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Live Bacterial Prophylactics in Modern Poultry

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Commercial poultry farms frequently use live bacterial prophylactics like vaccines and probiotics to prevent bacterial infections. Due to the emergence of antibiotic-resistant bacteria in poultry animals, a closer examination into the health benefits and limitations of commercial, live prophylactics as an alternative to antibiotics is urgently needed. In this review, we summarize the peer-reviewed literature of several commercial live bacterial vaccines and probiotics. Per our estimation, there is a paucity of peer-reviewed published research regarding these products, making repeatability, product-comparison, and understanding biological mechanisms difficult. Furthermore, we briefly-outline significant issues such as probiotic-label accuracy, lack of commercially available live bacterial vaccines for major poultry-related bacteria such as *Campylobacter* and *Clostridium perfringens*, as well research gaps (i.e., probiotic-mediated vaccine adjuvancy, gut-brain-microbiota axis). Increased emphasis on these areas would open several avenues for research, ranging from improving protection against bacterial pathogens to using these prophylactics to modulate animal behavior.

Keywords: probiotics, live vaccines, food safety, immunology, chickens

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INTRODUCTION

Poultry animals like layers and broilers are some of the most critical food animals, with 90 billion tons of chickens meat being produced globally per year (1) and 290 eggs consumed per capita in the United States (2). Over the years, poultry have been domesticated to maximize particular functions like meat and egg production. Although selecting for greater weight gain and egg-laying rates has improved poultry productivity, specific selection for bacterial diseases resistance has not been pursued as diligently. This is problematic, as poultry animals are becoming increasingly at-risk for bacterial infections given the push for cage-free rearing [reviewed by (3)] and serve as major reservoirs for foodborne pathogens like *Salmonella* and *Campylobacter,* contaminating their products [(4); reviewed in (5)]. Furthermore, the emergence of antimicrobial-resistant (AMR) bacterial pathogens threaten poultry animals and humans health alike (6). Specifically, avian pathogenic *Escherichia coli* (APEC), *Pasteurella multocida,* and *Mycoplasma gallisepticum* are causal agents of disease and mortality in poultry animals, which have the potential to harbor AMR genes [(7–9); reviewed in (10)]. Additionally, chickens are common carriers of bacteria like *Salmonella* and *Campylobacter*, which reside as commensals in their gastrointestinal tract [reviewed in (11, 12)]. However, these bacteria are frequent contaminators of poultry products and cause gastrointestinal disease in human consumers [reviewed in (13–15)]. Even worse, these microbes can horizontally-exchange AMR genes with commensals or other pathogens (16–18). This has created a dangerous situation in which bacterial pathogens (chicken and human alike) may become

highly-difficult to treat with conventional antibiotics. Thus, cost-effective additives that can boost resistance to pathogenic and AMR bacteria are needed to further optimize both poultry health and productivity.

Among the strategies currently used to promote productivity in animal agriculture includes use of live microorganisms. This includes live bacterial vaccines, which are attenuated bacteria typically used to immunize animals against particular pathogens [reviewed in (19)], and probiotics, which are live, non-attenuated microbes that confer health benefits to the animal host (20). Probiotics are typically delivered via feed, although spray and intraocular administration are commonly-used to deliver live bacterial vaccines. Additionally, both live bacterial vaccines and probiotics can be cultured *in vitro*, which drastically lowers production costs [reviewed in (21); reviewed in (22)]. In this review, we will outline live bacterial vaccines and probiotics commercially-available in poultry, describing the peer-reviewed studies using these commercial products in poultry animals. Additionally, we discuss probiotic labels and reliability-concerns. Lastly, this review will discuss the potential for novel live vaccines, synergism between live prophylactics, and a possible role for live prophylactics in less-studied biological mechanisms such as behavior.

LIVE BACTERIAL VACCINES

General

The earliest recorded live bacterial vaccination was in 1884, where Spanish clinicians utilized a weak *Vibrio cholerae* isolate to combat cholera outbreaks (23). Techniques for purposefully-attenuating bacterial strains while maintaining immunogenicity have been improved, using targeted modifications at the genetic level (24). Concerns over virulence-reversion by live bacterial vaccines have driven researchers to develop antigen-based vaccines incapable of sustaining disease. However, the successful development of antigen vaccines with long-term efficacy is relatively rare, mainly due to evolutionary adaptations by pathogens (i.e., antigenic loss/drift, serotype diversity) and design, as antigens have much-fewer epitopes compared to live bacteria vaccines, limiting protection against multiple, antigenically-diverse strains of a certain pathogen (25). Thus, live bacterial vaccines provide a lucrative alternative that can circumvent many issues with vaccination in poultry.

Like their wild-type counterparts, live bacterial vaccines can be easily cultured *in vitro* with low input costs, providing an inexpensive means of manufacturing large quantities of vaccine vs. the protein extraction steps required for antigen-based vaccines (21). Therefore, these vaccines can simultaneously prevent disease caused by their wild-type parent bacterium as well as additional pathogens (bacterial, viral, etc.) because cross-reactivity or via genetic insertions of genes encoding foreign antigen (24), creating an avenue for broad protection unachievable by many prophylactics currently available. In this review, we summarize characteristics and peer-reviewed findings for commercial live *Salmonella enterica*, *Escherichia coli*, *Mycoplasma gallisepticum*, and *Pasteurella multocida* vaccines available for poultry in **Table 1**.

COMMERCIAL LIVE BACTERIAL VACCINES

Salmonella

Although *Salmonella enterica* induces inflammatory in the chicken gut at an early age (46–48), this bacterium can persist by restructuring the intestinal environment to promote immunological tolerance, allowing for asymptomatically-shedding via feces from poultry animals [reviewed in (11)], resulting in potential contamination of meat and egg products. Human consumption of these contaminated poultry products is one of the major routes of salmonellosis incidence in the United States (49, 50). Live *Salmonella* vaccines are typically delivered orally via spray or drinking water to reduce *Salmonella* load in poultry. To improve food safety, live *Salmonella* vaccines are augmented with genetic deficiencies to limit intestinal replication while maintaining high levels of immunogenicity (24), although serotype and genetic attenuations are important drivers of vaccine efficacy (51, 52). Furthermore, these vaccines can be readily-modified to carry exogenous antigens (53, 54), enabling protection against additional pathogens.

