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Abstract

Modern laying hens selected to produce 450 to 500 eggs by 100 wk of age face sustained demands on skeletal reserves, hepatic lipid metabolism, and intestinal function. Maintaining productivity throughout this extended cycle requires nutritional interventions aligned with changing physiological demands. Probiotics offer diverse microbial functions that may support nutrient utilization and host physiology, but their efficacy depends on strain characteristics, dietary conditions, and supplementation schedules. This review seeks to examine the functional diversity of probiotic microbes and discuss reported probiotic responses within the context of changing age-specific physiological demands of the laying hen from pullet development through late lay. Five probiotic groups are considered, including spore-forming *Bacillus* spp.; lactic acid bacteria; obligate anaerobe *Clostridium butyricum*; commensal *Enterococcus*, *Bifidobacterium*, and *Propionibacterium*; and eukaryotic *Saccharomyces* and *Aspergillus* spp. Their strain-dependent activities include extracellular enzyme production, luminal acidification, butyrate production, microbial cross-feeding, and modulation of the intestinal redox environment. As to probiotic responses, stage-dependent priorities for probiotic evaluation were distinguished as follows: i) during the rearing and pre-lay phase (0 to 18 wk), microbial succession, intestinal barrier and immune maturation, skeletal development, and vaccine responsiveness were the major considerations; ii) during the early and peak-lay phase (~18 to 45 wk), nutrient utilization, productive performance, mineral metabolism, egg quality, and preservation of skeletal reserves became increasingly important; and iii) during the mid- and late-lay phase (~45 to 100 wk), maintaining intestinal barrier integrity, hepatic lipid homeostasis, antioxidant capacity, reproductive endocrine function, and egg-quality stability are central. Even though reported benefits vary among microbial strains, dietary conditions, and assessment periods, probiotic supplementation showed great potential to satisfy the changing physiological priorities of the hen due to age. Collectively, this review provides a framework for aligning probiotic selection, supplementation timing, and exposure duration with the changing physiological priorities of laying hens. However, it was observed that benefits within individual stages do not establish sustained efficacy across extended laying, and the need for long-term studies to determine whether stage-specific responses persist and contribute to cumulative lifetime outcomes is highlighted.

Keywords: eggshell quality, extended laying cycle, gut-bone axis, gut-liver axis, laying hens, probiotics

Introduction

Global demand for eggs continues to increase with population growth, emphasizing the importance of maintaining productive efficiency in commercial laying hens. The avian egg is a nutrient-dense and affordable dietary staple that

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provides high-biological-value protein, essential fatty acids, vitamins, and bioactive compounds [1]. To satisfy this global demand, commercial breeders have applied intensive multi-trait selection to maximize productive output. These efforts have produced strains capable of sustaining production up to 100 wk of age and yielding 450 to 500 marketable eggs through simultaneous selection for high peak and extended laying persistency [2]. However, sustaining this prolonged productive lifespan imposes a continuous metabolic strain, as dietary nutrients are partitioned toward egg production, potentially challenging somatic maintenance [3]. An important challenge of extended laying is maintaining egg quality while preserving hen health and welfare [4]. Daily eggshell formation requires the deposition of 2.0 to 2.5 g of calcium (Ca) within a narrow 16- to 20-h window, a demand that often involves mobilizing Ca reserves from medullary bone, since most shell calcification takes place in the dark when feed intake and the immediate supply of dietary Ca are limited [5].

When sustained across a 100-wk cycle, this chronic osteoclastic resorption progressively depletes both medullary and cortical bone, predisposing aging flocks to skeletal fragility and osteoporosis [6]. In a study evaluating late layers, groups with high egg production and good eggshell quality exhibited lower tibial ash weight and less medullary bone, although correlations between individual bone and egg-related parameters were generally weak [7]. Prolonged physiological stress may activate the hypothalamic-pituitary-adrenal (HPA) axis and increase circulating corticosterone, with potential consequences for feed intake, immune regulation, and metabolic homeostasis [8]. Concurrently, the intestinal microbiome undergoes age-associated shifts: while the peak-lay cecal microbiota is dominated by carbohydrate-fermenting taxa that support high energy metabolism, the late-lay phase is often characterized by dysbiosis, local oxidative stress, and impaired mucosal nutrient transport [9]. These skeletal, neuroendocrine, and intestinal changes highlight the importance of nutritional strategies that support gut barrier integrity and mineral bioavailability during extended laying cycles.

Alongside these physiological challenges, restrictions on antibiotic growth promoters (AGPs) have increased interest in AGP alternatives to support intestinal health and productivity. The European Union ban on AGPs in 2006, followed by regulatory restrictions in other regions, was prompted in part by concerns about antimicrobial resistance and its potential transmission through the food chain [10, 11]. However, reduced AGP reliance has increased challenges associated with subclinical dysbiosis and enteric disorders, highlighting the importance of gastrointestinal homeostasis for optimal poultry performance [10, 12]. Our group has previously defined gut health as the dynamic balance between dietary components, commensal microbiome, mucosal epithelial barrier, and host immune function that enables the gut to carry out its principal functions of nutrient digestion and absorption, maintain homeostasis, and

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withstand stressors [13]. Among feed additives, direct-fed microbials (DFMs), or probiotics, represent one of the most documented AGP alternatives [12]. Defined by the FAO/WHO as "live microorganisms that when administered in adequate amounts, confer a health benefit on the host," probiotics support host health through competitive exclusion, gut barrier maintenance, digestive enzyme production, antagonism towards pathogenic bacteria, metabolite production, microbial cross-feeding, and immune modulation [14].

Despite widespread investigation, many probiotic trials have focused on specific age ranges rather than evaluating responses across successive stages. Consequently, it remains unclear how these responses vary with age-dependent physiological changes. Furthermore, differences in supplementation duration and the limited availability of longitudinal evidence make it difficult to determine whether reported benefits persist across successive stages. Previous reviews, including that of Oketch and Heo [14], have broadly discussed the biological effects of feed additives in laying hens. The present review seeks to focus on the functional diversity of probiotic microbes and integrate reported effects within the context of changing physiological priorities of laying hens during the rearing and pre-lay (0 to 18 wk), early-to-peak laying (approximately 18 to 45 wk), and mid-to-late laying (approximately 45 to 100 wk) stages. Specifically, we examine whether reported responses support aligning microbial functions and supplementation schedules with stage-specific hen demands. This review emphasizes studies of probiotic interventions in laying hens that report bird age, supplementation duration, and physiological outcomes. Studies involving replacement pullets were considered for early developmental responses, whereas evidence from broilers, embryos, other animal models, and microbial culture systems was incorporated where direct evidence in laying hens was limited. However, findings from these models were used to provide mechanistic context and were not regarded as direct evidence of efficacy. Given the heterogeneity in probiotic strains, administration methods, experimental durations, and measured outcomes, quantitative comparisons of efficacy across studies were not attempted.

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Taxonomy and Functional Diversity of Avian Probiotics

Microorganisms investigated as probiotics in poultry differ substantially in their physiological characteristics, metabolic capabilities, and interactions with the host and resident microbiota [14]. Consequently, probiotics should not be treated as a uniform intervention, and taxonomic identity was reasoned to provide a useful starting point for examining how reported probiotic responses relate to the hen's changing physiological demands and the timing and duration of supplementation. Sporulation capacity, oxygen tolerance, substrate utilization, metabolite production, and survival during processing and gastrointestinal transit influence the delivery and potential activity of individual strains.

