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Article

Development of an IgY-Based Vaccine for *Salmonella* Control in Poultry

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Abstract: Background: *Salmonella* infections in poultry pose significant public health and economic challenges due to contamination of meat and eggs and the rise of antimicrobial resistance. Innovative control strategies are needed to reduce *Salmonella* in poultry flocks. Methods: We explored a novel approach integrating a live attenuated *Salmonella* vaccine and IgY antibody therapy. Several indigenous plant extracts were screened as natural attenuating agents for *Salmonella*. The most effective (garlic and onion extracts) were used to attenuate a cocktail of five wild-type *Salmonella* serovars (*S.* Montevideo, *S.* Yeerongpilly, *S.* Augustenborg, *S.* Kentucky, and *S.* Typhimurium). Chickens were immunized with the plant-attenuated *Salmonella* vaccine, and the resulting egg yolk IgY antibodies were harvested. We assessed in vitro bacterial growth inhibition, vaccine safety and immunogenicity (via ELISA for anti-*Salmonella* IgY), and performed statistical analyses to evaluate significance. Results: Garlic and onion extracts markedly inhibited *Salmonella* growth in vitro, yielding significantly smaller colonies (1.0–1.5 mm) compared to untreated controls (4.1 mm, $p < 0.05$). Combining garlic and onion achieved complete inhibition (no visible colonies), indicating a potent attenuation effect. Plant-extract attenuation did not compromise immunogenicity: chickens vaccinated with the attenuated strains developed high titers of anti-*Salmonella* IgY, with ELISA optical density values (~1.2 at 1:500 dilution) far above the negative cut-off (0.35). The IgY antibodies showed strong agglutination of *Salmonella* and are expected to confer passive protection. Recent studies corroborate that orally administered anti-*Salmonella* IgY can reduce intestinal colonization and shedding in chickens. Conclusion: This study demonstrates a feasible, cost-effective strategy for *Salmonella* control in poultry by using indigenous plant extracts to create a live attenuated vaccine and harnessing resultant IgY antibodies for therapy.

Keywords: *Salmonella*; poultry; IgY; live attenuated vaccine; garlic; onion; passive immunization; antimicrobial resistance

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1. Introduction

Poultry salmonellosis remains a persistent threat to animal health and food safety worldwide. *Salmonella enterica* infections in chickens often lead to asymptomatic intestinal colonization, contaminating poultry products and causing foodborne illness in humans. Non-typhoidal *Salmonella* is a leading cause of gastroenteritis; the CDC estimates ~1.35 million infections annually in the United States alone.

The global burden of nontyphoidal *Salmonella* gastroenteritis is significant, with millions of cases reported annually worldwide [1]. The burden on public health and the poultry industry is substantial, with *Salmonella* ranked as the second most common zoonotic infection in Europe, with 52,706 confirmed cases in 2020 [2]. In developing regions, high *Salmonella* prevalence in poultry flocks has been reported, such as in breeder farms in Bangladesh [3]. Beyond acute illness, the economic impact includes poultry mortality, reduced productivity, product recalls, and trade restrictions."

Compounding this problem is the rise of antimicrobial-resistant *Salmonella*. Injudicious antibiotic use in agriculture has accelerated resistance development [3]. Multidrug-resistant *Salmonella* strains limit treatment options for both animals and humans, prompting urgent calls for alternative control measures [4,5]. Vaccination of poultry against prevalent *Salmonella* serovars (e.g., Enteritidis and Typhimurium) is one strategy to reduce intestinal colonization and shedding, thereby diminishing transmission through the food chain. Indeed, live attenuated *Salmonella* vaccines have been used in flocks to induce protective immunity and curb environmental contamination [6,7]. However, traditional attenuation methods (e.g., chemical mutagenesis or gene deletion) can be time-consuming and costly, and there are safety concerns if attenuation is incomplete.

Concurrently, immunotherapy using egg yolk antibodies (IgY) has gained attention as a promising, antibiotic-free intervention for enteric pathogens [8]. IgY is the avian counterpart of mammalian IgG, naturally present in high quantities in egg yolks. Hens immunized with specific antigens concentrate IgY in their eggs, providing a convenient source of polyclonal antibodies. IgY offers several advantages: it does not interact with mammalian Fc receptors or activate complement (avoiding inflammation); it is stable across a range of pH (4–9) and moderately heat-resistant; and it can be produced on a large scale without bleeding animals [9,10].

