Intestinal microbiota of layer hens and its association with egg quality and safety

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ABSTRACT The intestinal microbiota has attracted tremendous attention in the field of the poultry industry due to its critical role in the modulation of nutrient utilization, immune system, and consequently the improvement of the host health and production performance. Accumulating evidence implies intestinal microbiota of laying hens is a potential mediator to improve the prevalent issues in terms of egg quality decline in the late phase of laying production. However, the regulatory effect of intestinal microbiota on egg quality in laying hens remains elusive, which requires consideration of microbial baseline composition and succession during their long lifespans. Notable, although Firmicutes, Bacteroidetes, and Proteobacteria form the vast majority of intestinal microbiota in layer hens, dynamic intestinal microbiota succession occurs throughout all laying periods. In addition to the direct effects on egg safety, intestinal microbiota and its metabolites such as short-chain fatty acids, bile acids, and tryptophan derivatives, are suggested to indirectly modulate egg quality through the microbiota-gut-liver/brain-reproductive tract axis. These findings can extend our understanding of the crosstalk between intestinal microbiota and the host to improve egg quality and safety. This paper reviews the compositions of intestinal microbiota in different physiological stages of laying hens and their effects on egg quality and proposes that intestinal microbiota may become a potential target for modulating egg quality and safety by nutritional strategies in the future.

Key words: intestinal microbiota, microbial succession, short-chain fatty acids, egg quality, layer hen

INTRODUCTION

As a cost-effective and superior resource of food protein, eggs are widely produced and consumed globally. The world egg production reached 76.50 Mt in 2018, with an increase of 50.12% from 50.96 Mt in 2000 (FAOSTAT, 2020). Egg production has met consumer demand while the improvement of egg quality is essential at present. In addition to eggshell quality, albumen quality, yolk quality, egg flavor, and egg safety are also concerned. However, due to the prolonged and high-intensity egg laying, adverse changes in intestinal health, liver metabolism, and oviducal immune function result in poor eggshell and albumen quality in the late phase of laying production (Wang et al., 2018; Feng et al., 2020), causing huge economic losses. Additionally, given the perception of animal welfare, the laying hen housing system is changing from cage rearing to cage-free laying hen management (Kidd and Anderson, 2019). This will increase laying hen exposure to pathogenic bacteria and parasites, challenging the physiology, performance, egg quality, and egg safety of laying hens (Jeni et al., 2021; Ricke et al., 2022). The disorder of intestinal microbiota indued by the invasion of pathogenic bacteria will pose serious threats to food safety due to the presence of foodborne pathogens in eggs (Gantois et al., 2009; Salihu et al., 2015). Thus, over the past 2 decades, numerous studies have tried various nutritional approaches to improve egg quality and safety, which found amino acids (Khattak and Helmbrecht, 2019), vitamins and minerals (Alagawany et al., 2020), natural plant bioactive compounds (Feng et al., 2021), prebiotics and probiotics (Khan et al., 2020), and enzyme (Lei et al., 2018) play vital roles in modulating production performance and egg quality of laying hens. Notably, improved egg quality was usually accompanied by the alteration of intestinal microbiota in these studies, suggesting critical interactions between the intestinal microbiota and nutritional responses, as well as the physiological health of layers.

It has been reported that the intestinal microbiota of layer hens had positive effects on the improvement of

nutrient utilization (Dai et al., 2020; Wang et al., 2020a), intestinal barrier (Khan et al., 2020; Miao et al., 2020), production performance (Shang et al., 2020; Wang et al., 2020c), as well as egg quality (Zhan et al., 2019; Feng et al., 2021). Indeed, intestinal microbes and their metabolites act as signaling molecules linking the gut, liver, brain, and reproductive tract (Nicholson et al., 2012; Cryan et al., 2020; Agus et al., 2021), which in turn have a direct or indirect impact on poultry health and egg quality. Thus, intestinal microbiota may act as a potential target for modulating egg quality and safety by nutritional strategies in the future. However, numerous studies imply that poultry age is the primary driver-force of changes in intestinal microbiota (Ballou et al., 2016; Park et al., 2017; Shi et al., 2019; Dai et al., 2020), which requires consideration of microbial baseline composition and succession during long lifespans to shape it effectively. Currently, the composition and function of the intestinal microbiota of poultry have been well characterized and clarified in broilers (Huang et al., 2018; Shang et al., 2018). Nevertheless, layers have longer lifespans, different genotypes, dietary requirements, and rearing conditions than broilers (Kers et al., 2018). Previous studies on layer microbiota were limited to a specific age and its regulatory effects on egg quality and safety remain unclear.

This review is hereby focused on the colonization characteristics of intestinal microbiota at different physiological stages of layer hens and their effects on egg quality and safety. This can extend our understanding of intestinal microbiota and host crosstalk, contributing to the development of nutrition strategies with targeted microbiota interventions to improve egg quality and safety of laying hens.

