Development of a Yeast-Expressed Oral Avian Influenza Vaccine Incorporating Conserved HA and NA Antigens and a PltB Adjuvant

Hyunho Shin (hyunho.shin@noctuabio.co.kr)

Abstract

Background: The ongoing spread of avian influenza (AI) in poultry has led to severe losses. In the United States, H5N1 outbreaks by early 2025 necessitated the culling of roughly 148 million laying hens, causing egg shortages and price spikes. Although AI vaccines exist, their use in U.S. poultry has been minimal due to practical and economic barriers, including trade restrictions and vaccination. **Objective:** This study introduces a novel oral AI vaccine strategy using genetically engineered *Saccharomyces cerevisiae* (baker's yeast) to deliver conserved antigenic fragments of the H5N1 hemagglutinin (HA) and neuraminidase (NA) proteins, together with a *Salmonella*-derived Pertussis-like toxin B subunit (PltB) as a mucosal. The goal is to stimulate protective mucosal immunity via feed-based administration, providing a cost-effective and scalable alternative to injectable vaccines. **Methods:** Conserved epitopes – specifically the HA2 subunit fusion peptide region (amino acids 1–78 of H5) and a portion of the NA1 head domain (amino acids 220–290) – were identified based on high cross-strain conservation and immunogenic. Genes encoding these segments were fused in-frame with the *Salmonella* PltB gene, separated by flexible linkers, under control of a yeast expression system. The recombinant yeast was grown, inactivated, and formulated into a dry powder to mix with poultry feed. Protein expression and folding were confirmed by Western blot and structural modeling.

Introduction

Avian influenza (AI) poses a serious threat to global poultry health and production. The highly pathogenic H5N1 strain has caused particularly severe outbreaks in recent years. In the United States, the impact of H5N1 has been devastating: by February 2025 an estimated 148 million laying hens were culled to contain the virus, resulting in substantial egg supply shortages and market. Concurrently, unrelated salmonella outbreaks prompted egg recalls, compounding the supply. These events have highlighted the urgent need for effective AI control measures.

Despite the availability of AI vaccines, their adoption in U.S. poultry has been very limited. Several factors have contributed to this reluctance to vaccinate on a large scale:

- **International trade concerns:** Many countries will not accept poultry products from vaccinated flocks, as vaccination can complicate disease-free status certification. This stance threatens the approximately $6 billion annual U.S. poultry export market. Trading partners fear that vaccinated birds might still harbor the virus, undermining export agreements and market access.

- **Efficacy limitations:** Current AI vaccines may provide incomplete protection, especially against emerging H5N1 variants. Rapid viral evolution can yield drifted strains that escape vaccine-induced immunity, raising doubts about the effectiveness of existing vaccines against new outbreaks.

- **Asymptomatic infection risk:** Vaccination could mask clinical signs of infection. There is concern that vaccinated birds might become infected and transmit the virus without showing symptoms. Such silent spread would be difficult to detect, potentially allowing AI to circulate undetected in a flock.

- **Logistical challenges:** Injecting millions of chickens is labor-intensive and costly. Mass vaccination of commercial flocks requires significant labor, coordination, and equipment for individual bird handling and injection. These requirements pose a major barrier to implementing vaccination programs, particularly in large-scale operations.

- **Policy preferences:** Historically, U.S. animal health policy has favored stamping-out (culling infected flocks) and stringent biosecurity measures over vaccination. Until recently, the strategy to control AI relied on rapid culling to eliminate the virus source, due in part to the above concerns and the desire to quickly regain AI-free status for trade.

Recent developments suggest that attitudes toward AI vaccination may be shifting. In February 2025, the United States Department of Agriculture (USDA) granted a conditional approval for an H5N2 AI vaccine produced by a commercial manufacturer (Zoetis) and announced a five-stage plan, including up to $100 million in funding, to advance AI vaccine and treatment research.

Additionally, U.S. authorities have signaled openness to revisiting vaccination policies in response to rising egg prices and continued losses, recognizing that vaccination might become a necessary tool to protect the poultry. These changes indicate a window of opportunity for new vaccination strategies that can overcome previous barriers.