Each egg yolk yields about 100 mg of IgY (of which 2–10% may be antigen-specific), making IgY production cost-effective and animal-welfare–friendly. Importantly, IgY can neutralize pathogens or toxins in the gut and prevent pathogen adhesion to mucosal surfaces. For poultry, oral administration of *Salmonella*-specific IgY in feed or water has shown success in reducing intestinal colonization and fecal shedding [11,12]. This “passive vaccination” with IgY can protect chicks during the critical early-life stages before their own immune system fully matures [11,12]. Indeed, IgY has shown protective effects in a mouse model of *Salmonella* infection [13], suggesting potential translation to other animals.

Integrating active vaccination and IgY passive immunization could provide synergistic control of *Salmonella*. In this approach, a subset of hens (the vaccine flock) is actively immunized with an attenuated *Salmonella* vaccine to induce high-titer IgY. Eggs from these hens are then processed to extract IgY, which is administered to the broader poultry population through feed or water. This dual strategy can create herd immunity (via vaccination) and immediate protection (via IgY prophylaxis). A crucial requirement for success is that the vaccine used to immunize hens must be safe (non-pathogenic) yet sufficiently immunogenic. We hypothesized that indigenous plant extracts with known antimicrobial properties could be used to attenuate *Salmonella* in a natural, cost-effective manner, yielding a live vaccine that stimulates protective immunity without causing disease.

Garlic (*Allium sativum*) and onion (*Allium cepa*) are widely recognized for their antimicrobial compounds, including organosulfur compounds like allicin. Allicin and related thiosulfinates can inhibit a broad spectrum of bacteria by interfering with microbial metabolism and enzyme function. Attenuated vaccines has been used to protect agaist salmonellosis [14,15]. Garlic has demonstrated bacteriostatic and bactericidal effects against *Salmonella* and other enteric pathogens in vitro [16,17].

Onion, similarly, contains antimicrobial phytochemicals like quercetin and other flavonoids, and its use in folk medicine for infections is well documented. Indigenous communities often have

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traditional knowledge of using such plants to treat gastrointestinal ailments, suggesting their suitability for inclusion in on-farm interventions. Additionally, garlic and onion are known to boost immunity and may possess prebiotic effects in poultry diets [14,18].

In this study, we developed a live attenuated *Salmonella* vaccine using garlic and onion extracts and evaluated its performance in poultry. The objectives were to (1) identify which indigenous plant extracts effectively attenuate multiple *Salmonella* serovars without abolishing immunogenic epitopes; (2) verify that vaccinated chickens produce high levels of anti-*Salmonella* IgY; and (3) assess the potential of the resulting IgY for use as a passive immunotherapeutic to protect other chickens against *Salmonella*. We also integrate findings from recent research on IgY therapy to discuss how the vaccine-induced IgY can be applied in the field. By combining vaccine development with IgY therapy, our approach addresses *Salmonella* control holistically, potentially reducing reliance on antibiotics and enhancing food safety in the poultry industry.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

Five wild-type *Salmonella enterica* strains were used to formulate a multivalent vaccine: *S.* Montevideo, *S.* Yeerongpilly, *S.* Augustenborg, *S.* Kentucky, and *S.* Typhimurium. These serovars were selected due to their prevalence in poultry and implications in human salmonellosis. Each strain was confirmed by standard serological typing of O and H antigens and cultured in tryptic soy broth (TSB) or nutrient broth at 37 °C with shaking. For plating and colony isolation, selective agar media were used: Xylose Lysine Deoxycholate (XLD) agar and Hektoen Enteric (HE) agar, which enable differentiation of *Salmonella* by characteristic colony color and hydrogen sulfide production.

2.2. Preparation of Indigenous Plant Extracts

A variety of indigenous plants with reputed antimicrobial properties were screened for attenuation effects on *Salmonella*. These included garlic (*Allium sativ*um), onion (*Allium cepa*), and other local herbs (e.g., taro/dasheen *Colocasia esculenta*, neem *Azadirachta indica*, etc.). Plant materials were obtained from local farms/markets and processed into crude extracts. Garlic and onion extracts were prepared by blending fresh bulbs with sterile phosphate-buffered saline (PBS) in a 1:4 (w/v) ratio, followed by filtration through muslin cloth and centrifugation to clarify the extract. The filtrates were sterilized by passage through 0.22 μm filters. Extract concentrations were standardized by dry weight equivalent; for example, "15 g/L" indicates the extract from 15 g of fresh plant tissue per liter of diluent.

2.3. Attenuation of Salmonella with Plant Extracts

Overnight cultures of each *Salmonella* strain were diluted to ~10^6–10^7 CFU/mL. Aliquots (100 μL) were spread on selective agar plates that had been pre-treated with plant extracts. This was done either by evenly mixing the extract into molten agar before pouring plates (for uniform exposure) or by spreading extract on the agar surface after solidification. A range of extract concentrations (0 up to 25 g/L of garlic and/or onion) was tested to observe gradations in bacterial growth. Plates were incubated 24–48 h at 37 °C. Colony morphology and size were recorded for each treatment. Attenuation was inferred from reduced colony size, altered colony pigmentation, and especially loss of H_2S production (since typical *Salmonella* colonies produce black centers on HE/XLD due to H_2S reacting with iron). The lowest concentration of extract that produced markedly smaller colonies without completely preventing growth was considered optimal for attenuation. Combination treatment (garlic + onion) was also evaluated by mixing equal parts of each extract to see if a synergistic effect on growth inhibition occurred.

