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Impact of Salmonella FimH Gene Insertion on Lactobacillus plantarum Growth and Expression for Oral Vaccine Development

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ABSTRACT

Salmonellosis poses a major threat to poultry health and food safety, driving the need for more effective and sustainable vaccines. In this study, we developed a recombinant *Lactobacillus plantarum*-based oral vaccine expressing the *Salmonella* FimH antigen to induce mucosal and systemic immunity in poultry. The pTRK892 shuttle vector was engineered to include a constitutive promoter (Ppgm1), a signal peptide (SP1), and anchoring motifs for efficient antigen expression and secretion. The recombinant plasmids (pTRK722 and pTRK722FimH Histag) were successfully transformed into *L. plantarum*, confirmed by PCR, restriction mapping, and sequencing. Western blot analysis verified the expression and secretion of the FimH fusion protein. Growth curve analysis revealed a moderate metabolic burden on recombinant strain, reflected in a slightly reduced growth rate compared to the control. This recombinant vaccine platform offers a safe, cost-effective, and scalable alternative for poultry immunization, with the potential to reduce *Salmonella* colonization and transmission, ultimately enhancing food safety and public health.

1. INTRODUCTION

Salmonellosis remains a major global health concern, especially in poultry, where it causes significant economic losses. *Salmonella*, a gram-negative, rod-shaped bacterium from the Enterobacteriaceae family, comprises over 2,500 serovars—with *S.* typhimurium and *S.* enteritidis being the most frequently associated with disease in both humans and animals (CDC). In poultry, factors such as farm hygiene and the extensive use of antibiotics have contributed to the emergence of multidrug-resistant strains, resulting in estimated annual losses ranging from US$64 to US$114 million (Angulo et al., 2000).

Vaccination remains the most promising strategy for controlling salmonellosis. Traditional approaches include killed, subunit, and live attenuated vaccines. Killed vaccines are advantageous due to their safety profile—there is no risk of reversion to virulence or environmental spread—but they often fail to elicit strong cell-mediated immunity and typically confer only short-lived protection (Barrow and Lovell, 1991; De Buck et al., 2004). Subunit vaccines, which use components such as outer membrane proteins, porins, toxins, or ribosomal fractions, have generally been less effective and are hampered by complex production processes (Mukkur et al., 1987). Although live attenuated vaccines can induce robust systemic and

mucosal immunity by expressing a broad array of antigens—including lipopolysaccharide, outer membrane proteins, heat-shock proteins, flagella, and fimbriae—the inherent risk of reversion to virulence remains a critical drawback (Tizard, 2004; McSorley et al., 2000; Sztein et al., 1994).

Among the various antigenic determinants, the fimbrial structures of *Salmonella* have garnered significant attention. The type 1 fimbrial protein of *S.* Typhimurium is encoded by the *fim* gene cluster (fimAICDHFZYW), with the FimH adhesin—a protein located at the tip of the fimbriae—playing a crucial role in binding to eukaryotic cells, particularly M-cells in Peyer's patches (Saini et al., 2009; Yeh et al., 2002). The high immunogenicity of FimH, demonstrated by its ability to induce a mucosal immune response following receptor-mediated uptake (Kisiela et al., 2009; Hase et al., 2009), makes it an ideal candidate for vaccine development.

Given the limitations of conventional vaccines, recombinant lactic acid bacteria (LAB) have emerged as promising vehicles for oral vaccine delivery. LAB such as *Lactobacillus plantarum* are natural inhabitants of the gastrointestinal tract, are generally recognized as safe (GRAS), and possess inherent probiotic properties. Moreover, *Lactococcus lactis* and other LAB strains have been successfully engineered to express a variety of heterologous proteins, including cytokines, bacterial and viral antigens, membrane proteins, and enzymes (Steidler et al., 2002; Nouaille et al., 2003). *Lactobacillus plantarum*, in particular, has been recognized as an effective delivery vector for oral vaccines (Vesa et al., 2000; Diep et al., 2009).

