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Original Research

Surface Display of Alpha-Toxin Hla$_{H35LH48L}$ on *Bacillus subtilis* Cells for Oral Vaccine Delivery in Mice

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ABSTRACT

Introduction: Surface display of proteins on *Bacillus subtilis* has emerged as a promising strategy in vaccinology, leveraging its safety, gastrointestinal resilience, and capacity for efficient antigen presentation. Targeting *Staphylococcus aureus*, a pathogen reliant on alpha-toxin (Hla) for virulence, this study focuses on a detoxified variant, Hla$_{H35LH48L}$, to potentially neutralize toxicity while preserving immunogenicity. We investigated *B. subtilis* as an oral vaccine vector to display Hla$_{H35LH48L}$ and elicit mucosal and systemic immune responses in mice. **Methods:** The *hla*$_{H35LH48L}$ gene was fused to the *yhcR* anchoring motif and integrated into the *amyE* locus of *B. subtilis* HT800F via double-crossover recombination, generating strain BsHT2315. Successful chromosomal integration was confirmed by PCR. Surface display of Hla$_{H35LH48L}$ was verified through Western blot and bacterial-enzyme-linked immunosorbent assay (bactELISA). Swiss mice were orally administered BsHT2315, wild-type *B. subtilis*, or PBS (control). Serum IgG and intestinal IgA levels were quantified by ELISA. **Results:** Western blot and bactELISA confirmed robust surface expression of Hla$_{H35LH48L}$ on BsHT2315. Oral immunization with BsHT2315 induced a significant two-fold increase in intestinal IgA compared to controls ($p < 0.05$), indicative of mucosal immunity. Serum IgG levels also showed a modest but significant elevation (1.5-fold, $p < 0.01$), suggesting systemic response activation. **Conclusion**: This study demonstrated the successful development of *B. subtilis* BsHT2315 as an oral vaccine vehicle for Hla$_{H35LH48L}$ delivery. The strain triggered potent mucosal and systemic antibody responses, underscoring *B. subtilis*'s potential for cost-effective, needle-free vaccine platforms. Future work will explore protective efficacy against *Staphylococcus aureus* infection and scalability for clinical translation.

Key words: alpha toxin, Staphylococcus aureus, HlaH35LH48L, Bacillus subtilis, cell surface, YhcR, oral vaccine

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INTRODUCTION

Gram-positive bacteria serve as highly effective hosts for surface protein display due to their structural and functional adaptability. Their permeable cell surfaces facilitate the anchoring of heterologous proteins with extended amino acid chains[1]. A single cytoplasmic membrane streamlines polypeptide translocation, while the thick peptidoglycan layer imparts resilience against environmental stressors[2]. Furthermore, their robust cell wall architecture supports diverse laboratory manipulations and practical uses, including oral delivery systems, positioning Gram-positive species as versatile platforms for biotechnological and medical applications. Among protein anchoring mechanisms, sortase-mediated covalent linkage is the most well-characterized[3,4]. Sortases, extracellular transpeptidases, cleave a conserved C-terminal LPXTG motif in target proteins, forming a transient acyl-enzyme intermediate that covalently attaches the protein to the peptidoglycan layer[5]. In biotechnological applications, foreign proteins are engineered for surface display by fusing an N-terminal signal peptide and a C-terminal cell wall anchoring domain (CWAD). CWADs typically feature three critical elements[5]: (i) a hydrophobic transmembrane domain, (ii) a positively charged tail to retain polypeptides intracellularly, and (iii) a pentapeptide sorting signal (*e.g.*, LPXTG, where X is any residue) that serves as a sortase substrate, enabling enzymatic cleavage and passenger domain translocation.

Bacillus subtilis, a Gram-positive model organism in biotechnology, is widely recognized for efficient surface protein display. In *B. subtilis*, the YhcS sortase and its cognate substrate YhcR mediate covalent attachment of heterologous proteins to peptidoglycan[6]. Unlike the canonical LPXTG motif, YhcR harbors an atypical LPDTS sorting signal[6]. Remarkably, despite this divergence, YhcR retains 5′-

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nucleotidase activity—a trait commonly linked to LPXTG-bearing proteins in other species[7]. Prior work demonstrated that a YhcR-α-amylase fusion protein, processed by YhcS, achieves robust surface display[6], suggesting that YhcR's sorting sequence can direct heterologous protein anchoring. As a Generally Recognized As Safe (GRAS) organism capable of surviving in harsh environments, including the gastrointestinal tract, *B. subtilis* holds promise for oral vaccine delivery. However, YhcR remains underexplored for antigen presentation, motivating this study's focus on exploiting YhcR to immobilize a mutant *Staphylococcus aureus* antigen on *B. subtilis* cell walls.