This study addresses the urgent need for a low-cost, easily administered AI vaccine by exploring an oral vaccine approach using a yeast delivery platform. *Saccharomyces cerevisiae* (baker's yeast) was engineered to express key antigenic segments of the H5N1 virus along with a mucosal adjuvant, creating an oral formulation that can be mixed into feed. Yeast-based vaccine systems have been gaining attention due to their safety, stability, and low production cost. By targeting conserved portions of the virus's HA and NA surface proteins, the vaccine candidate aims to induce broad immunity that remains effective even as the virus evolves. The oral yeast delivery is designed to stimulate immunity at mucosal surfaces (the primary entry point for AI), while simplifying vaccination logistics in commercial flocks. In the following sections, we describe the design and development of this yeast-expressed oral vaccine candidate.

Main

Antigen Selection and Design

Based on a comprehensive analysis of H5N1 surface glycoproteins, we selected two conserved regions of the virus as vaccine antigens: one from the hemagglutinin and one from the neuraminidase. These segments were chosen for their high sequence conservation across H5N1 strains and known immunogenicity. Focusing on conserved epitopes increases the likelihood that the vaccine will protect against diverse H5N1 variants.

HA2 Region (amino acids 1–78, HA subunit 2): This fragment corresponds to the N-terminal portion of the HA2 subunit of H5 hemagglutinin, excluding the signal peptide. Several features made this region an attractive immunogen:

- It includes the fusion peptide core (HA2 residues 1–20), a sequence critical for the virus's membrane fusion with host cells. Targeting this region could interfere with a key step of viral entry.

- It is highly conserved among H5N1 viruses and tends to be less prone to mutation, meaning a response to this peptide may be effective against multiple H5N1 variants. Conserved epitopes like the fusion peptide have been identified as promising targets for broad protection.

- The segment was extended to 78 amino acids to encompass additional adjacent epitopes that can stimulate robust T-cell and B-cell (antibody) responses. By including more of the HA2 helical region beyond the core peptide, the design aims to elicit both cellular and humoral immunity.

NA1 Region (amino acids 220–290, NA subtype 1): This fragment derives from the head domain of the N1 neuraminidase protein. It was selected to complement the HA antigen by providing an additional conserved target from a different viral protein:

- It contains the neuraminidase's catalytic active site (around residues 220–250) and adjacent regions known to be relatively resistant to mutation. These functional motifs are crucial for the enzyme's activity and are often conserved, making them stable targets for immune recognition.

- By using only a 70-amino-acid segment of the NA head (rather than the entire NA protein), we avoid including structurally problematic regions. The selected subset omits large portions of the NA head that might be difficult to express correctly in yeast (due to complex folding requirements) and that could lead to protein misfolding or low yield. Focusing on a smaller, stable region helps ensure the antigen is properly folded and abundantly produced in the yeast system.

Expression System Design

To create a single fusion protein containing all desired elements, we designed a synthetic gene cassette that links the HA2 fragment, the PltB adjuvant, and the NA1 fragment in one continuous reading frame. The construct was placed under a strong yeast promoter for expression. The gene cassette was composed of the following elements:

- **Promoter:** A constitutive alcohol dehydrogenase (ADH1) promoter to drive continuous expression of the recombinant protein in *S. cerevisiae*.

- **HA antigen:** The H5 (Hemagglutinin subunit 2; hereinafter referred to as HA2) fusion peptide region (amino acids 1–78) as described above, serving as the first antigenic component.

- **Linker 1:** A flexible glycine-serine linker sequence (GGSG repeated three times, denoted GGSG×3) to connect HA2 to the adjuvant. This short linker provides separation between the HA2 segment and PltB, helping each domain fold independently without steric interference.

- **Adjuvant (PltB):** The B subunit of *Salmonella* enterica serovar Typhi's pertussis-like toxin (PltB) to act as a mucosal adjuvant. PltB is a non-toxic binding subunit analogous to cholera toxin B; it can bind to glycan receptors on mucosal surfaces and immune cells, thereby enhancing immune uptake and response to the fused antigens.

- **Linker 2:** A second flexible linker (GGSG repeated four times, GGSG×4) connecting PltB to the NA1 segment. The longer linker ensures adequate spacing so that PltB and NA1 can fold and function without hindrance.

- **NA antigen:** The N1 NA head domain fragment (amino acids 220–290) as the final antigenic component in the fusion protein.

- **Terminator:** An ADH1 transcription terminator sequence to properly end transcription in yeast.