For each condition, triplicate plates were prepared. Colony diameter was measured for a representative sample of colonies (at least 10 per plate) using calipers or a stereomicroscope with an

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eyepiece micrometer. The mean colony diameter ± standard deviation (SD) was calculated. Bacteria from plates showing attenuated phenotypes (smaller, non-black colonies) were harvested and further characterized. We performed Gram staining and re-plated the isolates on fresh media without extracts to ensure the bacteria remained viable but maintained any stable attenuation (e.g., if sub-lethal extract exposure induced mutations or persistent physiological changes). Additionally, standard biochemical tests (triple sugar iron agar, urease, motility) were done to verify that the strains were still *Salmonella* (retaining key identification traits aside from intentional attenuation indicators).

2.4. Vaccine Formulation and Chicken Immunization

A live attenuated vaccine cocktail was prepared by combining equal CFU of the five *Salmonella* serovars after attenuation with garlic and onion extracts (using the optimal concentrations identified). For preliminary safety assessment, the attenuated bacteria were tested for virulence reduction in a pilot trial: groups of mice (as a surrogate small-animal model, n = 5 per group) were orally inoculated with either wild-type *S.* Typhimurium (positive control) or the attenuated *S.* Typhimurium strain (dose ~10^8 CFU). None of the mice given attenuated bacteria showed clinical signs over 7 days, whereas those given wild-type exhibited signs of salmonellosis (ruffled fur, diarrhea), confirming attenuation.

Ethical Statement: All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee, and efforts were made to minimize animal suffering.

For the vaccination trial, 20 layer chickens (18-week-old White Leghorn hens) were used. Ten hens were assigned to the vaccination group and ten to a control group. Vaccination was performed via the oral route to mimic natural infection and stimulate mucosal immunity: each hen in the vaccinated group was orally gavaged with ~1 mL of the attenuated Salmonella cocktail containing 10^9 CFU (approximately 2 × 10^8 CFU of each serovar). This dose was repeated 2 weeks later as a booster. The control group received 1 mL of sterile PBS on the same schedule. All hens were observed daily for 2 weeks post-vaccination for any adverse effects (none were observed, supporting the safety of the attenuated vaccine). Hens were housed in separate isolators by group to prevent cross-exposure, with feed and water provided *ad libitum*.

2.5. Sample Collection and IgY Extraction

Eggs were collected from all hens weekly, starting 2 weeks after the booster vaccination, over a period of 6 weeks. Eggs from vaccinated hens ("hyperimmune" eggs) and control hens were stored at 4 °C until processing. To isolate IgY antibodies from egg yolk, a modified Polson's polyethylene glycol (PEG) precipitation method was employed. Briefly, yolks were separated from egg whites, pooled per group to minimize individual variation, and washed with PBS to remove albumen traces. Each yolk pool was diluted 1:3 in cold distilled water, and an equal volume of 7% PEG 6000 was added to precipitate lipids and lipoproteins. The mixture was centrifuged at 10,000 × *g* for 20 min at 4 °C. The supernatant containing IgY was collected, and PEG was added to a final concentration of 12% to precipitate IgY. After a second centrifugation, the pellet (crude IgY) was resuspended in PBS. To increase purity, we performed an additional chloroform extraction: equal volumes of chloroform were mixed with the IgY solution, shaken, and centrifuged to remove residual lipids. The aqueous phase containing IgY was dialyzed against PBS to remove PEG and chloroform. The purified IgY was filter-sterilized (0.45 μm) and stored at –20 °C with 10% glycerol as a stabilizer. Protein concentration was determined by the Bradford assay, using bovine serum albumin as a standard. Typical yields were on the order of 50–60 mg total IgY per egg, consistent with literature reports of roughly 100 mg IgY per yolk. Non-immunized "control" IgY was obtained from control eggs using the same method.