For discussion, probiotic microbes can be broadly grouped into five functional categories: (1) endospore-forming, enzymatic *Bacillus* spp.; (2) lactic acid-producing non-spore-forming bacteria (LAB); (3) obligate anaerobic butyrogenic *Clostridium butyricum*; (4) colonization-centered commensals and metabolic cross-feeders (*Enterococcus* spp., *Bifidobacterium* spp., and *Propionibacterium* spp.); and (5) eukaryotic yeasts and filamentous fungi (*Saccharomyces* spp. and *Aspergillus* spp.). Their characteristic products and components, and proposed physiological roles are summarized in Table 1. Strain combinations may provide complementary activities, but broader taxonomic diversity does not necessarily confer greater efficacy [15, 16]. Metabolic cross-feeding, including the utilization of lactate and acetate by selected butyrate-producing bacteria, has been demonstrated in defined microbial systems [17]. Whether comparable interactions occur following supplementation in laying hens, and whether they contribute meaningfully to host responses, requires direct investigation. Demonstrating synergy requires comparisons with the individual components and an explicit test of whether the combined response exceeds the expected additive response.

Spore-Forming Bacteria (*Bacillus* spp.)

Bacteria of the genus *Bacillus* maintain a distinct processing advantage due to their capacity to transition into metabolically dormant endospores under environmental stress [18]. Thus, they can remain viable under transportation and storage, a key probiotic quality. Additionally, the multi-layered biophysical architecture of the endospore provides high resistance against thermal exposure, desiccation, mechanical shear forces, and gastric acid-bile barriers [19]. During feed manufacturing, hydrothermal processes such as steam conditioning (70 to 90°C) and pelleting routinely destroy vegetative probiotic cells. In contrast, the durability of *Bacillus* endospores preserves cell viability through these thermal hurdles, allowing subsequent germination and functional activity within the small intestine. Germination itself is a tightly regulated morphogenetic event: nutrient germinant receptors (GerA, GerB, GerK) embedded in the inner spore membrane recognize small-molecule signals such as L-alanine and inosine, triggering cortex-lytic enzyme activation, core rehydration, and subsequent outgrowth into metabolically active vegetative cells [20]. Processing tolerance and gastrointestinal activity should be evaluated separately when selecting strains for hen diets.

Beyond physical resilience, *Bacillus* species (including *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, and *B. coagulans*) also secrete an array of antimicrobial compounds, bioactive metabolites, and an array of extracellular enzymes, such as neutral and alkaline proteases, α-amylases, and non-starch polysaccharide (NSP)-degrading enzymes [18, 21, 22]. Extracellular enzymes produced by selected *Bacillus* strains may facilitate the breakdown of dietary substrates, although their effects on nutrient utilization depend on the strain and dietary conditions [14, 21, 22].

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Consistent with a potential contribution to nutrient utilization, *B. subtilis* supplementation from 19 to 48 wk of age improved apparent nutrient retention and production indices in laying hens [23]. Similarly, we previously determined the capacity of a dual-strain *B. subtilis* complex to increase the apparent ileal digestibility (AID) of dry matter, gross energy, and crude protein at 40 wk of age [18].

Notably, hen responses are not necessarily consistent across outcome domains. Oketch et al. [24] reported improved selected egg quality and tibial characteristics and altered enumerated cecal bacterial populations during 25 to 37 wk of age, but productive responses were less pronounced with a multi-strain *Bacillus* complex combining *Bacillus subtilis* PB6, *Bacillus subtilis* FXA, and *Bacillus licheniformis* G3. Select *Bacillus* strains also produce antimicrobial lipopeptides and other bioactive metabolites that may contribute to pathogen suppression and microbial interactions [25]. Recent studies also indicate that multi-strain *Bacillus* complex can improve skeletal and systemic outcomes in laying hens [18, 24]. Probiotic interventions involving *Bacillus*-based formulations have been associated with improvements in ileal villus height and the villus height-to-crypt depth (VH: CD) ratio, although changes in calculated villus absorptive surface area vary according to the intervention and assessment age [26-28]. Concurrently, *Bacillus* spp. support gut barrier function and systemic homeostasis, often reflected in lower inflammatory markers and selective *Lactobacillus* spp. enrichment [24, 29].

The magnitude of these *Bacillus*-based probiotic improvements often varies across studies depending on basal diet formulation, hen health status, and assessment period. Trials utilizing highly digestible corn-soybean meal diets under conditions of minimal physiological or environmental stress often show limited physiological improvements [24], underscoring the influence of probiotic efficacy being best expressed following a disturbance [14]. In addition, because combined-treatment designs generally do not isolate the individual contributions of each strain or demonstrate superiority over single-strain supplementation [18], these systemic benefits should be interpreted cautiously; specifically, improvements in intestinal morphology alone do not establish a direct causal mechanism for enhanced calcium and phosphorus deposition into skeletal tissues or the eggshell. Even so, multi-strain *Bacillus* formulations that target both substrate hydrolysis and physiological resilience may provide broad functional coverage and improved efficiency under commercial production conditions.

Lactic Acid Bacteria

Lactic acid bacteria (LAB), comprising the genera *Lactobacillus*, *Pediococcus*, *Streptococcus*, and *Enterococcus*, are Gram-positive fermentative members of the phylum *Bacillota* that produce lactic acid as their primary metabolic

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end-product [30]. Phylogenomic reclassifications have reorganized the genus *Lactobacillus* into 25 distinct genera, reassigning major avian species to *Lactiplantibacillus* (formerly *L. plantarum*), *Limosilobacillus* (formerly *L. reuteri*), and *Lacticaseibacillus* (formerly *L. casei*) [30]. The primary mechanism of LAB relies on rapid acidification: the accumulation of lactic and acetic acids that reduces luminal pH in the upper intestine and ceca, creating a microenvironment that suppresses acid-sensitive pathogens such as *Salmonella enterica* and *Escherichia coli* [21]. Furthermore, LAB stimulate mucosal immune development, upregulating epithelial MUC2 expression to thicken the protective mucus layer and induce secretory immunoglobulin A (sIgA) production [21, 31]. Specific LAB strains also produce bacteriocins, which are ribosomally synthesized antimicrobial peptides that target pathogen membranes: for example, plantaricin EF and plantaricin Pln from *Lactiplantibacillus plantarum* form pore-forming complexes dissipating proton motive force in *Listeria* and *Enterococcus* species [30, 32]. Additionally, mucosal adhesion by LAB is mediated through surface-layer proteins (SlpA), mucus-binding proteins (Mub), and moonlighting proteins such as elongation factor Tu (EF-Tu) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which facilitate competitive exclusion of enteropathogens from receptor sites on the epithelial glycocalyx [32].

In laying hens, *Pediococcus acidilactici* supplementation led to reduced yolk cholesterol [33]. A separate study using a composite formulation containing *Bacillus subtilis*, lactic acid bacteria, and *Saccharomyces* spp. reported stage-dependent changes in reproductive hormones and ovarian inflammatory gene expression, although these responses cannot be attributed to LAB alone [34]. However, the practical application of LAB as dry in-feed microbials faces several challenges. As non-spore-forming vegetative cells, LAB exhibit high sensitivity to heat, moisture, and mechanical pressure during feed conditioning and pelleting, leading to significant viability loss before consumption [21]. Furthermore, while homofermentative and heterofermentative LAB pathways produce high concentrations of lactate and acetate, they lack the enzymatic pathways for *de novo* butyrate synthesis. Although LAB generally do not produce butyrate directly, their fermentation products, including lactate and acetate, may serve as substrates for other butyrate-producing microorganisms [17]. Such cross-feeding has been demonstrated in defined microbial systems, but its magnitude and contribution to host physiology have not been established in laying hens. Combining LAB with prebiotic substrates may influence microbial fermentation and host responses [35, 36]; however, increased butyrate production through a specific cross-feeding pathway should not be assumed without direct experimental evidence. Therefore, LAB may be particularly suitable for early-life or water-based delivery and may also be combined with prebiotics or butyrogenic microorganisms to support complementary microbial functions.