INTESTINAL MICROBIOTA IN DIFFERENT STAGES OF LAYER HENS

Origin and Succession of Intestinal Microbiota in Laying Hens

It was previously accepted that chick embryos were sterile and the initial intestinal microbiota of chicks originated from a posthatch environment (van der Wielen et al., 2002). However, recent studies have argued the theory of sterility and reported the presence of diverse microbes in chick embryos using 16S rRNA sequencing technology (Ding et al., 2017; Lee et al., 2019; Akinyemi et al., 2020; Shterzer et al., 2020; Dai et al., 2021). Ding et al. (2017) collected a total of 12 fecal samples from the maternal hens, 51 embryo samples, and 113 chick samples under aseptic conditions and characterize the intestinal microbiota of maternal hens, embryos, and chicks. They found that there was a correlation coefficient of 0.40 in the microbiota composition between maternal and offspring embryonic intestines (Ding et al., 2017), indicating that maternal microbiota affects the microbial colonization in its offspring. A wide variety of microbes colonize in the oviduct of laying hens, such as Firmicutes, Proteobacteria, Bacteroidetes, and Acidobacteria (Lee et al., 2019; Wen et al., 2021). Interestingly, Shterzer et al. (2020) characterized the microbial composition along the oviduct and the intestine (jejunum and cecum) of broiler breeders at 37 wk of age based on amplicon sequence variant taxonomic assignment. They found a large overlap significant overlap between the intestinal microbiota and the oviducal microbiota, with 55 and 53% of amplicon sequence variants in the jejunum and cecum, respectively, shared with the magnum. Additionally, they concluded that higher microbe abundance in the jejunum and cecum resulted in a higher probability of being presented in the oviduct by the semi-log nonlinear regression analysis (Shterzer et al., 2020). Considering that the end of the reproductive and digestive systems of chicken open at the cloaca, intestinal microbes should travel the oviduct up to the infundibulum and establish a full oviductal microbiota (Lee et al., 2019; Shterzer et al., 2020). Because of the close contact between the egg and oviduct for about 24 h during egg formation (Hincke et al., 2012), microbes can be directly transmitted into the yolk, albumen, eggshell membrane, and eggshell. Recently, Lee et al. (2019) sampled oviducts, cloaca, eggs, and chicken embryos from specific pathogen-free layers to assess the impact of oviducal microbiota on the fertilized eggs and embryos by the SourceTracker analysis. They found that a total of 21 shared core genuses were identified among the oviduct, cloaca, eggshell, albumen, and the chick embryo (Lee et al., 2019). The eggshell microbiota contributed about 63% of the chick embryo and albumen microbiota, in comparison to the oviduct microbiota contributed 28% of the eggshell microbiota (Lee et al., 2019). This finding suggested the transfer of maternal oviduct microbiota to the embryo mainly occurs through the albumen and eggshell. It is consistent with the view that the fetus initially colonizes the microbiota from the uterus in mammals (Collado et al., 2016; Bi et al., 2021). Consequently, the vertical transmission route of microbiota has been established in poultry, starting from the maternal gut and passing through the oviduct to its offspring. However, microbial transmission appears to be selective from the maternal oviduct to the embryo. It is challenging to transfer Lactobacillus from the oviducal magnum to chick embryos (Lee et al., 2019), whereas pathogenic microorganisms such as Salmonella, Escherichia coli, and Campylobacter can be transmitted vertically (Kizerwetter-Świda and Binek, 2008). Studies on how microbes colonize from the egg to the embryonic intestine are still limited. It seems that chick embryos not only obtain microbes through the amniotic fluid but also absorb the yolk so that microbes in the egg can colonize the embryonic intestine.

The Embryonic Stage

The embryonic intestine microbiota was predominantly composed of Proteobacteria and Firmicutes at the phylum level. Proteobacteria showed the highest

abundance and accounted for more than 50% of all species (Ding et al., 2017), followed by Firmicutes (5% −21%), Acidobacteria (3%−16%), and Bacteroidetes (2%−5%) (Ding et al., 2017; Akinyemi et al., 2020). However, inconsistent results at the genus level were found in recent studies. Ding et al. (2017) reported that the core genus of embryonic gut microbiota was *Halomonas* (79%) while *Pseudomonas* (71%) and O*chrobactrum* (23%) dominated embryonic gut microbiota in other studies (Lee et al., 2019; Akinyemi et al., 2020). In addition to poultry breeds and hatching environments, the different hypervariable regions of the 16S rRNA gene for PCR also cause inconsistent results (Sperling et al., 2017). Moreover, there are dynamic alterations in the gut microbiota of chick embryos across the embryonic stage. An increased microbial population at the early embryonic stage was reported in chickens (Ding et al., 2017; Akinyemi et al., 2020), especially the Proteobacteria which was also the dominant bacterial taxa in the embryonic intestine of humans and mammals without intra-amniotic infection (Seferovic et al., 2019; Bi et al., 2021). Akinyemi et al. (2020) revealed that microbial functions of the chick embryos were enriched in the membrane transport, cellular processing and signal transduction, carbohydrate metabolism, amino acid metabolism, and genetic information pathways. Among them, the membrane transport pathway is closely related to host immune cell activation, while cellular processing and signal transduction is involved in the antigen presentation (Cella et al., 1997; Odamaki et al., 2016). Therefore, one hypothesis was that the high abundance of Proteobacteria could be associated with the development of the immune organ and system rather than permanent colonization (Hamburger and Hamilton, 1992; Oakley et al., 2014). Numerous pieces of evidence suggest that the microbial community is an essential driver of intestinal innate immune programming (Romano-Keeler et al., 2014; Levy et al., 2017), and plays a critical role in the differentiation and maturation of epithelial cells (Ivanov et al., 2009; Sommer and Bäckhed, 2013). Besides, aerobic lipid oxidation of yolk was the dominant way to supply energy for chick embryos accompanied by the extremely low carbohydrate metabolism (Yu et al., 2018), which can also explain the low abundance of Firmicutes at the embryonic stage (Polansky et al., 2015). However, the limited oxygen and other gas exchange capacity of chick embryos resulted in the shift of energy metabolism from aerobic oxidation to anaerobic glycolysis after the age of embryos 19 (Uni and Ferket, 2004). Simultaneously, the microbial population dropped drastically at the late embryonic stage and was accompanied by the absence of some microbes in chicks and maternal hens (Ding et al., 2017; Akinyemi et al., 2020). Interestingly, most of the microbes that gradually disappeared during the dramatic succession were facultative aerobes (Awad et al., 2016), suggesting these microbes were temporarily harbored in embryos and were influenced by the physiological metabolism shift during embryonic development (Ding et al., 2017).