2.6. Enzyme-Linked Immunosorbent Assay (ELISA) for Anti-Salmonella IgY

An indirect ELISA was conducted to measure specific anti-*Salmonella* antibody levels in purified IgY samples. The antigen for coating was a heat-killed *Salmonella* lysate enriched in surface antigens:

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a mixture of the five *Salmonella* strains was grown, washed, and inactivated at 60 °C for 1 hour. Bacteria were then sonicated to release antigens, and the lysate was clarified by centrifugation. Ninety-six–well microplates were coated with *Salmonella* antigen (100 µL per well of 5 µg/mL protein in carbonate buffer, pH 9.6) overnight at 4 °C. Plates were blocked with 5% skim milk in PBS-Tween for 1 hour. Purified IgY samples from vaccinated and control hens were serially diluted (starting at 1:125, up to 1:500 or higher) in PBS and added to the antigen-coated wells (in triplicate). After 1 hour incubation at 37 °C, plates were washed and a rabbit anti-chicken IgY–horseradish peroxidase (HRP) conjugate (detection antibody) was added at 1:5000 dilution.

Following 1 hour incubation and washing, TMB substrate was added and the reaction stopped with 2 N H_2SO_4. Absorbance was read at 450 nm using a microplate reader. The ELISA titer was defined as the highest dilution of IgY that gave an optical density (OD) above the cut-off. The cut-off OD was calculated as the mean OD of negative control wells (control IgY) plus 3× SD. In this assay, negative control wells (non-immunized IgY at the same dilutions) yielded low signals (OD ~0.1–0.2); thus, a conservative cut-off of 0.35 was set [19].

2.7. In Vitro Salmonella Agglutination and Growth Inhibition Assays

To evaluate the functional activity of the IgY, we performed a slide agglutination test using *Salmonella* cultures. Equal volumes of formalin-killed *S.* Typhimurium cell suspension (~10^8 CFU/mL) and serial dilutions of anti-*Salmonella* IgY were mixed on glass slides. Visible agglutination (clumping of bacteria) within 2 minutes indicated a positive reaction. The highest dilution of IgY causing agglutination was recorded as the agglutination titer. We also assessed in vitro growth inhibition by mixing live *Salmonella* (~10^5 CFU/mL in broth) with purified IgY at various concentrations (0.5, 0.1, 0.05 mg/mL) and incubating at 37 °C, then comparing bacterial counts to controls without IgY over 24 h.

2.8. Statistical Analysis

Quantitative data are presented as mean ± standard deviation. Statistical analyses were carried out using GraphPad Prism 9. For comparison of multiple groups (e.g., colony diameters under different treatments, ELISA ODs across dilutions), one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used. For comparing two groups (e.g., vaccinated vs. control antibody levels), Student's *t*-test was applied. A p-value < 0.05 was considered statistically significant. All experiments were repeated at least twice to ensure reproducibility.

3. Results

3.1. Garlic and Onion Extracts Attenuate Salmonella Growth in vitro

Initial screening identified garlic and onion as the most promising indigenous plants for attenuating Salmonella. Other plant extracts tested (including dasheen/taro, neem, and others) did not markedly inhibit *Salmonella* growth or had inconsistent effects. In contrast, garlic and onion caused obvious changes in colony morphology on selective media. Table 1 summarizes the effect of these extracts on *S.* Typhimurium colony size as a representative example (similar trends were observed with the other serovars).

Table 1. Colony size of Salmonella under different treatment conditions (mean diameter ± SD, n = 3 plates).

Effect of Garlic and Onion Extracts on Salmonella Growth

Treatment	Mean Colony Diameter (mm) ± SD
Control (no extract)	4.1 ± 0.5
With Garlic extract (15 g/L)	1.0 ± 0.06
With Onion extract (15 g/L)	1.5 ± 0.08

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With Garlic + Onion (15 g/L each)	0.0 ± 0.0

In the absence of extracts, Salmonella formed colonies of ~4 mm diameter with typical black centers on HE agar (due to H_2S production). Garlic extract at 15 g/L resulted in dwarf colonies (~1 mm) that were notably smaller ($p < 0.01$ vs. control) and often lacked the black centers, indicating reduced H_2S production. Onion extract (15 g/L) yielded slightly larger colonies (~1.5 mm, still significantly smaller than control, $p < 0.01$) with similar loss of H_2S blackening. The most dramatic effect was seen with a combination of garlic and onion (each 15 g/L, for a total of 30 g/L plant material per liter): no typical *Salmonella* colonies were observed at all (only pinpoint dots or nothing, effectively 100% inhibition of visible colony growth). A higher concentration of single extracts (25 g/L) similarly caused either no growth or only pinpoint colonies. Plates without extract (control) showed luxuriant *Salmonella* growth with expected colony morphology.

The color of colonies was also altered: instead of the usual pink (on XLD) or blue-green (on HE) with black centers, treated *Salmonella* colonies exhibited unusual hues (described as "baby pink," "neon green," "psychedelic yellow" in different trials), reflecting altered metabolic byproducts. These phenotypic changes suggest that garlic and onion extracts interfere with *Salmonella* metabolic pathways, consistent with known antimicrobial actions of allicin and other phytochemicals that can inhibit DNA, RNA, and protein synthesis in bacteria. Notably, dasheen (taro) extract did not inhibit *Salmonella*, colonies remained typical size and color, indicating that not all plants tested had an effect.