Staphylococcus aureus, a commensal bacterium colonizing human skin and nasal passages, causes infections ranging from mild (*e.g.*, skin abscesses) to life-threatening (*e.g.*, endocarditis, bacteremia, and toxic shock syndrome)[8]. The rise of multidrug-resistant strains and the pathogen's multifactorial virulence complicate treatment and vaccine development[9]. Alpha-toxin (Hla), a pore-forming cytolytic protein expressed by ~83% of pathogenic *Staphylococcus aureus* isolates[10], disrupts host barriers by lysing eukaryotic cells[11,12]. Structural studies identified HlaH35L—a hemolytically inactive mutant with a histidine-to-leucine substitution at position 35—as a protective antigen in murine models of acute infection[13,14]. To prevent potential reversion, a second mutation (H48L) was introduced, yielding the double mutant $Hla_{H35LH48L}$ as a stable toxoid[15]. In this study, we engineered a novel *B. subtilis* strain displaying $Hla_{H35LH48L}$ on its surface via YhcR-mediated anchoring. Oral immunization of mice with this strain elicited elevated serum IgG and intestinal IgA titers, suggesting its potential as an oral vaccine platform. These findings underscore the utility of *B. subtilis*-YhcR for antigen delivery and warrant further investigation into mucosal immunization strategies.

METHODS

Bacterial Strains and Growth Conditions

The bacterial strains used in this study were *Escherichia coli* OmniMAX^{TM} (Invitrogen) for cloning and *Bacillus subtilis* HT800F[16] as an expression host. Competent cell preparation for both *E. coli* and *B. subtilis* followed established protocols[17,18]. Cells were cultured in LB medium supplemented with antibiotics (100 μg/mL ampicillin for *E. coli* or 10 μg/mL chloramphenicol for *B. subtilis*) at 37°C with shaking. All plasmids and strains are listed in **Table 1**.

Plasmid and Strain Construction

To facilitate the anchoring of heterologous protein on the *B. subtilis* cell wall, the vector pHT1796, containing the yhcR sequence, was used as a template in the study. The target gene $hla_{H35LH48L}$ was initially amplified from the synthesized plasmid pHT2328 using the primers ON2336 and ON2335, as detailed in **Table 2.** After digestion with the two restriction enzymes, *BamHI* and *AatII*, both the target gene and the template pHT1796 were ligated using *T4* DNA ligase to construct the plasmid pHT2315. The ligated plasmid pHT2315 was transformed into *E. coli* OmniMAX^{TM}, and cells containing the plasmid were screened on LB agar plates supplemented with ampicillin. The constructed plasmid was subsequently confirmed via colony PCR and sequencing.

Following cloning, the fusion gene *yhcR*-$hla_{H35LH48L}$ from pHT2315 was naturally transformed into the chromosome of *B. subtilis* HT800F. The resulting strain, in which the fragment was integrated through double crossover at the *amyE* locus, was designated as BsHT2315. Bacterial cells were screened on LB agar plates containing chloramphenicol, and colonies were further validated by PCR.

For each colony, three pairs of primers were used to ensure correct integration. Details of all primers, including their nucleotide sequences and binding sites, are provided in **Table 2**. All bacterial strains, including the newly constructed BsHT2315, were stored at −80°C for further analysis.

Expression in *B. subtilis* Vegetative Cells

The BsHT2315 bacterial cells encoding recombinant $Hla_{H35LH48L}$ were pre-cultured in 4 mL of LB broth supplemented with chloramphenicol until the OD_{600} reached approximately 2–3. The suspension cells were then sub-cultured into 10 mL of LB broth containing chloramphenicol and shaken at 37°C and 220 rpm until the OD_{600} reached 0.8. Next, the suspension was sub-cultured into 10 mL of LB medium and incubated at 37°C with shaking at 220 rpm until an OD_{600} of 0.8 was achieved. Subsequently, the culture was induced with IPTG at concentrations of 0 mM, 0.1 mM, and 1 mM and incubated for 20 hours at 30°C.

For Western blot preparation, cell pellets were collected by centrifugation at 10,000 rpm for 2 minutes. For whole-bacterial cell enzyme-linked immunosorbent assay (bactELISA), the culture was collected with 30% glycerol added as a preservative. All samples were stored at -80°C.