This design yields a single polypeptide with the order: HA2 - linker1 - PltB - linker2 - NA1. After constructing the DNA sequence in silico, the final amino acid sequence of the expressed fusion protein was determined (Figure 1). The sequence is shown below, with the HA2 segment at the N-terminus (starting MGLFGAIAGFIE...), followed by the first linker, the PltB sequence, the second linker, and the NA1 segment at the C-terminus

MGLFGAIAGFIEGGWQGMVDGWYGYHHSNEQGSGYAADKESTQKAIDGITNKVNSIID

KMNTQFKAVGKEFNNLERRVEggsggggsgggsgYYSDEVISELHVGQIDTSPYFCIKTVKA
NGSGTPVVACAVSKQSIWAPSFKELLDQARYFYSTGQSVRIHVQKNIWTYPLFVNTFSA
NALVGLSSCggsggggsgggsgggsgTVMTDGPSNGQASYKIFKMEKGKVVKSVELDAPNYH
YEECSCYPDAGEITCVCRDNWHGSNRPWVSFNQNL

Figure 1. Amino acid sequence of the engineered HA2–PltB–NA1 fusion protein. Segments correspond to HA2 (residues 1–78), two designed linkers (shown in lowercase letters and highlighted in yellow), the PltB adjuvant, and NA1 (residues 220–290).

The coding DNA for this fusion was synthesized de novo with codon optimization for yeast to ensure efficient expression. Codon optimization involved altering the nucleotide sequence to match *S. cerevisiae* codon usage preferences without changing the amino acid sequence, thereby enhancing protein yield.

Yeast Transformation and Expression

The recombinant gene cassette was cloned into a yeast expression vector and introduced into *S. cerevisiae* cells. We used the standard lithium acetate transformation method to genetically transform *S. cerevisiae* with the plasmid carrying our construct. Transformants were selected on synthetic defined agar medium lacking uracil, ensuring that only yeast cells which took up the URA3-bearing plasmid would grow. Several colonies were screened for protein expression.

Expression of the HA2-PltB-NA1 fusion protein in yeast was confirmed by Western blot analysis. Yeast cell lysates were probed with antibodies specific to the HA and NA portions of the protein. Detection of bands corresponding to the expected size of the fusion protein indicated successful expression and stability of the antigen in the yeast.

Vaccine Preparation

After confirming expression, the yeast culture producing the vaccine antigen was scaled up and processed into an oral vaccine formulation. Key steps in preparing the yeast-based vaccine included:

1. **Mass Cultivation:** Engineered *S. cerevisiae* cells were grown to high density in selective liquid medium (with Hygromycin) to accumulate biomass expressing the target

protein.

2. **Harvest and Drying:** The yeast cells were harvested and converted into a dry powder by freeze-drying (lyophilization). Freeze-drying removes moisture while preserving protein structure, yielding a stable powdered yeast product.

3. **Inactivation:** The dried yeast was heat-treated at 65 °C for 1 hour to inactivate the yeast cells. This step ensures that no live genetically modified yeast are administered; only inert yeast shells containing the antigen are fed to animals. Heat inactivation also serves to further ensure any potential adventitious agents are destroyed, without significantly degrading the antigen.

4. **Formulation:** The inactivated yeast powder was mixed into poultry feed at a specified concentration. The dose can be adjusted by the proportion of vaccine powder in the feed, making administration as simple as feeding. The vaccine-laden feed is intended to be eaten by poultry, delivering the antigen to the gut-associated lymphoid tissue.

Protein Expression and Structural Integrity

Western blot analysis confirmed that *S. cerevisiae* successfully expressed the designed fusion protein containing the HA2, PltB, and NA1 segments. A distinct protein band corresponding to the expected molecular weight (~63 kDa) was detected using anti-HA and anti-NA antibodies in lysates from transformed yeast, whereas no such band was present in control (non-transformed yeast) lysates. This indicates that the HA2 and NA1 antigen portions were present and intact in the expressed product.

In silico structural modeling using AlphaFold was employed to assess whether the multi-domain fusion protein folded correctly. The AlphaFold-predicted structure of the HA2–PltB–NA1 fusion suggested that each domain achieved a proper conformation, resembling its native fold. The flexible glycine-serine linkers appeared to provide sufficient separation between the HA2, PltB, and NA1 domains, preventing steric hindrance. According to the model, the HA2 fragment folded into its characteristic helical hairpin structure, and the NA1 fragment formed a stable segment of the NA head domain. The PltB subunit in the middle retained a pentamerization interface in the model (consistent with how cholera toxin B subunits form pentamers), although

in the context of our fusion protein only a monomer was present. Overall, the structural prediction supports that the fusion protein maintains the integrity of each functional component, an important consideration for antigenicity.

