poultry production has increased rapidly, and this movement has been promoted by global events, such as the prohibition or decline in the use of antibiotic growth promotants in poultry feeds. There has been a persistent search for alternative feed additives, and probiotics have shown that they can restore the composition of the gut microbiota, and produce health benefits to the host, including improvements in performance. Probiotics have shown potential to increase productivity in poultry, especially in flocks challenged by stressors. However, the outcomes of probiotic use have not always been consistent. There is an increasing demand for well defined products that can be applied strategically, and currently, probiotic research is focusing on delineating their mechanisms of action in the gut that contribute to an improved efficacy. In particular, mechanisms involved in the maintenance and protection of intestinal barrier integrity and the role of the gut microbiota are being extensively investigated. It has been shown that probiotics modulate intestinal immune pathways both directly and through interactions with the gut microbiota. These interactions are key to maintaining gut homeostasis and function, and improving feed efficiency. Research has demonstrated that probiotics execute their effects through multiple mechanisms. The present review describes recent advances in probiotic use in poultry. It focuses on the current understanding of gut homeostasis and gut health in chickens, and how it can be assessed and improved through supplementation of poultry diets with probiotics in poultry diets. In particular, cellular and molecular mechanisms involved in the maintenance and protection of gut barrier structure and function are described. It also highlights important factors that influence probiotic efficacy and bird performance.

**Keywords:** microbiota, mode of action, mucosal integrity, intestinal health, immunomodulation, necrotic enteritis, broiler performance.

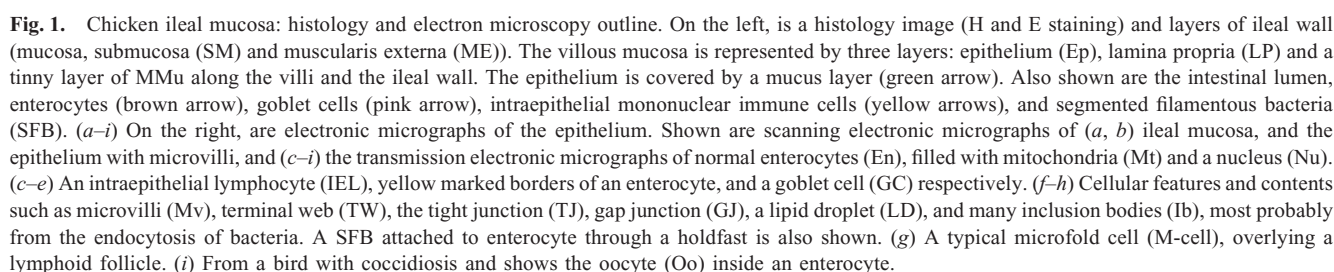
Received 30 December 2020, accepted 5 April 2021, published online 17 June 2021

## Introduction

The gastrointestinal tract (GIT) or gut of all species is a tubular organ of great structural and functional complexity, as has been detailed elsewhere (Scanes and Pierzchala-Koziec 2014; Denbow 2015; Furness and Cottrell 2017; Pluske *et al.* 2019). An important part of the GIT is the small intestine, the segment where the majority of the digestion and absorption of nutrients takes place. Briefly, the intestinal mucosa (which consists of epithelium with a core of lamina propria) projects into the gut lumen through villi, and each villus serves as a functional absorptive unit (Fig. 1). The chicken intestinal epithelium is a single layer of columnar cells, and comprises enterocytes (absorptive cells), goblet (mucus-producing) and enteroendocrine cells, and various intraepithelial immune cells. The mucosa is covered by mucus (a complex hydrated gel that protects epithelial cells from chemical, enzymatic, microbial, and mechanical damage) and mucus-linked specific commensal bacteria. An intact epithelial barrier is essential for gut physiology and immunity or GIT

homeostasis (de Santa Barbara *et al.* 2003; Kastl *et al.* 2020). The epithelial cells linked by intercellular junctions and the mucus layer are crucial components of the intestinal barrier that selectively permits the movement of ions, nutrients and water, but restricts the translocation of microbes and toxins from the lumen, thus playing a fundamental role in maintaining gut health of the host. However, the entire integrity of the barrier is sustained by a complex network of regulatory pathways that interact with the microbiota, luminal contents or digesta, and host mucosa.

The GIT ecosystem is a delicate balance among the microbiota, the intestinal epithelium and host immunity. The gut lumen wall covered by the mucus layer surrounds the intestinal contents, a mixture of nutrients, metabolites, and a diverse microbial community, that work in concert to maintain GIT homeostasis or health. Shifts in intestinal microbial composition of a chicken can be caused by stress emanating from diet, such as changes in dietary ingredients, antigens in the feed, microorganisms associated with the diet,



systemic disorders. Moreover, subclinical infections, and chronic, low-grade intestinal inflammation, may also cause similar alterations of the epithelial barrier without clinical manifestations, and these may be difficult to diagnose.

The future Nobel laureate Elie Metchnikoff, a Russian working in Paris at the turn of the 20th century, was the first to suggest a role for the gut microbiota in health. However, a brief note in *Nature* in 1973 by the Finnish scientists Esko Nurmi and Marjatta Rantala, describing their research to control *Salmonella* infections in poultry with bacterial cultures, rekindled interest in the use of direct-fed microbials or probiotics, in animals (Nurmi and Rantala 1973). Rob Cumming and his group at the University of



New England pioneered the application of ‘competitive exclusion’ (Lloyd *et al.* 1977; Soerjadi *et al.* 1978) in Australia. Since then, many investigators have shown that probiotics can restore the composition of the gut microbiome, and introduce beneficial functions to microbial communities, and in doing so, they prevent or reduce gut inflammation and intestinal infection and improve bird performance (Eckert *et al.* 2010; Mountzouris *et al.* 2010; Cengiz *et al.* 2015; Huff *et al.* 2015; Latorre *et al.* 2015; Park and Kim 2015; Forte *et al.* 2016; Bai *et al.* 2017; Pereira *et al.* 2019; Yadav and Jha 2019; Shini *et al.* 2020b; Zaghari *et al.* 2020).

The use of probiotics in poultry production has increased rapidly. This reflects the efficacy of most probiotic products in the field, acceptance by consumers of a product that is widely consumed in society, and the need to find additional feed additives following the banning or decline in the use of antibiotic growth promotants (AGPs) globally. Interestingly, probiotics, not unlike AGPs, do improve animal performance and both classes of feed additives initiate these improvements through reducing inflammation in the gut (Niewold 2007; Mountzouris *et al.* 2019). There is an increasing demand by feed companies and poultry producers for well defined antibiotic alternatives that can be applied strategically without significant increase in feed costs. Currently, probiotic research is focussed on delineating intestinal mechanisms of action with the objective to improve efficacy and feed utilisation.

The present review describes recent advances in probiotic use in poultry, and examples relating to the control of necrotic enteritis (see Box 1, Fig. 2) are cited frequently. It focuses on our current understanding of gut health, how to assess it in chickens, and highlights important mechanisms of probiotic action that appear to improve gut health. In particular, cellular

and molecular mechanisms involved in the maintenance and protection of gut barrier structure and function are described.

## Gut health

‘Gut health’ is a term that is used very frequently in the human and animal literature, especially when describing the outcomes of probiotic use. However, the definition is often very broad and nebulous. In the human health literature, the role of probiotics is seen as facilitating gut homeostasis, primarily through interactions with the gut microbiota (Watnick and Jugder 2020). As the gut is a very dynamic environment, the term gut homeostasis may be a more precise way in which to describe such an environment than the more general term, gut health; the terms are used here interchangeably.

### Importance of gut health

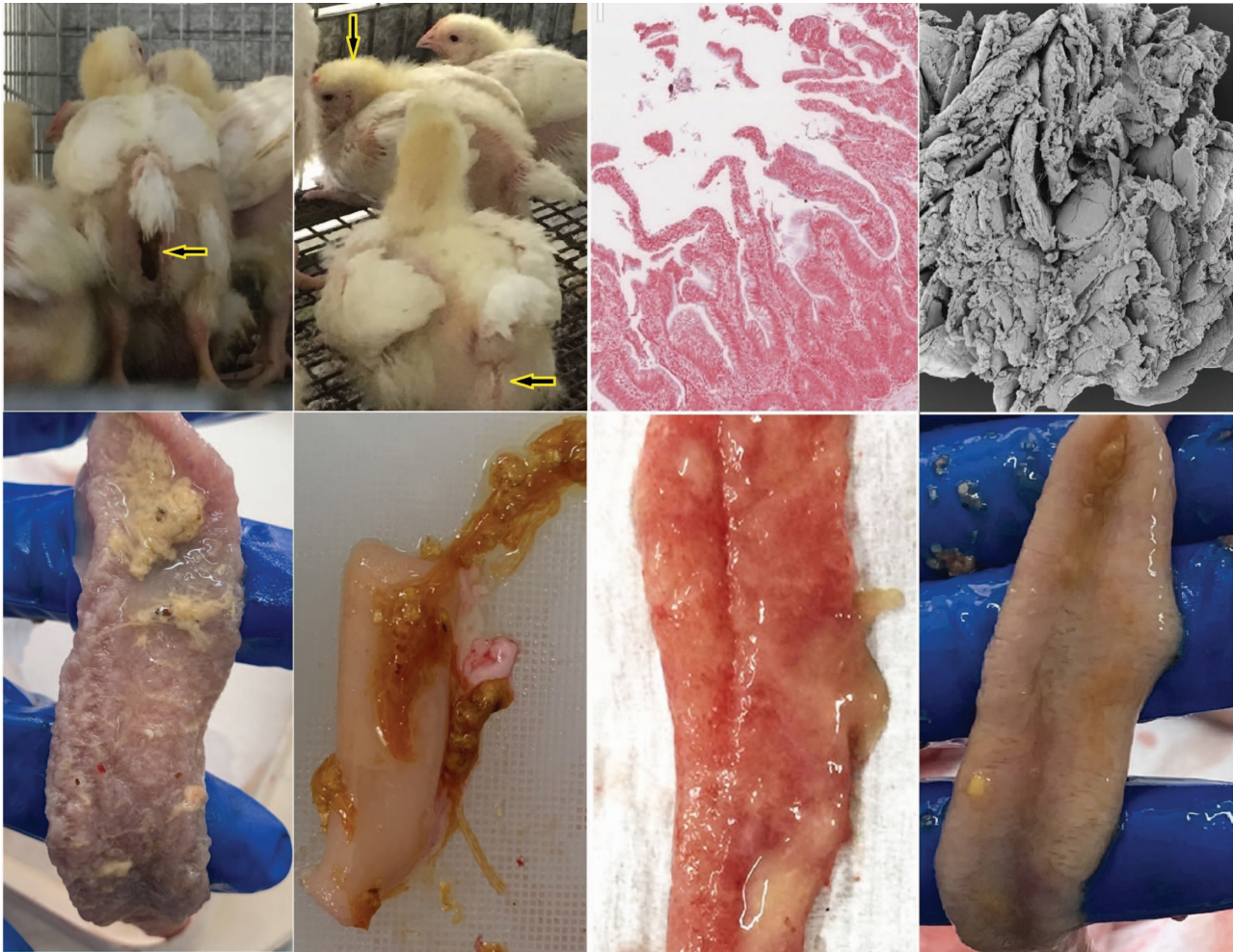
The GIT has a central role in nutrient digestion and absorption, host metabolism and energy generation, intestinal barrier integrity, mucosal immunity and providing a niche for a stable microbiota (Kogut and Arsenault 2016). For poultry, a ‘healthy gut’ means the absence of abnormal or damaged structures and functions, so that the bird is protected from pathogens and is able to digest feed and absorb nutrients efficiently to achieve optimum performance. There is increasing evidence that probiotics can make an important contribution to keeping a gut healthy by the maintenance of gut homeostasis (Bajagai *et al.* 2016; Cameron and McAllister 2019; Zommiti *et al.* 2020). Optimal gut health is crucial not only for the health, performance and welfare of production animals, but it also contributes to the environment because it improves feed efficiency, reduces use of AGPs, and sustains food safety and human health.