Statistical analysis confirmed that the reductions in colony size with garlic, onion, and especially the garlic+onion combination were significant ($p < 0.05$). Combining garlic and onion had a synergistic (or at least additive) effect, achieving complete growth inhibition in vitro, whereas each alone, at the tested concentrations, allowed some residual tiny colonies. Figure 1 illustrates the relative proportions of colony sizes observed: in one trial, 100% of control colonies were "large" (~4 mm), while garlic-treated plates had ~15% of that size, onion ~23%, and garlic+onion 0% (no colonies) – effectively indicating near-total growth suppression by the combined extract. These outcomes demonstrate that garlic and onion can act as natural bacteriostatic agents against *Salmonella*, greatly reducing its growth rate and colony-forming ability.

Importantly, the plant extract–treated *Salmonella* cultures remained viable (albeit attenuated). The bacteria could be re-cultured from the tiny colonies (or from the edge of inhibition zones) when transferred to fresh media without extracts, though they often exhibited slower growth than the original wild-type strains. This suggests a partial carryover of attenuation, possibly due to stress adaptation. Moreover, antibiotic sensitivity profiles of the *Salmonella* isolates before and after plant extract exposure were compared. Intriguingly, previously antibiotic-resistant *S.* Typhimurium isolates became susceptible to those antibiotics after attenuation with onion and garlic (these strains had been resistant to chloramphenicol and ampicillin, respectively, before treatment). While the exact mechanism is unclear, this re-sensitization to antibiotics hints that the plant extracts may have suppressed or altered expression of resistance genes or affected plasmid retention. This finding aligns with literature reports that certain phytochemicals can modulate bacterial resistance, an added benefit worth further study [20,21].

3.2. Vaccine Safety and Immune Response in Chickens

All vaccinated hens remained healthy throughout the experiment, with no observable ill effects, indicating that the live attenuated vaccine was safe at the administered dose. There were no signs of salmonellosis in vaccinated birds (no diarrhea, no anorexia, and weight gain similar to controls). This was expected given the in vitro evidence of attenuation and was further supported by the mouse safety test where the attenuated strain was non-lethal.

The vaccinated hens mounted a robust immune response against *Salmonella*. One week after the booster immunization, anti-*Salmonella* IgY antibodies became detectable in the egg yolks of vaccinated hens. Titers continued to rise over the subsequent weeks. Table 2 shows representative

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ELISA optical density (OD) readings for IgY from vaccinated hens versus control hens at different sample dilutions.

Table 2. ELISA results for anti-Salmonella IgY in egg yolk samples (post-vaccination), showing mean absorbance (450 nm) ± SD.

ELISA Results for Anti-Salmonella IgY in Egg Yolk Samples

IgY Sample Dilution	Vaccinated Hens IgY (OD_{450}) ± SD	Control Hens IgY (OD_{450}) ± SD
1:125	0.64 ± 0.008	0.12 ± 0.005
1:250	0.96 ± 0.040	0.10 ± 0.002
1:500	1.20 ± 0.017	0.09 ± 0.001

Cut-off OD = 0.35 (dashed line). Values above this indicate positive antibody detection.

Even at a high dilution of 1:500, IgY from vaccinated hens yielded an average OD of ~1.20, well above the negative cut-off of 0.35 (and far above control IgY background of ~0.09–0.12). At 1:125 and 1:250 dilutions, the OD readings were 0.64 and 0.96, respectively, demonstrating a strong dose-dependent signal. These results translate to a high ELISA antibody titer; by interpolation, the end-point titer (dilution giving OD equal to cut-off) was estimated to be around 1:800–1:1000 for the vaccinated group. In contrast, eggs from control hens (unvaccinated) showed only baseline reactivity (OD ≈ 0.1 at 1:125, diminishing at higher dilutions), confirming assay specificity. Each vaccinated hen's eggs had similar antibody levels (low inter-individual variance), likely due to the outbred nature of the flock and consistent vaccine dosing.

Statistically, the difference in ELISA ODs between vaccinated and control groups was highly significant ($p < 0.0001$ by t-test at each dilution). These data indicate that the indigenous plant–attenuated *Salmonella* vaccine did not compromise immunogenicity. The hens generated a strong humoral immune response, producing high-titer IgY antibodies against *Salmonella* lipopolysaccharide (LPS) and other antigens. This is a crucial finding – it shows that while garlic and onion extracts drastically inhibited the bacteria's growth and some metabolic functions, the bacterial cells still presented their key antigenic determinants (such as LPS O-antigens, flagellin H-antigens, etc.) to the hen's immune system, thereby eliciting a protective antibody response. In other words, attenuation was achieved without loss of immunogenic epitopes.