This study explores a novel approach to vaccine design by utilizing recombinant *L. plantarum* engineered to express the *Salmonella* FimH antigen. By modifying the pTRK892 plasmid system to include a constitutive promoter (Ppgm1), a signal peptide (SP1), and various anchoring motifs, we aimed to optimize antigen expression and secretion. The study further evaluates the balance between robust antigen expression and its impact on bacterial growth— a critical factor for ensuring the viability and efficacy of a live vaccine. Ultimately, the objective is to develop a vaccine that elicits both systemic and mucosal immune responses while being safe, effective, and cost-efficient for the poultry industry.

2. MATERIALS AND METHODS

Plasmid Construction

The cloning strategy was based on modifying the pTRK892 shuttle vector, originally designed for expression in both *E. coli* and lactic acid bacteria. First, the native reporter gene (βglucuronidase, GusA3) and the phosphoglycerate mutase (pgm) region were excised using NcoI and HindIII (or alternatively EcoRI and HindIII) to create an opening in the vector. Into this site, a DNA fragment containing the signal peptide sequence (SP1 from *Lactobacillus plantarum* LP0373), *Salmonella* FimH gene, and anchoring motifs CWA Histag was inserted. Gibson Assembly was used to seamlessly fuse these elements with the pTRK892 backbone, resulting in the construction of the recombinant plasmids pTRK722Histag. The assembly was performed following the manufacturer's protocol, and the reaction conditions were optimized to ensure high-efficiency overlap extension and fusion of the DNA fragments.

Transformation and Verification

The recombinant plasmids were first introduced into *Escherichia coli* for amplification and initial verification. Chemically competent *E. coli* MC1061 cells were prepared using a standard $CaCl_2$ protocol. Plasmid DNA (5 µl) was added to 50 µl of competent cells, followed by incubation on ice for 30 minutes, a heat shock at 42°C for 45 seconds, and a recovery period in LB broth at 37°C for 1 hour. Transformed cells were then plated on LB agar containing erythromycin (150 µg/ml) (Figure 1), and successful transformants were identified by restriction mapping and PCR amplification of the insert. The expected fragment sizes were confirmed by agarose gel electrophoresis, and select clones were further validated by sequencing .

Subsequently, verified plasmids were electroporated into *Lactobacillus plantarum* ATCC8014. For these transformations, electrocompetent *L. plantarum* cells were prepared by growing the strain in MRS broth at 30°C until an OD_{600} of 0.8, followed by centrifugation and sequential washes with chilled 10 mM $MgCl_2$ and a 0.5 M sucrose/10% glycerol solution. The cells were then resuspended in the sucrose/glycerol solution. For transformation, approximately 1–2 µg of plasmid DNA was mixed with 50 µl of competent *Lactobacillus* cells in chilled cuvettes (0.2 cm gap) and electroporated into these cells at 1200 V, immediately recovered in pre-warmed MRS medium for 3 hours at 30°C, and plated on erythromycin-containing agar. A single colony was cultured overnight at 37°C, after which plasmid extraction was performed using a modified Qiagen kit (Buffer P1 replaced with a *Lactobacillus* Resuspension Buffer containing 10 mM Tris-HCl, 10 mM EDTA, 50 mM NaCl, 20% sucrose, and 10 mg/ml lysozyme). Finally, the plasmid was confirmed by PCR amplification of the FimH and Sanger sequencing.