### Box 1. Necrotic enteritis

Necrotic enteritis (NE) is a significant enteric infection of poultry, primarily seen in broiler chickens, but the disease has also been reported for commercial layers raised on the ground, cage reared replacement pullets and turkeys. Other avian species can be affected. In broiler chickens, *Clostridium perfringens*, a normal occupant of the digestive tract, is the major aetiological agent for NE (Parish 1961; Long and Truscott 1976; Kaldhusdal and Hofshagen 1992). However, NE is a multifactorial disease and it occurs when gut conditions are altered in favour of clostridia proliferation, leading to the onset of NE. For example, coccidiosis (a parasitic disease of the intestinal tract of poultry caused by coccidian protozoa of *Eimeria* species) is believed to be one of the major predisposing factors for NE outbreaks (Al-Sheikhly and Al-Saieg 1980; Hermans and Morgan 2003; Collier *et al.* 2008). Other predisposing factors include the addition of fishmeal to the diet (Wu *et al.* 2010; Wu *et al.* 2014; Rodgers *et al.* 2015), feedstuffs containing high amounts of water-soluble non-starch polysaccharides, such as barley, rye and wheat (Kaldhusdal and Hofshagen 1992; Riddell and Kong 1992; Kaldhusdal and Skjerve 1996; Annett *et al.* 2002; Jia *et al.* 2009); in general, any factor that induces stress and immunosuppression in chickens and disrupts the homeostasis of the gut ecosystem contributes to the risk of NE in a flock.

NE is characterised by distended intestines containing gas and or fluids, and lesions such as patches of necrotic tissue on the intestinal mucosa (Fig. 2). NE often develops as an acute disease, with birds dying within a day after clinical signs are observed (e.g. ruffled feathers, depression, and diarrhoea; Fig. 2). Flock mortality can be as high as 10–30%, if untreated. The subclinical form of NE is associated with subtle clinical signs or no clinical signs; however, chronic damage of the intestinal mucosa (i.e. intestinal lesions) can be found after necropsy or histopathology (Kaldhusdal and Hofshagen 1992; Gholamiandehkordi *et al.* 2007; Park *et al.* 2008; Wu *et al.* 2010; Smyth 2016; Kogut *et al.* 2018; Shini *et al.* 2020b). Subclinical NE is difficult to diagnose, but can spread through flocks, resulting in substantial production losses (Skinner *et al.* 2010) due to malabsorption and reduced performance.

Historically, NE was effectively controlled by adding antimicrobial growth promoters (AGP) to broiler feed (Prescott *et al.* 1978; Elwinger *et al.* 1992, 1998). However, it has become one of the most significant broiler diseases globally, after the banning or reduced use of most AGPs in poultry diets. Current strategies focus on introducing alternatives to AGPs and managing predisposing factors, rather than trying to eliminate *Clostridium*. In the case of NE outbreaks associated with clinical signs and increased mortality, birds can still be treated therapeutically with antibiotics such as bacitracin, lincomycin, oxytetracycline or virginiamycin.



**Fig. 2.** The assessment of gut health using clinical signs, necropsy and histopathology. First row from the left to the right, pictures showing 14-day-old chicks; a bird with pasty vent, and a 21-day-old bird with clean vent, and a slower-growing bird with ruffled feathers (all yellow arrows). Next to it, images from histology and electron microscopy from birds with subclinical necrotic enteritis (NE). Second row from the left to the right, images from gross pathology of the ileum of birds with subclinical NE, watery diarrhoea, digesta mixed with bile, hyperaemic mucosa covered with a creamy content, and mucosa with a tan to yellow pseudomembrane referred to as 'Turkish towel' due to necrosis.

An undeniable feature of probiotic use is the broad spectrum of applications that have resulted in positive animal performance outcomes. Increases in egg and milk production, gut health and disease reduction, leg health and lameness reduction, reproductive health, meat quality and metabolic homeostasis, have all been demonstrated experimentally following feed supplementation with probiotics (Mountzouris *et al.* 2010; Wideman *et al.* 2012; Shini *et al.* 2013, 2020b; Zheng *et al.* 2014, 2016; Latorre *et al.* 2015; Bajagai *et al.* 2016; Angelakis 2017; Gadde *et al.* 2017; Cameron and McAllister 2019; Park *et al.* 2020; Wang *et al.* 2020; Zommiti *et al.* 2020). Together, this is a very strong endorsement for the role of probiotics in promoting gut health, and animal health and productivity. Moreover, probiotic use will also have a positive impact on animal and bird welfare (Bryden *et al.* 2021). Importantly, there is increasing evidence that the gut microbiota plays a central role not only in physical, but also mental wellbeing, with obvious implications for bird

health and welfare (Kraimi *et al.* 2019). Nevertheless, as we discuss below, there are instances when probiotics do not show any effect in improving animal and bird productivity.

#### Assessment of gut health

One difficulty to improving gut health with probiotics has been the lack of reliable methods or biomarkers to assess intestinal health or test probiotic efficacy. This assessment is of a particular importance in the case of subclinical infections and chronic intestinal inflammation (Kogut and Arsenault 2016; Kogut *et al.* 2018; De Meyer *et al.* 2019; Shini *et al.* 2020b). A bird with a subclinical infection or intestinal inflammation does not display signs of the disease, but epithelial damage and malabsorption can occur. For example, during subclinical necrotic enteritis (NE), birds may appear normal or have very mild clinical signs; however, a damaged intestinal epithelium can be found



after necropsy and histopathology or electron microscopy (Kaldhusdal and Hofshagen 1992; Teshfam and Rahbari 2003; Gomide *et al.* 2004; Zekarias *et al.* 2005; Gholamiandehkordi *et al.* 2007; Park *et al.* 2008; Wu *et al.* 2010; Smyth 2016; Kogut *et al.* 2018; Shini *et al.* 2019, 2020a, 2020b, 2021). Subclinical intestinal infections are difficult to diagnose, but can spread through flocks; therefore, they are one of the most important problems in the poultry industry because of high economic losses. A standard evaluation methodology for intestinal health status needs to be established.

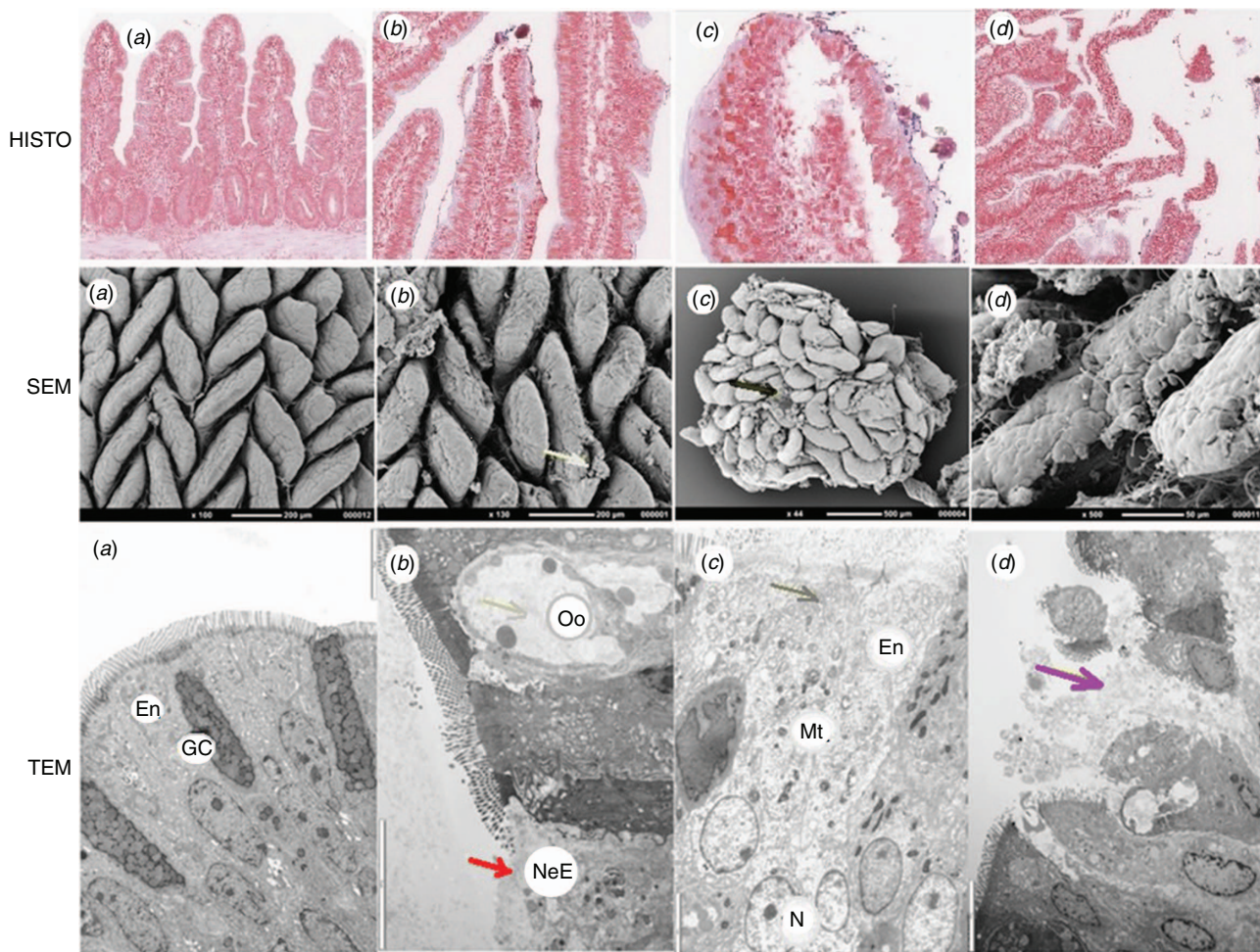
What to assess and how? There are visual clues and aspects of performance efficiency that might suggest gut health problems in a flock. Observations of compromised chicken performance, increased flock morbidity, evidence of diarrhoea or birds with pasty vents (Fig. 2), and the occurrence of wet droppings and wet litter (Dunlop *et al.* 2016; Kaldhusdal *et al.* 2016; De Cesare *et al.* 2017; Kumar *et al.* 2018; Shini *et al.* 2020b) may indicate disturbances to gut health and should be investigated to minimise the possibility of reduced flock welfare and productivity. In addition to performance and visual signs, necropsies of dead or culled birds can indicate the presence of any clinical or subclinical gut problems. As presented in Fig. 2, small and apparently normal birds may appear 'healthy,' but their intestinal mucosa might be 'disturbed', and this can be determined only at necropsy.