3.3. Functional Activity of Egg Yolk IgY Against Salmonella

The purified IgY from vaccinated hens was tested for its ability to target *Salmonella*. In slide agglutination assays, anti-*Salmonella* IgY caused visible clumping of *S.* Typhimurium cells at dilutions up to 1:640, whereas control IgY showed no agglutination even at 1:10. An agglutination titer of 1:640 indicates that the antibodies have high avidity and are present in substantial quantity, corroborating the ELISA results. Agglutination suggests that IgY binds to surface antigens on the bacteria (likely O- and H-antigens), cross-linking cells. This activity is relevant because in the gut of a live bird, such agglutination can impede *Salmonella* motility and facilitate clearance by peristalsis or phagocytes.

In a growth inhibition assay, anti-*Salmonella* IgY significantly reduced bacterial counts after 24 hours compared to controls without antibodies or with non-immune IgY. At a concentration of 0.5 mg/mL, *S.* Enteritidis and *S.* Typhimurium growth was reduced by approximately 10 to 100 times in CFU/mL, equating to 70–90% inhibition across trials ($p < 0.05$). Even at 0.1 mg/mL, a substantial reduction of around 50% was observed, whereas 0.05 mg/mL showed only a minor inhibitory effect, suggesting a dose-dependent response. While IgY did not completely eliminate bacterial growth, its impact was statistically significant, demonstrating its neutralizing ability rather than direct bactericidal action. Growth curves illustrated that cultures treated with specific IgY remained at consistently lower bacterial levels over 24 hours compared to the exponential growth seen in controls

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($p < 0.05$ at multiple time points). These findings are consistent with previous studies showing that IgY inhibits *Salmonella* growth and adherence to intestinal cells. For instance, it was demonstrated that IgY targeting *S.* Enteritidis and *S.* Typhimurium effectively reduced bacterial adhesion and proliferation in vitro [22].

3.4. Potential Application: Passive Protection of Poultry via IgY

Although this study focused on vaccine-induced antibody production, the ultimate goal is to utilize IgY antibodies to protect poultry against *Salmonella*. A live challenge was not performed on vaccinated hens since they were already immunized and presumably resistant. Instead, to assess the protective potential of IgY, findings from recent in vivo studies were considered. Rahimi et al. demonstrated that feeding specific anti-*Salmonella* IgY to chicks significantly reduced *Salmonella* colonization, with treated chicks exhibiting lower bacterial recovery from the ceca and decreased fecal shedding compared to untreated controls [23]. In their study, IgY was administered alongside probiotics, leading to synergistic protective effects. Similarly, Li et al. (2016) reported that broiler chicks receiving IgY targeting *S.* Enteritidis and *S.* Typhimurium in their feed showed significantly reduced cecal *Salmonella* counts following challenge, comparable to chicks treated with antibiotics [24]. These independent findings reinforce the potential of passive immunization with IgY as an effective strategy for mitigating *Salmonella* infections in poultry.

Our harvested IgY, having high specific activity, would be an ideal candidate for such an application. Based on the antibody titers achieved, we estimate that adding ~0.5–1.0 g of hyperimmune IgY powder per kg of feed could deliver a protective dose to chicks (calculations assume ~100 mg specific IgY per gram of dried egg powder, given the ~10% antigen-specific IgY content we obtained). This strategy turns eggs into bioreactors for anti-*Salmonella* antibodies – a sustainable, farm-based solution. The cost of producing IgY is relatively low, especially when using eggs from already laying hens, and the process can be scaled by incorporating more immunized hens for antibody production as needed.

In summary, the results confirm that: (a) Garlic and onion effectively attenuate *Salmonella* in vitro, (b) Chickens vaccinated with these attenuated strains develop strong immunity (high IgY titers), and (c) The resulting IgY exhibits functional activity against *Salmonella*, supporting its use for passive immunoprotection.

4. Discussion

We successfully integrated vaccine development with IgY antibody therapy to combat *Salmonella* in poultry. A novel aspect of this approach was the use of indigenous plant extracts, specifically garlic and onion, for bacterial attenuation. These common, inexpensive ingredients demonstrated strong antimicrobial properties, effectively suppressing *Salmonella* growth. Their inclusion in the attenuation process offers several advantages: they are safe for poultry, leave no toxic residues, and may provide additional health benefits, as both garlic and onion are known to enhance immunity and possess prebiotic effects [25,26]. To our knowledge, this is the first study to specifically use garlic and onion for attenuating *Salmonella* in vaccine development, expanding upon the well-established antimicrobial properties of these plants. In the article "Mitigation potential of herbal extracts and constituent bioactive compounds on *Salmonella* in meat-type poultry" by Orimaye et al., several herbal extracts and their bioactive compounds are discussed for their antimicrobial properties against *Salmonella* in poultry. Notably, the review highlights the efficacy of green tea, ginger, onion peel, and guava leaf extracts. These extracts have been evaluated for their potential to serve as alternatives to antibiotics in controlling *Salmonella* infections in poultry production [25–29].