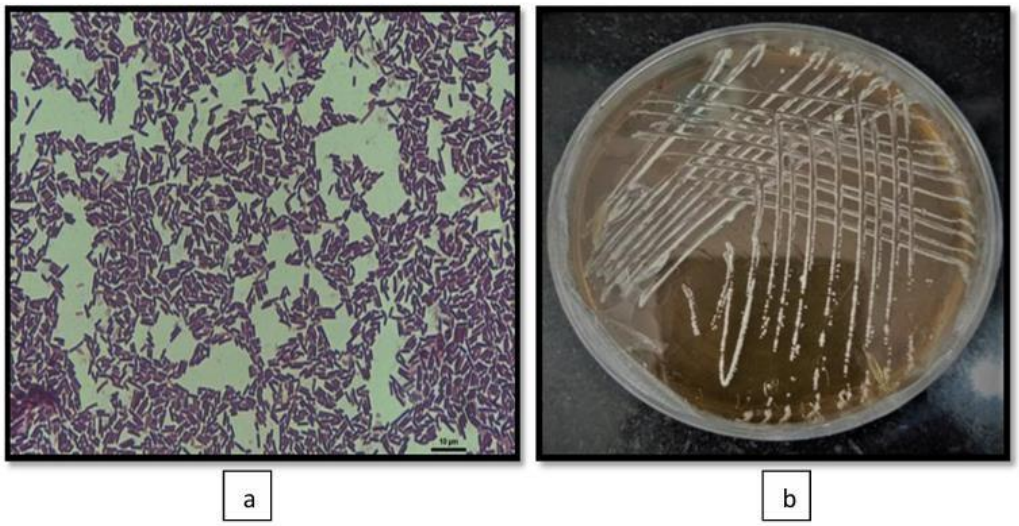

Fig. 1: Identification of *Lactobacillus plantarum*

a) Gram Staining purple-coloured Gram-positive rods under 40X

b) b) *Lactobacillus plantarum*-white small size colony on MRS agar.

Protein Expression Analysis

For evaluation of FimH fusion protein expression, recombinant *Lactobacillus* cells harbouring the pTRK722Histag constructs were cultured to mid-log phase, and cells were harvested by centrifugation. The cell pellet was resuspended in lysis buffer (containing SDS, protease inhibitors, and appropriate loading buffer), and lysates were prepared by mechanical disruption and sonication. The supernatant was collected following centrifugation at 12000 rpm on 4 ^{0}C for 10 minutes to remove cellular debris.

Protein sample supernatant was separated by SDS-PAGE using a 12% polyacrylamide gel. Separated proteins were then transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 hour at room temperature to minimize nonspecific binding. For detection, membranes were incubated overnight at 4°C with primary antibodies specific for the Histag diluted in blocking buffer. After extensive washing with TBST, membranes were incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature. Protein bands were visualized using an enhanced chemiluminescence (ECL) substrate, and images were captured using a gel documentation system.

Growth Curve Analysis

To assess the impact of heterologous FimH expression on bacterial fitness, growth curve analysis was performed for recombinant and control strains. Recombinant *Lactobacillus plantarum* strains harbouring the pTRK722 Histag construct, as well as natural *L. plantarum* controls, were cultured in MRS broth. Cultures were inoculated to an initial optical density (OD_{600}) of approximately 0.05 and incubated at 37°C without shaking.

At predetermined time intervals, typically every 2 hours, aliquots were taken to measure OD_{600} using a spectrophotometer. These measurements allowed the plotting of growth curves to monitor bacterial proliferation over time. The resulting data revealed that recombinant strains expressing the FimH fusion protein exhibited a slower growth rate compared to the control strains, suggesting that the metabolic burden imposed by continuous heterologous protein synthesis may affect cellular fitness. The growth curves were subsequently analysed to determine the lag phase duration, exponential growth rate, and stationary phase onset. These parameters provided quantitative insight into the physiological impact of the recombinant expression system, informing future optimization strategies for vaccine vector development.

3. RESULTS

Plasmid Construction and Verification

Recombinant plasmids were successfully constructed by modifying the pTRK892 backbone. Initially, the native β-glucuronidase (GusA3) reporter gene and the phosphoglycerate mutase (pgm) region were excised using NcoI and HindIII, creating the appropriate cloning site for the heterologous DNA fragment. This fragment contained the *Salmonella* FimH gene, a signal peptide (SP1 derived from *Lactobacillus plantarum* LP0373), and distinct anchoring motifs (CWA Histag).