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Butyrate-Producing Anaerobes (*Clostridium butyricum*)

Clostridium butyricum is a Gram-positive, obligate anaerobic, endospore-forming bacterium belonging to the family Clostridiaceae [37]. Unlike vegetative anaerobes, the endospores of *C. butyricum* withstand pelleting heat, gastric acidity, and bile salts, allowing viable delivery to the anaerobic distal gastrointestinal tract [37]. Upon germination in the ceca, *C. butyricum* ferments complex carbohydrates into short-chain fatty acids (SCFAs), predominantly butyrate and acetate, through the acetyl-CoA/butyryl-CoA pathway [37]. In this pathway, two molecules of acetyl-CoA are condensed via thiolase to form acetoacetyl-CoA, reduced through 3-hydroxybutyryl-CoA dehydrogenase and crotonase to butyryl-CoA, and finally converted to butyrate via either butyryl-CoA: acetate CoA-transferase or the phosphotransbutyrylase-butyrate kinase system. The CoA-transferase route is particularly significant in the cecal ecosystem because it simultaneously regenerates acetyl-CoA from external acetate, thereby linking butyrate production to substrate-level cross-feeding from acetate-producing commensals. Butyrate provides an important metabolic substrate and signaling molecule for intestinal epithelial cells.

In laying hens, dietary *C. butyricum* interventions have been associated with improved intestinal morphology and AID of AA [38–40], although outcomes do not independently verify direct enterocyte proliferation or reduced mucosal maintenance costs. Beyond morphological changes, *C. butyricum* supplementation has been investigated for its influence on upregulating tight junction proteins, specifically claudin-1, occludin, and zonula occludens-1 (ZO-1), which together limit the paracellular translocation of luminal endotoxins (lipopolysaccharides; LPS) into the bloodstream [41]. Butyrate-mediated regulation of epithelial barrier function may involve histone deacetylase-dependent signaling pathways that influence tight-junction gene expression [35, 41]. Supplemental *C. butyricum* supplementation has been associated with changes in inflammatory indicators, including serum IL-6 concentrations and the heterophil-to-lymphocyte ratio, as well as antioxidant-related parameters in poultry [40, 42, 43]. The potential relevance of these functions changes with physiological context. In late-laying hens, probiotic combinations containing *C. butyricum* have been associated with reduced defective egg rates and improved intestinal morphology [26]. Although barrier-related benefits have been reported following *C. butyricum* supplementation, the specific pathways connecting these responses to calcium transporter regulation in the oviduct shell gland remain unclear.

Commensal Cross-Feeders

Commensal non-spore-forming probiotics occupy a specialized niche in the avian gut, functioning through adherence and metabolic cross-feeding networks. Although taxonomically classified with LAB, *Enterococcus* species

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(such as *E. faecium* and *E. faecalis*) also function as key commensal colonizers that demonstrate tolerance to acid and bile fluctuations through F1F0-ATPase proton pumps and bile salt hydrolase (BSH) activity that deconjugates bile salts, reducing their detergent toxicity and allowing passage through the upper gut, despite the absence of endospores [44]. The primary protective mechanism of *Enterococcus* spp. involves epithelial surface colonization: surface-anchored LPXTG-motif proteins and adhesion factors mediate mucosal attachment and auto-aggregation [45]. This physical presence helps block enteropathogens from epithelial receptor sites, while the secretion of classes I to IV bacteriocins disrupts target bacteria membranes through receptor-mediated pore formation [46].

Strictly anaerobic *Bifidobacterium* species utilize the fructose-6-phosphate phosphoketolase (F6PPK) pathway to ferment dietary glycans into acetate and lactate at a characteristic 3:2 molar ratio [47]. In this pathway, fructose-6-phosphate is cleaved by F6PPK into erythrose-4-phosphate and acetyl phosphate; the former is recycled through transaldolase and transketolase reactions, while the latter yields acetate with ATP generation, ultimately producing 2.5 mol ATP and 1.5 mol acetate plus 1 mol lactate per mol glucose, which represents a higher ATP yield than the 2.0 mol ATP generated via classical homofermentative glycolysis. Acetate and lactate produced by *Bifidobacterium* spp. may serve as substrates for butyrate-producing microbes [35, 48]. However, much of the mechanistic evidence for these pathways comes from microbial systems rather than direct laying hen investigation [16, 17], and changes in the abundance of these microbial groups should not be interpreted as evidence of metabolite exchange, increased butyrate production, or improved intestinal barrier function.

Complementing this network, selected *Propionibacterium freudenreichii* strains can utilize lactate and generate propionate and acetate through the Wood-Werkman cycle, in which methylmalonyl-CoA carboxyl transferase participates in cyclic carboxyl-group transfer reactions [49]. Absorbed propionate enters portal circulation and is converted to propionyl-CoA, which undergoes biotin-dependent carboxylation toward methylmalonyl-CoA followed by vitamin B12-dependent conversion toward succinyl-CoA, thereby providing a potential microbial contribution to hepatic substrate metabolism [50]. Certain strains have been reported to produce low-molecular-weight factors that stimulate *Bifidobacterium spp.* growth in vitro [51]. However, whether these interactions contribute to metabolite exchange or measurable physiological benefits in laying hens remains unclear. Nevertheless, because these obligate anaerobes are sensitive to environmental oxygen and feed processing heat, delivering them in viable form remains a technological challenge. Consequently, current feeding strategies often aim to stimulate these indigenous populations indirectly via prebiotics or synbiotic strategies.

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Eukaryotic Yeasts and Fungi

Eukaryotic microbes, including yeasts (*Saccharomyces cerevisiae*, *Saccharomyces boulardii*) and filamentous fungi (*Aspergillus oryzae*), differ substantially in composition and mode of action. Live cultures, inactivated cells, isolated cell-wall fractions, fermentation products, and enzyme preparations should be considered separately [52, 53]. Although oxygen availability is an important determinant of intestinal ecology [54], direct evidence that supplemental live yeast lowers intestinal redox potential and promotes obligate anaerobic populations in laying hens remains limited. Metabolic modeling has suggested that the effects of fungal interventions on poultry microbial communities depend on microbial strain, resident microbiota, and dietary conditions [55]. In contrast, inactivated yeast cell-wall preparations contain mannoproteins, β-glucans, and chitin, although their relative proportions vary depending on the yeast species and preparation [53]. Mannan-rich yeast cell-wall products, including mannan oligosaccharides (MOS), may interfere with the intestinal adhesion of susceptible type 1-fimbriated *Salmonella* strains through mannose-dependent binding [56]. β-glucans can modulate innate immune responses through pattern-recognition pathways; however, the specific receptors and downstream mechanisms involved in β-glucan recognition remain incompletely characterized in chickens [57]. Live yeasts and inactivated yeast cell-wall preparations are to be evaluated separately to validate efficacy in laying hens. Additionally, selected fungal preparations may provide enzyme-related functions that complement those of bacterial probiotics. Filamentous fungi such as *Aspergillus oryzae* also produce non-starch polysaccharide-degrading enzymes that break down fibrous plant cell walls, aiding fiber utilization from alternative feedstuffs [58, 59]. Yeast-based supplements and fungal fermentation products have been investigated for their effects on laying performance and egg quality [58, 59]. However, these responses should be interpreted within the context of the specific preparation used, as live fungal cells, fermented feed ingredients, and isolated microbial components are not equivalent interventions. Integrating eukaryotic cultures with spore-forming probiotics can provide a complementary approach that addresses both mechanical pathogen exclusion and enzymatic nutrient breakdown.