In addition to observations, intestinal health can be assessed using invasive tools that are necessary for a definite diagnosis, such as blood testing (haematology and serology), necropsy and histopathology or even electron microscopy for ultrastructure evaluations (Chen *et al.* 2015; Smyth 2016; Baxter *et al.* 2019; Shini *et al.* 2019, 2020a, 2020b, 2021). These direct tests provide more conclusive details. Studies conducted with a broiler model of subclinical NE (Shini *et al.* 2020b) have demonstrated that histopathology of the ileal mucosa helps quantify villus morphological alterations (Fig. 3, HISTO: a–d), and demonstrate focal erosion of epithelial cells and the fusion of adjacent villi, in otherwise apparently healthy birds. Histopathology of NE-challenged birds helped detect the successful infection with *Eimeria*, and oocyst formation underneath the epithelium (Fig. 3, HISTO: b, c). Consequently, the epithelial detachment in sheets and mucus production occurred and predisposed colonisation by *Clostridium perfringens* (Cp) deeper into the mucosa and crypts, causing focal necrosis (Shini *et al.* 2020a; Fig. 3 HISTO: c, d, h, l). The presence of Cp, intimately associated with necrotic lesions of intestinal mucosa, was also essential for the diagnosis of NE, and Gram-staining of ileal tissue permitted identification and enumeration of Cp (Fig. 3, HISTO: b, d; Shini *et al.* 2020b). The electron microscopy study of ileal tissue allowed the examination of enterocyte condition and demonstrated the value of this approach, as birds with subclinical NE displayed only very mild or no signs of the disease. In terms of ultrastructural examination of mucosa, the condition of epithelium and enterocytes, and their content and features, such as mitochondria, lysosomes, endoplasmic reticulum, tight junction (TJ) and microvilli can show the state of mucosa health (Fig. 3 SEM: a–d; TEM: a–d, 4). Other cells and structural components of the intestinal

mucosal barrier such as mucus-producing cells (goblet cells), immune-competent cells, i.e. intraepithelial lymphocytes and tissue-related immune cells (macrophages and heterophils), and microbial presence, provide evidence of involvement of inflammatory and immune defence mechanisms in the pathogenesis of NE (Shini *et al.* 2020a, 2020b, 2021).

When evaluating intestinal health, both the intestinal wall and the luminal contents must be considered. The luminal contents or digesta contain a variety of microbial and chemical components such as bacteria, nutrients, endogenous secretions, other dietary ingredients, antigens and substances delivered with diet or generated in the GIT tract by digestion and microbial metabolism; all are potential biomarkers of gut health. In the case of intestinal disturbances, inflammatory metabolites can be assessed in the digesta or excreta. Most of these components are known to participate in the stimulation of gut mucosal defence, and mucosa growth and regeneration by directly stimulating enterocytes or acting on the enteric nervous system and modulating a variety of GIT functions (Furness and Cottrell 2017; Pluske *et al.* 2019). Metabolome analysis using biological specimens, such as digesta, excreta, blood or gut tissue, can target metabolites and explore disease-related or dysregulated metabolic pathways and their biological implications for gut health (Celi *et al.* 2019). However, here is a need for reliable and rapid tests for gut health that can be conducted on farm. In this regard, excreta is an invaluable source of information of clinical and subclinical infections. Faecal droppings can be checked for abnormalities in colour, consistency and content, such as excessive water, fat, mucus, gas bubbles and undigested feed particles. Inflammatory metabolites in excreta show promise as potential biomarkers for the evaluation of intestinal barrier function in broilers; metabolites include ovotransferrin (Goossens *et al.* 2018), cloacal immunoglobulin A (IgA); (Baxter *et al.* 2019), fibronectin, intestinal alkaline phosphatase and lipocalin-2 (Barekatain *et al.* 2020). Detection of such biomarkers could be incorporated into an excreta dipstick to be used on-farm for a quick gut health screening test.

Two simple, but very important, physicochemical parameters of digesta that affect microbial activity, digestion and absorption in the small intestine, are viscosity and pH. Many grains (wheat, barley, rye, triticale and oats) contain soluble fibre or non-starch polysaccharides (NSP), that interact with mucus and secretions in the GIT to form a hydrocolloidal layer and impair digestion and absorption (Knudsen 2014; Bederska-Łojewska *et al.* 2017). Moreover, prolonged exposure to NSP can induce a low-grade sterile inflammatory response (Rubartelli *et al.* 2013). Intestinal viscosity may also influence the host resistance to diseases. The supplementation of diets rich in viscous grains with probiotics and exogenous enzymes, such as xylanase, partially breaks down NSP, and reduces digesta viscosity, and decreases the proliferation of undesirable microbes such as *Escherichia coli* and *Clostridium perfringens* (Latorre *et al.* 2015), and parasites such as *Ascaridia galli* (Dänicke *et al.* 2009). The measurement of digesta viscosity is a possible biomarker of gut health in poultry. However, greater standardisation of how digesta viscosity is measured and



**Fig. 3.** Histology and electron microscopy images from control, necrotic enteritis (NE)-challenged, NE and H57 and H57 birds. Control chicks (21-day-old) were fed a basal wheat/soybean diet (not supplemented with the probiotic H57) and not treated with any pathogen; NE chicks received a co-infection with *Eimeria* vaccine and *Clostridium perfringens* (Cp) and were fed the basal diet not supplemented with the probiotic H57; NE and H57 chicks were exposed to *Eimeria* vaccine and Cp, and fed the basal diet supplemented with the probiotic H57; H57 chicks were not treated with any pathogen, but were fed the basal diet supplemented with the probiotic. Histopathology (HISTO, Gram stain) showing (a) normal villi from a control chicken, and (b, c) swollen and damaged tip of villi with the presence of *Eimeria* oocytes and *Clostridium perfringens*, and (d) damaged mucosa from chickens with subclinical NE. (b–d) Scanning electron micrographs (SEM) from (a) a H57-fed chicken, and (b–d) chickens with NE, showing damaged tip of a villus or a group of villi. Transmission electron micrographs (TEM), showing enterocytes (En) and goblet cells (GC) in NE birds (a), and (b) in birds with subclinical NE, showing an enterocyte undergoing necrosis (NeE), an oocyte (Oo), enterocytes with damaged microvilli (red arrow) and (c, d) damaged mitochondria (Mt), and a necrotic area of the epithelium (purple arrow).

the results interpreted is required (Bedford 2018). The pH decreases as the digesta passes from the crop into the proventriculus and gizzard, and then becomes progressively less acidic when it reaches the small intestine (Jeurissen *et al.* 2002; Denbow 2015). Many probiotics decrease the ileal pH in broilers, and this mechanism is related to increases in commensal microbial growth or destruction of pathogenic bacteria (see section *Mechanisms of probiotic action*). However, it has been suggested that digesta pH measurement must be conducted in a manner that avoids artefacts that can be introduced by exposing digesta to air or touching the intestinal wall with the pH probe (Jeurissen *et al.* 2002).

Finally, an increase of metabolites or nutrient concentration in the excreta can reflect gut problems and therefore could serve as biomarkers. For example, excess of undigested feed particles, or starch, fat or unabsorbed bile acids/salts in excreta might indicate problems with digestion and absorption or permeability in the small intestine. In the case of bile, the majority of bile acids (over 90%) are actively absorbed in the distal ileum, and only less than 10% are lost in the excreta (Scanes and Pierzchala-Koziec 2014; Ticho *et al.* 2019); an excess in excreta could be an indicator of reduced enterohepatic recycling. However, a word of caution with the use of excreta biomarkers. The avian excreta sample is



a mixture of faeces and urine, and contains metabolites from the ileum, and those excreted in urine or generated by bacterial fermentation in the caeca. Thus, avian excreta values will therefore reflect both GIT and post-absorptive metabolism problems. For example, calcium concentrations in excreta reflect both incomplete feed digestibility and urinary excretion. The relative importance of both routes of excretion will depend on gut homeostasis and the metabolic status of the bird (Li *et al.* 2017).

Poultry house dust may be another source in which to measure markers for subclinical infection. Ahaduzzaman *et al.* (2021) found that in the case of NE-subclinical and NE-clinical flocks, high levels of *Eimeria* spp. and *C. perfringens* were detected in dust after inoculation, followed by a gradual decline over time, while in the control flock, *C. perfringens* and netB were detected at low levels. Recent research has also shown improved litter quality and decreased ammonia emissions after feeding probiotics to poultry. Dietary *B. subtilis* supplementation reduced ammonia emission in laying hens, by improving the activity of enzymes and N utilisation (Zhang *et al.* 2012). Park *et al.* (2016) also reported that a shift of excreta faecal microbial composition following *E. faecium* supplementation in laying hens and *B. subtilis* in broilers (Park and Kim 2015) was accompanied by increased nutrient retention and reduction in nutrient excretion, leading to improved nutrient digestibility and reduced excreta ammonia emissions. Ducatelle *et al.* (2018) proposed that volatile organic compounds (VOCs) from chicken excreta were potential novel markers of intestinal health, whereas previously, VOCs were considered only for their contribution to malodorous and environmental pollution. Although, it had been shown that different VOC profiles occur in chickens with and without *Campylobacter* infection (Garner *et al.* 2008). Six compounds (hexanal, E-2-octenal, pyrrole, ethyl ethanoate, methyl alcohol and 2-heptanone) were identified and used together, to classify excreta samples as positive or negative for *Campylobacter*. These biogases were recommended as biomarkers for *Campylobacter* infection.