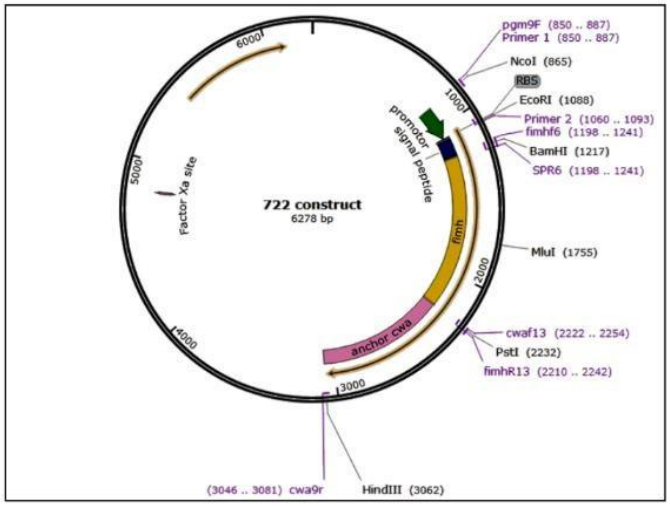

Fig.2: Schematic overview of the expression vectors pTRK722

Promotor pgm, Ppgm1(Green coloured), Ribosomal binding site (grey coloured), signal peptide SP1(blue coloured), FimH1 (yellow), Anchor motiff CWA-Histag (pink).

Gibson Assembly was employed to seamlessly fuse the FimH expression cassette into the pTRK892 vector, generating the plasmid pTRK722 Histag as shown in Figure 2. Successful assembly was initially confirmed in *E. coli* MC1061 by restriction mapping, which demonstrated the expected fragment sizes (4080 bp and 1434 bp), corresponding to the different backbone preparations) when analysed by agarose gel electrophoresis (Figure 3). PCR amplification of the insert further confirmed the presence and correct orientation of the FimH cassette. Selected clones were additionally validated by DNA sequencing, which confirmed that all fusion junctions were intact and that the construct was free of mutations.

Fig 3: Plasmid pTRK722 and its Restriction Enzyme Mapping:

Agarose gel electrophoresis depicts Plasmid pTRK722 isolated from MC1061 cell strain against 1kb DNA marker Thermo scientific, L2 HindIII digested 6278bp, L3- HindIII and NcoI digested 4081bp (backbone) and 2197 bp (insert).

Protein Expression Analysis

Western blot analysis confirmed the successful expression and secretion of the FimH fusion protein in recombinant *Lactobacillus plantarum* ATCC 8014 (NCK1733) strain. SDS-PAGE analysis revealed distinct protein bands corresponding to the expected molecular weights. the cell pellet fraction contained recombinant *L. plantarum* cell pellet extracts purified with a Histag column, showing a prominent band at approximately 68 kDa, while as the cell pellet extract from natural *L. plantarum* purified with the same column, but no FimH band was detected.

In the subsequent Western blot analysis of cell pellet fractions, in figure 3, lanes L1 contains pre-stained protein markers (Thermo Scientific) for reference, while lane L2 and L3 shows the recombinant *L. plantarum* cell lysate purified with the Histag column, displaying a clear band at approximately 68 kDa, confirming the presence of the FimH fusion protein. In the supernatant fractions, lanes L4 and L5 shows lysate of natural *L. plantarum*, with no specific bands, confirming the lack of FimH expression in these strains.

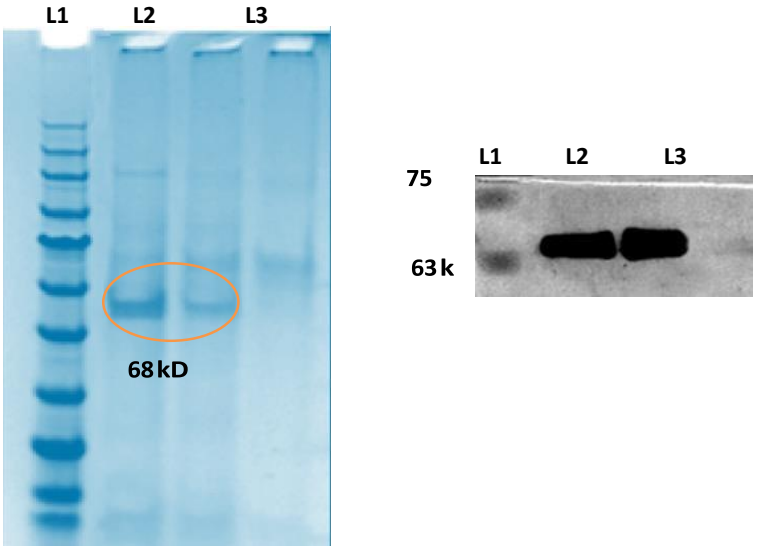

Figure 4. Western blot analysis of *Lactobacillus plantarum* strains.