Before the Laying Period

The chicken intestinal microbiota is a microenvironment with continuous succession to maintain dynamic balance (Lu et al., 2003). The intestinal microbial composition in chickens is influenced by several factors such as genotypes, sex, dietary patterns, and the rearing environment (Zhu et al., 2002). At present, the composition and function of the intestinal microbiota have been well characterized and clarified in broiler chicks (Huang et al., 2018; Shang et al., 2018), which have different microbial diversity and community composition compared to those of layers (Pandit et al., 2018). The early microbial succession of the broiler intestine can be divided into 3 stages: the first stage is dominated by vertical transmission and rapid colonization of *Streptococcus, Shigella*, and *Escherichia coli*. The second stage is characterized by the rapid growth of Lachnospiraceae and Ruminococcaceae from 4 d posthatch. In the third stage, the relative abundance of Clostridium decreases with the appearance of Bacilliales, Bacteroidales, and Pseudomonadales from 10 d posthatch (Jurburg et al., 2019). Nevertheless, studies about intestinal microbiota composition and succession in layers are limited due to longer lifespans.

To consider changes in physiological characteristics and nutritional requirements of pullets, the prelaying period (0−16 wk) is divided into 3 stages, including digestive and immune system development (0−6 wk), bone development (7−12 wk), and reproductive system development (13−16 wk) (Adil and Magray, 2012). Likewise, the composition of the intestinal microbiota of pullets undergoes characteristic succession with changes in host growth and development (Li et al., 2018). After the embryonic microbial succession, the low microbial diversity in newly-hatched layer chicks is predominantly composed of Proteobacteria and Firmicutes, including Enterobacteriaceae, Clostridiaceae, Streptococcus, Enterococcaceae, Lachnospiraceae, and Ruminococcaceae (Ballou et al., 2016; Dai et al., 2020). After 2 wk posthatch, the relative abundance of Proteobacteria in the cecal microbiota decreased from 52.33% to 1.11%, while the relative abundance of Firmicutes increased from 17.13% to 67.38%. Meanwhile, the relative abundance of Bacteroidetes increased from 0.31% to 24.49% (Ballou et al., 2016; Dai et al., 2020). The development of the intestinal immune system has been shown to parallel the development of intestinal microbiota (Ekino et al., 1980). Therefore, short-term colonization of the intestine with a high abundance of Proteobacteria in layer chicks may be associated with activating the intestinal immune response and promoting immune system development (Oakley et al., 2014). Furthermore, a previous study found that the early succession of intestinal microbiota was synchronized with intestinal development of layer chicks, and the enriched Firmicutes, *Lactobacillus, Enterococcus*, and *Ruminiclostridium* were identified as biomarkers for accelerated intestinal development by linear discriminant analysis (Dai et al., 2020). In addition, Firmicutes can ferment dietary

nondigestible carbohydrates into short-chain fatty acids (**SCFA**) such as butyric acid, providing energy to enhance the intestinal barrier (Peng et al., 2009). Lachnospiraceae and Ruminococcaceae were also confirmed to be highly correlated with feed efficiency in poultry (Singh et al., 2012). Accordingly, the predominant bacterial taxa rapidly shift from Proteobacteria to Firmicutes in the early intestinal microbiota, contributing to carbohydrate absorption to provide more energy for the intestinal development of layer chicks (Polansky et al., 2015; Dai et al., 2020). The relative abundance of Bacteroidetes in the cecum of layer chicks increases over wk 3 to 6 posthatch. Subsequently, a relatively stable intestinal microbiota dominated by Firmicutes and Bacteroidetes is established (Dai et al., 2020; Liu et al., 2020b). Bacteroidetes can also provide energy for the host intestine by utilizing complex polysaccharides to produce propionic and butyric acids (Gibiino et al., 2018). Bacteroidetes proliferate with the increasing complexity and maturity of intestinal microbiota, and its abundance gradually becomes consistent with that of Firmicutes. This finding indicates that the microbial energy supply pathway gradually shifts from mainly relying on the butyric acid produced by Firmicutes to synergistic effects, which may be adapted to the physiological needs of layers with slower growth (Polansky et al., 2015). In addition, one of the characteristics of Firmicutes is the ability to express *L*-fucose isomerase, whereas Bacteroidetes express xylose isomerase. The site-specific or age-specific proteoglycan expressions of the fucosylation or xylosylation in intestinal mucus may be the conditions for microbial colonization (Polansky et al., 2015; Richards et al., 2019). In general, the intestinal microbial succession of layer chicks is first characterized by the vertical transmission of Proteobacteria as the dominant microbes, followed by the rapid colonization of Firmicutes and Bacteroidetes during the brooding period.

During the pullet period, the abundance of Bacteroidetes in layers, including *Alistipes, Bacteroides* and *Barnesiella* gradually decrease in layers, accompanied by a renewed increase in the abundance of Firmicutes (Li et al., 2018; Neijat et al., 2019). The critical period for the rapid development of the bones in layers occurs at 7 to 12 wk posthatch (Adil and Magray, 2012). An increase in Firmicutes indicates a corresponding increase in the content of butyric acid as a fermentation product. Butyric acid can bind to the G protein-coupled receptor 43 (**GPR43**) on dendritic cells and activate Wnt signaling pathway in bone marrow stromal cells by increasing the production of TGF-β by Tregs, causing their proliferation and differentiation into osteoblasts (Zaiss et al., 2019). Therefore, the periodic increase in the abundance of butyric acid-producing bacteria may reflect the physiological needs of rapidly developing bones during the pullet period. In addition, the physiology of layers changes significantly near the laying period, as evidenced by the enhancement of lipid metabolism and nutrient utilization (Li et al., 2017), driving the succession of intestinal microbiota. A more mature and stable intestinal microflora has been established at 16 wk of layers' age. The

relative abundance of Bacteroidetes increased again, accompanied by an increase in *Lactobacillus* abundance and microbial community diversity. These changes can reduce intestinal lumen pH to inhibit colonization of pathogenic bacteria while enhancing nutrient utilization, consequently supporting the increased egg production in the laying period (Videnska et al., 2014; Neijat et al., 2019).

