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Novel strategies for *Salmonellosis* **risk reduction in poultry.**

Saima Ansari¹ , Vaishali Nirmalkar² , Rasika Pawar1*

¹Department of Microbiology, Smt. Chandibai Himathmal Mansukhani College, Ulhasnagar, District: Thane- 421003, Maharashtra, India. ²Department of Botany, K.M.E Society's G.M. Momin Women's College, Bhiwandi, District: Thane- 421302, Maharashtra, India. *Department of Microbiology, Smt. Chandibai Himathmal Mansukhani College, Ulhasnagar, District: Thane- 421003, Maharashtra, India. Phone: + 91- 9869118328. E-mail: rasikapawarchm@gmail.com

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ABSTRACT:

Poultry farming is one of the fastest-growing sectors in India. Poultry immunity, health, and production are the variables that challenge the future growth of the poultry industry. Consumer confidence, product quality and safety, types of products, and the emergence and re-emergence of diseases will continue to be significant challenges to the current situation and the strategic future of the industry. Poultry farming faces substantial challenges related to maintaining high levels of immunity and health among birds, directly impacting production and consumer confidence. The industry must address the emergence and reemergence of diseases to ensure the quality and safety of poultry products for consumers. Foodborne and zoonotic diseases are strictly linked with poultry. Eradication, elimination, or control of foodborne and zoonotic pathogens is extremely important in the poultry industry. *Salmonellosis* is a common foodborne disease affecting humans and animals. *Salmonella's* incidence in poultry significantly impacts the economy and growth of poultry farming. In this article, we shed light on the importance of exploring alternative interventions, such as probiotics, to combat antibiotic-resistant *Salmonella* infections in poultry, enhancing bird health and consumer safety. Implementing strategic measures to reduce *Salmonella* prevalence improves public health outcomes and contributes to the overall success and resilience of the poultry industry in India. These interventions are primarily used to control antibiotic-resistant *Salmonella* colonization in poultry food. Effective control of foodborne and zoonotic pathogens like *Salmonella* is crucial for the economic sustainability and growth of poultry farming in India.

 Keywords: Chicken, *Salmonella,* Probiotics, Poultry.

INTRODUCTION

Ensuring the quality and safety of animal products is a crucial focus for the poultry industry, shaping its strategic future. Disease control, high productivity, high-quality products, and low prices are the goals of current poultry farming. (Hafiz and Atiya, 2020). However, animal-derived bacteria (Sweeney et al., 2018), primarily transmitted via food or via direct contact with animals, have become problematic (Marshall & Levy, 2011). Some food-borne bacteria, such as *Salmonella typhimurium* or *Escherichia coli*, isolated from chicken intestines are threat to food industry (Castellanos et al., 2017).

In 1885, American scientist Daniel E. Salmon isolated intestinal bacteria from the intestines of pigs. In 1900, French bacteriologist Joseph Leon Marcel Lignères proposed naming cholera *"Salmonella"* in honor of Daniel Salmon (El-Saadany et al., 2022). *Salmonella* belongs to the Enterobacteriaceae family. *Salmonella* is a Gram-negative, facultative anaerobic, rod-shaped, non-spore-forming bacterium (Gut et al., 2018). *Salmonella* is catalase-positive and oxidase, negative. It hydrolyzes urea using citrate as the sole carbon source (Vijaya et al., 2018). Contaminated water, soil, and wastewater are sources of *Salmonella* (Li et al., 2013). Animal products contaminated with *Salmonella* are responsible for 3% of foodborne illnesses worldwide, infecting 80 million people and killing 155,000 humans (Abd El-Hack et al., 2021a). Non-typhoidal *Salmonella*, *Salmonella enterica* serovar *Typhimurium*, is commonly found in food source all over the world (Havelaar et al., 2015) and has the most significant impact on human health compared to other contaminated foods (Kirk et al., 2015Scallan et al., 2015). A recent study by the World Health Organization's Foodborne Disease Burden Epidemiology Reference Group also found *Salmonella enterica* serovar *Typhi* to be the pathogen responsible for most deaths from foodborne illness (Havelaar et al., 2015). A recent literature study investigated the characteristics of some probiotic *Enterococcus* strains (Laukova et al., 2008a; Laukova et al., 2008b) and the production of bacteriocins of these strains thought to prevent *Salmonellosis* (Simonova & Laukova, 2007). Poultry birds do not necessarily get infected by eating food contaminated with *Salmonella*. However, humans get infected by consuming contaminated chicken carcasses with *Salmonella* (Wibisono et al., 2020). This article focuses on the risk of *Salmonellosis* in poultry and the use of probiotics in its treatment.