Detection of FimH fusion protein by Western blotting. Comparison of recombinant *L. plantarum* ATCC 8014 (NCK1733) strains expressing the FimH antigen with natural *L. plantarum* . SDS -PAGE and Western blot analysis show FimH fusion proteins (L2 and L3) produced by recombinant *L. plantarum* , purified using a His -tag column from cell pellets. L4 represents the cell pellet of natural *L. plantarum* (NCK1733), while L1 is a pre -stained protein ladder (Himedia), revealing the expected ~63 kDa band.

Growth Curve Analysis

Recombinant *Lactobacillus plantarum* strains harbouring the pTRK722 Histag constructs were compared with natural *L. plantarum* strains to evaluate the impact of heterologous FimH expression on bacterial growth. Growth curves, measured by optical density (OD_{600}) at regular intervals, revealed that the recombinant strains exhibited a prolonged lag phase and a lower exponential growth rate compared to controls. In particular, the maximum OD_{600} reached by the recombinant strains was significantly reduced, indicating that the continuous synthesis and secretion of the FimH fusion protein imposed a metabolic burden on the host cells. These findings suggest that while the expression cassette efficiently drives antigen production, it concurrently compromises bacterial fitness—highlighting the need for further optimization to achieve a balanced expression system.

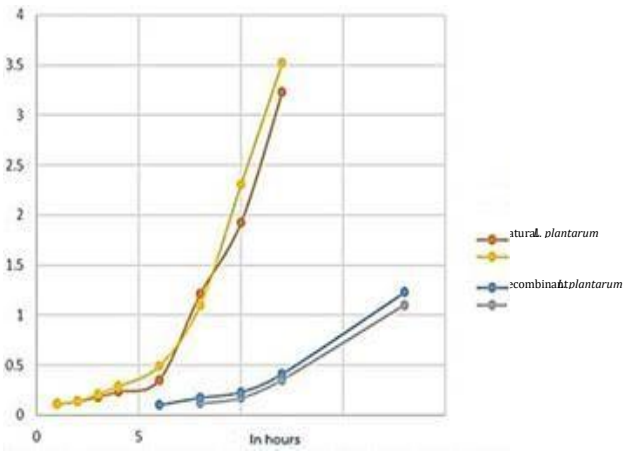

Fig 5: Growth Curves for different *Lactobacillus* Harbouring Plasmids.

The growth curve of *L. plantarum* harbouring the plasmids pTRK722-Histag showed a slower growth in comparison with the natural *L. plantarum* control.

4. DISCUSSION

In this study, we successfully engineered recombinant *Lactobacillus plantarum* strains capable of expressing and secreting the *Salmonella* FimH antigen using modified pTRK892-based plasmids. The results demonstrate that the designed expression cassettes effectively drive heterologous antigen production under the control of the constitutive Ppgm1 promoter. However, the study also highlights a trade-off between robust protein expression and the metabolic burden on the host bacteria, as evidenced by the slower growth rates observed in recombinant strains.

The pTRK892 shuttle vector, originally designed for dual-host expression in *E. coli* and lactic acid bacteria, was successfully modified by removing the native β-glucuronidase (GusA3) reporter gene and the phosphoglycerate mutase (pgm) region. This allowed the insertion of the *Salmonella* FimH gene along with a secretion signal peptide (SP1) and anchoring motifs (CWA Histag). Gibson Assembly facilitated the seamless fusion of the expression cassette into the vector, generating the recombinant plasmid pTRK722 Histag. The successful construction of the plasmids was confirmed by restriction mapping, verifying the fidelity of the genetic insertions.