Interpreting Probiotic Responses Across the Laying Hen Life Cycle.

Probiotic responses are herein considered within three intervals: rearing and pre-lay from hatch to approximately 18 wk, early-to-peak lay from approximately 18 to 45 wk, and mid-to-late lay from approximately 45 to 100 wk. These boundaries reflect broad changes in reproductive status, nutritional demands, and physiological priorities rather than strict biological transitions, as the timing of sexual maturity and subsequent production patterns may vary among laying hen strains and management conditions. A response measured in an older hen after a short intervention differs

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conceptually from a response following continuous exposure since rearing. Likewise, different significance levels at separate ages do not establish that treatment efficacy differs with age. Both the age at intervention and the experimental duration were considered to distinguish stage-specific responses from effects sustained across multiple production stages. Reported age ranges and supplementation durations are summarized in Table 2.

Rearing to Pre-lay (Day 0 to 18 wk): Microbial Succession, Intestinal and Immune Development, and Skeletal Preparation

The rearing and pre-lay period encompasses rapid intestinal development and mucosal immune maturation occurring during the first weeks after hatch. Consequently, early-life probiotic intervention often targets five key biological domains: accelerating early microbial succession, stimulating gut-associated lymphoid tissue (GALT) maturation, regulating developmental inflammation, expanding villus absorptive surface area, and improving vaccine responsiveness. Early-life outcomes on microbial succession, mucosal immune responses, intestinal morphology, and growth following probiotics are summarized in Table 3. In conventional systems, transport and processing may delay access to feed for approximately 48 to 72 h, potentially affecting early nutrient intake, microbial colonization, and immune development [60]. In broilers, functional maturation of gut-associated lymphoid tissue occurs in two stages during the first and second weeks after hatch, as reflected by interleukin-2 (IL-2) and interferon-γ (IFN-γ) mRNA expression and age-dependent changes in lymphocyte populations [61]. Although early microbial establishment is particularly relevant immediately after hatch, later pullet development also requires adequate nutrient intake, readiness for lay, body-weight composition and uniformity, and skeletal development. Probiotic responses during this interval should be evaluated against developmental outcomes rather than microbial measurements alone. Because mechanistic studies specific to pullets remain limited, evidence from broiler and general chicken models is included.

Microbiome studies indicate that early intervention can influence intestinal microbial succession and community development during early life [62-64]. Early intestinal microbial development is closely associated with mucosal immune maturation [65]. In addition, in ovo administration of prebiotics and synbiotics has been reported to influence the composition and distribution of immune cells within gut-associated lymphoid tissues in broilers [66]. In unimmunized chicks, early probiotic administration increased serum IgG and IgM responses to selected antigens and intestinal IgA responses, suggesting an influence on natural antibody production [67]. Because the window of colonization is transient, probiotic interventions may be particularly effective when applied before native microbiota achieves structural stability and competitive exclusion capabilities. Microbial colonization and rapid tissue expansion

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inherently induce a degree of inflammation, requiring tight mucosal regulation to prevent structural damage and energy loss [62, 65]. Reported cytokine responses to probiotics often vary depending on flock health status and sampling age. In early-life chicken models, selected *Lactobacillus* interventions are associated with reduced expression of inflammatory cytokines, including IFN-γ, IL-1β, and IL-8 [63]. Early microbial interventions are associated with improved intestinal villus morphology and goblet cell numbers in broiler chicks [68]. Nevertheless, faster microbial succession, higher microbial diversity, or lower expression of an inflammatory cytokine should not automatically be interpreted as improved development. Beneficial responses require interpretation relative to bird health, immune competence, and growth. Similarly, reduced inflammatory signaling may reflect altered immune regulation but does not necessarily indicate improved protection against infection.

As to layer-specific evidence, probiotic spraying of incubating eggs, followed by post-hatch dietary supplementation, improved embryonic development and growth-related outcomes in White Leghorn pullets up to 5 weeks of age [69]. During rearing, pullets commonly receive multiple vaccinations against economically important pathogens, including Newcastle disease virus (NDV) and, depending on regional vaccination programs, other viral and bacterial diseases. Probiotics are reported to modulate humoral and cellular immune responses in layer- and meat-type chickens [70]. Furthermore, a study involving 42-wk-old laying hens reported increased antibody titers against NDV and a reduced heterophil-to-lymphocyte (H/L) ratio with probiotics [71]. However, findings obtained from mature hens cannot be directly extrapolated to vaccine responsiveness during pullet rearing. The next step would be to evaluate vaccine-specific antibody titers alongside the H/L ratio to determine whether early probiotic supplementation influences immune responsiveness and physiological stress-related indicators in pullets.

An important frontier that warrants further attention would be looking into evidence of carryover effects into lay, even though it is appreciated that improved early-life indicators do not necessarily translate to subsequent performance improvements. Direct evidence linking rearing-phase probiotic supplementation to laying outcomes remains limited. In a study comparing early-life (1 to 18 wk), laying-phase (19 to 52 wk), and continuous probiotic supplementation, early-life supplementation alone was associated with higher laying rates during the early and peak laying periods [28]. Another long-term study evaluated the effects of continuous probiotic supplementation on production performance till 58 weeks of age [34]. However, neither study establishes whether the benefits of early-life intervention explicitly result in improved performance, and carry-over effects are suggested to be further investigated.

From a practical perspective, in-feed supplementation allows for repeated administration during pullet rearing, although probiotic viability during feed processing must be considered. Earlier interventions, including in ovo

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inoculation and spraying of incubating eggs, may influence initial microbial colonization, but efficacy in replacement pullets remains insufficiently established. For example, combined egg spraying and subsequent in-feed supplementation improved growth-related outcomes in pullets up to 5 wk of age [69], whereas studies involving in ovo administration in broilers and egg spraying in other chicken models have demonstrated changes in early microbial colonization and immune-related responses [63, 64]. Therefore, no single delivery method can currently be recommended as the most effective strategy for improving probiotic responses during growth to pre-lay. Future studies should directly compare delivery routes in layer-type pullets and evaluate performance-related outcomes, including body weight and composition, abdominal fat accumulation, age at first egg, early egg production and quality, skeletal health, and the potential persistence of probiotic effects after withdrawal.

Early to Peak-laying (~18 to 45 wk): Nutrient Utilization, Productive Performance, and Mineral Allocation

The transition into early and peak oviposition marks a major physiological shift in nutrient partitioning and allocation. Sexual maturation induces rapid functional enlargement of the oviduct, establishes a continuous ovulatory sequence, and elevates metabolic calcium demands two- to threefold to sustain eggshell calcification [28]. During this period, the gastrointestinal tract must process elevated daily feed intake to meet the competing demands of somatic maintenance, sustained egg production, and skeletal conservation. Accordingly, relevant outcomes during this stage appear to be mainly performance-related, including laying performance, nutrient utilization, mineral metabolism, eggshell characteristics, and albumen quality. Representative probiotic interventions and their reported physiological and production-related outcomes during this phase are summarized in Table 4. Hen-day egg production, egg mass, and FCR are practical measures for evaluating productive responses to probiotic supplementation during early-to-peak laying. Dietary *Bacillus*-based direct-fed microbials are often reported to enhance HDEP and egg mass while lowering FCR and the proportion of unmarketable eggs [18, 27]. *Pediococcus acidilactici* supplementation reduced downgraded egg percentage [33], whereas *Enterococcus faecium* supplementation improved productive performance while reducing excreta ammonia emissions [72]. These production responses, when observed, may be mediated in part by changes in intestinal microbial ecology, mucosal function, and nutrient utilization [12, 30].