Indian Scenario

Salmonellosis in India is a prevalent disease affecting humans and animals. *Salmonellosis* in poultry is not endemic but is on the rise and significantly impacts the economy and growth of poultry farming. The incidence of *Salmonellosis* in India is high but has not yet been fully reported due to the limited number of laboratories monitoring the birds (Rajagopal & Meaney, 2013).

Classification of *Salmonella* **species**

The genus *Salmonella* is split into two species, *Salmonella enterica,* and *Salmonella bongori*, based on differences in temporal analysis. The intestinal *Salmonella* group is divided into six subspecies: *Salmonella enterica subsp. enterica, Salmonella enterica subsp. Salamae, Salmonella enterica subsp. Arizona, Salmonella enterica subsp. Diarizonae, Salmonella enterica subsp. Houtenae* and *Salmonella enterica subsp. Indica* (Lan et al. 2009). Kauffman and White's phylogenetic-based subspecies classification provides additional concepts for classifying *Salmonella* into serotypes based on the significant antigenic determinants: somatic (O), capsule (K), and flagella (H). Heat-stable somatic O-antigen is an oligosaccharide component of lipopolysaccharide found in bacterial membranes. Heat-labile H antigens are found on bacterial flagella. Surface K antigen is a heat-sensitive polysaccharide found on the surface of the bacterial capsule and is the most common antigen for *Salmonella* serotypes (S.K.Eng et al., 2015). *Salmonella* species specific to avian hosts include *S. gallinarum* and *S. pullorum*. *Salmonella* infection is usually transmitted from chickens via the fecal-oral route by ingesting contaminated food or water (Ferrari et al., 2019). Contamination occurs in two ways: direct contact with animals and the environment or indirect contact via food (e.g., milk, eggs, and meat) with contaminated products (e.g., stainless steel, milk jugs, and knives) (Kebede et al., 2022).

Diagnosis

Water and feed samples can be collected and tested for the presence of *Salmonella*. Sterile Cotton swabs samples are collected from laying cages, breeder nests, or hatchery machines. Rapid *Salmonella* detection techniques including Widal tests, enzyme immunoassays,

antigen capture assays, DNA probes, and immunofluorescence are routinely used for detection. (www.poultrydvm.com)

Salmonella **antibiotic resistance**

Certain bacterial species, like lactic acid bacteria, carry resistance genes and might serve as reservoirs of resistant genes for enteropathogens. Namely, tetracycline, vancomycin, and erythromycin resistance genes have been identified. Alternatives like prebiotics, probiotics, and essential amino acids (Abou- (Kassem et al., 2021a; Arif et al., 2021; Alagawany et al., 2021a), polyphenols, biologically synthesized nanoparticles, organic acids, essential oils, (Saad et al., 2021a,b), bioactive peptides (El-Saadony et al., 2021a,b; Saad et al., 2021c), herbal extracts (Abou-Kassem et al., 2021b; El-Saadony et al., 2021c), enzymes (Llamas-Moya et al., 2019), bioactive plant compounds (El- Saadony et al., 2021d; Reda et al., 2021a), and phytogenic compounds (Abdelnour et al., 2020a,b; Ashour et al., 2020, 2021; Abd Elkader et al., 2021; Abdel Moneim et al., 2021; Abd El Hack et al., 2021c,d) are used to improve poultry performance and human health using safe and natural products. Probiotics are generally derived from lactic acid bacteria isolated from fermented dairy products, sausages, and raw meat products, including poultry, beef, and pork (V.T. Nair et al., 2018). Antibiotic-resistant *Salmonella* cause the contamination of the farm environment and water systems (H.Y. Done et al., 2015). The use of fecal waste as manure in agricultural lands can be a reason for the spread of antibiotic resistance species of *Salmonella.* Antibiotic-resistant foodborne pathogens, namely *Salmonella, E. coli,* and *Shigella,* have often been recovered from fresh products that are available locally (S. Liu et al., 2017). Using pesticides causes soil contamination with livestock feces, and the spraying or irrigation of contaminated water causes the spread of resistant bacteria to fruits, vegetables, and fresh produce (V.T. Nair et al., 2018).