Western blot analysis revealed that *L. plantarum* strain harboring pTRK722-Histag produced the FimH fusion protein efficiently. The presence of the FimH antigen in cell lysates confirmed that the secretion signal (SP1) effectively directed protein export, while the anchoring motifs ensured stable surface display. The consistent band intensities detected across independent replicates indicate a stable and reliable expression system. Importantly, the absence of detectable bands in control strains confirmed the specificity of the recombinant antigen production.

The use of a constitutive promoter (Ppgm1) ensured continuous expression of the antigen without the need for external inducers. This promoter, derived from *Lactobacillus gasseri*, has been reported to drive strong, stable expression in lactic acid bacteria, making it a suitable choice for live oral vaccine candidates (Duong et al., 2011). The observed secretion and cell surface display of FimH are critical for efficient interaction with M-cells in the gastrointestinal tract, enhancing antigen uptake and stimulating mucosal immunity (Hase et al., 2009; Kisiela et al., 2011).

Despite the efficient expression of the antigen, growth curve analysis revealed a metabolic cost associated with high-level protein production. Recombinant *L. plantarum* strains exhibited a prolonged lag phase and a slower exponential growth rate compared to the non-recombinant controls. This reduced growth fitness is likely due to the energy and resources redirected toward the synthesis, secretion, and anchoring of the heterologous protein. Similar metabolic burdens have been reported in other studies involving recombinant LAB, where continuous protein production affects bacterial replication and viability (Steidler et al., 2002; Nouaille et al., 2003).

The growth impairment observed is consistent with previous reports demonstrating that highlevel protein expression can lead to cellular stress, protein misfolding, or secretion system overload, ultimately slowing bacterial proliferation (van Rooijen et al., 1992; Voskuil and Chambliss, 1998). These findings highlight the importance of optimizing expression levels to maintain bacterial fitness, a key consideration for the development of live bacterial vaccines.

The observed secretion and surface display of the FimH antigen in *L. plantarum* indicate that the recombinant strains have potential as oral vaccine candidates. LAB-based vaccines offer several advantages, including their GRAS status, ease of oral administration, and ability to elicit mucosal immunity (Steidler et al., 2002; Wells et al., 1993). The efficient secretion of FimH in this study suggests that the recombinant strains could effectively interact with M-cells in the Peyer's patches, promoting transcytosis and subsequent immune activation (Hase et al., 2009).

However, the reduced growth rates highlight the need for further optimization. Strategies such as promoter fine-tuning, codon optimization, or the use of inducible expression systems could help balance antigen production with bacterial viability. To enhance the effectiveness of the recombinant LAB vaccine, future studies should focus on several key strategies. First, optimizing promoter strength by employing regulated or inducible promoters could help minimize the metabolic burden during bacterial growth and trigger antigen expression only upon reaching the target site, thereby improving bacterial viability and vaccine efficacy (Sorvig et al., 2003; Mathiesen et al., 2008). Second, codon optimization of the FimH gene to match the preferred codon usage of *L. plantarum* may enhance translation efficiency and reduce cellular stress, leading to improved protein yields. Finally, in vivo validation will be essential to evaluate the vaccine's capacity to elicit both mucosal and systemic immune responses, as well as its effectiveness in preventing *Salmonella* colonization. These approaches will collectively refine the recombinant LAB vaccine, making it more effective and practical for use in disease prevention.

This study demonstrates that recombinant *Lactobacillus plantarum* strains expressing the *Salmonella* FimH antigen can effectively produce and secrete the antigen, making them promising candidates for oral vaccine delivery. However, the

associated metabolic burden highlights the need for expression system optimization. With further refinement, LAB-based vaccines could provide a safe, efficient, and cost-effective solution for preventing poultry salmonellosis and reducing the risk of transmission to humans.

Data Availability

NA

Author contributions

J.N., S.A.K, designed the experiments and wrote main text S.N.M., P.J. and S.M.A helped in the experiments and preparing the figures and reference formatting. All authors reviewed the manuscript and approved the final manuscript.

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Competing interests

The author(s) declared no conflicts of interest with respect to their search, authorship, and/or publication of this article.

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