### *Cut microbiota*

We have known for many years that there is microbial activity throughout the avian digestive tract from the presence of volatile or short-chain fatty acids (Annison *et al.* 1968). With the advent of molecular techniques, it has been shown that there is a diverse microbiota throughout the avian GIT (Rychlik 2020), which has a determinant role in gut health (Broom 2019). The ‘elephant in the room’ in relation to gut homeostasis is the microbiota with its greatest concentration in the caeca. In birds, the caeca are important sites of fermentation. Compared with other parts of GIT, the caecum contains a more diverse and stable microbial community, including anaerobes (Oakley *et al.* 2014). However, gut health evaluations target the distal portion of the small intestine or ileum. This section of the GIT has a vital role in digestion, absorption and mucosal immunity (see Box 2) and it is rich in microbiota (less rich and diverse microbial populations than in caeca). The importance of gut microbes to bird performance and productivity is starting to be unravelled using bacterial 16S rRNA (rRNA) gene sequencing (Stanley *et al.* 2014; Kollarcikova *et al.* 2019), but our understanding of the diversity of the microbiota from different GIT segments is still very limited. However, the composition and diversity of mucosa-associated microbiota seems to be a more interesting indicator of intestinal homeostasis, due to its close proximity to the intestinal tissue and its important biological role in the development of mucosal immunity (Shang *et al.* 2018) and bird physiology through the endocrine responses of the microbiota (Villageliu and Lyte 2017).

A diverse and normal (or commensal) microflora (microbiome) is associated with the epithelial and mucosal surfaces of a chicken’s body (including the skin, gut, cloaca, oral cavity, upper respiratory tract, oviduct and lungs), which is a complex invisible organ that is integrated into the biology of the host. The role is to maintain homeostasis and protect against invading microbes. This normal microflora is usually stable, with specific genera populating various body regions during particular periods in the bird’s life. It is represented by a large variety of microbial communities, involving several

### **Box 2. Ileum**

The ileum is the most distal section of the small intestine and extends from vitelline diverticulum (formerly Meckel’s diverticulum) to the ileo-caecal junction. It has an important role in digestion and absorption, and mucosal immunity. The ileum exhibits several unique features of the mucosal immune system, or gut-associated lymphoid tissues (GALT). These features include lymphoid cells located in the epithelial lining (intraepithelial lymphocytes and Paneth cells), the immune cells in the lamina propria, as well as specialised lymphoid structures, such as Peyer’s patches covered by microfold cells (M-cells), which all play roles in the regulation and stimulation of gut immune defence (Allaire *et al.* 2018). Conditions in the ileum are more favourable for microbial growth (including growth of probiotic bacteria) than in the more proximal small intestine (duodenum and jejunum). The pH (close to neutral), and a longer transit time through the ileum create a suitable environment for microbial growth and metabolism (Booijink *et al.* 2010; Gerritsen *et al.* 2011). Lu *et al.* (2003) studied ileal microbiota and found that *Lactobacillus* was the major group (70%), followed by members of the family *Clostridiaceae* (11%), *Streptococcus* (6.5%) and *Enterococcus* (6.5%). The presence of segmented filamentous bacteria (SFB), *Candidatus savagella*, a unique group of commensal bacteria, attached to the ileal epithelium during the first 3–4 weeks of a bird’s life, reinforces the ileum’s critical role in innate and adaptive immunity (Goodwin *et al.* 1991; Ericsson *et al.* 2014). Hence, many researchers select the ileum as an organ of interest, when evaluating gut health. Likewise, those interested in nutrient metabolism often sample from the ileum as it is the last segment of the small intestine where digestion and absorption is essentially complete. However, the jejunum is the segment of small intestine where most absorption occurs, and the caecum, a part of large intestine, contains the highest microbial cell densities in the GIT.

thousands of different taxonomic units of bacteria, fungi, viruses, bacteriophages, archaea and eukaryotes. The chick's microbiome is partly acquired from the hen and egg, but is greatly influenced by environmental factors (Ding *et al.* 2017). The bacterial species found in the chicken gut include four predominant bacterial phyla (*Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*), which account for more than 99.42% of the total sequences (Oakley *et al.* 2014; Sun *et al.* 2018). *Lactobacilli*, *Streptococci* and *Escherichia coli* are found in small numbers in the gut. The gut microbiota is constantly reshaped by contact with the outside environment and might be damaged when its collective population structure is altered (Baquero and Nombela 2012). Particular interest has been paid to the bacterial communities or 'microbiome' of the gut, as it confers health benefits to the host, such as aiding in the digestion and absorption of nutrients, contributing to the construction of the intestinal epithelial barrier, the development and function of the host immune system, and competing with pathogenic microbes to prevent their harmful propagation (Kogut and Arsenault 2016; Shang *et al.* 2018; Kogut 2019). The gut microbiota is altered by diet, pathogens, antimicrobials, and other nutritional, hormonal, or both internal and external behavioural factors (Oakley *et al.* 2014; Diaz Carrasco *et al.* 2019; Haberecht *et al.* 2020). Both pathogenic and beneficial microbes can be found in the lumen of the gut or on the gut's mucosal surface (Jeurissen *et al.* 2002). The composition of microflora at each site could be slightly different and determined by different factors (Shang *et al.* 2018); however, they influence each other and are collectively responsible for the gut health status. In saying this, when sampling, care should be taken to assess the microbiota on both sites, as there could be significant differences between lumen and mucosa sampling sites.

Discussions of the microbiota normally centre on single organisms, with the implication that 'bugs act alone'. However, there is increasing evidence that many bacteria form biofilms and the ecological niche, so formed, provides mutual benefits to the participating bacteria. Biofilm formation is usually considered a precursor to intestinal disease, but this is hotly debated (Tytgat *et al.* 2019). Low doses of antibiotics induce bacterial biofilm, and antibiotic resistance and infection recurrence can be connected to biofilm formation. Biofilm-forming bacteria are highly organised in multicellular bacterial structures on the intestinal absorption surfaces, thus preventing normal functions in the ileal mucosa. For *C. perfringens*, biofilm formation could play a role in the development of NE because a biofilm can help bacteria adhere to surfaces, and this facilitates colonisation and infection (Charlebois *et al.* 2014). In humans, there is evidence for anti-biofilm activity of probiotic spore-forming bacilli (*Bacillus amyloliquefaciens* B-1895) against clinical and animal urinary tract infection isolates such as *P. mirabilis* (Algburi *et al.* 2020).

Gut dysbiosis, resulting from changes in composition and function of the gut microbiota and disruption of gut barrier function, has been reported in poultry. It has been triggered by non-infectious and infectious factors, including the ban on AGPs in poultry feed and administration of antibiotics at

therapeutic doses (Ducatelle *et al.* 2018). Metabolite concentrations affected by gut microbiota dysbiosis, such as short-chain fatty acids (SCFAs) and secondary bile acids influence host metabolism. Microbial fermentation of carbohydrates results in the production of a range of SCFAs, predominately acetate, propionate, butyrate and lactate (Annison *et al.* 1968; Macfarlane and Macfarlane 2012). The SCFAs, especially butyrate, are the preferred substrate for the epithelial cells, and are associated with cell proliferation, differentiation, and apoptosis, increased MUC2 gene expression, and antioxidant activity; all play a part in the integrity of the gut barrier (Lee and Hase 2014). Therefore, disturbances of gut health will be reflected in increased or decreased intestinal permeability to metabolites generated in the gut. High serum d-lactate concentrations are indicative of increased intestinal permeability in laying hens (Lei *et al.* 2013), and increased d-lactate concentrations have been observed after lipopolysaccharide challenge in broilers (Wu *et al.* 2013). Gut dysbiosis also generates toxic metabolites from protein fermentation and the impaired intestinal barrier may permit translocation of these toxins into the systemic circulation (Yadav and Jha 2019).

There is no doubt that feed additives and supplements such as probiotics, prebiotics, organic acids, and exogenous enzymes can modulate the intestinal microbial community of the host to promote health (Adeola and Cowieson 2011; Kim *et al.* 2011; Teng and Kim 2018; Araujo *et al.* 2019; Pereira *et al.* 2019; Yadav and Jha 2019; Haberecht *et al.* 2020) and are especially recommended for use in poultry to build or re-establish normal flora during periods of stress and other challenges that cause immunosuppression.

## Probiotics, performance and health

In the past 20 years, there has been an exponential increase in the use of probiotics in animal agriculture. The estimated global market value of probiotic supplementation in animal feed is some US\$4.6 billion (MarketsandMarkets 2019), and the benefits of probiotic application have been demonstrated for animal and bird performance, as described above. Nevertheless, Applegate *et al.* (2010) raised important areas of concern for the use of probiotics by industry, including unfamiliarity with product, overselling of product effects, product inconsistency, lack of documented physiological and microbial effects *in vivo*, and lack of documented persistence. In the intervening decade, research has improved industry's confidence in the application of probiotics. However, there are still many areas of uncertainty and this is exacerbated by the number of probiotic products on the market with impressive claims of efficacy, many with little proof. Some areas of probiotic use and application that require further delineation are discussed in this section.

### Probiotics and AGPs

The Food and Drug Administration (FDA) recommends 'overall reduction in use of all classes of medically important antimicrobials in food-producing animals; complete restriction of use of all classes of medically important antimicrobials in food-producing animals for



growth promotion, and complete restriction of use of all classes of medically important antimicrobials in food-producing animals for prevention of infectious diseases that have not yet been clinically diagnosed' (FDA 2017). Concerns about drug-resistant superbugs and the lack of new antibiotics for treating human and animal diseases, have led to the reduction in the use, or in some countries, explicit banning, of antibiotics and other antimicrobial agents in food-producing animals. This situation has created a void for those involved in animal agriculture and promoted a research environment seeking to find alternative strategies for the maintenance of gut homeostasis. Probiotics have shown many positive effects in maintaining and improving gut health by reducing inflammation, as do antibiotics (Niewold 2007; Mountzouris *et al.* 2019). However, it is likely that both groups of compounds reduce inflammation by different mechanisms. It should be pointed out that probiotics are not a therapeutic replacement or substitute for antibiotics or other antimicrobials; it will take more than one product (probiotic, prebiotic, phytogetic, organic acid, essential oil or a combination thereof) to replace the beneficial effects that AGPs delivered in the past.