Antibiotic Alternatives against *Salmonella*

Many interventions have targeted antibiotic-resistant *Salmonella*. However, it is understood that the interventions used against non-antibiotic-resistant bacteria could work equally well against resistant *Salmonella*. Here are a few interventions that could attack antibioticresistant *Salmonella. Salmonella* is mainly treated with antibiotics, including ciprofloxacin, ceftriaxone, and ampicillin (Obaidat & Stringer, 2019). This antibiotic resistance is

responsible for the failed treatment of *Salmonella* in clinics, resulting in high mortality and morbidity. Overuse of antibiotics is also associated with gut dysbiosis and induces other disorders, such as inflammatory bowel disease or allergies (Schulfer et al., 2018). Consequently, probiotics have been identified as promising solutions in *Salmonella's* preventive and therapeutic treatment. Nowadays, the world is directed to limit the usage of antibiotics. Hence use of probiotics is considered safe as they have many benefits for humans and animals (Hill *et al*., 2014). Many probiotic strains also inhibit *Salmonellosis* (Adetoye *et al*., 2018; Pradhan et al., 2019).

Direct-Fed Microbials

The use of probiotics in poultry has steadily increased due to the growing demand for antibioticfree poultry and their well-documented benefits. By 2018, the probiotics market in poultry had reached 80 million USD, with projections indicating growth to 125 million USD by 2025 at a compound annual growth rate of 7.7% (Ahuja K. *et al.*, 2021). Benefits of probiotics in poultry include enhanced growth and laying performance, improved gut health, boosted immunity, and increased beneficial microbiota. Probiotics for livestock are termed direct-fed microbials or DFM. They are introduced into poultry via diets or water or administered to developing embryos through in ovo feeding technology (Pender *et al* 2017). DFMs, as feed additives containing beneficial microbes, they complement antibiotic usage, restore gut functions, and enhance animal performance (Bernardeau *et al*., 2013). They have gained popularity due to their ability to prevent bacterial gut infections and modulate immunity (Lee *et al*., 2017). In contrast, probiotics offer a wider range of benefits as functional foods, reducing disease risk and promoting overall health (Roberfroid et al., 2002). Commercial probiotic products often contain multiple strains to provide synergistic effects, with commonly used genera including *Bifidobacterium, Lactococcus, Lactobacillus, Bacillus, Streptococcus,* and yeast such as *Candida*. Selection criteria for probiotic strains include their tolerance to gastrointestinal conditions, adhesion ability to the gastrointestinal mucosa, and competitive exclusion of pathogens (Gadde *et al*., 2017). Additionally, probiotics are chosen based on their survival during manufacturing, transportation, storage, and application processes, ensuring viability and desired characteristics (Bajagai *et al*., 2016). Probiotics such as *B*. *subtilis*, *Lactobacillus* strains, *Saccharomyces* (probiotic yeast), and *Aspergillus oryzae* have antimicrobial properties against pathogenic bacteria such as *Salmonella spp*. (V.T.Nair *et al.*, 2018).