In the Laying Period

Age-linked variations in the microbial composition of layers intestinal have been defined in recent studies (Videnska et al., 2014; Li et al., 2018; Liu et al., 2020b; Xiao et al., 2021). The composition of the gut microbiota fluctuates substantially before the laying period while it is relatively stable in the laying period, which may be attributed to the management system of laying hens (Joat et al., 2021). Laying hens acclimatize to the rearing conditions after moving to the production shed, which remains constant till the end of the production cycle. However, the endocrine and sex hormone changes at the onset of laying may potentially influence the intestinal microbiota in laying hens (Lumpkins et al., 2008). For example, *Lactobacillus, Lactococcus*, and *Bifidobacterium* were more abundant in laying hens with low body weight and high production performance (Zhao et al., 2013; Wang et al., 2020c). Although Firmicutes was still the dominant phylum in the early laying period, Bacteroidetes overtook Proteobacteria as the second most abundant phylum (Joat et al., 2021). Moreover, the intestine of laying hens was also rapidly colonized with some bacteria that were not present before the laying period, such as *Synergistetes, Fusobacteria*, and *Elusimicrobia* with 1.0 to 2.0% relative abundance (Videnska et al., 2014). Compared with the brooding and pullet period, a broader microbial diversity of the intestine is observed in the laying period (Videnska et al., 2014). In the peak production period (30−50 wk), Bacteroidetes (47.5%−62.0%), Firmicutes (30.8%−60.4%), Proteobacteria (2.0%−10.0%), and Fusobacteria (2.0%−5.0%) formed the vast majority of microbiota (Guo et al., 2018; Hamid et al., 2019; Miao et al., 2020; Shang et al., 2020; Joat et al., 2021). Notably, the relative abundance of Firmicutes decreases gradually in the mid laying period, whereas the increased abundance occurs in Bacteroidetes. Accordingly, Ruminococcaceae (16.7%−28.8%), Rikenellaceae (12.5% −30.0%), Bacteroidaceae (12.5%−27.5%), Lachnospiraceae (6.7%−21.2%), and Lactobacillaceae (2.5% −10.0%) dominate gut microbiota at the family level. Among them, the most abundant bacterial genuses are *Bacteroides* (11.1%−32.5%), *Rikenella* (10.1%−15.0%), *Lachnospira* (5.0%−8.0%), and *Lactobacillus* (2.0%−10.0%) (Guo et al., 2018; Hamid et al., 2019; Miao et al., 2020; Shang et al., 2020; Joat et al., 2021). In addition, the relative abundance of Bacteroidetes overtake Firmicutes and become the dominant phylum in the late laying period, accompanied by a renewed decrease in the abundance of Proteobacteria and Fusobacteria (Joat et al., 2021).

Collectively, Firmicutes, Bacteroidetes, and Proteobacteria form the vast majority of intestinal microbiota across the whole laying period. Furthermore, the relative abundance of Bacteroides increase in the early laying period, accompanied by a decrease in the relative abundance of Firmicutes, until they reach a balance in the peak production period. In the late phase, the relative abundance of Bacteroides exceeds that of Firmicutes (Figure 1).

EFFECTS OF THE INTESTINAL MICROBIOTA OF LAYING HENS ON EGG QUALITY AND SAFETY

Vertical Microbiota Transmission Directly Affects Egg Quality and Safety

Egg contaminations with pathogenic bacteria such as *Escherichia* and *Salmonella* can lead to foodborne infection in human consumers (Wen et al., 2021). In addition to environmental factors, the microbiome on the eggshell surface assists in establishing maternal-offspring contact by vertical microbiota transmission (van Veelen et al., 2018). The laying hen can pass intestinal and cloacal microbes to its offspring during oviposition, as the egg is exposed to cecal secretions in the cloaca. Previous studies reported that Firmicutes were the dominant bacterial taxa on the eggshell surface, making up approximately 50% of the total phylum (Neira et al., 2017; Shi et al., 2020). The cloacal microbial community of laying hens is also largely dominated by Firmicutes (Wen et al., 2021), suggesting vertical microbiota transmission is key to the contamination of eggshell and egg safety (Trudeau et al., 2020). Therefore, modulating the intestinal microbiota will be an effective way to reduce the risk of spreading foodborne pathogens in humans. As the first barrier, the eggshell microbiota plays an important role in preventing pathogenic bacteria through competitive exclusion. Compared with eggs that did not contact the cloaca and were removed by dissection, the oviposited egg surface had a distinct microbiota and a higher bacterial load with a density of 0.75 per 1800 μm^2 (Bunker et al., 2021). Generally, fungi can multiply rapidly by catabolizing nutrients in eggs and producing harmful toxins and an unpleasant odor (Chang et al., 2021). Recently, Bunker et al. (2021) confirmed that eggshell microbes had antifungal properties by fungal attachment assays. Microbes can produce hydrolytic enzymes such as chitinase or protease to degrade mycelia and disrupt fungal growth (Gutiérrez-Román et al., 2015). In addition, it was found that microbes on the eggshell surface like *Salmonella*
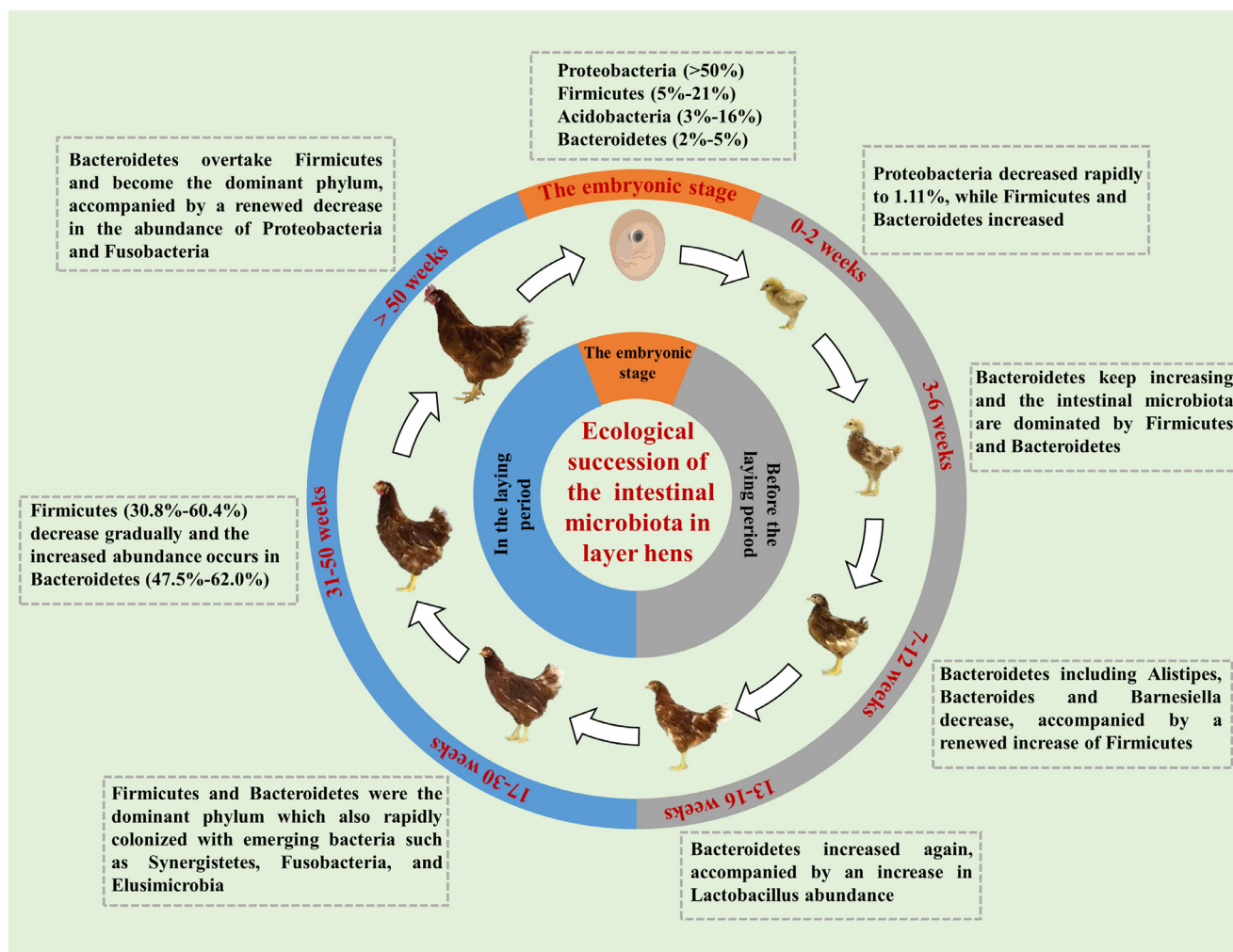