Increasing evidence suggests that probiotics assist in the maintenance of intestinal integrity. There is a plethora of terminology that is used to describe probiotic effects on intestinal mucosa, including, maintenance, improvement, enhancement, alleviation, control and prevention of infections. All of these terms indicate that the probiotic has either prevented a pathogen from causing intestinal damage (maintained mucosa), or regenerated or improved damaged mucosa. However, there is still much to be explored on probiotic- and host-related factors that affect the outcomes of probiotic use. The benefit of probiotic use is most obvious in chickens challenged by stress such as infection rather than when chickens are maintained under optimal conditions, as is also the case with AGPs. Previous research conducted with challenged chickens (by heat or cold stress) or with experimentally infected birds, showed that probiotics improved growth or feed conversion ratio (FCR; Mountzouris *et al.* 2010; Huff *et al.* 2015; Park and Kim 2015; Abudabos *et al.* 2016; Shini *et al.* 2020b). In most of cases, intestinal health of challenged birds was maintained or improved, and this could have been the reason for improved performance in many trials with challenged birds. We have demonstrated with electron microscopy that birds challenged with subclinical NE and fed the probiotic *Bacillus amyloliquefaciens* strain H57 (H57), maintained the cellular architecture of the ileal epithelium comparable to control birds, whereas birds without H57 supplementation had a damaged and disintegrated mucosa (Shini *et al.* 2021); NE-challenged and H57-ed birds also showed a significant decrease in FCR against NE (1.28 vs 1.36), and achieved a FCR similar to control birds (1.28 vs 1.27 respectively; Shini *et al.* 2020b).

#### *Do probiotics always work?*

A large number of investigators have used diets supplemented with probiotics and studied their efficacy on bird performance; however, the outcomes have not always been consistent

(Bajagai *et al.* 2016). There have been mixed reports on the effects of probiotics on broiler performance. Some investigators have reported positive effects on bird performance (Palamidi *et al.* 2016; De Cesare *et al.* 2017; Pereira *et al.* 2019), while others have not seen any significant probiotic effect (Olnood *et al.* 2015b; de Souza *et al.* 2018; Zarei *et al.* 2018; Araujo *et al.* 2019). Similar outcomes have been reported in humans, with investigators explaining that 'probiotics don't work for everyone,' and suggesting factors such as strain of probiotic organism, dose, route and period of administration, and health conditions of the host (Islam 2016), as being responsible for these discrepancies. These factors could also apply to production animals.

While effects of probiotics in ameliorating gut infections are well established (Higgins *et al.* 2010; Menconi *et al.* 2011; Huff *et al.* 2015; Forte *et al.* 2016; de Souza *et al.* 2018; Shini *et al.* 2020b, 2021), other beneficial effects on liver, bone and muscle, egg and meat quality (Watkins and Kratzer 1983; Mutuş *et al.* 2006; Lutful Kabir 2009; Wideman *et al.* 2012; Zheng *et al.* 2014, 2016; Cengiz *et al.* 2015; Bai *et al.* 2017; Yan *et al.* 2019) have been reported and should be considered when probiotics are applied. All these effects contribute to enhanced performance and profitability, and improved health and welfare of flocks. However, many of these claimed benefits of probiotic use are difficult to quantify in a production setting and require further investigation.

#### *Is it best to use multi-strain probiotics?*

Commercially available probiotics contain different bacterial species and strains that are often isolated from a variety of habitats. Some products contain single species (strains), some contain multiple species (strains), with others containing multiple isolates of the same species (Smith 2014; Bajagai *et al.* 2016; Aalaei *et al.* 2019). There are other products that contain probiotic organisms in combination with prebiotics, organic acids, essential oils and phytochemicals, but discussion of these mixtures is outside the scope of the present review. The probiotic strain is considered essential for effectiveness. A single-strain probiotic can be very effective if the bacterial strain is carefully selected for the circumstances under which it will be used. The selection of a probiotic strain should take into account the relevant functional properties of the strain, and whether it can be considered safe for poultry and human consumption; this principle would also apply when designing a multi-strain (multi-species) probiotic. Multiple species probiotics most frequently have various combinations of *Lactobacilli* (often a few different species or strains), *Streptococcus* species, *Bifidobacterium* species, *Enterococcus* species and *Bacillus* species. The relevance on using a multi-strain (multi-species) probiotic lies in the fact that some bacterial strains (species) perform some functions, whereas others perform different functions, all contributing to an overall beneficial outcome to meet the expectations for the use of the probiotic. Moreover, not all isolates or strains of the same bacterial species have equal efficacy as a probiotic (Liu *et al.* 2010). *Bacillus* spp. probiotics have been used successfully due to spore formation and their survival rate and persistence in the GIT

(Cutting 2011). Many *Bacillus* spp. produce enzymes and bacteriocins, potentially enhancing their mode of action in the gut (Cutting 2011). There are many commercial products that are single- or multi-strain probiotics, but the benefits of using more than one strain or species in a single product has not been clearly established (Zhao *et al.* 2013). However, single-strain probiotics have unique properties, and using a single-strain probiotic makes it easier to differentiate the effectiveness and understand the mode of action of a probiotic. Therefore, single-strain probiotics are easier to patent, whereas multi-strain probiotics are not, often lacking clinical studies for the correct combination of strains. Examples of microorganisms in single- and multi-strain/species probiotic products, commercially used in animal feeds, including poultry, have been detailed in a report by Bajagai *et al.* (2016), along with the effect on poultry (broilers and laying hens) performance.

#### *What other factors determine probiotic efficacy?*

There is general agreement among researchers and manufacturers for more quality control studies regarding formulation, route of administration and delivery system, dose and dosage regimen of strain-specific probiotic products. These studies should be undertaken before the release of a commercial product (Weese and Martin 2011) and are crucial for appropriate application and achieving the desired health and performance outcomes (Kechagia *et al.* 2013; de Simone 2019).

Administration of probiotics on farms can be via feed or drinking water; adding to feed is the most commonly used method for poultry. Heat-stable probiotic products can be added to feed before pelleting or applied post-pelleting (spray-coating), or delivered through litter. Olnood *et al.* (2015b) showed that different routes (feed, water, litter, and gavage) for administering *L. johnsonii* did not significantly influence broiler growth performance. Furthermore, there were no statistically significant differences among the various routes of administration on the gut microflora, but individual oral application (gavage) resulted in the greatest reduction in intestinal counts of *Enterobacteria* and *C. perfringens*, an outcome regarded as a key attribute for probiotic application in poultry diets (Olnood *et al.* 2015b). In another study, administration in drinking water appeared to be superior over supplementation in feed, in terms of performance and immune competence of birds (Karimi Torshizi *et al.* 2010).

As for dosage, FAO/WHO guidelines emphasise that 'when administered in adequate amounts, probiotics confer a health benefit to the host' (FAO/WHO 2006; Morelli and Capurso 2012). The dose of a probiotic is the number of viable microbial cells in a given product, derived by plate counting the total number of colony-forming units (CFUs) in a given volume, and expressed as CFU/g or mL. The minimum effective concentration of probiotics in feed or water is debated; however, it is generally accepted that probiotic products should have a minimum concentration of  $10^6$  CFU/g or mL. For humans, it is recommended that a total of  $10^8$ – $10^9$  probiotic microorganisms need to be taken daily for

the probiotic to be effective (Kechagia *et al.* 2013; Forssten and Ouwehand 2020). However, clinical studies have shown that the concentration of a probiotic needs to be  $10^6$  CFU/mL in the small intestine and  $10^8$  CFU/g in the colon to obtain a clinical effect in the gut (Minelli and Benini 2008). For poultry, probiotic application is based on the manufacturer's recommendations for dose and duration of administration. In a recent study (S. Shini, unpubl. data), a diet supplemented with graded doses of H57 at 0,  $10^6$ ,  $10^7$  and  $10^8$  CFU/g feed was fed to broiler chickens and half of the birds were challenged with subclinical NE. The data showed that there was a dose effect. Birds challenged with NE and fed H57 at  $10^7$  and  $10^8$  CFU/g feed, had similar growth rates, and these birds performed better than did birds fed H57 at 0 and  $10^6$  CFU/g feed. FCR was significantly improved in birds challenged with NE and fed the highest dose of H57. From these data, it appears that there is a threshold dose for performance in challenged birds and it was concluded that the dose of  $10^7$  CFU/g feed would be satisfactory for field application, as it improves the performance of challenged birds and is more economical. At necropsy, the lower dose ( $10^6$  CFU/g feed) of H57 also showed benefits on mucosal health of challenged birds. Tomaszewska *et al.* (2018) demonstrated that the influence of probiotic administration on tibia geometry was also dose-dependent in female turkeys.

Other factors that could influence probiotic efficacy are poultry species, the breed, age, and health or disease conditions. Most probiotic work with poultry is undertaken with broilers, but the same response may not occur in other avian species (Edens 2003). Similar improved growth rates from probiotic use are seen in chickens and turkeys (Smith 2014), but less in ducks where overfeeding causes significant changes to metabolism, microbial diversity and growth (Even *et al.* 2018). The use of different broiler breeds could also have been the reason for contrasting results when using the same probiotic. For example, studies with an *E. faecium* strain showed different results on performance when conducted with different broiler breeds; Cao *et al.* (2013) found an effect on growth in male Cobb broilers, while Zhao *et al.* (2013) did not find any effect in Ross broilers. The use of probiotics has shown to be more effective in young birds, as this coincides with the critical period of GIT microbial colonisation (Applegate *et al.* 2010). This is very important for chicks hatched from artificially incubated eggs, thus lacking contact with hens, resulting in delayed development of the intestinal microflora. As birds grow, the microflora becomes more diverse and tends to become relatively stable. Diet content is also likely to be a contributing factor to probiotic efficacy (Lutful Kabir 2009).

The results of many clinical investigations in humans suggest that probiotics may be useful in preventing and treating various health conditions and diseases; however, efficacy depends on the health and disease status, including clinical indications (acute or chronic). There is evidence that probiotics are effective for acute infectious diarrhoea, antibiotic associated diarrhoea, *Clostridium difficile* associated diarrhoea, hepatic encephalopathy, ulcerative colitis, irritable bowel syndrome, functional gastrointestinal disorders, and necrotising enterocolitis; conversely, there is



evidence that probiotics are not effective for acute pancreatitis and Crohn's disease (Wilkins and Sequoia 2017). In poultry, enteric diseases have become a major concern after the exclusion of AGPs, and feed additives including probiotics have had varying degrees of success in combating these diseases, probably due to the factors outlined above, and the stress and disease challenges faced by birds on farm.

### Mechanisms of probiotic action

Probiotics contain live bacterial cultures, but how they induce their effects remains unclear. This is a complex area of biology in which there are multiple players; consequently, many possible modes of action and pathways are involved. Several mechanisms have been established or proposed and it is likely that the beneficial effects of probiotics are mediated by multiple mechanisms, and some of these mechanisms are outlined below.