Megan® Vac-1 is a Δ*cya*Δ*crp S.* Typhimurium vaccine [parent strain Δ3761 or UK-1 (55)], genetically-attenuated to knockout adenylate cyclase (Δ*cya*) and cAMP receptor protein (Δ*crp*). These mutations reduce pathogenicity and persistence of this live vaccine in the intestine while maintaining high immunogenicity, as demonstrated by the decrease of a challenge *Salmonella* invasion and intestinal colonization in vaccinated layer pullets (26). However, the protection of this vaccine against *Salmonella* appears to be inconsistent. A previous study testing protection against a wild-type *S.* Typhimurium strain in broiler chicks found the Megan® Vac-1 only reduced challenge *Salmonella* load in one of the two challenge experiments, although the failure in the first experiment may have been related to *in ovo* antibiotic administration (27). Furthermore, the vaccine strain was frequently-recovered from internal organs and ceca of vaccinated birds (27), although sampled animals were only 1-week-old and thus are not representative of broilers at final slaughter. In support of this, Dórea and colleagues determined that Megan® Vac-1 significantly-reduced detection of *Salmonella* in commercial broiler carcasses, minimizing carcass condemnation (28).

Poulvac® ST (Zoetis) is another metabolically-attenuated *S.* Typhimurium strain with Δ*serC* (phosphoserine aminotransferase) and Δ*aroA* (3-phosphoshikimate 1-carboxyvinyltransferase) deletions. Despite these deletions, Poulvac® ST is still immunogenic, inducing anti-lipopolysaccharide IgA and IgY responses in intestinal washes at day 13 (29) despite a reduction in ileal macrophages and $CD4^+$ T cells (30). Furthermore, vaccinated broilers had reduced *S.* Heidelberg loads in the ceca when challenged at 21 days old (31). This response may have been facilitated by recruitment of intestinal $CD8^+$ T cells (30), which have been previously demonstrated to improve *Salmonella* clearance in chickens (56). However, this vaccine was unable to reduce *Salmonella* Heidelberg load in the ceca when challenged at 3 days (31). This may be due to serovar-specific, as Bailey and colleagues

TABLE 1 | Summary of live bacterial vaccines commercially available for poultry application.

Commercial live bacterial vaccine	Company	Species (strain), additional attenuations	Peer-reviewed findings
AviPro® Megan® Vac 1	Elanco	*Salmonella* Typhimurium, Δ*cya*Δ*crp*	Decreased *Salmonella* invasion and intestinal colonization (26) Vaccine efficacy may be inconsistent and recoverable from internal organs (27) Decrease *Salmonella* detection in commercial carcasses (28)
Gallivac® SE	Merial select	*Salmonella* Enteritidis, Δ*ade*Δ*his*	Induces IgA and IgY in intestinal washes; Reduced Typhimurium and Enteritidis in ceca and internal organs (29) Reduced intestinal macrophages and CD4+ cells but increased CD8+ cell recruitment (30) Reduced *S.* Heidelberg in ceca when challenged at 21 but not 3 days post-hatch (31)
Poulvac® ST	Zoetis	*Salmonella* Typhimurium, Δ*serC*Δ*aroA*	Reduced *S.* Typhimurium load in liver and ceca (32) Increased inflammatory gene expression in splenic cells (33)
Poulvac® *E. coli*	Zoetis	*Escherichia coli*, O78 serotype, Δ*aroA*	Reduced O78 APEC bacterial load in internal organs (34) Did not protect chickens against O1 APEC; spray administration had superior protection against O78 APEC challenge vs. drinking water (35)
MG TS-11	Merial select	*Mycoplasma gallisepticum* (TS-11 strain)	Protection against R-strain *M. gallisepticum* challenge without change in productivity (36, 37)
MYCOVAC-L®	Merck	*Mycoplasma gallisepticum* (Intervet 6/85 strain)	Improved vaccine viability in PBS vs. distilled water (38) Protective immunity against *M. gallisepticum,* vaccination at recommended-dose may reduce egg production (39)
Poulvac® MycoF	Zoetis	*Mycoplasma gallisepticum* (F strain)	Protection against *M. gallisepticum*-induced airsacculitis (40) Intraocular vaccination induces greatest immune response (41)
AviPro® MG-F	Elanco	*Mycoplasma gallisepticum* (F strain)	Protection against M. gallisepticum-induced airsacculitis (40) Lless antibody production vs. MycoF (42) induced superior immune responses vs. TS-11 and MYCOVAC-L® (43, 44)
M-NINEVAX®-C	Merck	*Pasteurella multocida* (M-9 strain)	Potent antibody response against *P. multocida* (45)
PM-ONEVAX®-C	Merck	*Pasteurella multocida* (PM-1 strain)	Protection against *P. multocida* and high antibody titer (45)

found that Poulvac® ST alone did reduce challenge *Salmonella* Typhimurium and Enteritidis invasion of internal organs and ceca colonization in 3 and 13-day-old chicks (29).

Unlike the previously-described *Salmonella* vaccines, Gallivac® SE (Merial Select) is a *S.* Enteritidis strain (Δ*ade*Δ*his*) developed via chemical mutagenesis. Similar to the other vaccines, Gallivac® SE can provide protection against non-Enteritidis serovars, as orally-delivered Gallivac® SE reduced *S.* Typhimurium burden in the liver and ceca up to week 71 in layer hens vs. unvaccinated hens (32). Although live *Salmonella* vaccines are normally given orally, intraocular administration of Gallivac® SE increased IFNΔ, IL-8, and iNOs production by splenic cells (33), suggesting that this vaccine is capable of inducing robust immune responses, which extend from mucosal barriers. Unfortunately, to the authors' knowledge, these are the only two peer-reviewed studies which investigated the immunological potential of Gallivac® SE *in vivo*.