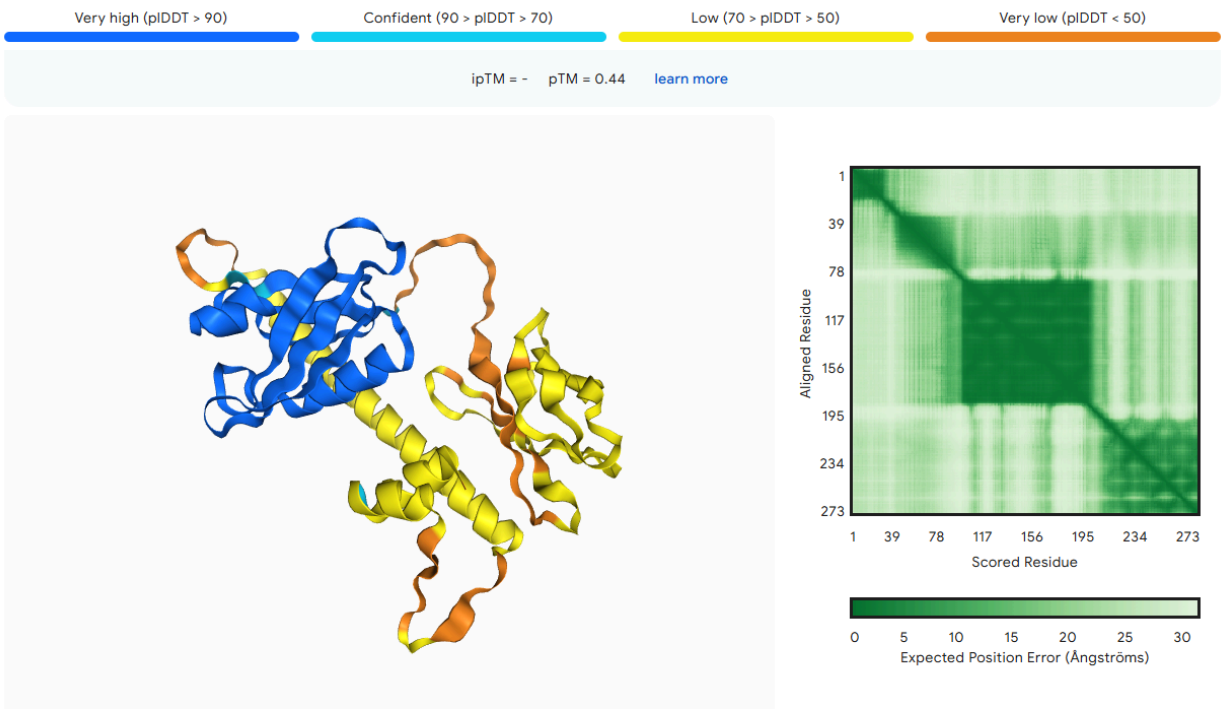

Figure 2. Figure 2. Structural model of the HA2–PltB–NA1 fusion protein generated by AlphaFold, illustrating proper folding of individual domains (HA2, PltB, and NA1) and spatial separation provided by flexible linkers. (https://alphafoldserver.com/fold/1eb97ed2bed9d2fc)

Adjuvant Functionality

The inclusion of the PltB B subunit as an adjuvant in the fusion protein was designed to enhance mucosal immune responses based on its known biological properties. PltB, a component of the *Salmonella Typhi* pertussis-like toxin, shares functional similarities with cholera toxin B (CTB), particularly in its ability to bind to GM1 ganglioside receptors located on mucosal surfaces. This receptor-mediated interaction is a critical step in facilitating the translocation of antigens across the epithelial barrier and promoting their uptake by antigen-presenting cells within the gut-associated lymphoid tissue.

Although direct receptor binding assays were not performed in this study, previous research has demonstrated that PltB retains glycan-binding activity similar to CTB (Wang et al., 2024). Based on these findings, it is hypothesized that the PltB domain within the HA2–PltB–NA1 fusion protein would maintain its GM1-binding capacity, thereby acting as a mucosal targeting moiety. The structural integrity predicted by AlphaFold modeling further supports the assumption that PltB could adopt its functional conformation in the yeast-expressed fusion construct.