#### *Modulation of intestinal microbiota*

The gut microbiota is one component of an animal's microbiota that colonises all epithelial and mucosal surfaces of the GIT. Overall health and wellbeing is dependent on a balanced gut microbiota and delivery to the host of microbial-derived metabolites. Probiotics are considered to be one of the best alternatives to antibiotics, especially in terms of modulating the intestinal microbiota. The inclusion of probiotics in the diet has shown to help maintain or rebuild the intestinal microbiome by stimulating the growth of beneficial indigenous microorganisms such as *Lactobacilli* and *Bifidobacteria* (Gagliardi *et al.* 2018). These bacteria have the ability to limit the direct contact of pathogenic bacteria with the epithelium by competitive exclusion for access to mucosal surfaces, nutrients, and the creation of harsh environmental conditions in the lumen for pathogens. Competitive exclusion has been found to be effective in inhibiting intestinal colonisation by *Salmonella*, *Shigella*, *Clostridium* and *Listeria* (Naidu *et al.* 1999; Bermudez-Brito *et al.* 2012; Park and Kim 2015). A probiotic strain of *L. plantarum* has been reported to induce MUC2 and MUC3 mucins, thus inhibiting the adhesion of pathogenic *E. coli* (Mack *et al.* 1999). It has been observed that many probiotic bacteria show a greater capacity to adhere to the chicken's intestinal mucosa than do pathogens and, therefore, displace them (Collado *et al.* 2005). It has also been demonstrated that many probiotics favour the survival of beneficial bacteria such as *Lactobacilli* because they decrease intestinal pH (Olnood *et al.* 2015a; Shini *et al.* 2020b; Zaghari *et al.* 2020). Lactic acid production is increased by *B. amyloliquefaciens*, which most probably explains the drop in ileum pH (Wu *et al.* 2011; Salim *et al.* 2013; Shini *et al.* 2020b) associated with feeding this probiotic species.

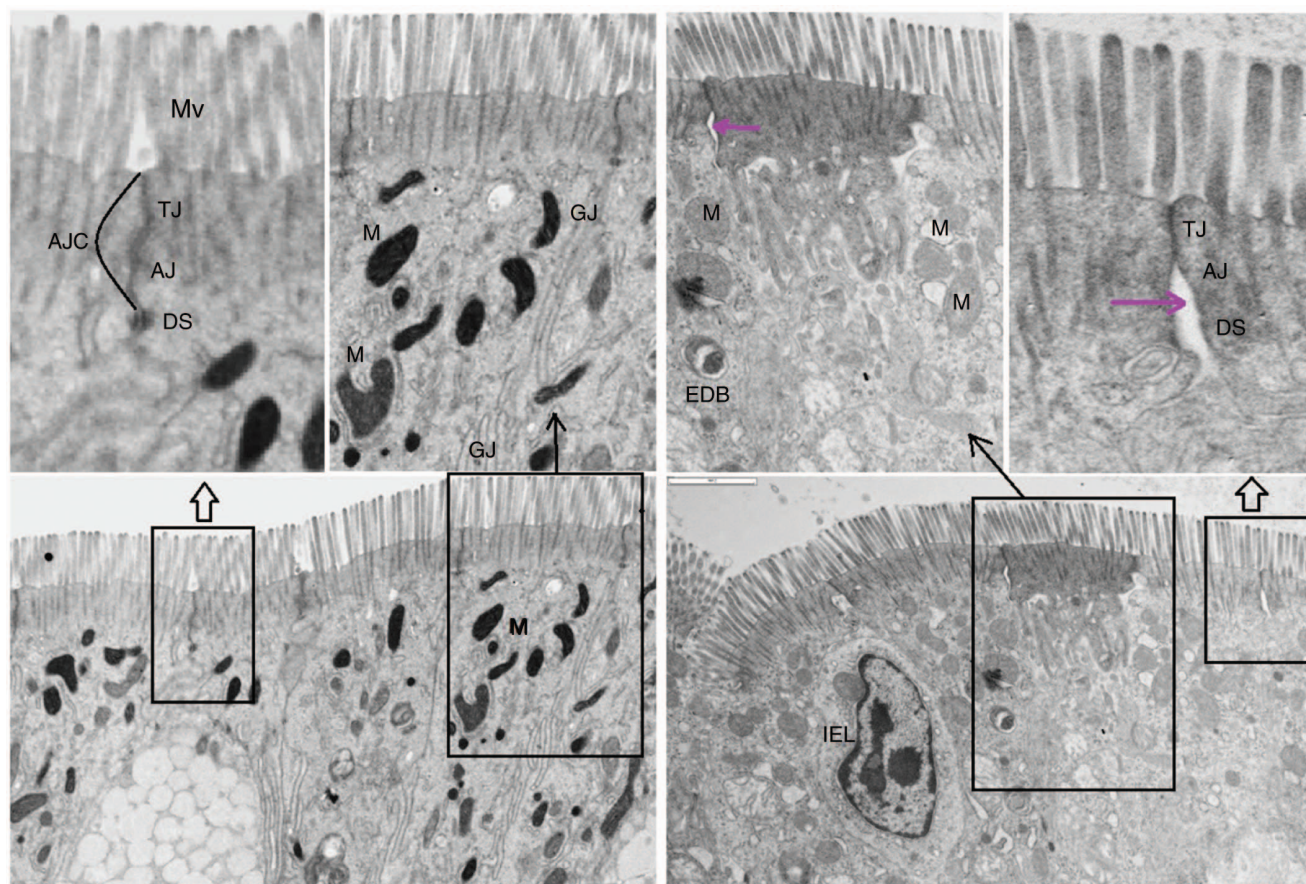
Probiotics can modify the ecosystem of the GIT by the production and release of enzymes, bacteriocins, and other secondary compounds into the gut. For example, many *Bacillus* spp., including *B. amyloliquefaciens*, produce large quantities of extracellular enzymes such as amylase, protease and lipase, and some cellulases and xylanases (Elshaghabee *et al.* 2017), that can assist by increasing nutrient digestibility

and deactivating anti-nutritional factors present in poultry diets (Yadav and Jha 2019). Similar enzymes are routinely added to poultry diets to improve nutrient digestibility and to degrade anti-nutritive factors (Bedford 2000, 2018; Ravindran 2013). Probiotics have also been shown to increase the abundance of segmented filamentous bacteria (SFB), the indigenous bacteria that are found in ileal microflora of chicks (Shini *et al.* 2021). Liao *et al.* (2012) manipulated the colonisation of SFB in chickens by feeding *Lactobacillus delbrueckii* at hatch, resulting in SFB colonisation occurring 4 days earlier, with implications for regulation of the immune response, and this mechanism is explored below.

#### *Maintenance of intestinal barrier integrity*

The intestinal epithelium is a dynamic bidirectional layer that functions as a frontier-barrier, accepting or refusing movement of intraluminal particles into adjacent enterocytes and underlying microvasculature. The junctional complexes between enterocytes maintain the integrity of the epithelial barrier by regulating paracellular permeability and are composed of TJ, gap junctions, adherens junctions (AJ), and desmosomes as shown in Fig. 4. The TJ and AJ constitute the apical junctional complex, the primary structure that regulates the intestinal barrier. Tight junctions include four integral transmembrane proteins (occludin, claudin, junctional adhesion molecule, and tricellulin) that interact with the actin cytoskeleton (Ulluwishewa *et al.* 2011). The DS and GJ are involved in cell-cell adhesion, and intracellular communication respectively. The paracellular barrier in normal intestinal tissue is characterised by high expression levels of TJ proteins and a low paracellular permeability (or a tight epithelium), while mucosal inflammation is frequently associated with decreased expression of junctional proteins (or a leaky epithelium). The integrity of the mucosal barrier is important, for the digestion and absorption of nutrients, and it is considered the first line of defence against pathogens and toxic molecules. The disruption of its integrity is the primary cause of disturbed physiology, and inefficient feed utilisation. Hence, there is great interest to understand how probiotics can assist in improving gut mucosal integrity, barrier function and nutrient metabolism.

Several mechanisms appear to be associated with the role of probiotics in improving mucosal barrier integrity, and protecting it from a variety of insults. It has been suggested that probiotics enhance the mucosal barrier by increasing the production of mucus, inhibiting bacterial translocation, and strengthening TJ in a manner similar to that of normal gut microbiota (La Fata *et al.* 2018). There is also a growing body of evidence indicating that bioactive molecules released by some probiotics activate various cell-signalling pathways that strengthen the TJ and preserve mucosal barrier function (Rao and Samak 2013). Changes in absorption occurring in coccidiosis and NE have been linked to changes in intestinal permeability, a feature of intestinal barrier function (Williams 2005; Ducatelle *et al.* 2018). Many luminal and systemic factors can independently influence



**Fig. 4.** Transmission electron micrographs of the apical enterocyte region from the ileal epithelium of healthy and unhealthy 21-day-old chicks. Magnified areas from a normal epithelium (top row, two first images) show details of the apical junctional complex (AJC) region with tight junction (TJ) and adhesive junction (AJ); the desmosome (DS) and microvilli (Mv), mitochondria (M) and gap junction (GJ) are also shown. The images next are from chickens with subclinical necrotic enteritis (NE), displaying intact microvilli, but swollen or damaged mitochondria (M), widening (sacculation) of the AJC in the AJ and DS areas (purple arrow), and electron-dense bodies (EDB) and an intraepithelial lymphocyte (IEL) migrated from lamina propria (bottom row, image left).

barrier function and cause leakage of plasma proteins and diarrhoea (Quigley 2016; Camilleri 2019). Electron microscopy using a NE broiler model (co-infection with coccidiosis and Cp) demonstrated enterocyte injury and loss of cellular integrity (damage of intercellular connections) within the epithelium due to erosions and ulcerations caused by *Eimeria* oocytes and Cp (Figs 3, 4). It was thought that barrier dysfunction contributed to diarrhoea via a leak flux mechanism. Birds challenged with NE and treated with a probiotic H57 had preserved ileal mucosa and normal enterocytes and TJ (Shini *et al.* 2020a).