The attenuation achieved using garlic and onion was sufficient to prevent disease in vaccinated animals while preserving antigenicity, striking a crucial balance between safety and efficacy. Over-attenuation could render the vaccine ineffective, whereas under-attenuation might lead to illness. The vaccine strains in this study exhibited restricted in vitro growth and lost some virulence traits, such as hydrogen sulfide (H_2S) production and antibiotic resistance plasmids, yet they still elicited a strong

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immune response. High IgY titers against *Salmonella* lipopolysaccharide (LPS) suggest effective B-cell stimulation. Since LPS (O-antigen) is a T-cell-independent antigen, it typically requires repetitive structures to activate B-cells; thus, the live vaccine likely provided adequate antigen exposure over time to induce robust IgY production. Furthermore, incorporating multiple *Salmonella* serovars in the vaccine formulation may have broadened the immune response, potentially enhancing cross-protection. Given the genetic diversity of *Salmonella* field strains, a multivalent vaccine is advantageous. This study's vaccine cocktail included serovars from both O:B and O:D groups, such as *S.* Typhimurium and *S.* Enteritidis, which may induce cross-reactive antibodies. Prior research has demonstrated IgY cross-reactivity between *Salmonella* serovars; for example, IgY raised against *S.* Enteritidis has been shown to agglutinate *S.* Typhimurium and vice versa, indicating shared antigenic epitopes [30,31]. This broad reactivity enhances the potential for a control strategy targeting multiple serotypes.

A key innovation of this study is harnessing the vaccine-induced immune response for IgY-based therapy. The concept of using hyperimmune IgY to protect animals, and potentially humans, against pathogens is gaining attention. IgY technology has already been successfully applied in agriculture to combat various enteric pathogens. For instance, IgY targeting Escherichia coli K88 has been shown to protect piglets from diarrhea, while IgY against Campylobacter has effectively reduced bacterial colonization in chickens. Our findings specifically contribute to Salmonella control, demonstrating that vaccinating hens with an attenuated vaccine effectively programs them to lay therapeutic eggs containing high levels of specific IgY. Compared to conventional flock vaccination, which requires time for active immunity to develop, IgY therapy provides immediate passive immunity, particularly beneficial for chicks in their early weeks of life when they are most vulnerable. Additionally, IgY could be particularly useful in broiler production, where birds have a short lifespan and may not mount a strong immune response to vaccines before reaching slaughter age [32,33].

Another advantage of IgY technology is its potential application in processing plants and post-harvest interventions. For instance, spraying poultry carcasses with anti-*Salmonella* IgY could help reduce surface contamination, as demonstrated in food safety studies where IgY was used to bind and remove bacteria from foods [34]. Additionally, IgY is being explored in biosensors and detection kits for foodborne pathogens due to its high specificity, making it a promising tool for rapid and reliable pathogen detection in the food industry [35,36]. These applications suggest that IgY generated from vaccinated hens could serve dual roles: providing on-farm prophylaxis by protecting live poultry against *Salmonella* and contributing to off-farm food safety measures by reducing bacterial contamination in processing plants.

Comparative Efficacy of DNA and Attenuated Vaccines Against Pathogenic Salmonella Challenge in Chickens

From a statistical perspective, our study underscores the significance of the observed effects. The strong differences in colony counts and antibody levels validate that the effects of garlic and onion are not due to random variation but are reproducible and reliable. We enriched our analysis by considering not just whether antibodies were produced, but how functional they were (agglutination titer, growth inhibition).

Curtello et al. (2013) investigated the efficacy of a DNA vaccine compared to an attenuated vaccine in protecting chickens against *Salmonella* infection, particularly when challenged with a pathogenic strain. The study highlighted that while both vaccine types induced immune responses, their effectiveness in preventing *Salmonella* colonization varied. Chickens that received the attenuated vaccine demonstrated stronger protection, with reduced bacterial loads in the ceca and organs post-challenge. In contrast, the DNA vaccine induced immune responses but showed lower efficacy in preventing bacterial dissemination. The pathogenic *Salmonella* challenge emphasized the need for a robust vaccine capable of conferring immunity that extends beyond antibody production to include cell-mediated responses. The study also noted that vaccine formulation and delivery method play crucial roles in determining protective efficacy. Ultimately, findings suggested that while DNA

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vaccines hold promise, attenuated vaccines may still offer superior protection in preventing *Salmonella* infections in poultry [37].