Accordingly, a dual-strain *Bacillus subtilis* complex increased the AID of dry matter, gross energy, crude protein, Ca, and P in laying hens at 40 wk of age [18]. Notably, AID estimates the disappearance of measured nutrients before the terminal ileum [73] and does not account for endogenous nutrient losses, and should not be interpreted as a direct measure of true enzymatic digestion or intestinal absorption. In addition, the effects of *Pediococcus acidilactici*

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supplementation on productive performance and egg quality have been evaluated in laying hens fed diets with different energy densities [74], highlighting the importance of dietary conditions when interpreting probiotic responses. Although microbial enzyme activity, reduced digesta viscosity, and changes in intestinal morphology have been proposed as potential mechanisms, increases in AID alone do not give deeper mechanistic insight into nutrient utilization [12, 18, 22]. Understanding how probiotics influence nutrient utilization will require investigation to link nutrient digestibility responses to specific mechanisms while accounting for the composition of the basal diet.

Microbial SCFA production has been proposed as a potential link between intestinal fermentation and mineral metabolism [75], although increased cecal SCFA concentrations alone do not demonstrate enhanced intestinal calcium or phosphorus absorption. In laying hens, xylo-oligosaccharide supplementation was associated with increased cecal butyrate concentrations and circulating 1,25-dihydroxyvitamin D3 [76]. In a separate study involving 50-wk-old laying hens fed diets formulated with calcium and phosphorus levels approximately 10% below their requirements, *Pediococcus acidilactici* supplementation increased Ca and P retention and circulating calcitriol concentrations [77]. Composite probiotic supplementation has also been associated with stage- and intestinal segment-dependent changes in the mRNA expression of mineral transport-related genes, including transient receptor potential vanilloid 6 (TRPV6), plasma membrane calcium ATPase 1b (PMCA1b), and sodium-dependent phosphate transporter IIb (NaPi-IIb) [28], alongside altered ion transporter and eggshell matrix-related gene expression in the uterine shell gland [78]. However, while several studies have reported concurrent improvements in tibial characteristics following probiotic administration [18, 24], direct causal evidence confirming that microbial SCFAs mediate mineral transporter expression remains to be established in laying hens.

Improvements in intestinal mineral utilization, when demonstrated, may reduce reliance on skeletal mineral reserves but cannot eliminate the physiological requirement for medullary bone mobilization during eggshell formation. Eggshell mechanical properties, along with internal albumen and yolk characteristics, represent the final physical indicators of successful nutrient and mineral utilization. During peak lay, each egg requires approximately 2.0 to 2.5 g of calcium within a 16- to 20-h period [28, 77]. Probiotic interventions involving *Bacillus* spp. or *Pediococcus acidilactici* have been associated with improvements in selected eggshell traits and reductions in defective eggs [18, 33, 79]. Studies involving hens beyond 45 wk of age have also reported improvements in eggshell ultrastructural characteristics, including effective layer thickness and calcite column organization, following probiotic supplementation [79, 80]. Beyond eggshell quality, probiotic supplementation has been associated with variable Haugh unit responses [81] and improvements in albumen height [82], as well as alterations in circulating lipid profiles

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and egg yolk composition [18, 24]. However, the specific mechanisms linking gut microbial modulation, such as bile salt hydrolase activity or gut-liver metabolic signaling, to albumen synthesis and yolk lipid deposition remain underexplored. Additionally, a 52-d supplementation trial beginning at 200 d of age reported reductions in serum ALT, AST, and MDA concentrations, together with increased total antioxidant capacity, following supplementation with compound probiotics containing *Bacillus subtilis* (1×10^9 CFU/g), *Lactobacillus plantarum* M8 (1×10^9 CFU/g) and *Saccharomyces cerevisiae* [82]. Overall, the physiological relevance of supplementation during early-to-peak lay depends on whether changes in nutrient utilization support egg output and quality while maintaining skeletal health.

Mid to Late-laying (~45 to 100 wk): Intestinal Integrity, Hepatic Lipid Homeostasis, and Extended Laying.

From mid-lay onwards, age-associated physiological changes become increasingly relevant to hepatic lipid metabolism, antioxidant capacity, reproductive endocrine function, and egg quality [83, 84]. It is known that meeting the physiological requirements of extended laying and maintaining acceptable egg quality alongside skeletal preservation and metabolic resilience will depend on the capacity to maintain the health of tissues and organs involved in egg production and skeletal balance [14]. Table 5 summarizes five key outcome domains for evaluating probiotic responses during mid-to-late lay: hepatic lipid metabolism, serum biochemical markers of metabolic status, antioxidant defenses and oxidative damage, reproductive endocrine function, and the incidence of defective eggs. However, age-related physiological changes do not necessarily occur uniformly across tissues, limiting the use of chronological age alone to characterize physiological status and predict responses to probiotic supplementation. Hepatic lipid accumulation is a major metabolic concern during extended laying and is associated with alterations in lipid metabolism [84, 85]. However, hepatic steatosis should be distinguished from fatty liver hemorrhagic syndrome (FLHS), as hepatic lipid accumulation alone does not establish the presence of hemorrhagic liver pathology. Systemic antioxidant defenses (T-AOC, SOD, GSH-Px) also decline as lipid peroxidation products (MDA) accumulate, indicating an imbalance between reactive oxygen species generation and antioxidant capacity [83, 84]. In addition, aging lowers circulating E2, reducing the stimulus for medullary bone turnover and shell gland calcification [84]. These physiological changes underscore the importance of evaluating probiotic responses using metabolic, reproductive, and production-related outcomes rather than relying on individual biochemical indicators alone.

Hepatic triglyceride (TG) accumulation is an important metabolic alteration observed in aging hens, although its relationship with intestinal microbial changes and gut–liver communication remains underexplored. Specifically, 75-wk-old laying hens accumulated higher hepatic total TG and cholesterol than 28-wk-old birds, alongside lower

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circulating estradiol and depleted antioxidant enzymes [84]. By 83 wk of age, hepatic lipid synthesis declines alongside reduced serum 17β-estradiol, estrogen receptor expression, and antioxidant capacity, showing that late-phase yolk precursor formation is constrained by hormonal and oxidative factors [83]. Hepatic steatosis has been associated with alterations in intestinal microbial composition, suggesting a relationship between intestinal dysbiosis and hepatic lipid metabolism [85]. In a 45-d feeding trial beginning at 63 wk of age, supplemental *Lactobacillus rhamnosus* GG reduced serum free fatty acids and hepatic TG concentrations [80]. In hens aged 65–76 wk, *Akkermansia muciniphila* supplementation reduced abdominal fat deposition and serum and hepatic cholesterol concentrations, alongside changes in cholesterol synthesis-related gene expression [86]. Furthermore, a 12-wk feeding trial beginning at 50 wk of age reported reductions in circulating lipid concentrations and hepatic lipogenic gene expression (ACC-α and SREBP1) in selected groups receiving combined supplementation with quercetin and *Ligilactobacillus salivarius* [87]. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities are frequently measured as biochemical indicators of hepatic status in poultry; however, they should be interpreted cautiously as they do not sufficiently demonstrate improved hepatic health [84].