Coccidiosis and NE-related diarrhoea and malabsorption have been associated with reduced nutrient digestion and performance (Turk 1972; Witlock and Ruff 1977). However, epithelial damage in *Eimeria* infected birds appears to be less severe than in NE birds; therefore, a higher absorptive area is available, contributing to a slightly better performance in *Eimeria*-infected birds than in NE-challenged birds (Shini *et al.* 2020b). In our experiments with H57, it was demonstrated that the probiotic can maintain the intestinal epithelial barrier by improving

epithelial cell morphology, in particular mitochondria, and preserving the enterocyte apical junctional complex (which includes TJ; Fig. 4; Shini *et al.* 2021). Mitochondria have a crucial role in the regulation of gut functions such as intestinal barrier integrity and mucosal immune responses (Jackson and Theiss 2020). Enterocyte mitochondria are involved in the regulation of numerous aspects of cellular activity, including, but not limited to, apoptosis,  $\text{Ca}^{2+}$  signalling, and redox homeostasis of the cell (Kang and Pervaiz 2012). Our findings suggest that the maintenance of the epithelial barrier is an energy-dependent process, thus swollen, irregular, vacuolated, or cristae-damaged mitochondria (observed in NE birds), are associated with loss of ATP (ATP) generation and release of oxygen radicals in enterocytes. Subsequent apoptotic necrosis of enterocytes and impaired energy metabolism of epithelial cells evoke a variety of insults, allowing spread of infection. Similar degenerative changes and mitochondrial dysfunction, including oxidative stress and impaired ATP production, are also observed in the intestines of patients with inflammatory bowel disease. In an experiment with broiler chicks, an early

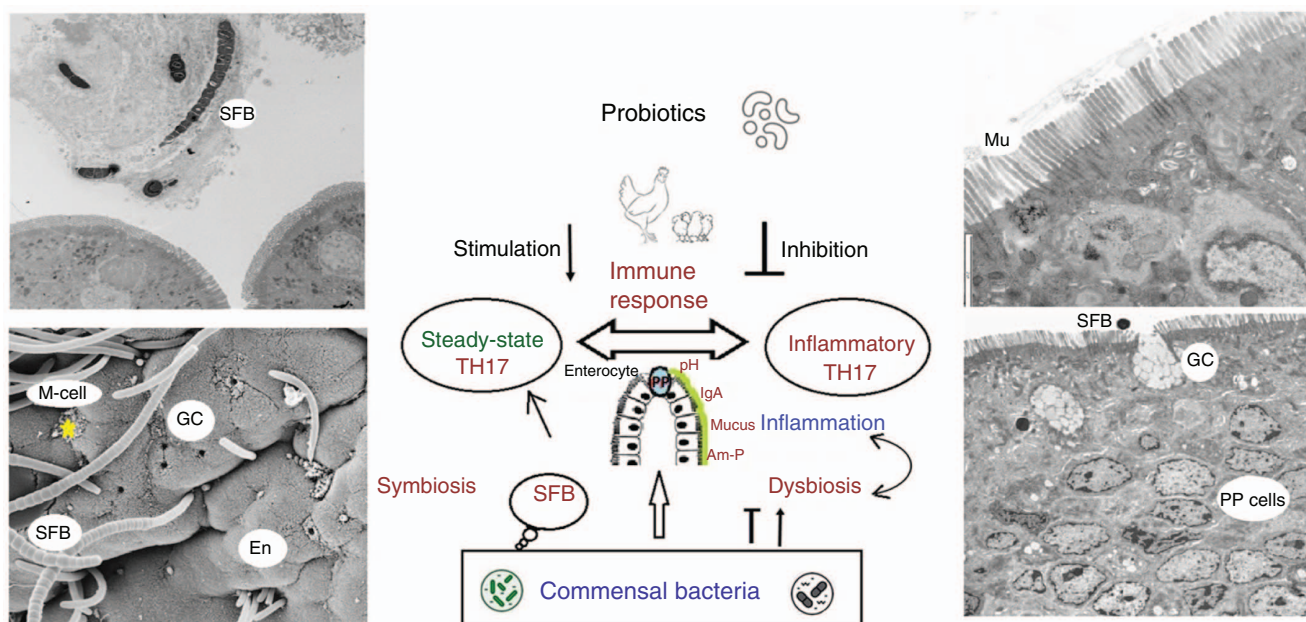


infection with *Salmonella enterica* induced paracellular transport leakage and mucosal barrier dysfunction, but these adverse effects were prevented by the administration of a probiotic culture derived from poultry gut proprietary strains of lactic acid bacteria (Prado-Rebolledo *et al.* 2017). It has also been shown that *B. subtilis* 747 improved epithelial barrier integrity of chickens by elevating occludin concentrations in the TJ (Park *et al.* 2020).

#### Modulation of inflammatory and immune responses

Probiotics influence host immunity, without invading host tissues. Probiotics are living microorganisms and as such the intestinal mucosa responds to probiotic exposure through innate and acquired immune mechanisms, as it does for other 'foreign agents.' However, the response to commensal and probiotic bacteria is a 'good thing,' and it accounts for a substantial part of their immunomodulatory effects. The reaction starts with an inflammatory response that activates immune cells, cytokines and chemokines, acute phase proteins and other signalling mediators to protect the body (Abdulkhaleq *et al.* 2018). Inflammation is a complex and fine-tuned mechanism and may act as both a 'friend and foe' (Kjekshus 2015). Normally, it is followed by resolution, and this is an active and highly regulated cellular and molecular process required to protect against deleterious consequences of the inflammatory response (Serhan *et al.* 2007). Unresolved inflammation can lead to intestinal or system diseases, and increases in energy expenditure for

maintaining a chronic immune response. Hence, it affects bird health and performance. The exposure to pathogens induces the intestinal defence mechanisms and the first response is the innate (non-specific) response, including the inflammatory response. Innate immune cells (including dendritic cells) can respond and detect microbial fragments through pattern recognition receptors, and mount a robust immune response against pathogens. In fact, most probiotics appear to be potentially well tolerated by immune cells (Plaza-Díaz *et al.* 2017). Probiotics are considered to be regulators of inflammation (Lescheid 2014; Peng *et al.* 2020). Multiple mechanisms of action have been suggested to explain the protective and anti-inflammatory effects of probiotics in modulating intestinal inflammation. Probiotics enhance production of SCFAs with anti-inflammatory properties (e.g. butyrate), as well as increase synthesis of antimicrobial peptides (bacteriocins) that influence inflammation resolution pathways in the mucosa (Wang *et al.* 2016; Tarradas *et al.* 2020). It has been proposed that probiotics that target the innate immune system and stimulate it, could be more advantageous. This is because this type of response is quick, multicomponent and non-specific (Swaggerty *et al.* 2019), offering local and systemic benefits, especially for young birds. Subsequently, components of acquired (specific) immune response would be induced, such as B- and T-lymphocytes and their products, (antibodies and interleukins), and a more specific immune response mounted.



**Fig. 5.** Schematic diagram illustrating potential mechanisms involving probiotic bacteria in partnership with commensal bacteria, including segmented filamentous bacteria (SFB), to maintain a balanced immune response, thereby contributing to intestinal homeostasis. Probiotics suppress pathogenic Th17 cells and induce steady-state Th17 cells. The relationship between probiotics and Th17 cells is dependent on host conditions/state, whether at steady or inflammation (dysbiosis) state. Probiotics also improve the colonisation and subsequent adhesion of commensal bacteria, including SFB, on epithelial cells (enterocyte), which directly influence several protective immune responses, such as the development of lymphoid tissue (Payer's patches, PP), stimulation and the accumulation of Th17 cells in the mucosa, the secretion of immunoglobulin A (IgA) and antimicrobial proteins (Am-P) within the mucus layer, and intraepithelial lymphocytes and M-cells (yellow star) barrier protection. The electron micrographs (left and right) present evidence of cells and ultrastructural features involved in the mode of action of probiotics as modulators of intestinal immune response.

The intestinal surface is also protected from invasion of foreign antigens by an efficient protective layer of 'partners in slime', including a mucus layer (which contains mucin and glycocalyx layers) and chemical barriers such as antimicrobial proteins (Johansson *et al.* 2013). Other mucosal partners, including antigen specific secretory IgA, and mucus associated microbiota (commensal bacteria, e.g. SFB), all play an important role in the initiation of an integration of an immune response (Fig. 5). In experiments with the probiotic H57, we demonstrated that birds exposed to NE and supplemented with H57 produced a better immune response than NE-challenged birds not supplemented with H57; more precisely, birds treated with H57 had increased abundance of goblet cells and intraepithelial lymphocytes (Shini *et al.* 2020b), and elevated levels of defensin (Gal-6) mRNA expression (S. Shini, unpubl. data). An exciting aspect of the experiments with H57 was finding SFB, and evaluating its abundance and morphology. Interestingly, there were more SFB attached to villi in birds exposed to 'foreign microorganisms' (pathogens and the probiotic), i.e. NE, NE-H57 and H57 birds, than in control birds. However, the abundance was lower in H57 birds than in NE-exposed birds (Shini *et al.* 2021). It appeared that H57 regulates the abundance of SFB, in NE-challenged H57-fed birds, most probably by suppressing pathogenic Th17 cells and inducing steady-state Th17 cells (Fig. 5).

Segmented filamentous bacteria (SFB) are intestinal commensal microorganisms with important roles in host immunology and physiology (Box 3; Ivanov and Littman 2010; Ericsson *et al.* 2014), and they are host specific. SFB are found on the ileal mucosa (Fig. 3), and participate in the activation of naïve CD4<sup>+</sup> T cells to become Th17 cells, which are capable of producing IL-17 cytokines with a proinflammatory role in the generation of the mucosal (IgA) immune response (Sczesnak *et al.* 2011). Probiotics favouring SFB could therefore have an effect in stimulating the immune response in the gut. There is evidence that probiotics have an anti-inflammatory effect, which is a consequence of downregulation of IL-17 production and other proinflammatory Th17-secreted cytokines (Tanabe 2013). IL-17 is beneficial in controlling

dysbiosis in the gut, but may be harmful if dysregulated (Fig. 5). In the case of overgrown SFB, accumulation of Th17 cells in the ileum could lead to damaging inflammatory effects, as was the case with NE-challenged birds. Supplementation with the probiotic H57 limited the SFB expansion and the Th17 associated proinflammatory response in NE-H57 birds (Fig. 5). It is suggested that probiotics (through beneficial bacteria, and SFB) induce a moderate steady-state Th17 cellular response, and an appropriate production of IL-17, while pathogenic-induced inflammatory Th17 cells are involved in the development of inflammation and dysbiosis of resident microbes (Tanabe 2013; Flannigan and Denning 2018).