Escherichia coli

One of the major drivers of mortality and carcass condemnation in poultry, APEC are a major problem in commercial production (57). In addition, APEC are characterized by the possession of large virulence plasmids that often carry numerous resistances to antibiotics and heavy metals [reviewed in (58–60)]. These plasmids can be horizontally-transferred to other gut commensals as well bacteria like *Salmonella* (61), making the reduction of APEC in poultry a major priority. Given their antigenic variability (62), vaccines with broad protection have proved problematic. Notably, APEC colonize the gastrointestinal tract as commensals (63, 64) and only cause colibacillosis when they translocate the lung epithelium upon fecal aerosolization (65, 66). Thus, orally-delivered live vaccines are a feasible strategy to reduce abundances of these microbes in the gut while also inducing systemic immunity for extraintestinal resistance.

As of this review, Poulvac® *E. coli* (Zoetis) is the only live *E. coli* vaccine for poultry on the market. Poulvac® *E. coli* has a O78 serotype and, similar to Poulvac® ST, is a Δ*aroA* mutant. When implemented in broilers, this vaccine increased the number of healthy carcasses and reduced collibacillosis of a O78 APEC field isolate compared to non-vaccinated controls (67). Similarly, Poulvac® *E. coli* decreased bacterial load of an O78 APEC in internal organs compared to non-vaccinated controls, possibly related to improvements in *E.coli* O78 antigen-specific IgY serum levels and splenocyte proliferation (34). However, this protection appears to be serotype-specific, as Poulvac® *E. coli* did not confer any protection against challenge with an O1 APEC serotype (35). Furthermore, route appears to be a major determinant of efficacy, as Poulvac® *E. coli* administered to broilers via drinking water did not confer any protection to an O78 APEC, whereas coarse spray-administration did (35).

Mycoplasma gallisepticum

The etiological agent of chronic respiratory disease and infectious sinusitis in poultry animals [reviewed in (68); reviewed in (69)], *M. gallisepticum* is a major cause of carcass condemnation, reductions in egg-laying efficiency and weight gain, and mortality

in commercial poultry (70–73). Given its antigen variability, details regarding its entire pathogenesis are unclear (74). Initially, *M. gallisepticum* binds to sialic acid residues on lung epithelial cells (75) and can cause damage high inflammatory damage locally (76) or in deeper lymphoid tissues like the bursa of Fabricius (77). Although birds at all ages are susceptible to this bacterium, immunocompromised birds are especially at-risk for infection (78). Currently, live vaccines are typically used to prevent *M. gallisepticum* infection in poultry.

MG TS-11 (Merial Select) is a live attenuated strain of *M. gallisepticum* that is delivered via intraocular route (i.e., eyedrop). Its complete genome sequence is available to the public (79). This vaccine strain can prevent development of clinical airsacculitis, peribronchitis, and interstitial pneumonia via R-strain *M. gallisepticum* challenge without reducing egg-laying productivity (36, 37). More recently, research groups have sought to improve the efficacy of the TS-11 vaccine. Muneta and colleagues found that a recombinant TS-11 expressing IFNΔ increased cellular immunity via increased splenocyte-IFNΔ production and a non-edematous infiltration of heterophils into the trachea mucosa (36). Furthermore, TS-304, a TS-11 derivative that expresses the cytadherence molecule GapA, was shown to be more efficacious than TS-11 at a lower dose (80) likely related to its ability to more-effectively improve tracheal barrier function (76).

MYCOVAC-L® (Merck) is an attenuated 6/85 strain of *M. gallisepticum*. Similar to TS-11, its complete genome sequence is readily-available (38). Typically delivered via spray, rehydration of the vaccine via distilled water (standard practice for many bacterial vaccines) results in much lower MYCOVAC-L® viability vs. resuspension in PBS (38). In addition, although vaccination dose at the manufacturer's recommendation confers protective immunity against virulent *M. gallisepticum*, egg production may be negatively-impacted. However, hens previously vaccinated with fifteen times the recommended dose did not exhibit any deficiencies in egg-laying efficiency and produced more antibodies (39), suggesting a greater inoculum concentration is needed to negate certain side effects of MYCOVAC-L®.

Poulvac® MycoF (Zoetis) is an F strain of *M. gallisepticum* typically administered via spray. Using spray, Evans and colleagues showed MycoF-vaccinated animals did not exhibit *M. gallisepticum*-induced airsacculitis compared to control animals (40). Similar to MYCOVAC-L®, resuspension medium prior to MycoF immunization had a major impact on viability and antibody production immediately post-vaccination (81). When given via intraocular route, MycoF demonstrated protection against spread of *M. gallisepticum* in a co-mingled poultry system (82), suggesting that different vaccination routes may deliver similar success. However, when delivered in the same study via eyedrop, nares, or orally, intraocular MycoF vaccination induced the greatest antibody response (41), although this study did not investigate differences in *M. gallisepticum* resistance *in vivo*.

Another F strain vaccine, AviPro® MG-F (Elanco) was similarly able prevent airsacculitis via *M. gallisepticum* challenge (40). Although recommended delivery is in drinking water, Evans and colleagues found that when delivered via spray, MG-F delivered similar protection against *M. gallisepticum* infection as MycoF. Additionally, MG-F induced less antibody production vs. MycoF at one and ten-times recommended dose (42). However, MG-F induced superior immune responses compared to TS-11 and MYCOVAC-L® live vaccines (43, 44), suggesting that these *M. gallisepticum* vaccines induce immune responses in a vaccine strain-specific manner.

Pasteurella multocida

In the 1880s, Louis Pasteur developed one of the earliest live bacterial vaccines by isolating avian *P. multocida*, the etiological agent of fowl cholera, and using old cultures for immunization (83). Although a commensal member of the oropharyngeal microbiota, *P. multocida* can become an opportunistic pathogen in the respiratory tract (84). If able to bypass the lung epithelium, it can induce a highly-lethal septicemia (i.e., fowl cholera), causing major economic losses in poultry production (85, 86), though turkeys are more-affected (85). Thus, wing-web immunization of live *P. multocida* vaccines, superior to bacterin-based vaccines for this pathogen (87), is the most common method of prophylaxes against this pathogen. Although the exact mechanisms for protection are somewhat unclear, these live vaccines can induce broad protection independent of serotype and lipopolysaccharide composition (88). However, to the author's knowledge, very little peer-reviewed research has been performed using these *Pasteurella* live vaccines.