Figure 1. Intestinal microbial compositions in different stages of layer hens.

and *Escherichia* directly affect the eggshell quality and accelerate the formation of egg translucency through bacterial penetration (Chousalkar et al., 2010). Conversely, the eggshell characteristics and antimicrobial molecules may determine the eggshell surface microbiota (Réhault-Godbert, 2021). Certain matrix proteins distributed from the outer cuticle to the inner membranes of eggshells reinforce the antimicrobial properties of eggs (Gautron et al., 2007). The association between ovocalyxin-32 and egg quality, including eggshell color, eggshell strength, eggshell stiffness, blood, and meat spots, was revealed by quantitative trait locus searching (Takahashi et al., 2010). Moreover, ovocalyxin-36 is a prominent protein expressed in the oviduct at the shell mineralization stage (Gautron et al., 2007). As it is similar to lipopolysaccharide-binding proteins and bactericidal permeability-increasing proteins, ovocalyxin-36 is related to the natural defense of eggshells (Yin et al., 2020). The purified ovocalyxin-36 can inhibit the growth of *Staphylococcus aureus* and demonstrate immune regulatory functions in the LPS challenged model (Kovacs-Nolan et al., 2014). Compared with the magnum and uterus, some of the avian beta-defensin family members including AvBD-1, AvBD-7, AvBD-3, and AvBD-10 were over-expressed in the oviducal isthmus (Yin et al., 2020). This implies that the eggshell membrane also plays a defensive function against further pathogenic invasion (Hincke et al., 2012). In addition, pigments were secreted from the oviducal epithelium into the uterine fluid and then deposited on the eggshell (Sparks, 2011). A recent study found that the abundance of oviducal *Staphylococcus* was positively associated with the darker brownness of eggshells (Wen et al., 2021). Notably, *Staphylococcus* has been demonstrated to biosynthesize uroporphyrin and coproporphyrin (De la Fuente et al., 1986), which were part of pigments affecting the eggshell color. Overall, the eggshell not only is a barrier reducing the contamination chances of eggs but also provides potential crosstalk between microbes and host to modulate egg quality and safety.
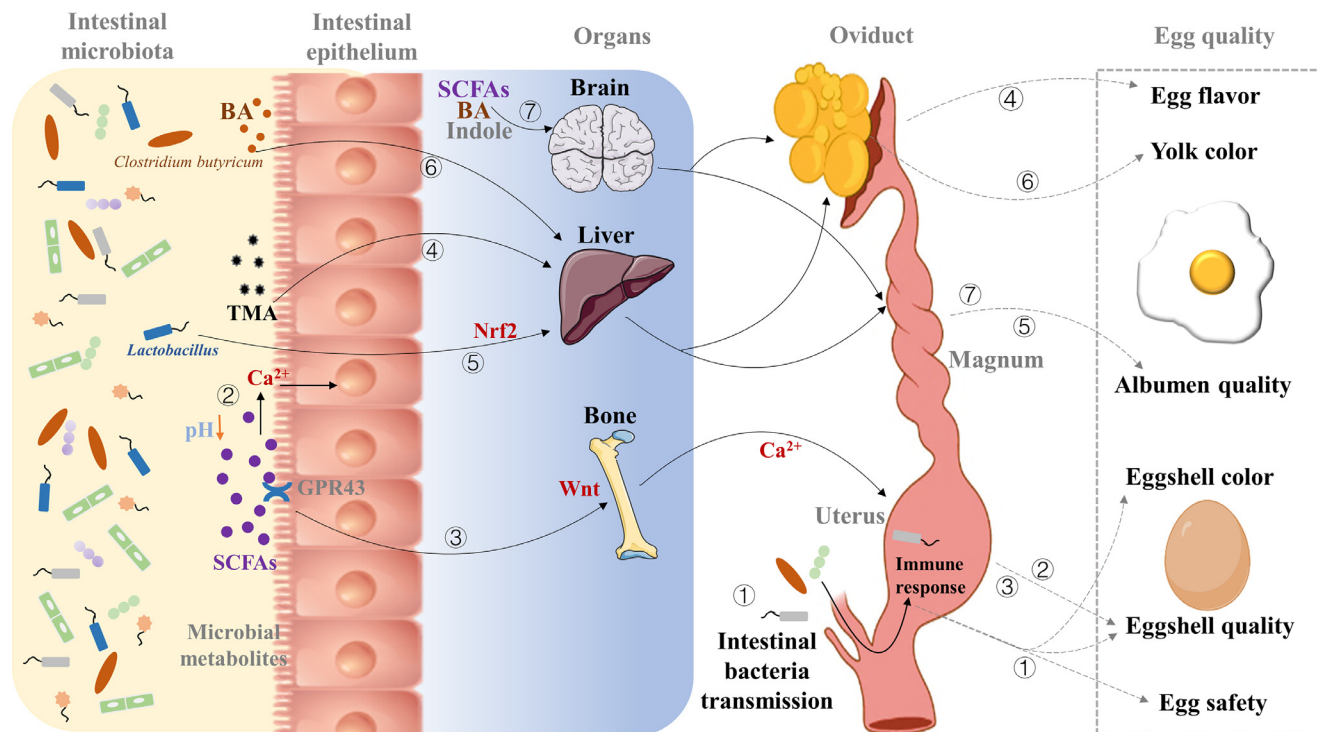
Notably, microbes in the internal contents of eggs can be passed down from the maternal oviduct during egg development (Trevelline et al., 2018). Proteobacteria were the most abundant phylum in the internal contents of eggs (Vieira et al., 2019), which coincided with the highest abundance of Proteobacteria in the oviduct of laying hens (Wen et al., 2021). When laying hens are infected with pathogenic bacteria like *Salmonella*, intestinal *Salmonella* will spread to the oviduct through the cloaca due to the unique anatomical structure of poultry (Gantois et al., 2009). Furthermore, *Salmonella* alters the expression of *TLRs, NLRs, AvβDs*, and cytokine family genes in the oviduct, resulting in the decline of egg quality (Zhang et al., 2019). Finally, the presence of foodborne pathogens in eggs poses serious threats to food safety (Gantois et al., 2009; Salihu et al., 2015). Additionally, early microbial colonization of chick embryos by vertical transmission is an essential driver of intestinal innate immune programming in heredity. Thus, future studies are required to understand the mechanism of vertical microbiota transmission to improve egg quality and safety by shaping the oviducal microbiota.