Impact of Probiotics on Gut

In chickens, *Salmonella* first attaches to the cecal epithelial cells and then spreads to the liver, spleen, and oviduct. In pigs, early *Salmonella* infection disrupts microbiome composition and functionality, principally at the ileum, and in humans, it may affect different sites (Argüello *et al*., 2018). Consequently, probiotic strains may work at different locations (jejunum, ileum, colon, and cecum) in different hosts. Genetically modified *L. casei* promotes overall bacterial species diversity and increases the abundance of *Lactobacillus* and *Bifidobacterium* in the cecum in mice (Peng *et al*., 2019). Probiotics act with different mechanisms of action in their host, such as competitive exclusion, the release of bacteriostatic and bactericidal agents, immune modulation, lowering intestinal pH, and improvement of the intestinal mucosal barrier (Alagawany *et al*., 2021b; El-Saadony *et al*., 2021).

Probiotics play significant roles as normal commensals. They exert their therapeutic properties against *Salmonella* in four ways (Gut et al., 2018). Firstly, they protect the tight junction in the gut and modulate the host's innate and acquired immunity (Pedicord et al., 2016; Thiemann et al., 2017). Second, they directly compete with *Salmonella* for niches and nutrients (Lam & Monack, 2014). Third, they produce antimicrobial molecules which inhibit *Salmonella* (Garcia-Gutierrez et al., 2019). Fourth, they regulate *Salmonella's* virulence by regulating the expression of virulence genes (Tanner et al., 2016).

Probiotics can be regulators, producers, and residents after being administered. As a modulator, it effectively regulates either the host or the pathogen. *L. casei* modulates host immunity by regulating the expression of intestinal inflammation-related cytokines (Peng et al., 2019, Muyyarikkandy & Amalaradjou, 2017). As producers, probiotics produce metabolites that may be signals to stimulate host immunity or substances to inhibit *Salmonella* colonization and growth. For example, *L. pentosus* AT6 and its cell-free culture supernatants inhibit *Salmonella* growth, adhesion, and invasion (Liu et al., 2018). As residents, probiotics can reduce *Salmonella* through physical repellence and colonization resistance (Ubeda et al., 2017).

Selecting the Right Probiotics

Probiotics can be either prokaryotes or eukaryotes. Species belonging to *Bacillus spp., Bifidobacterium spp., Clostridium spp., Escherichia coli Nissle 1917, and Lactobacillus spp., yeast,* are most commonly used (Kanmani et al., 2013). Conventional probiotics, such as *Bifidobacterium spp. and Lactobacillus spp.,* harbor the most well-characterized probiotic strains and are widely commercialized. These probiotics reduce more than 90% of caecal *Salmonella* load and prevent invasion of organs (Gut et al., 2018). In contrast to conventional probiotics, several next-generation probiotics have recently been identified, such as *Akkermansia muciniphila, Eubacterium hallii, and Faecalibacterium prausnitzii* (Almeida *et al.*, 2019).

Genetically modified probiotics have attracted much interest due to their advantages and strengthened effects (Barra et al., 2020). Two approaches are used to construct genetically modified probiotics: mutation and overexpression. Considering the infection stages of *Salmonella*, probiotic mutants with high adaptability may be more suitable for prevention and specifically targeted overproduced probiotics may be more effective in therapy. For example, the expression of microcin H47 in probiotic *E. coli* inhibits *Salmonella* growth (Palmer et al., 2018). Genetically modified probiotics may be the best option for *Salmonella* intervention. Synthetic biology explores diverse biosynthetic pathways and provides versatile engineering toolboxes for probiotic strain improvement (Yadav & Shukla, 2020). These toolboxes consist of genetic circuits, different delivery systems, and many genomeediting tools, which strongly accelerated the development of advanced, genetically modified probiotics (Bober et al., 2018; Ozdemir et al., 2018). However, a newly developed strategy called inducible plasmid self-destruction provides a novel genome-editing tool for simple gene knockout and knock-in in *Lactobacilli* and *Bifidobacteria* (Zuo et al., 2019). All these advances will strengthen the prophylactic and therapeutic activity of genetically modified probiotics on *Salmonella*.

How Should Probiotics Be Formulated?