M-NINEVAX®-C (Merck) is an M-9 vaccine strain used in vaccinating commercial turkey flock against *P. multocida* and in combination with other live vaccines (89). Of the few studies using this vaccine, Sharaf and colleagues found this vaccine induced a potent antibody response against *P. multocida* (45). Similarly, PM-ONEVAX®-C, a PM-1 strain, induces protection against *P. multocida* challenge *in vivo*, accompanied with a high antibody titer (90). Unfortunately, no peer-reviewed studies on these live vaccines have been performed in the last two decades.

PROBIOTICS

General

Probiotics are live microorganisms including bacteria (i.e., *Lactobacillus acidophilus*) and yeast (i.e., *Saccharomyces cerevisiae*) that are commonly supplemented in poultry feed to improve animal well-being through a variety of mechanisms. Probiotics have a variety of functions in host, which are mainly triggered by their outer membrane composition and metabolic outputs. In this section, we will discuss major classes of probiotics used in poultry and their general functions. Furthermore, we will summarize the findings of peer-reviewed studies using commercial probiotic products, organized by probiotics composed of a single class or mixture of classes (**Table 2**). This review will be limited to the effects of these commercial products on host immune function, productivity measures, and bacterial resistance (specifically, intestinal colonizers like *Salmonella*, *Campylobacter*, *E. coli,* and *Clostridium perfringens*). Given the limited focus on mechanisms with these commercial probiotics

TABLE 2 | Summary of commercial probiotics available for poultry application.

Probiotic class	Commercial product	Microbial taxa (per label)	Peer-reviewed findings
LAB	FloraMax®-B11	*Lactobacillus salivarius, Pediococcus parvulus*	Immunomodulation and reduced intestinal NFκB transcription (91) Reduced colonization of *S*. Enteritidis and improved barrier function (92) Reduced *S.* Enteritidis, *E. coli*, and *C. jejuni in vitro* (93) Improved gut morphology and decreased *Salmonella* (94) Increased weight gain, reduced *Clostridium perfringens* and necrotic enteritis-induced mortality (95) Sequencing methods yield different taxonomic identifications (93)
	Cylactin®	*Enterococcus faecium* NCIMB 1045	Improved body weight, reduced colonization of *Clostridium* spp. and *E. coli* in excreta and intestine; increased levels of lactate, short-chain and branched-chain fatty acids (96) No change in intestinal *S.* Enteritidis load (97)
Bacillus subtilis	GalliPro®	*Bacillus subtilis* DSM 17229	Improved performance and decreased ammonia emission (98) Reduced *Salmonella* colonization (99) Increased body weight, feed conversion, and crude protein liberation (100) Complete elimination of *Clostridium perfringens* colonization in ileum (101)
	CloSTAT®	*Bacillus subtilis* PB6	Increased body weight, feed intake; no change in ileal lactobacilli nor *Clostridium perfringens* (102) Reduced mortality against *E. coli* (103) Reduced *Clostridium perfringens* in ileum (102)
	Norum™	*Bacillus amyloliquefaciens* AM0938 *Bacillus amyloliquefaciens* JD17 *Bacillus subtilis* AM1002	*In vitro* reduction of *Salmonella*, *E. coli, Clostridium difficile* (104) Reduced gut leakage (105–107) Decreased necrotic enteritis lesions (105) Lower horizontal transfer (108) and liver translocation (104) of *E. coli*
Mixture	Lavipan®	*Lactobacillus casei* LOCK 0915, *L. lactis* IBB 500, *Carnobacterium divergens* S-1, *L. plantarum* LOCK 0862, *Saccharomyces cerevisiae* LOCK 0141	Limited colonization of *Campylobacter* and *Salmonella* Enteritidis (109) Improved villi width and surface area in duodenum, jejunum, and ileum (110) Reduced *Clostridium* and *E. coli* (96)
	PrimaLac®	*Lactobacillus acidophilus*, *L. casei, Enterococcus faecium, Bifidobacterium bifidum*	Limited colonization of *Salmonella* and *E. coli* (111)*, Campylobacter jejuni* (112), *Clostridium perfringens* (144) No changes in ceca lactobacilli (113, 115) Age-dependent alterations in immune gene expression via *in ovo* (116)
	MicroGuard®	*Bacillus licheniformis, B. megaterium, B. mesentricus, B. polymyxa, B. subtilis, Saccharomyces boulardii, Bifidobacterium bifidum, Lactobacillus acidophilus, L. bulgaricus, L. plantarum, Streptococcus faecium*	Improved broiler performance; reduced *Salmonella* Enteritidis and *E. coli* (117)
	Gro-2-Max®	*Lactobacillus acidophilus*, *Pediococcus pentosaceus, P. acidilactici, Bacillus subtilis, Saccharomyces cerevisiae*	Increase in intestinal Enterobacteriaceae (118, 119) although this finding is inconsistent (119) Adjuvant activity against *Salmonella* and APEC using live *Salmonella* vaccine (118) Reduced total triglycerides, low-density lipoprotein cholesterol; altered circulatory immune parameters (119) Label inaccuracy (*Saccharomyces pastorianus* vs. *S. cerevisiae*) (118)

in poultry, this review will outline observed outcomes in-general in peer-reviewed studies.