Beyond hepatic lipid metabolism, maintaining intestinal barrier function and limiting oxidative damage are additional priorities with age. Hens at 75 weeks of age exhibit increased MDA concentrations and reduced GSH-Px and SOD activities in both serum and liver, accompanied by transcriptional downregulation of Nrf2, NQO1, and SOD1 [84]. This hepatic oxidative imbalance parallels the decrease in circulating 17β-estradiol [83]. Increased oxidative stress may contribute to hepatic and ovarian dysfunction during aging. However, the extent to which oxidative damage directly mediates changes in yolk precursor synthesis and follicular development in laying hens remains to be established [83]. In a long-term study involving a composite probiotic formulation, serum T-AOC and T-SOD increased, whereas GSH-Px activity was lower in the supplemented group during peak and late lay; ovarian inflammatory gene responses also varied among production stages [34]. In 48-wk-old layers, *Bacillus subtilis* reduced duodenal MDA while upregulating occludin expression, suggesting a potential association between intestinal barrier integrity and oxidative status [29]. In a 16-wk trial beginning at 44 wk of age, supplementation with *Lactobacillus acidophilus* and *Pediococcus acidilactici* via drinking water improved antioxidant status and increased serum IgG concentrations [88], while synbiotics containing *Bacillus amyloliquefaciens* and inulin decreased ovarian oxidative stress and supported follicle survival in hens from 80 to 94 weeks of age [89].

Reproductive endocrine function provides another frontier for evaluating probiotic responses in late lay. Circulating estradiol (E2) coordinates the liver-blood-ovary axis by stimulating hepatic yolk precursor synthesis (ApoVLDL-II,

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VTG-II), upregulating renal 1α-hydroxylase to produce active 1,25-dihydroxyvitamin D3 for intestinal calcium absorption, and promoting shell gland cellular activity for biomineralization [90]. In older hens, circulating E2 declines, accompanied by lower hepatic estrogen receptor (ERα, ERβ) expression and reduced yolk precursor transcription [83, 84]. Estradiol is involved in the regulation of active vitamin D metabolism and calcium homeostasis in laying hens. An age-comparison study reported changes in the balance of estrogen receptor subtypes in the shell gland and reduced duodenal carbonic anhydrase activity, which coincided with changes in eggshell quality and bone strength [91]. Probiotics are reported to potentiate changes in circulating reproductive hormones and ovarian function in laying hens. A long-term feeding study extending to 58 wk of age reported increased circulating E2 during early lay, whereas E2 and FSH did not differ at later assessment [34]. Furthermore, supplemental *Bacillus amyloliquefaciens* and inulin were associated with increased reproductive hormone concentrations, improved ovarian antioxidant status, and changes in yolk precursor-related gene expression in hens as at 82 to 94 wk of age [89]. The same study also conducted a separate fecal microbiota transplantation experiment, providing additional evidence that microbial communities may contribute to reproductive responses. Nevertheless, the specific microbial metabolites and signaling pathways linking intestinal microbial changes to hepatic and ovarian function were not established.

Maintaining egg output and acceptable egg quality while reducing unmarketable egg rate remains a central practical outcome in extended laying. Eggshell quality and the incidence of defective eggs are economically relevant during extended laying and contribute to flock replacement decisions. In addition, age-related changes in eggshell quality and skeletal strength are important concerns during extended production. However, their trajectories are not necessarily linear. In one study, shell quality and bone strength had deteriorated by 49 wk of age but were subsequently maintained at comparable levels through 70 wk [91]. This pattern highlights the importance of identifying physiological factors associated with egg-quality maintenance rather than assuming an inevitable, continuous decline. Age-associated changes in intestinal physiology and microbial composition have also been linked to declining eggshell quality. A recent multi-omics study comparing young and older laying hens identified associations among *Ligilactobacillus salivarius*, microbial metabolites, host CALB1 expression, and eggshell quality [92]. In a feeding trial involving 450-d-old hens (approximately 64 wk of age), supplementation with *C. butyricum* and *B. subtilis* for 4 weeks reduced the proportion of sandpaper-shelled eggs from 42.51% to 28.02%, accompanied by improvements in ileal morphology and changes in intestinal microbial composition [26]. These findings suggest potential benefits for shell quality during later production, although they do not establish the underlying mechanism of the response.

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Conclusions and Future Perspectives

This review sought to discuss reported probiotic responses within the context of changing age-specific physiological demands of the laying hen from pullet development through late lay. It was determined that functional diversity of probiotic microorganisms provides a rationale for evaluating their application in relation to the changing physiological demands of laying hens. Selected strains are often reported to influence substrate utilization, intestinal microbial activity, barrier-related responses, and host metabolism at different stages of the laying cycle. However, benefits observed with one formulation or production stage cannot be assumed to apply to another. Reported responses are to be interpreted according to strain identity, dietary conditions, physiological status, supplementation timing, and exposure duration. Within this framework, it is suggested that stage-dependent physiological priorities should guide selection of targeted research outcomes with probiotic applications. During rearing to pre-lay, the principal question is whether intestinal and immune responses contribute to pullet growth and development, skeletal preparation, and subsequent onset of lay. During early-to-peak lay, nutrient utilization, productive performance, mineral allocation, egg quality, and skeletal preservation are central. During mid-to-late lay, intestinal integrity, hepatic lipid homeostasis, oxidative resilience, reproductive function, and egg-quality stability become increasingly important.

Translating these priorities into effective supplementation strategies requires distinguishing physiological responsiveness from the effects of exposure duration and continued administration. Studies comparing feeding schedules or following hens across stages provide useful evidence [28, 34], including reported benefits after withdrawal of early-life supplementation [28]. Future experiments should build on this evidence through controlled comparison of supplementation timing and duration, with post-withdrawal assessments to establish persistence. Where feasible, follow-up toward 100 wk should prioritize cumulative marketable egg production, egg mass, feed efficiency, shell defects, skeletal health, mortality, and economic return. Consistent reporting of strain identity, viable dose, product composition, delivery route, diet, and bird characteristics would strengthen comparisons across studies. Component treatments are also necessary to test additivity or synergy, while functional measurements linking microbial activity, metabolites, and host responses are needed to evaluate proposed mechanisms involving cross-feeding, mineral transport, and gut–liver–ovary communication. Physiologically targeted supplementation is a promising approach, but current evidence does not establish a program that consistently sustains benefits through 100 weeks of age. Its practical value requires long-term studies demonstrating that stage-specific responses translate into reproducible improvements in lifetime productivity, economic return, and hen health and welfare.