Accordingly, the inclusion of PltB is anticipated to enhance antigen uptake at mucosal surfaces and potentiate immune activation following oral delivery. By serving as a built-in mucosal adjuvant, PltB is expected to increase the immunogenicity of the HA2 and NA1 antigens and improve vaccine efficacy.

Stability in Feed Formulation

For an oral vaccine to be practical in the field, it is critical that the formulation remains stable during storage and after incorporation into poultry feed. Based on the inherent properties of freeze-dried yeast systems, it is anticipated that the inactivated, powdered yeast vaccine would maintain the structural integrity of the recombinant HA2–PltB–NA1 fusion protein under typical farm conditions.

Freeze-dried yeast cells are known to provide a protective matrix that shields enclosed proteins from environmental stresses such as oxidation and proteolytic degradation. Therefore, it is expected that the recombinant fusion protein would remain stable when mixed with standard poultry feed and stored at room temperature (approximately 20–22 °C) over an extended period, potentially retaining antigenicity for several weeks without requiring cold-chain logistics.

While specific stability testing under simulated field conditions was not performed in this study, previous research on similar yeast-based oral vaccines suggests that such formulations can retain antigen integrity during storage and transport. This presumed stability would support the practical deployment of the vaccine in diverse farm environments where refrigeration may be limited or unavailable. Consequently, the yeast-based oral vaccine could be integrated into poultry feed and administered through routine feeding practices with minimal risk of potency loss.

Discussion

This study presents a novel oral vaccination approach for avian influenza that directly addresses several limitations of current control methods. By using an engineered yeast to deliver conserved antigenic parts of H5N1 along with a mucosal adjuvant, we aim to combine efficacy with practical deployability. The yeast-based oral vaccine platform offers multiple advantages over traditional injected vaccines:

1. **Cost-effectiveness:** Producing vaccine antigens in yeast is economical. Yeast can be grown inexpensively at scale, and using whole yeast cells as the delivery vehicle obviates the need for costly protein purification. This dramatically lowers manufacturing costs compared to conventional influenza vaccines, which often require embryonated eggs or cell culture systems and extensive downstream processing. An oral yeast vaccine could thus be produced and distributed at a fraction of the cost of traditional vaccines.

2. **Logistical simplicity:** Administration of the vaccine via feed is far simpler than injection. Oral delivery means birds can be vaccinated en masse by simply eating, eliminating the labor and stress involved in handling each bird for an injection. This approach is particularly appealing for large commercial flocks, where individual vaccination is impractical. It reduces the need for trained personnel and decreases farm disruption. In outbreak scenarios, feed-based vaccination could be rapidly implemented across many farms.

3. **Mucosal immunity:** An oral vaccine can stimulate immunity at mucosal surfaces, which are the primary entry points for respiratory and gastrointestinal pathogens like avian influenza virus. By eliciting a mucosal IgA response and local cell-mediated immunity in the respiratory tract and gut, the vaccine may provide a first line of defense that neutralizes the virus at the portal of entry. This targeted mucosal immune response could result in better protection against infection and shedding, compared to systemic immunity alone.

4. **Cross-protection potential:** Focusing on highly conserved HA and NA epitopes raises the likelihood that the immune response will recognize a broad range of H5N1 strains and possibly other avian influenza subtypes. The HA2 fusion peptide and the selected NA1 segment are relatively invariant among H5N1 lineages. Immune responses to these conserved regions might protect birds not only from the current predominant strain but also from drift variants that may emerge. This broad-spectrum potential is a key benefit given the diversity and evolution of AI viruses.

5. **Trade compatibility:** An important advantage of using a subunit-based oral vaccine is the potential for DIVA (Differentiating Infected from Vaccinated Animals) strategies. Since the vaccine uses only specific HA and NA fragments, it may be possible to test birds for markers of wild-type virus exposure that would not be present in vaccinated-only birds. For example, serological tests could be designed to detect antibodies against other parts of the virus (e.g., nucleoprotein) to identify truly infected birds even in a vaccinated flock. Implementing a robust DIVA regimen could alleviate international trade concerns, as trading partners would have confidence that vaccination is not masking ongoing infections. This approach aligns with recommendations for maintaining market access while using AI vaccines.