Lactic Acid Bacteria

Lactobacillus, *Enterococcus*, and *Pediococcus* are gut commensals and examples of lactic acid (i.e., lactate in the ionized form)-producing bacteria (LAB), which protect against pathogens by several mechanisms. LAB are frequently used by poultry producers in part due their ability to produce several digestive enzymes (amylases, chitinases, lipases, phytases, and proteases), which greatly enhance the digestive process and improve feed conversion [reviewed in (120)]. Lactate is the major product of sugar metabolism across all LAB (121). Lactate can inhibit pathogenic bacterial growth by lowering the pH of the intestinal environment (122) or directly by disturbing normal bacterial metabolism (123). Select LAB also produce inhibitory compounds like bacteriocins, which are bactericidal compounds that target specific microorganisms (124). LAB can also directly stimulate immune cells via secretory factors (125) and toll-like receptor stimulation (125, 126). Given this

wide array of functions, LAB are common components in several commercial probiotics used in poultry agriculture. Some examples of commercial LAB products for poultry animals are discussed below.

FloraMax®-B11 is a probiotic supplement composed of *Lactobacillus salivarius* and *Pediococcus parvulus*. Upon oral challenge with *Salmonella* Enteritidis, broilers fed FloraMax®-B11 showed reduced colonization of *Salmonella* Enteritidis, improved gut barrier function, and reduced percentages of heterophils, lymphocytes, eosinophils, and basophils of peripheral blood compared to control broilers (92). Given the role of immune inflammation in clearing intestinal *Salmonella* (127) and observed-reduction of circulatory immune cells, this suggests this product may have directly-reduced *Salmonella* load in the intestine. This mechanism of direct competition is supported in which each probiotic bacterium in FloraMax®-B11 directly-reduced *Salmonella* Enteritidis, *E. coli*, and *Campylobacter jejuni* growth *in vitro* (93). Although Prado-Rebolledo and colleagues did not investigate phenotypic changes in these immune cells, FloraMax®-B11 reduces intestinal gene expression associated with the NFκB complex and aldose reductase (91), suggesting this probiotic also reduces expression of inflammatory genes. In combination with the perinatal supplement EarlyBird (Pacific Vet Group USA Inc.), FloraMax®-B11 was shown to improve gut morphology and significantly decrease *Salmonella* recovery, incidence, and horizontal transmission to broiler chicks (94). Lastly, broilers supplemented with FloraMax®-B11 showed significant body weight gain, lower total *Clostridium perfringens* (the causal agent of necrotic enteritis), and lower necrotic enteritis-induced mortality when compared to control broilers after *C. perfringens* challenge (95).

Cylactin® is composed of a single LAB, *Enterococcus faecium* NCIMB 1045 (128). Implementation of Cylactin® to the diets of broilers has shown to have positive effects on average body weight, greatly-decreased counts of *Clostridium* spp. and *E. coli* in intestinal tract and excreta compared to controls, and had improved lactate production as well as short-chain and branched-chain fatty acids (96). However, Cylactin® alone did not reduce *Salmonella* Enteritidis load in the layer intestine (97). Although tested in a non-avian model, administration of Cylactin® in the diet of piglets showed significantly reduced mucus-adherent extraintestinal pathogenic strains of *E. coli* (129), suggesting that this probiotic could have direct effects on APEC found in the chicken intestine.

Bacillus

Similarly to LAB, *Bacillus* species secrete digestive enzymes that improve feed conversion and competitive exclusion, which limit the ability of pathogens to invade the host (130–132). However, *B. subtilis* specifically limits pathogen colonization by production and secretion of lipopeptides and other antimicrobial compounds, as 4–5% of a *B. subtilis* genome is devoted to the production of antimicrobials [reviewed in (133)]. In contrast to LAB, *B. subtilis* can form endospores (134), improving their survival in the harsh conditions of the intestinal tract and food preparation processes better than other probiotics (135, 136). *B. subtilis* has also been shown to alter the morphology of the intestinal tract via elevated villi height and increased villi height-to-crypt depths (137), increasing the surface area for nutrient absorption. Notably, the host immune response toward *B. subtilis* is driven based on whether it is in its metabolically-inactive (i.e., endospore) or active (i.e., vegetative) state, as T cell differentiation was driven toward inflammatory, intracellular T_H1 responses and extracellular T_H2 responses via sporous and vegetative *B. subtilis*, respectively (138). Thus, de-sporulation in the intestine is a critical factor that could have major consequences on the host immune response.

GalliPro® consists of a single strain, *B. subtilis* DSM 17229 which improved performance, and reduced ammonia emission from the excreta in broilers (98). GalliPro® has been shown to reverse loss of splenic mass in *Salmonella*-infected birds, although no immune parameters were changed when non-infected birds were fed this probiotic (67). Furthermore, GalliPro® increased the liberation of crude protein from the diet, consequently decreasing broiler feeding costs and increasing body weight and feed conversion ratios (100). However, this study did not show whether GalliPro® was directly involved in this liberation or indirectly through a shift in the microbiota. Addition of GalliPro® to feed reduced *Salmonella* in cecum samples and greatly reduced *Salmonella*-positive drag swabs when compared to control broilers (99). Lastly, GalliPro® facilitates complete elimination of *C. perfringens* colonization in the ileum of challenged birds (101).

CloSTAT® contains a single strain of *B. subtilis*, PB6. When included to the diet of *C. perfringens*-challenged broilers at 1×10^9 CFU CloSTAT®/g feed, these broilers had statistically increased body weight and feed intake counts compared to challenged broilers without probiotics (139). However, CloSTAT® supplementation did not significantly change bacterial load of lactobacilli nor *C. perfringens* in the ileal digesta (139).When investigating the mortality rates from *E. coli* challenge comparing broilers fed CloSTAT®, control, and antibiotic growth promoters, CloSTAT® showed reduction comparable to the antibiotic growth promoter (both significantly compared to control) (103). Similarly to GalliPro®, CloSTAT® also reduced *C. perfringens* colonization of the ileum upon challenge (102).

Norum™ is a direct-fed microbial culture that consists of two *B. amyloliquefaciens* strains (AM0938 and JD17) Addition of Norum™ has shown an increase in productivity parameters like body weight, body weight gain and feed conversion (105, 140). Norum™ greatly reduced the gut permeability and leakage of mucosal, immunological effectors like IgA into serum (105). In a necrotic enteritis model in which birds were challenged by *Salmonella* Typhimurium, *Eimeria maxima*, and *Clostridium perfringens* at days 1, 13, and 18–19 post-hatch, respectively, Norum™ significantly improved lesion scores (105). Lastly, *in ovo* administration of Norum™ to the feed greatly decreased the horizontal transmission of virulent *E. coli* and infection of broiler chickens during hatch, possibly through alterations of microbiota composition and community structure (108).