Effects of Microbial Metabolites on Egg Quality

Microbiota-derived metabolites produced mainly through saccharolytic fermentation of carbohydrates, notably short-chain fatty acids (**SCFAs**) such as acetate, propionate, butyrate, and lactate have been implicated in host-microbiota crosstalk (Morrison and Preston, 2016). SCFAs can improve intestinal nutrient utilization by stimulating intestinal epithelial cell proliferation and differentiation, and further increasing the intestinal villus height and absorptive surface area (De Vadder et al., 2014). Calcium is the most abundant mineral element in eggshells, accounting for 37% of the dry weight of eggshells. The declined utilization of intestinal calcium was recognized as the dominant reason for the poor eggshell quality in the late phase production of laying hens (Al-Batshan et al., 1994). The improvement in intestinal structure induced by SCFAs is favorable to calcium absorption, increasing calcium deposition into the eggshell and improving eggshell thickness (Feng et al., 2021). However, there is almost void of calcium in the intestinal contents about 4 to 5 h after feeding. To avoid dietary calcium deficiency during intense eggshell calcification, the development of medullary bone occurs at the onset of sexual maturity. Large amounts of calcium are stored in the medullary bone and are released into the blood for eggshell formation during the night hours of laying hens' photoperiod (Bar et al., 1998). Nevertheless, long-term and intense laying behavior will cause osteoporosis simultaneously with poor eggshell quality in the late phase of laying hens (Hanna, 2019). SCFAs have been shown to play an important role in the prevention and treatment of bone metabolism-related diseases (Zaiss et al., 2019). SCFAs directly induce metabolic reprogramming of osteoclast precursor cells, resulting in enhanced glycolysis and decreased oxidative phosphorylation, thereby inhibiting osteoclast differentiation (Montalvany-Antonucci et al., 2019). On the other hand, SCFAs like butyric acid can bind to GPR43 to promote the differentiation of auxiliary CD4+ cells to Treg, and activate Wnt signaling in the bone marrow stromal cells to proliferate and differentiate into osteoblasts (Zaiss et al., 2019). Therefore, the regulation of SCFAs on bone resorption and bone formation is of great significance for improving the homeostasis of bone metabolism, eggshell quality, and prolonging the laying phase of aged laying hens. Another potential mechanism involves SCFAs lowering the pH of the host intestinal lumen and increasing calcium solubility, thus further increasing dietary calcium utilization in laying hens (Gultemirian et al., 2014). Overall, SCFAs as microbial metabolites affect calcium utilization and deposition by regulating the homeostasis of systemic calcium metabolism, thereby improving eggshell quality (Figure 2).