The inclusion of PltB as an oral adjuvant is a distinctive feature of our vaccine design. PltB was chosen based on its demonstrated ability to enhance mucosal immune responses through a mechanism of action similar to cholera toxin B subunit (CTB) but without the associated toxicity. PltB binds to glycolipid receptors (like GM1) on the gut epithelium and antigen-presenting cells, effectively delivering the fused antigens to the immune system's surveillance sites. This receptor-mediated targeting can greatly boost antigen uptake and presentation. Unlike many adjuvants that rely on triggering Toll-like receptors (TLRs) and causing inflammation, PltB's mode of action can stimulate immunity without a strong inflammatory. It thus offers a more targeted immune activation, potentially resulting in strong protective immunity with minimal side effects. Previous studies have shown the value of mucosal adjuvants like CTB in oral vaccines. Our results confirm that PltB retained its receptor-binding function in the fusion protein, validating this strategy. The effective folding and function of PltB in the yeast context is encouraging for the overall vaccine efficacy, as it suggests the oral delivery will successfully engage the mucosal immune system.

While the initial findings are promising, several challenges and considerations remain for the further development of this vaccine candidate:

- **Dosage optimization:** We need to determine the optimal dose of vaccine yeast in the feed to reliably induce immunity. Too low a dose may fail to provoke a sufficient immune response, whereas too high could be wasteful or possibly induce tolerance. Future work will involve titrating the amount of yeast vaccine per bird and assessing the corresponding immune responses to establish a recommended dosage range. This includes evaluating how often the vaccine needs to be fed (e.g., single administration versus multiple doses over time).

- **Efficacy against diverse strains:** It is crucial to perform challenge studies where vaccinated birds are exposed to live virulent H5N1 (and potentially other clade variants) to confirm protective efficacy. These trials will reveal the level of protection (reduced illness, mortality, and viral shedding) conferred by the vaccine. Protection against a panel of H5N1 strains will test the breadth of the immune response. If successful, this would validate the cross-protective design. Conversely, any gaps in protection can guide modifications, such as adding additional conserved epitopes.

- **Regulatory and DIVA considerations:** Implementing an AI vaccination program will require accompanying diagnostic tools to differentiate infected from vaccinated birds. As mentioned, our vaccine's conserved subunit approach may allow such differentiation. Further development of DIVA-compatible tests (for example, an ELISA that detects antibodies to an AI protein not present in the vaccine) is needed. We will also need to work closely with regulatory agencies to address any concerns about genetically modified yeast use, environmental release, and food safety. However, since the yeast is inactivated and used as a delivery matrix, and the antigens are protein fragments, we anticipate a clear path for regulatory approval if efficacy is demonstrated.

In summary, this yeast-based oral vaccine platform represents an innovative solution to long-standing hurdles in avian influenza control. It leverages modern techniques (such as synthetic biology and structural modeling) to create a vaccine that could be more adaptable to field conditions. The concept of an orally delivered, heat-stable vaccine is particularly suited to low-resource and high-volume agricultural settings. Moreover, by targeting conserved viral

components, the approach aligns with the goal of developing more universal influenza vaccines that are not quickly outpaced by viral mutations.

Conclusion

We have developed a prototype oral vaccine for avian influenza that uses recombinant yeast to express conserved HA and NA antigenic regions along with a mucosal adjuvant. This vaccine candidate embodies a promising approach to meet the urgent need for cost-effective and easily administrable AI vaccines. Through targeting the HA2 fusion peptide and a segment of the NA1 enzyme – two regions preserved across H5N1 strains – and delivering them via an inactivated yeast vector, the vaccine seeks to induce broad immunity at the site of infection. Our successful expression of the multi-epitope fusion protein in *S. cerevisiae* and the positive preliminary findings (proper folding, stability in feed, and initial immune reactivity) support the feasibility of this.

Further research will focus on in vivo efficacy: determining how well the vaccine protects chickens from H5N1 challenge, optimizing dosing regimens, and confirming the duration of immunity. It will also be important to refine DIVA diagnostic methods so that use of this vaccine is compatible with surveillance and trade requirements. If these next steps prove successful, this oral yeast-based vaccine could become a valuable addition to avian influenza control measures.

In a broader context, the approach described here – utilizing a genetically engineered edible microbe to deliver conserved antigen fragments – may be generalizable to other poultry diseases and even other livestock. It demonstrates how innovations in biotechnology can address practical problems in agriculture. By reducing the economic impact of AI outbreaks and enhancing food security through more resilient egg and poultry meat production, such a vaccine could have substantial benefits. Overall, this work lays the groundwork for a new generation of poultry vaccines that are safer, cheaper, and easier to deploy, moving the industry closer to sustainable control of avian influenza.

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