Bifidobacterium, *Saccharomyces*, and Multi-Species Probiotics

To the authors' knowledge, there are no commercial poultry probiotics solely-constituted of *Bifidobacterium* spp. However, *Bifidobacterium* spp. are widely used in combination with *Lactobacillus* probiotics (ex: PrimaLac®) and other combination products (ex: MicroGuard®). *Bifidobacterium* directly affects IgA secretion in the gut (141) as well as stimulates professional phagocytes and pancreatic elastase production via secretion of the serine protease inhibitor Serpin (142). This pro-inflammatory mechanism action suggests that *Bifidobacterium* Serpin-production is involved in the homeostasis of the gut microbiota. Additionally, *Bifidobacterium* spp. produces acetate and lactate, which are subsequently-used by microbial gut fermenters to produce butyrate and propionate (143). These two short-chain fatty acids (SCFAs) promote colonic regulatory T cell differentiation (144, 145) as well as increase bactericidal functions of intestinal macrophages (146). Furthermore, the high GC content of the *Bifidobacterium* genome interacts with TLR9 that is present on the surface of mammalian immune cells (141, 147), although it is not clear whether *Bifidobacterium* DNA has a similar effect on the avian analog TLR21 (148).

Although the scope of this review is live bacterial prophylactics, the eukaryotic *Sacchormyces* species *S. cerevisiae* and *S. boulardii* [although *S. boulardii* is arguably a sub-species of *S. cerevisiae* (149)] are widely-implemented in poultry probiotic mixtures (i.e., Gro-2-Max® and MicroGuard®, respectively) and thus will be briefly-mentioned. Despite these two species being highly-similar, *S. boulardii* has greater heat and acid tolerance vs. *S. cerevisiae*, making it more competitive in the gut microenvironment [reviewed in (150)]. Additionally, both *Saccharomyces* species increased SCFA production via shifts in the microbiome (151, 152). Furthermore, *S. cerevisiae* and *S. boulardii* can directly-eliminate pathogens via secretory antimicrobials (153, 154). However, only *S. boulardii* appears to possess membrane-associated inulin, which can agglutinate pathogens (155, 156).

Lavipan® consists of several LAB (*Lactobacillus casei* LOCK 0915, *Lactobacillus lactis* IBB 500, *Carnobacterium divergens* S-1, and *Lactobacillus plantarum* LOCK 0862, all at 1×10^9 CFU/g product) and *Saccharomyces cerevisiae* LOCK 0141 (1×10^7 CFU/g) and was shown to competitively exclude pathogenic bacteria such as *Campylobacter* spp. and *Salmonella* Enteritidis (109). This probiotic also improved villi morphometric parameters (i.e., villus width and surface area) of the duodenum, jejunum, and ileum compared to control group (110). Lavipan® supplementation also caused reduced *Clostridium* spp. and *Escherichia coli* when compared to the control broilers, which was increased with the addition of prebiotics (i.e., raffinose family oligosaccharides) (96), which are non-viable food components like that improve host health via direct modification of the commensal microbiota (157). Thus, adding prebiotics to commercial probiotic products may improve health outcomes in poultry animals.

PrimaLac® is composed of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Enterococcus faecium*, and *Bifidobacterium bifidium*, all at 1×10^6 CFU/g (158). The use of PrimaLac has been shown to limit the colonization of *Salmonella* and *E. coli* (111) as well as *C. jejuni* (115). However, this probiotic does not induce any changes in ceca lactobacilli (113, 115). Supplementation of this probiotic to broilers *in ovo* produced an upregulation of *iNOS*, crucial for improving macrophage-killing of bacteria, in the ileum at day-of-hatch. However, later time points observed PrimaLac®-mediated downregulation of immune genes encoding toll-like receptors, cytokines, and iNOS in the ileum and ceca tonsil (116). The addition of PrimaLac® to the feed of turkey poults reduced *Salmonella* colonization upon challenge when compared to the control birds (111). When compared to an antibiotic growth promoter and control groups, addition of PrimaLac® increased reduction of *C. perfringens*, as well as improved broiler performance (114).

MicroGuard® contains 11 microorganisms (*Bacillus licheniformis, B. megaterium, B. mesentricus, B. polymyxa, B. subtilis, Saccharomyces boulardii, Bifidobacterium bifidum, Lactobacillus acidophilus, L. bulgaricus, L. plantarum,* and *Streptococcus faecium*) (159). The addition of MicroGuard® to the commercial broilers increased final bodyweight, weight gain, high density lipoprotein, triglyceride, and antibody titers against Newcastle disease and avian influenza levels (117). The addition of MicroGuard® also limited colonization of both *Salmonella* Enteritids and *E. coli* due to the above mentioned mechanisms, competitive exclusion, and possibly the production of bacteriocins (117).

Lastly, Gro-2-Max® is a multi-species probiotic product containing LAB (*Lactobacillus acidophilus*, *Pediococcus pentosaceus, P. acidilactici*), *Bacillus subtilis*, and *Saccharomyces cerevisiae*. When comparing route and length of treatment, Gro-2-Max® supplementation via food or water had general physiological impacts like reduced total triglycerides, low-density lipoprotein cholesterol, circulating lymphocytes, and viral vaccine-specific antibody titers. Additionally, ceca *Enterobacteriaceae* levels were inconsistently increased or decreased by Gro-2-Max®, regardless the route of inoculation (119). Our team has recently demonstrated changes in chicken intestinal *Enterobacteriaceae* levels via Gro-2-Max®, with layers only fed Gro-2-Max® exhibiting increased *Enterobacteriaceae* fecal shedding compared to the control birds (160). Furthermore, layers showed increased resistance to both APEC and *Salmonella* Kentucky when fed with both live *Salmonella* vaccine and Gro-2-Max® (118), suggesting this probiotic has adjuvant activities.