Investigations with *Salmonella* have found that probiotics stimulate the gut immune system, protecting against infection, by improving the phagocytic activity of peritoneal and spleen macrophages, increasing the antibody response, and improving protection against *Salmonella* infection (Martin Manuel *et al.* 2017). *Lactobacillus* sp. was used as a vehicle for an orally administered avian influenza virus vaccine, and was found to be effective in inducing the systemic and mucosal immune responses with higher anti-haemagglutinin-specific IgA and IgG concentrations (Wang *et al.* 2013). It was suggested that the acquired immune responses were dependent on the *Lactobacillus* as a recombinant. Lee *et al.* (2010) studied the effects of *Bacillus* spp. as direct-fed microbials on immune characteristics in broiler chickens, and found that cytokine mRNA concentrations (IL-13, IL-6, IL-17 and IL-10) in intraepithelial lymphocytes were increased, decreased or unchanged, depending on the strain used. It was suggested that the direct-fed microbials in the present study showed immunomodulating effects and enhanced host protective immunity against enteric pathogens in broiler chickens. Earlier studies by (Chichlowski *et al.* 2007) had suggested decreased expression of IL-6 (a proinflammatory cytokine), and increased expression of IL-10 (an anti-inflammatory cytokine). Whereas Haghighi *et al.* (2008) associated the repression of IL-12 and IFN- $\gamma$  (proinflammatory cytokines) expression with the effect of probiotic-mediated reduction and intestinal colonisation of *Salmonella typhimurium*. Further studies are required to

### Box 3. Segmented filamentous bacteria

*Candidatus savagella* or segmented filamentous bacteria (SFB), a genetic relative of the genus *Clostridium*, are unique microorganisms identified as Gram-positive, anaerobic, spore-forming bacteria, colonising the ileum of many young vertebrates, including chickens (Klaasen *et al.* 1992; Ericsson *et al.* 2014). SFB are host-specific indigenous bacteria ranging from 0.7 to 1.8  $\mu$ m in diameter and up to 80  $\mu$ m in length and have important roles in modulating host immunology and physiology (Ivanov and Littman 2010; Ericsson *et al.* 2014). SFB are involved in regulating postnatal development and maturation of immune responses in the gut of mammals and some birds (chicken and turkey). SFB have a unique morphology, life cycle and binding location. They intimately interact with the host, most notably, firmly attaching to epithelial cells of the distal ileum by one end of the filament (holdfast) to form multicellular filaments, as well as single 'holdfasts' and spores, i.e. vegetative and dormant reproductive cells (Chase and Erlandsen 1976).

SFB have gained attention due to their capacity to induce and stimulate multiple types of intestinal lymphoid tissue (Peyer's patches and IEL). SFB are found attached to the ileal mucosa (Fig. 3), and stimulate helper 17 (Th17), which are capable of producing IL-17 cytokines with a proinflammatory role in the generation of the mucosal (IgA) immune response (Ivanov and Littman 2010; Ericsson *et al.* 2014; Hedblom *et al.* 2018). As part of the commensal microbiota, SFB have a key role in post-hatching maturation of gut immunity, especially mucosal immunity, and participate in the activation of naïve CD4<sup>+</sup> T cells to become Th17 cells, which are capable of producing IL-17 cytokines (Sczesnak *et al.* 2011). Chicken SFB (Fig. 5) have an important role in the critical transition period from innate and maternal immunity to adaptive immunity, especially during the first 3 weeks post-hatch. In chickens, *B. amyloliquefaciens*, (H57) reduced the SFB expansion and potentially down-regulated the Th17 associated proinflammatory response (Shini *et al.* 2021).



determine the precise action of probiotics on the immune response. These findings will be key to delineating the immune pathways modulated by probiotics, and in identifying more adequate approaches to optimise gut health and performance in poultry.

#### *Improvement of oxidative status*

Several studies claim that probiotic supplementation improves antioxidant activity and reduces cell damage caused by oxidation (Aluwong *et al.* 2013; Persichetti *et al.* 2014; Wang *et al.* 2017). Probiotic lactic acid bacteria modulate the redox status of the host via their metal ion-chelating ability, antioxidant systems, regulation of signalling pathways, enzyme producing ROS, and intestinal microbiota. An increase in the activity of antioxidative enzymes protects cells from oxidative stress-induced damage. Most of investigators measure the antioxidant activity using the content of serum (total antioxidant capacity), superoxide dismutase (SOD) and glutathione peroxidase to assess the antioxidant effect of probiotics. However, most of studies have been conducted *in vitro* and, it is difficult to relate these results to reactions *in vivo*.

Cao *et al.* (2019) used the supernatant from ileum mucosa to determine the myeloperoxidase, nitric oxide, SOD, catalase and malondialdehyde after treatments with dietary *L. plantarum* 1.2567. They observed significantly improved anti-inflammatory and antioxidant activity in broiler chickens with Cp-induced NE. Another investigator used two different probiotics (*Bacillus subtilis* and *B. cereus*) and did not find any antioxidant effect using total antioxidant capacity, SOD, and hydrogen peroxide measurements (Abudabos *et al.* 2016). A probiotic containing both *Clostridium butyricum* and a combination of *Saccharomyces boulardii* and *Pediococcus acidilactici* was beneficial when added to the diets of the laying hen, as it enhanced intestinal development and improved antioxidant activity in the gut (Xiang *et al.* 2019). The probiotic treated hens exhibited decreased levels of reactive oxygen species (ROS) in ileum and caecum, and reduced malondialdehyde in serum (Xiang *et al.* 2019).

In our experiments with H57, we did not measure any antioxidative biomarker; however, we assessed the effect of probiotic H57 indirectly by evaluating enterocyte morphology and mitochondrial abundance and morphology. The ultramicroscopy of the ileal epithelium showed severe enterocyte damage, and loss of cellular integrity in NE birds. Mitochondria, in particular, had morphological alterations; they were irregular in form, containing electron-lucent regions of matrix, swollen or damaged cristae, most probably due to oxidative stress, resulting in increased ROS exposure and lowered ATP production (Figs 3, 4). In control, H57 and NE-H57 birds, most mitochondria were round or elongated, with mild or no structural damage, and displayed well preserved structure (Fig. 4). Mitochondrial damage and dysfunction were found in NE birds, which impaired ATP production, causing cell necrosis. Dietary addition of H57 appears to maintain mitochondrial morphology and improve their efficiency in terms of mitochondrial ROS production and

scavenging, most probably due to release of enzymes and improved digestion and absorption of nutrients (Shini *et al.* 2019).

#### *Modification of digestion and metabolism*

Since the early studies of Marie Coates and her colleagues with germ-free birds, there has been an appreciation of the importance of the gut microflora to the nutrition of the host (Coates 1986). Many commensal bacteria, including probiotics, produce enzymes to breakdown feed substrates to permit bacterial metabolism, and these enzymes may also facilitate digestion by the host (Bajagai *et al.* 2016; Rowland *et al.* 2018). In addition to enzymes, probiotics secrete many other compounds that can interact directly or indirectly with the nutritional status of the host. The SCFA butyrate is a good example and can act as an energy source for enterocytes as mentioned above. As discussed in the present review, probiotics improve the integrity of epithelial barrier, consequently increasing nutrient absorption and, ultimately, improving feed efficiency. Probiotics can indirectly modify the gut homeostasis by helping in the assimilation of nutrients through activation of the transport system from ATP generated by improved digestion. A significant sign of reduced digestion and absorption is the change in excreta quality (also discussed above) and the presence of watery excreta or undigested feed particles in the excreta. An absence of pasty vent in chickens indicates normal digestion and absorption. De Cesare *et al.* (2017) demonstrated that supplementation with *Lactobacillus acidophilus* D2/CSL (CECT 4529) at the recommended dietary dosage of  $1 \times 10^9$  CFU/kg feed significantly improved nutrient digestibility, particularly crude fat and protein, and reduced the incidence of pasty vent in broiler chickens. In our study with H57, broiler chicks exposed to NE and fed the probiotic H57 had a significant improvement of FCR, and a low pasty vent occurrence (Shini *et al.* 2020b). The modification of digestion and metabolism by probiotics is very complex, with both direct and indirect aspects (the probiotic itself or probiotic-derived products) and occurs in conjunction with the activities and actions of the normal microbiota and microbiota-related products. It involves many pathways, which influence the overall metabolism of the host and are responsible for feed efficiency.

#### **Concluding overview and future directions**

Following the reduction or ban of AGPs in poultry feed, there has been worldwide interest in the application of alternative feed additives, especially probiotics. Recent advances on the effects of probiotics in improving poultry gut health and bird productivity have been discussed and the following was concluded from the review:

- (1) Gut health is a complex phenomenon that includes all the biological structures of the intestinal tract and their functions, along with the genetic and physiological makeup of the host, the diet being consumed, and the intestinal microbiota. The dynamic interactions of these different components determine gut homeostasis and the health status of the gut.

- (2) Achievement of gut homeostasis involves several physiological, microbiological, immunological and physical functions that maintain intestinal dynamic balance or homeostasis.
- (3) Determination of gut health or homeostasis is difficult to ascertain, especially when disturbances are chronic or in the case of subclinical disease. Physical appearance of the bird may give an indication that there is GIT disturbance. Excreta is an invaluable source of information of clinical and subclinical infections and biomarkers are being developed for leaky gut.
- (4) Biomarkers are indirect measures but for determination of subtle changes in gut structure, electron microscopy is the method of choice. Using electron microscopy has also provided insights into how the action of a probiotic maintains gut wall integrity.
- (5) The mechanisms of probiotic action are complex and include local and systemic changes, and corresponding effects on the gut microbiota, intestinal barrier integrity, immunity, oxidation status and feed efficiency.
- (6) Gut homeostasis is an important link to bird productivity, but there are circumstances when productivity gains are not seen when diets are supplemented with probiotics. Many factors are likely to contribute to this outcome, but a key factor is a stressful environment; a situation in which probiotics enhance bird performance.

The increasing demand for probiotics by the poultry industry demonstrates the benefits that probiotics can bring to poultry productivity. Despite the great deal of research that has been published on probiotics and avian gut health, as outlined above, many questions remain unanswered. Advances in microbial and molecular methods will increase our understanding of the factors that determine gut homeostasis, in particular the interactions of diet, indigenous microbiota and host metabolism, especially of the intestinal mucosa. Such studies should clarify mechanisms of probiotic action and define mechanistic attributes of different probiotic organisms. This should provide insights into why probiotics are not uniformly successful in the field and inform the development of next-generation probiotics. It will also provide basic information for studies that combine different feed additives to achieve gut homeostasis. To accomplish these outcomes, there will need to be rigorous *in vitro* and *in vivo* testing under standard conditions that represent field conditions. This will be greatly enhanced by the development of relevant biomarkers that access the efficacy of probiotics. It is important that future specific probiotic products are designed for strategic field application.

### Conflicts of interest

Both authors are members of the Editorial Board of *Animal Production Science*. Neither were involved in the review and editorial process for this paper. The authors have no further conflicts of interest to declare.

### Declaration of funding

Funding for the research work on probiotic *Bacillus amyloliquefaciens* strain H57 was provided by an Advance

Queensland Innovation Partnership grant awarded to the Queensland University of Technology and the University of Queensland, along with their collaborating industry partners Ridley AgriProducts Pty Ltd, Bioproton Pty Ltd and Kennedy Creek Lime Pty Ltd.

### Acknowledgements

The authors acknowledge the facilities, and the scientific and technical assistance, of the Australian Microscopy & Microanalysis, St Lucia Campus, and the Poultry Research Group, Gatton Campus, University of Queensland. WLB had the great privilege to receive his initial training in poultry science and nutrition by Professor Rob Cumming and Professor David Farrell, respectively.

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Handling editor: Frank Dunshea