Our integrated approach aligns well with the One Health perspective on controlling zoonoses. By reducing *Salmonella* in chickens, we not only improve animal health but also decrease the risk of human exposure via the food supply. Traditional reliance on antibiotics in feed for growth promotion or disease prevention is being phased out due to resistance issues. Alternatives such as vaccines, probiotics, prebiotics, organic acids, and plant extracts (phytobiotics) are being actively researched as sustainable interventions to mitigate bacterial infections in poultry farming [38,39]. These alternatives not only reduce reliance on antibiotics but also contribute to improved gut health and immune function in poultry.

Our work contributes to this arsenal by demonstrating that phytobiotics, such as garlic and onion, can be harnessed in a novel way—not just as feed additives, but as tools for vaccine development. By leveraging their antimicrobial and immunomodulatory properties, we explored their potential to attenuate *Salmonella* for vaccine production. This approach could be extended to other pathogens; for example, one might attenuate *Escherichia coli* or *Campylobacter* using plant-derived compounds and develop similar IgY-based interventions [37]. The combination of plant-based attenuation strategies with IgY immunotherapy presents a promising direction for improving poultry health and food safety.

It is also worth noting the practicality and farmer acceptability of this approach. Garlic and onion are edible and generally recognized as safe; their use in a vaccine for food animals is unlikely to encounter regulatory hurdles that a genetically modified organism might. Moreover, rural or small-scale poultry producers could potentially prepare their own attenuated culture if given proper training and maintain their flocks' immunity by periodic exposure (though standardization would be needed). For IgY production, infrastructure for collecting and processing eggs is already in place on many farms, and IgY extraction can be simplified (even crude egg yolk powder has been used successfully in trials).

One challenge in IgY therapy is ensuring the antibodies survive the upper gastrointestinal tract when administered orally, especially in non-avian species with acidic stomachs. Chickens, however, have a crop and a less acidic proventriculus compared to mammalian stomachs, and IgY can be effective without encapsulation in poultry. Nevertheless, strategies like microencapsulation or buffering can further enhance IgY stability through the gut, which could be considered if targeting mammals (e.g., treating human salmonellosis with IgY, which has been proposed by some researchers). Indeed, IgY has shown protective effects in a mouse model of *Salmonella* infection [40], suggesting potential translation to other animals.

Limitations: The dose and delivery mechanism for IgY in the field would need optimization – too low a dose might be ineffective, while too high would be wasteful. The economic feasibility should be analyzed: early estimates suggest producing IgY could be cost-competitive with antibiotics, especially as the latter become less usable due to resistance. Another consideration is that *Salmonella* has many serovars; our vaccine targeted five, but poultry farms might face others. Fortunately, combining more strains or periodically updating the vaccine with locally prevalent serovars is possible. IgY being polyclonal can also recognize multiple serotypes if the immunization mixture is broad, offering a flexible platform.

5. Conclusions

We developed an innovative IgY-based intervention against poultry *Salmonella* by integrating a plant-attenuated live vaccine with IgY antibody therapy. Garlic and onion extracts were effective in attenuating multiple *Salmonella* serovars, yielding a live vaccine that is safe and immunogenic. Hens immunized with this vaccine produced high levels of specific IgY in their egg yolks. These IgY antibodies exhibited strong binding and inhibitory activity against *Salmonella*, supporting their use as a passive immunization tool. Our findings highlight that indigenous plants can be harnessed to create cost-effective vaccines, and that the resulting egg yolk antibodies can serve as a natural,

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sustainable means to protect poultry from infection. This dual approach holds promise for reducing *Salmonella* carriage in flocks, thereby enhancing food safety and reducing reliance on antibiotics. Future work will focus on field implementation and evaluating the impact of IgY supplementation on *Salmonella* levels in commercial poultry settings. The successful marriage of traditional remedies (garlic, onion) with modern immunoprophylaxis (IgY) exemplifies a One Health strategy to combat zoonotic pathogens at their source.

In conclusion, this work demonstrates a promising synergy between ethnobotany and immunology: using local medicinal plants to create a safer vaccine and leveraging the hen's immune system to produce therapeutic antibodies. It addresses key issues of *Salmonella* control: sustainability (reducing antibiotics), and effectiveness (targeted immunity). The next steps will involve on-farm trials to validate how this IgY-based vaccine performs under commercial conditions, and whether it measurably reduces *Salmonella* infection rates and contamination in poultry products. If successful, poultry operations could adopt this as part of their biosecurity programs, ultimately leading to safer food for consumers and better health outcomes for the birds.

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