FUTURE DIRECTIONS FOR LIVE PROPHYLACTICS

Although much progress has been made in protecting poultry against bacterial disease, the movement of poultry animals to cage-free facilities has driven an increase in bacterial infections [reviewed by (3)], which pose risks to both animal and human health. Although the previously-described commercial vaccines and probiotics are used in practice, there are emerging technologies and strategies to improve food safety that warrant

discussion. For the duration of this review, we will highlight issues in probiotic label-accuracy, novel yet non-commercial live vaccine strategies, and research gaps where the effects of probiotics and live vaccines are largely-understudied.

Probiotic Product Label Reliability

Although probiotics are widely-implemented in animal agriculture, label accuracy is a major concern that can drastically-influence product efficacy and health outcomes in poultry animals. More than 28% of the commercial cultures intended for human or animal use were misidentified at the genus or species level through rapid detection methods (161). Looking specifically at poultry probiotics, Redweik and colleagues use PCR to confirm the identification of all probiotic bacteria in Gro-2-Max® but detected *Saccharomyces pastorianus* (not *S. cerevisiae* as advertised) (160). Using four different methods to taxonomically-identify LAB present in FloraMax®-B11, Menconi and colleagues found that each method produced mixed results (93). Although 16S sequencing was the most accurate method used in this study, it is nearly impossible to speciate bacteria via 16S sequencing unless they are highly-characterized [reviewed in (162)], demonstrating its limited application. Using whole-genome shotgun sequencing is a far more accurate tool in addressing current labeling issues and false positive results for species not listed as components (163). Finally, another major concern is the accuracy of bacterial concentrations in these commercial products, as total viable cell counts often do not correspond with the concentrations given on the label (164). Altogether, it is imperative that researchers studying commercial probiotic activities in poultry verify label accuracy to improve repeatability.

Novel Live Vaccine Strategies

Although live vaccine technologies for *Salmonella*, APEC, *Mycoplasma gallisepticum,* and *Pasteurella multocida* are commercially-available for poultry animals, there is no commercial live vaccines for *Campylobacter* nor *Clostridium* available. *Campylobacter*, a major foodborne pathogen responsible for intestinal and extraintestinal disease in humans (165, 166), typically colonizes the chicken gut as a commensal (167). Despite several studies evaluating the use of whole-cell *Campylobacter* vaccines (168–171) and antigen-based vaccines (172–177), there is no vaccine commercially-available for *Campylobacter* reduction in the intestine. A major issue with orally-delivered, live *Campylobacter* vaccines may arise in distinguishing between vaccine and pathogenic strains during meat processing. To avoid this issue, one solution could be to use another vaccine strain that is genetically-modified to express conserved *Campylobacter* antigens. Several studies have explored the use of *Lactococcus lactis* (178), *Salmonella* (179), and *E. coli* (180) to carry these antigens for anti-*Campylobacter* immune development. However, a major limitation to using antigen-based strategies against *Campylobacter* is that they are highly, antigenically-variable between strains (181), making the identification of a conserved target difficult.

Although necrotic enteritis is a major cause of mortality and reduced productivity in young birds (182, 183), no vaccine is available against its causative agent *Clostridium perfringens*. Non-virulent *C. perfringens* can be used to promote intestinal immunity against pathogenic strains (184). Furthermore, *Salmonella* vaccines carrying recombinant *C. perfringens* antigens have been successful in potent protection against necrotic enteritis (185, 186). Thus, there is much potential for a live, oral vaccine that can protect against *C. perfringens*-induced necrotic enteritis, which might be further-improved through support with probiotics like Cylactin∆, GalliPro®, and CloSTAT® which, on their own, offer protection (96, 101, 102).

Research Gaps

Most studies evaluate probiotic and live vaccine-efficacy by comparing mono-treated animals vs. non-treated controls. While this experimental design is a crucial first-step in identifying the usefulness of a live prophylactic, this format is not representative of natural commercial conditions and it ignores the impact other vaccines, feed, etc. may have on the animal's response to that live prophylactic of-interest. This is of extreme-importance, as commercial farms routinely use a wide repertoire of prophylactics (live, inactivated, and subunit alike) on their poultry animals without knowing how they might improve or nullify each other's effects. Probiotics are widely-reported to serve as biological, vaccine adjuvants [reviewed in (187)]. However, the role of probiotics in vaccine-responsiveness is largely-understudied in poultry. As mentioned briefly, efficacy and weight gain of a live, recombinant *Campylobacter* vaccine was drastically-improved in broilers which were also given *Anaerosporobacter mobilis* as a probiotic (180). This improvement in vaccine response is even found for live vaccines outside the scope of this review. The protection against the eukaryotic pathogen *Eimeria* was highest when a live coccidiosis vaccine was combined with probiotics (188). Use of Gro-2-Max∆ in combination with a live *Salmonella* vaccine improved resistance to both intestinal *Salmonella* Kentucky colonization and extraintestinal infection by an O78 APEC (118). This latter study suggests that probiotics can even exert their benefits outside of the intestine, potentially through activation of immune phagocytes via TLR-dependent pathways (126). Another commercial probiotic (Cylactin®) also may be a useful vaccine adjuvant, as combining this product with the live *Salmonella* vaccine Gallivac® SE increased *Salmonella*-specific IgA in layers (97). Thus, it is imperative that future studies look at the synergistic-effect other prophylactics may have on one another. Given the expensive nature of trying to fully-model the spread of prophylactics used in poultry agriculture, one could feasibly use commercially-available birds already given their respective prophylactics prior to experimental treatment.