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Tables and Figures

Table 1. Structural characteristics, microbial products and components, proposed physiological mechanisms, and reported production-related responses of major microbial groups in laying hen nutrition

Microbial Group / Intervention Type	Representative Taxa	Technological / Structural Traits	Characteristic Microbial Products and Components	Key Modes of Action & Physiological Mechanisms	Target Layer Production Traits & Impacts	Key References
Spore-Forming Bacteria	*Bacillus subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, *B. coagulans*	Dormant endospores; highly resistant to feed pelleting heat (70–90 °C), steam, shear, and acid	Secreted enzymes: proteases, amylases, xylanases, and β-glucanases (strain-dependent)	NSP-degrading enzyme activity; potential reduction in digesta viscosity; reported changes in intestinal villus morphology	↑ Apparent ileal digestibility; ↓ FCR; ↑ Selected skeletal traits; ↑ eggshell thickness	[18, 22-24]
Lactic Acid Bacteria (LAB)	*Lactiplantibacillus*, *Limosilobacillus*, *Lacticaseibacillus*, *Pediococcus* spp.	Vegetative non-spore formers; heat-sensitive; require stabilization/encapsulation	Fermentation metabolites: lactate and, depending on the species and fermentation conditions, acetate	Potential modulation of intestinal luminal pH; pathobiont suppression; Potential modulation of mucosal barrier and immune-related markers	↓ Defective egg rates; ↓ Yolk cholesterol	[21, 31, 33, 93]
Butyrate-Producing Anaerobes	*Clostridium butyricum*	Endospore-forming, obligately anaerobic bacteria; spores may improve survival during feed processing and gastrointestinal transit	Fermentation metabolites: butyrate and acetate	Butyrate-associated epithelial support; reported changes in selected tight-junction and antioxidant-related markers	Study- and time-dependent changes in eggshell strength and Haugh units; ↓ Pimpled-egg rate	[26, 40, 42, 43]
Commensal Cross-Feeders	*Enterococcus* spp., *Bifidobacterium* spp., *Propionibacterium* spp.	Non-spore commensals; *Bifidobacterium* strictly anaerobic; require stabilization	Fermentation metabolites: lactate, acetate, or propionate (taxon-dependent); bacteriocins: enterocins (selected *Enterococcus* spp.)	LPXTG-mediated adhesion and enterocin production in selected Enterococcus strains; F6PPK-mediated fermentation in *Bifidobacterium*; propionate production and potential metabolic cross-feeding	Potential support of butyrate-producing communities; potential contribution to host substrate metabolism	[44, 46, 50]
Eukaryotic Yeasts & Fungi	*Saccharomyces cerevisiae*, *S. boulardii*, *Aspergillus oryzae*	Distinct properties of live fungal cells and nonliving cell-wall preparations; product-dependent	Cell-wall constituents: β-glucans and mannans; extracellular enzymes (fungal	Proposed redox-related effects of live yeasts; potential inhibition of type 1 fimbriae-mediated pathogen adhesion by MOS; extracellular fiber-	↑ HDEP and egg mass in selected yeast-supplementation studies; ↑ Eggshell	[52, 53, 58, 59]

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	processing tolerance	preparations, product-dependent)	degrading enzymes in selected fungal preparations	traits with fungal-fermented feed products

The listed microbial products and components include fermentation metabolites, secreted enzymes, bacteriocins, and structural cell-wall constituents. These categories should not be interpreted interchangeably as metabolic end products. Their occurrence and biological activities may vary depending on the microbial strain, preparation, and experimental conditions. Reported outcomes are specific to the tested strains, formulations, and assessment periods. Effects observed with mixed formulations should not be attributed to individual microbial components without appropriate comparative evidence. MOS, mannan-oligosaccharides; LAB, lactic acid bacteria.

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Table 2. Comparison of intervention timing and exposure duration in representative probiotic and related microbial-intervention studies involving laying hens

Ref.	Probiotic intervention	Age at intervention	Supplementation duration	Age at final assessment
[69]	*Lactobacillus* spp.	Pre-hatch / hatch	Egg spraying; in-feed up to 5 wk	5 wk
[28]	*Bacillus* + LAB + yeast	Day 1 or 19 wk, depending on group	1-18 wk, 19-52 wk, or continuous	52 wk
[34]	Composite probiotics	5 wk	54 wk	58 wk
[18]	Dual-strain *B. subtilis*	18 wk	22 wk	40 wk
[23]	*B. subtilis* DSM 29784	19 wk	30 wk	48 wk
[33]	*P. acidilactici*	22 wk	24 wk	46 wk
[24]	Multi-strain *Bacillus*	25 wk	12 wk	37 wk
[94]	Multi-strain *Bacillus*	25 wk	12 wk	37 wk
[82]	Compound probiotics	200 d	52 d	~36 wk
[86]	Live or pasteurized *A. muciniphila*	29 wk	18 wk	46 wk
[38]	*C. butyricum* / *Brevibacillus*	32 wk (enrolled at 30 wk)	12 wk	44 wk
[40]	*C. butyricum* + FOS	31 wk (enrolled at 30 wk)	12 wk	43 wk
[74]	*P. acidilactici*	32 wk	16 wk	47 wk
[72]	*E. faecium*	40 wk	6 wk	46 wk
[29]	*B. subtilis*	48 wk	9 wk	57 wk
[42]	*C. butyricum*	48 wk	10 wk	58 wk
[87]	*L. salivarius* + quercetin	50 wk	12 wk	62 wk
[80]	*L. rhamnosus* GG	63 wk	45 d	~69 wk
[26]	*C. butyricum* + *B. subtilis*	450 d	4 wk	~68 wk
[86]	*A. muciniphila*	65 wk	12 wk	76 wk
[89]	*B. amyloliquefaciens* + inulin	82 wk	12 wk	94 wk

Age at intervention refers to the age at the initiation of probiotic administration unless otherwise indicated. In studies involving multiple supplementation schedules, the intervention periods are reported separately. Approximate ages at final assessment were calculated from the reported experimental durations where necessary. Reference [28] reports the laying-phase assessment of a cohort previously supplemented during rearing. For References [38] and [40], hens were enrolled at 30 wk of age, and the approximate ages at the start of active supplementation were inferred after a 2-wk acclimation/feed transition period and a 1-wk feed transition period, respectively. Reference [86] includes two separate experimental cohorts involving a diet-induced fatty liver hemorrhagic syndrome model and older laying hens. LAB, lactic acid bacteria; FOS, fructooligosaccharides.

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Table 3. Summary of probiotic applications during the growing and pre-lay phase (Day 0 to 18 weeks) on intestinal physiology and immunocompetence in poultry

Target Domain	Experimental Model / Breed	Probiotic Taxa & Intervention Route	Dosage / Regimen	Primary Physiological & Immunological Responses	References
Microbial Succession	Broiler embryos and chicks	*Lactobacillus* cocktail	In ovo (ED 18), post-hatch, or combined administration	↑ Cecal *Lactobacillus*, ↓ *Klebsiella*, ↓ *Enterococcus*	[63]
Microbial Succession	Incubating eggs	*Enterococcus faecalis*	Egg spraying on embryonic days 12, 15, and/or 17	Earlier intestinal colonization by the administered probiotic and changes in jejunal microbial community development	[64]
GALT & Mucosal sIgA	1-d-old chicks	Direct-fed microbials (multi-strain)	In-feed post-hatch	↑ Intestinal sIgA, ↑ systemic natural IgG/IgM against novel antigens	[67]
GALT & Mucosal sIgA	Chicks (ED 12 to D 21)	Prebiotics and synbiotics	*In ovo* delivery	↑ Cecal tonsil T/B-cell infiltration, ↑ ileal T cells, ↑ mucosal sIgA	[66]
Cytokine Balance	Broiler embryos and chicks	*Lactobacillus* species	In ovo (ED 18), post-hatch, or combined administration	↓ Cecal tonsil IFN-γ, IL-1β, and IL-8 expression following early probiotic intervention	[63]
Mucosal Morphology	0 to 42-day-old chicks	Defined pioneer commensals (*Bacteroidia*, *Clostridia*)	Single oral gavage at hatch	↑ Jejunal VH (1.14–1.46×), ↑ ileal VH (1.17×), ↑ goblet cell density	[68]
Pullet Growth and Development	White Leghorn embryos and pullets (up to 5 wk)	*L. paracasei* and *L. rhamnosus*	Egg spraying during incubation; in-feed supplementation up to 5 wk	↑ Hatchability, ↑ hatchling weight, ↑ pullet growth and tibia measurements	[69]
Humoral and Cellular Immune Responses	Layer- and meat-type chickens	*Lactobacillus* spp.	Discontinuous low-dose	Modulation of humoral and cellular immune responses	[70]

ED, embryonic day; GALT, gut-associated lymphoid tissue; sIgA, secretory immunoglobulin A; VH, villus height.
Evidence derived from broiler and general chicken models is included to illustrate early-life microbial and immunological responses; however, these findings should not be directly extrapolated to long-term laying performance in replacement pullets.