According to Almeida et al., formulating viable probiotics while enabling cost-effective biomass yield is critical for product development in translational applications (Almeida et al., 2019). Regarding *Salmonella* prevention, conventional probiotics formulated as foods or drinks containing 10^6 CFU/g or 10^6 CFU/mL viable cells may be acceptable. However, nextgeneration or genetically modified probiotics are more promising in curing *Salmonella* infections. Probiotics can be formulated as concentrated pills or capsules containing more than 10^9 CFU/g or even more cells (O'Toole et al., 2017).

Overview of some critical probiotics studied for the reduction of

Salmonellosis

Lactobacillus reuteri

L. reuteri produces lactic acid, ethanol, acetate, hydrogen peroxide, carbon dioxide, reuterin, and retericylin (Yu et al., 2007). All the above biomolecules are implicated in the inhibition of the growth of *E. coli, S. typhimurium, Helicobacter pylori, Staphylococcus epidermidis, Staphylococcus aureus,* and Rotavirus (Seo et al., 2010). Additionally, chickens supplemented with 10⁸ CFU/mL *L*. *reuteri* revealed reduced lesion scores in birds infected with *C*. *perfringens*, suggesting that *L*. *reuteri* can modulate the innate immune response by regulating inflammatory cytokines and inhibiting *C. perfringens* proliferation (Cao et al., 2012).

E. coli Nissle **1917**

Forkus et al reported on technology to reduce *Salmonella enteritidis* in poultry by using *in vitro* experiments and an animal model of 300 turkeys. They engineered the probiotic *E. coli* Nissle 1917 to express and secrete the antimicrobial peptide. *Salmonella* was rapidly cleared from the ceca of the birds administered with the modified probiotic compared to the treatment groups (Forkus B.*et al.,* 2017).

Pediococcus acidilactici

P. acidilactici is a facultative anaerobe (Lin et al., 2008). *Pediococci* suppresses enteric pathogens' growth by producing lactic acid and bacteriocins as pediocins. Dietary supplementation with *Pediococcus acidilactici*, mannan-oligosaccharide, and butyric acid reduces colonization of *S. typhimurium* and improves broiler chickens' growth performance (Jazi et al., 2018).

Enterococcus faecium

E. faecium is a gram-positive, facultative anaerobe, and *E*. *faecium* secretes bacteriocins (Kang & Lee, 2005). According to Beirao et al., after vaccination, *E*. *faecium* improves layer chickens' immune and health status with live attenuated *S*. *Enteritidis* vaccine. (Beirao et al., 2018).

Lactococcus lactis subsp. lactis.

Likewise, Sabo et al., 2020 isolated *L. lactis* subsp. *Lactis* from the chicken cecum and screened for their antagonistic effect towards gut pathogens. The study demonstrated a high potential of the strain to be used as probiotics in poultry feed, a valuable alternative to replace use of antibiotics against *Salmonella heidelberg* (Sabo, S.d.S., et al.,2020).

Bifidobacterium animalis

B. animalis is an anaerobic, Gram-positive, rod-shaped bacterium found naturally in the intestines of rabbits, chickens, and humans (Sanchez et al., 2007). Incorporating *Bifidobacteria* and *Lactobacillus* with broiler feed reduces the colonization of infected *Salmonella enterica* and improves broiler health (Sharkawy et al., 2020).

Lactobacillus fermentum **1.2133**

According to Wang et al, 2023, *Lactobacillus fermentum* 1.2133 has antibacterial activity against *Salmonella pullorum* CVCC533. Also, the cell-free supernatant of *Lactobacillus fermentum* 1.2133 has bactericidal effect on the *Salmonella pullorum* CVCC533, a biofilm forming pathogen. It significantly reduced the number of *Salmonella* and aerobic bacteria in the chicken duodenum, ileum, and cecum, including *Escherichia and Shigella*, and improved *Lactobacilli* count in the gut (M. Wang et al.,2023).