Although parameters such as weight gain, food-conversion, egg laying efficiency, and bacterial resistance are commonly-used to study prophylactic-efficacy, there are many other mechanisms in which these live microbes could affect the host. Gut bacteria play a major role in the maturation of the enteric nervous system (189) and mediate animal behavior via the gut-brain-microbiota axis (190–192). These interactions are largely-driven by the ability of probiotic bacteria, *Salmonella*, and *E. coli* to directly synthesize and respond-to neurochemicals

through a bidirectional communication network called microbial endocrinology (193–195). Animal models have demonstrated the ability for probiotics like *Lactobacillus* and *Bifidobacterium* (196, 197) as well as *C. perfringens* (198) to modulate behavior, although only the latter has been shown in chickens. Recently, a Δ3761-derived *Salmonella* vaccine and Gro-2-MaxΔ were shown to modulate gut catecholamine (but not serotonin) metabolism in layer pullets, depending if the live prophylactics were given individually or in combination (160). Altogether, these findings suggest that the prophylactics used may have a direct impact on animal behavior. Thus, a novel target for live prophylactics could be to manipulate poultry animals into exhibiting positive behaviors (feeding, dust-bathing) while mitigating negative social behaviors like pecking. However, a major consideration is whether effects of these live prophylactics on the gut-brain-microbiota axis are maintained by chickens with different gut microbiotas. Given the variability of the chicken gut microbiome due to factors like geographical location, litter, breed, and feed [reviewed by (199)], it is very possible that other commensal bacteria might nullify, reduce, or amplify the effects live prophylactics might have on animal behavior and neurochemical metabolism.

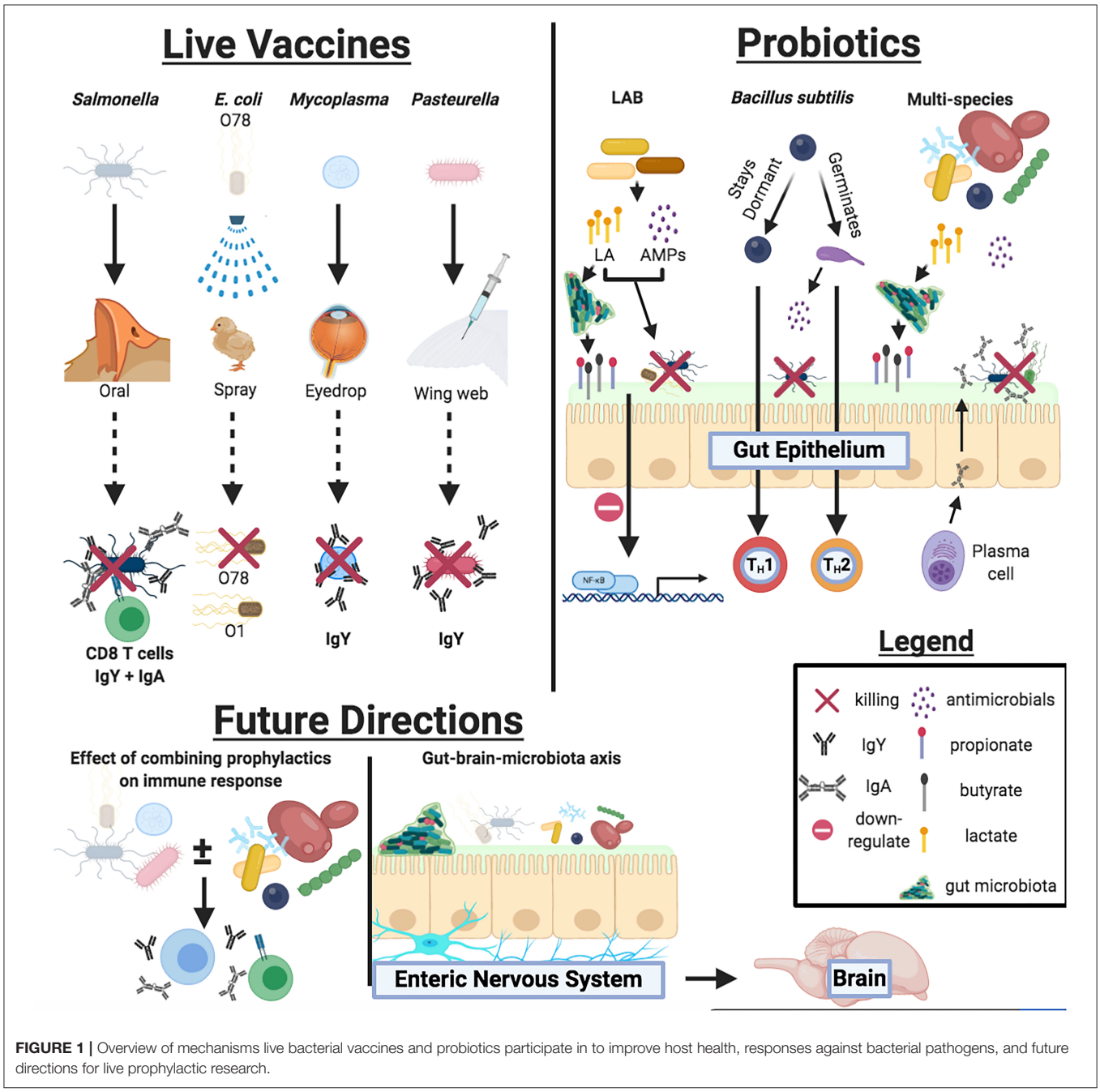

FIGURE 1 | Overview of mechanisms live bacterial vaccines and probiotics participate in to improve host health, responses against bacterial pathogens, and future directions for live prophylactic research.

CONCLUSIONS

Commercial live bacterial vaccines and probiotics offer several advantages in improving poultry health against bacterial disease and colonization (summarized in **Figure 1**) However, a paucity of peer-reviewed research studies, inconsistencies with product labels, limited cross-protection against certain pathogens, and a vague understanding of synergistic effects when using multiple prophylactics have encumbered our ability to optimize poultry health. Additionally, it is crucial that future studies must investigate whether these live prophylactics may facilitate animal behavior changes via the gut-brain axis (**Figure 1**), providing a convenient means of improving social behaviors among poultry flock.

AUTHOR CONTRIBUTIONS

GR and JJ wrote manuscript and developed figures and tables. MM revised manuscript. GR and MM provided funding and conceptualized review topic. All authors contributed to the article and approved the submitted version.

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