The presence of a fishy odor in eggs seriously affects the egg quality and flavor, which is associated with abnormally elevated trimethylamine (**TMA**) levels. Since excessive TMA cannot be metabolized, it is

Figure 2. Effects of the intestinal microbiota of layers on egg quality and safety. Suggested mechanisms of microbial action are as follows: ① Intestinal bacteria can spread to the oviduct through the cloaca leading to egg contaminations, and accelerating the formation of egg translucency through bacterial penetration. In addition, oviducal bacteria biosynthesize pigments affecting the eggshell color. Moreover, oviducal pathogenic bacteria activate the TLR4/NF-κB signaling pathway inducing the synthesis of IL-1β and IL-6, which affects the eggshell ultrastructure by inhibiting the protein expression of a calcium-binding protein and Ca^{2+} transport. ② Short-chain fatty acids (SCFAs) produced by intestinal microbiota reduce the luminal pH and enhances calcium solubility, utilization, and deposition to improve eggshell thickness. ③ SCFAs bind to G protein-coupled receptor 43 (GPR43) to activate Wnt signaling in the bone marrow stromal cells to improve the Ca^{2+} homeostasis of bone metabolism and eggshell quality. ④ Trimethylamine (TMA) produced by intestinal microbiota is deposited in egg yolks, resulting in an unpleasant fishy odor. ⑤ Intestinal probiotics can activate the Nrf2 signaling pathway in the liver to regulate the antioxidant status and metabolic functions, which may improve albumen quality. ⑥ Bile acids (BA) produced by intestinal microbiota modulate lipid metabolism and intestinal absorption to improve the egg yolk color. ⑦ SCFAs, BA, and indole derivatives can directly interact with intrinsic enteric neurons and gut-innervating vagal and spinal afferents to regulate the secretion of estradiol, which regulates the secretion of proteins in the oviducal magnum and finally completes the regulation of albumen quality.

gradually deposited in egg yolks, resulting in an unpleasant fishy odor (Honkatukia et al., 2005). In addition to chicken monooxygenase 3 enzyme activity, the abnormally increased TMA level is also mainly derived from the degradation of choline and other dietary TMA precursors by intestinal microbiota (Honkatukia et al., 2005; Rath et al., 2017), and the reduction of TMA oxide through bacterial reductase (Barrett and Kwan, 1985). Studies found that the TMA level in egg yolks was positively correlated with the TMA level in the cecum (Wang et al., 2016), and the fishy odor in eggs disappeared after the cecum of laying hens was removed (Pearson et al., 1983). Further studies found that Firmicutes and Proteobacteria were positively correlated with the TMA level in the cecum, whereas Bacteroidetes were negatively correlated with the TMA levels (Long et al., 2017). Analyses of bacterial genomes revealed that the gene cluster of choline utilization is widely distributed across Firmicutes and Proteobacteria, but it is absent in Bacteroidetes (Martínez-del Campo et al., 2015). Firmicutes and Proteobacteria are commonly considered TMA-producing bacteria (Long et al., 2017). Therefore, the intestine microbiota-mediated TMA formation suggests its essential role and potential targets to improve

egg flavor (Figure 2). This hypothesis is also supported by the decreased fishy eggs by antibiotics supplementation to disturb intestinal microbiota (Zentek, 2003).

Moreover, microbial metabolites can modulate lipid metabolism to affect egg quality. The disturbance of lipid metabolism is common in the late phase production of laying hens (Wang et al., 2020b). The liver is the main organ of lipid metabolism in poultry, and induced fat accumulation and hepatic dysfunction can affect not only the yolk color but also the cholesterol deposition in egg yolks (Qiu et al., 2021). Due to extremely low contents of dietary cholesterol, almost all of the cholesterol is synthesized endogenously in the liver and enriched into egg yolks via the blood. Furthermore, enzymatic oxidation of cholesterol in the liver generates numerous distinct bile acids (**BA**), which are metabolized in the intestine by the gut microbiota (Wahlström et al., 2016). In addition to its function as a detergent facilitating digestion and absorption of dietary lipids, BA can also act as an effective ligand activating farnesoid X receptor (**FXR**) to modulate lipid metabolism (de Aguiar Vallim et al., 2013). Recent studies found that supplemental *Clostridium butyricum* modulated lipid metabolism of aged laying hens via shaping BA profiles and enhancing intestinal absorption to improve

the egg yolk color (Wang et al., 2020a,b) (Figure 2). The alteration of BA induced by the intestinal microbes is dependent on the secretion of bile salt hydrolase (**BSH**) to generate unconjugated free BA through deconjugation (Wahlström et al., 2016). Similarly, the BSH produced by *Lactobacillus* can regulate bile acid enterohepatic circulation to improve cholesterol metabolism, which subsequently contributes to reducing the cholesterol levels in serum and egg yolks (Choe et al., 2012; Hou et al., 2020).

Effects of Microbial-Mediated Immune Responses in the Oviducal Mucosa on Eggshell Quality

It is well documented that microbes can be sensed by the pattern recognition receptors of immune cells such as macrophages and dendritic cells to establish interactions with the host (Agostinis et al., 2019). This process is involved in regulating innate and adaptive immune responses in the host endometrium. Notably, the maintenance of host mucosal homeostasis requires a dynamic balance between anti-inflammatory and proinflammatory cytokines generated in the microenvironment (Maillard and Snapper, 2010). The expression of *IL-1β* and *IL-6* proinflammatory cytokines and *CX3CL1* chemokine in the oviducal uterine mucosa of laying hens is increased during eggshell mineralization. Furthermore, the expression of *TGF-β2* anti-inflammatory cytokine is also significantly increased during the initial stage of eggshell mineralization (Elhamouly et al., 2018). However, when attacked by pathogenic bacteria or certain gram-negative bacteria, the innate immune system of oviducal tissue recognizes microbial-associated molecular patterns including molecules from the microbial cell wall (peptidoglycan) and cell membrane (lipopolysaccharide) through Toll-like receptors. This induces the synthesis of proinflammatory cytokines *IL-1β* and *IL-6* by activating transcription factors *NF-κB* and *AP-1* (Yoshimura, 2015), resulting in an imbalance between anti-inflammatory and proinflammatory factors in uterine mucosal tissues. Further studies revealed that the proinflammatory cytokines *IL-1β* and *IL-6* affected the eggshell ultrastructure by inhibiting the protein expression of a calcium-binding protein (*CABP-D28K*) and Ca^{2+} transport in the oviduct uterine mucosa of laying hens (Nii et al., 2017). This is probably due to the critical roles of *IL-1β* and *IL-6* in promoting hematopoiesis and protein degradation (Narsale and Carson, 2014), interfering with the effect of matrix proteins and the efficiency of inorganic ion supply during eggshell mineralization. Furthermore, the dysregulation of organic matrix protein synthesis and the compromised immune function in the oviducal uterus of aged laying hens result in the variation of eggshell ultrastructure and mechanical properties. The latter may be the main reason for the decline in eggshell quality in the late phase production of laying hens (Feng et al., 2020). Recently, Feng et al. (2021) found that dietary oregano essential oil improved epithelial barrier functions and mucosal