Synergistic effect of lactic acid bacteria against *Salmonella* **infection in chicken**

Upadhaya et al, reported the beneficial effects of locally isolated probiotic strains, *Bacillus subtilis* RX7 and *Bacillus methylotrophicus* C14, on egg production, excreta, and intestinal

microbiota of laying birds challenged with *S. gallinarum* KVCC BA 0700722 (Upadhaya et al., 2016)*.*

In vitro and in vivo studies conducted by Shanmugasundaram et al, to study the effects of supplementation on *Salmonella enteritidis* proliferation indicated that *L. reuteri, P. acidilactici, B. animalis, and E. faecium* can colonize the chicken intestine successfully and have the capacity to decreases the proliferation of *S*. *enteritidis*. They confirmed that the supplementation improves the body weight and feed intake and reduces the colonization of *Salmonella* in the cecal content of broiler chickens. (Shanmugasundaram R. et al., 2019)

Chang et al demonstrated that feed containing probiotic-supplemented basal diet containing multi-strain probiotics, namely *Lactobacillus acidophilus* LAP5*, L. fermentum* P2*, Pediococcus acidilactici* LS*,* and *L. casei* L21 increased *Lactobacillaceae* and reduced *Enterobacteriaceae* in the ceca. The administration of multi-strain probiotics modulated intestinal microbiota, gene expression of tight junction proteins, and immunomodulatory activity in broiler chickens (Chang et al., 2020)

Kowalska J.D. et al., 2020 fed three strains of *Lactobacillus sp.: L.rhamnosus LOCK 1131, L.casei LOCK 1132*, and *L. paracasei LOCK 1133*. They possess antagonistic activity towards a broad spectrum of environmental *Salmonella enteritidis* species. (Kowalska J.D. et al., 2020)

Likewise, Raheleh ostovan et al., 2021 used a mixture of *B. subtilis* PY79 and *B. subtilis* ATCC 6633. They used their antimicrobial substances against *S. typhimurium*. They found a significant reduction in the invasive ability of *S. typhimurium* to Caco-2 cells by employing *B. subtilis* probiotics (Raheleh Ostovan et al .,2021). *B. subtilis KATMIRA1933* is a probiotic that can produce bacteriocins (M.H. Tazehabadi et al.,2021). *B. amyloliquefaciens B-1895* is another probiotic with antimicrobial properties. It produces a variety of proteolytic enzymes, which have been shown to have activity against the foodborne pathogen *Listeria monocytogenes* (Algburi et al., 2020b). Studies reported that when used as probiotics in poultry, they have different impacts on rooster sperm production, egg production, hatching egg quality, egg hatchability, etc. (Mazanko et al., 2018). According to M.H. Tazehabadi et al., a mixed strain of *B*. *subtilis KATMIRA1933* and *B*. *amyloliquefaciens* B-1895 inhibit biofilm formation of several *Salmonella* serovars. They not only act as a beneficial feed additive for poultry to increase their physiological parameters, but also as an influential antimicrobial producers and prophylactic agents against *Salmonella* (M.H. Tazehabadi et al.,2021)

Table 1: List of probiotics organisms used in poultry.

Conclusions and Future Directions

The problem of antibiotic resistance is that antibiotic-resistant clones of several major pathogens, particularly *Salmonella*, have increasingly been found in foods, which include poultry, retail meat products and seafood. All determinants of maximal vital resistance, which include those conferring resistance to β-lactams, extended-spectrum β-lactams,

fluoroquinolones, aminoglycosides, tetracyclines, and chloramphenicol, have been identified in different *Salmonella* serovars isolated from the food supply. It is increasingly clear that antibiotic resistance will remain an obstacle for the foreseeable future. These studies have demonstrated the effectiveness of probiotic manipulation as part of prevention or internal treatment in competition with *Salmonella* infection. There are different mechanisms by which probiotic can additionally exert their results. They include nonimmune mechanisms and modulation of mucosal and systemic immune responses. Moreover, one or more probiotic lines in a fermented product can increase the beneficial properties of the contained probiotic traces. One crucial goal of the poultry industry is the reduction of foodborne pathogens, namely *Salmonella spp.*, *Campylobacter spp*., and *C.perfringens*. Through the introduction of probiotic bacteria in diets; these pathogenic microorganisms, the primary cause of diseases in humans transmitted by contaminated foods can be eliminatd.

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