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780 Table 4. Summary of probiotic applications during early and peak lay (~18 to 45 weeks) on productive performance, nutrient digestibility, and egg quality.

Target Domain	Breed / Age	Probiotic Taxa & Composition	Inclusion Level	Key Zootechnical & Physiological Response	Reference
Production & FCR	ISA Brown (25–37 wk)	*Bacillus*-based direct-fed microbials	5 g/kg feed	↑ HDEP, ↑ egg mass, ↓ FCR (weeks 32–35)	[94]
Production & FCR	Hy-Line Brown (40–46 wk)	*Enterococcus faecium* DSM 7134	0.01% in-feed	↑ HDEP, ↑ egg weight, ↓ excreta ammonia emissions	[72]
Production & FCR	Hy-Line Brown (18–40 wk)	Dual-strain *Bacillus subtilis* (597 & 600)	0.01% in-feed	↑ HDEP, ↑ egg mass, ↓ FCR, ↓ egg loss rate (weeks 29-40)	[18]
Nutrient Digestibility	Hy-Line Brown (18–40 wk)	Dual-strain *Bacillus subtilis* (597 & 600)	0.01% in-feed	↑ AID of DM, GE, CP, Ca, and P; ↑ ileal VH and VH: CD ratio	[18]
Mineral Transporter Expression	Lohmann Brown (1–52 wk)	Composite probiotics	In-feed	Stage- and intestinal segment-dependent changes in TRPV6, PMCA1b, and NaPi-IIb mRNA expressi	[28]
Shell Mechanical Traits	Hy-Line Brown (32–47 wk)	*Pediococcus acidilactici*	In-feed (M-LEDp and LEDp)	↑ Shell thickness, ↓ FCR with bioequivalence between the probiotic-supplemented low-energy diet (LEDp) and the unsupplemented moderately low-energy diet (M-LED)	[74]
Shell Mechanical Traits	Hy-Line Brown (22–46 wk)	*Pediococcus acidilactici*	PA1: 100→50 mg/kg feed (12 wk each); PA2: 100 mg/kg feed (24 wk)	↑ Eggshell thickness; ↓ downgraded eggs (39% and 52% relative reductions in PA1 and PA2, respectively)	[33]
Shell Mechanical Traits	Hy-Line Brown (18–40 wk)	Dual-strain *Bacillus subtilis* (597 & 600)	0.01% in-feed	↑ Shell breaking strength, ↑ shell thickness, ↑ tibia breaking strength & ash	[18]
Shell Mechanical Traits	Hy-Line Brown (25–37 wk)	B. subtilis (PB6 and FXA) + B. licheniformis (G3)	5 g/kg feed	↑ Eggshell breaking strength, color, and thickness	[24]
Egg Quality & Endocrine Responses	Tianfu green-shell layers (23 and 27 wk assessments)	B. subtilis + LAB + Saccharomyces spp.	10 g/kg feed	No significant difference in Haugh units; ↑ eggshell thickness and serum E2 during early lay; stage-dependent yolk cholesterol responses	[34]
Albumen Quality & Lipids	Hy-Line Brown (18–40 wk)	Dual-strain *Bacillus subtilis* (597 & 600)	0.01% in-feed	↑ Haugh unit, ↑ albumen height, ↓ serum TG/VLDL-c, ↓ yolk cholesterol	[18]
Serum Biochemistry & Antioxidant Status	Hy-Line Brown (29-36 wk)	*B. subtilis* + *L. plantarum* M8 + *S. cerevisiae*	0.5 g/kg feed	↓ Serum ALT, AST, MDA; ↑ T-AOC	[82]

781 AID, apparent ileal digestibility; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CP, crude protein; DM, dry matter; E2, estradiol; FCR, feed conversion
782 ratio; FSH, follicle-stimulating hormone; GE, gross energy; HDEP, hen-day egg production; MDA, malondialdehyde; T-AOC, total antioxidant capacity; TG, triglycerides;
783 VH, villus height; VH:CD, villus height-to-crypt depth ratio; VLDL-c, very-low-density lipoprotein cholesterol.

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Table 5. Summary of probiotic applications during mid-to-late lay (~45 to 100 weeks) on gut-liver axis, endocrine stability, and shell defects

Target Domain	Breed / Age	Probiotic / Microbial Intervention	Dosage / Form	Key Systemic, Hepatic & Terminal Reproductive Responses	Reference
Endocrine and antioxidant responses	Tianfu green-shell layers (5-58 wk; observations at 58 wk)	Composite probiotics	Continuous in-feed, 54 wk	↑ Serum T-AOC and T-SOD; ↓ GSH-Px during later laying stages; no significant late-lay E2 or FSH differences	[34]
Intestinal barrier and redox status	Lohmann Pink-shell (48-57 wk)	B. subtilis	In-feed, 9 wk	↑ Duodenal occludin mRNA; ↓ duodenal MDA	[29]
Lipid metabolism	Laying hens (50-62 wk)	L. salivarius + quercetin	Polyphenol–probiotic combination, 12 wk	↓ Serum TG; ↓ hepatic TG, TC, and ACC-α/SREBP1 mRNA expression in HD and BM groups	[87]
Hepatic lipid metabolism	Jinghong No. 1 (63-69 wk)	L. rhamnosus GG	In-feed, 45 d	↓ Serum free fatty acids; ↓ hepatic TG	[80]
Defective egg rate	Hy-Line (64-68 wk)	C. butyricum + B. subtilis	In-feed, 4 wk	↓ Sandpaper-shelled egg rate; ↑ ileal villus indices	[26]
Hepatic lipid metabolism	Hy-Line Brown (65-76 wk)	A. muciniphila	Lyophilized preparation, in-feed	↓ Abdominal fat and hepatic cholesterol indices	[86]
Ovarian and endocrine function	Hy-Line Brown (82-94 wk)	B. amyloliquefaciens + inulin	Dietary synbiotic (5, 10, or 15 mg/kg feed), 12 wk	High-dose BAI: ↑ ovarian antioxidant status, follicle numbers, and circulating reproductive hormones	[89]
Microbiota-mediated reproductive responses	Hy-Line Brown (560 d at enrollment; approximately 80-88 wk overall)	Microbiota from synbiotic-treated donors	FMT by oral gavage for 4 wk after preparatory treatments	Changes in egg production, ovarian function and yolk precursor-related gene expression	[89]

The reported ages refer to the actual experimental periods or assessment ages rather than the entire 100-wk production cycle. Reference [34] represents a longitudinal feeding study spanning rearing and laying stages. Reference [86] includes a separate diet-induced FLHS experiment involving younger hens (29–46 wk), which should not be conflated with the older laying-hen cohort. Reference [89] includes separate dietary synbiotic supplementation and fecal microbiota transplantation (FMT) experiments. These interventions should not be interpreted as equivalent. E2, estradiol; FMT, fecal microbiota transplantation; FSH, follicle-stimulating hormone; MDA, malondialdehyde; TC, total cholesterol; TG, triglycerides.

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