immune status by altering microbial composition and decreasing Shigella abundance, thus favoring eggshell quality of late-phase laying hens. The balance of intestinal microbiota can effectively reduce the transfer of pathogenic bacteria to the oviduct causing inflammation and immune responses. Thus, we hypothesize that microbial-mediated immune responses in the oviducal mucosa have a potential impact on the eggshell quality, contributing to the nutritional improvement of eggshell quality via oviducal microbiota of laying hens.

Effects of the Microbiota-Gut-Liver/Brain-Reproductive Tract Axis on Egg Quality

After intensive metabolism during peak production, attenuation of the antioxidant function in aged laying hens results in excessive reactive oxygen species disrupting the balance of the host redox system (Liu et al., 2018). Numerous age-related diseases are associated with elevated levels of oxidatively modified proteins (Stadtman, 2001). Thus, impaired liver metabolism in aged laying hens may lead to poor albumen quality, including the decrease in albumen height and Haugh unit (Wang et al., 2018). It was found that dietary probiotics or tea polyphenols improved albumen quality by altering intestinal microbiota composition and its metabolites to regulate the antioxidant status and metabolic functions of the liver (Wang et al., 2018; Zhan et al., 2019; Wang et al., 2020b). In fact, the intestine and liver are anatomically and physiologically connected, which has been called the gut-liver axis (Ohtani and Kawada, 2019). As an important gut-liver axis mediated factor, the microbiota affects the intestine and distant organs via systemic circulation. For example, the intestinal microbiota induced upregulation of the Nrf2 antioxidant and xenobiotic response of the liver in a germ-free mouse model. Moreover, oral delivery of *Lactobacillus* protected against oxidative liver injury by producing 5-methoxyindoleacetic acid to potently activate Nrf2 in the liver (Saeedi et al., 2020). Indeed, bacterial tryptophan catabolites like indole can be sensed by enteroendocrine cells to activate enteric and vagal neuronal pathways, achieving the remote regulation of the liver (Ye et al., 2021). Likewise, BA are important microbial metabolites involving the regulation of lipid metabolism and inflammatory response, and key mediators in the gut-liver crosstalk. BA promoted fatty acid oxidation in the liver by activating intestinal and hepatic FXR and inducing the expression of the peroxisomal proliferator-activated receptor (Schneider et al., 2018). It further inhibits the expression of key genes for lipid synthesis, thereby reducing plasma triglyceride and cholesterol levels (Xi and Li, 2020), suggesting the potential of bile acids as important targets for regulating egg yolk lipids.

In addition, it is well-known that the development of ovarian follicles and production performance in laying hens are mainly regulated by the hypothalamus (gonadotropic releasing hormone)-pituitary (luteinizing and

follicle-stimulating hormone)-gonad (estrogen) axis. Significant differences in the intestinal microbiota of laying hens with different production performance have been found in the previous study (Elokil et al., 2020). Interestingly, fecal microbiota transplantation from high-yield hens improved production performance in low-yield hens (Wang et al., 2020c). Notably, the abundance of some intestinal microbes was positively correlated with serum follicle-stimulating hormone, luteinizing hormone, and estradiol levels (Liu et al., 2020a), indicating the microbiota-brain crosstalk is involved in regulating the production performance in laying hens. Generally, intestinal microbes and their metabolites, like SCFAs, BA, and indole derivatives, can signal via enteroendocrine cells and enterochromaffin cells to regulate the secretion of neuropeptides (Agirman and Hsiao, 2021). Moreover, they can directly interact with intrinsic enteric neurons and gut-innervating vagal and spinal afferents to regulate neurotransmitters such as aminobutyric acid and 5-hydroxytryptamine (Cryan et al., 2020). Furthermore, the hypothalamus can modulate oviducal health in laying hens through the brain-reproductive tract axis, in particular, regulating the secretion of proteins in the oviducal magnum by changing the secretion of estradiol, and finally completing the regulation of albumen quality (Gaub et al., 1990). Overall, the microbiota-gut-liver/brain axis may be proposed as a new and systematic strategy to improve egg quality and safety (Figure 2), but further research to understand the mechanisms underlying is still needed.

CONCLUSIONS AND FUTURE RESEARCH

The analysis of the colonization and succession patterns of intestinal microbiota is critical for our understanding of intestinal microbiota ecology and targeted microbiota interventions. The available literature suggests that microbial succession in different physiological stages of layers seems to correspond to changes in organ development and metabolic functions, including driving immune system development, promoting nutrient utilization and bone development, and maintaining production performance. In addition to direct effects on the egg quality and safety via vertical transmission route in the intestine-oviduct-egg, intestinal microbiota and its metabolites such as SCFAs, BA, and tryptophan derivatives are indirectly involved in regulating egg quality through the microbiota-gut-liver/brain-reproductive tract axis. Nevertheless, the connection between intestinal microbiota and egg quality remain rather tentative, due to numerous conclusions being summarized using correlation analysis in previous studies. Therefore, a comprehensive understanding of interactions between intestinal microbiota and egg quality is needed. We suggest that future research should focus on the critical periods of albumen secretion and eggshell mineralization to explore relevant molecules and signaling pathways based on the microbiota-gut-liver/brain-reproductive tract axis. It will contribute to extending our understanding of intestinal

microbiota and host crosstalk to improve egg quality and safety in the late phase production of laying hens.

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DISCLOSURES

The authors declare that there is no conflict of interest.

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