Table 1: List of plasmids and bacterial strains in this study

Plasmids / Strains	Description	Reference
pHT2328	Template plasmid used to obtain the hla$_{H35LH48L}$ gene	Our lab's stock
pHT1796	The integration vector contains Pgrac100-MCS-yhcR118 and *amyE* locus, used as the backbone	Our lab's stock
pHT2315	The integration vector contains Pgrac100-hla$_{H35LH48L}$-yhcR118 and *amyE* locus	This study
BsHT1796	*B. subtilis* strain with the integration of Pgrac100-MCS-yhcR118 into the chromosome at the *amyE* locus, used as the control	Our lab's stock
BsHT2315	*B. subtilis* strain integration of Pgrac100-hla$_{H35LH48L}$-yhcR118 into the chromosome at the *amyE* locus	This study

Table 2: List of primers used in the study

Primers	Oligonucleotide sequences (5' – 3')	Purpose	Amplicon length (bp)
ON2336 ON2335	ACTGTCGCTTCCAAGACGTCGTTTGTCATT AAAGGAGGAAGGATCCATGAAAAC	Amplify hla$_{H35LH48L}$	993
ON2332 ON2335	GATCTTCTTTAATTGGGTCTTCCGTCCCCG AAAGGAGGAAGGATCCATGAAAAC	Colony PCR for *E. coli*	1151
ON469 ON2137	GGCGTTCTGTTTCTGCTTCG CTGTTTGTGATGGTTATCATGCAGGATTG	Confirm integration at 5'amyE site	1097
ON925 ON2378	GAATTAGCTTGGTACCAAAGGAGGTAAGG CTCAACTGTCGCTTCCAAGACG	Confirm the presence of yhcR-hla$_{H35LH48L}$ after integration	1122
ON2331 ON470	GACGGAAGACCCAATTAAAGAAGATCCAA AACCCGCTCCGATTAAAGCTAC	Confirm integration at 3'amyE site	1477

Detection of Hla$_{H35LH48L}$ in recombinant *B. subtilis* strain by Western Blot

Pellets containing cell wall–bound proteins were incubated at 37°C for 30 minutes in 100 μL of lysis buffer (0.25 M sucrose, 25 mM Tris-HCl, pH 7.2) supplemented with 3 μL of 50 mg/mL lysozyme. The samples were then combined with 5× loading buffer and heated at 95°C for 5 minutes. After separation by SDS-PAGE, proteins were transferred to a nitrocellulose membrane.

For Western blot analysis, reactive bands were detected using an anti-Hla primary antibody (raised in mice) at a dilution of 1:20,000, followed by incubation with a rabbit anti-mouse IgG–HRP conjugate (Sigma) at a dilution of 1:40,000. Signal detection was performed using Pierce^{TM} ECL Western Blotting Substrate (Thermo Fisher Scientific). The BsHT1796 strain and the attenuated toxin Hla$_{H35LH48L}$ were utilized as controls in the assay.

Confirmation of Hla$_{H35LH48L}$ cell wall surface display by bactELISA

Whole-bacterial cell ELISA (bactELISA) was employed to verify the anchoring of *Hla*$_{H35LH48L}$ on the *B. subtilis* cell wall. This technique was referenced in the study by Cumming *et al.*[19]. Briefly, BsHT1796 and BsHT2315 vegetative cells, serving as analytes, were resuspended in 200 μL of coating buffer (100 mM $NaHCO_3$, pH 9.6) and directly coated onto a microplate (Thermo Scientific^{TM} Nunc^{TM} MicroWell^{TM} 96-Well Microplates) overnight at 4°C. The samples were washed twice with 100 μL of 0.1% PBS-Tween and then incubated in blocking buffer, consisting of PBS-Tween supplemented with 5% (w/v) skim milk, at room temperature for 1 hour. After washing twice, 50 μL of anti-Hla primary antibody (diluted

1:10,000) was applied to each well and incubated for 2 hours at room temperature. Next, 50 μL of rabbit anti-mouse IgG–HRP conjugate (Sigma), diluted 1:40,000, was added. The plates were incubated with the conjugate for 2 hours at room temperature, washed, and then incubated with 50 μL of TMB Liquid Substrate for ELISA (Sigma) for 20 minutes. Finally, 50 μL of 1N HCl was added to stop the reaction. Absorbance was measured at OD_{450} using a BMG Labtech CLARIOstar plate reader. Each sample was replicated three times, and statistical analysis was performed using a one-way ANOVA test in GraphPad Prism software (USA).

Oral administration in mice

Each experimental group consisted of five 6-week-old female Swiss mice. Oral immunizations were administered on days 0, 14, and 28 using 250 μL of *B. subtilis* vegetative cells (strains BsHT1796 and BsHT2315) at an optical density at 600 nm (OD_{600}) of 60, diluted in 1X PBS. The control groups comprised mice receiving either BsHT1796 or PBS via oral gavage. Blood and stool samples were collected on days 21, 35, and 42, while small intestine samples were collected on day 42. For analysis, 0.6 g of feces or small intestine tissue was treated with 500 μL of 1X PBS containing 0.2 mg/mL PMSF, homogenized, and centrifuged to obtain the supernatant.

Indirect-ELISA

A 96-well plate (Thermo Scientific^{TM} Nunc^{TM} MicroWell^{TM}) was coated with 50 μL of $Hla_{H35LH48L}$ protein (produced by our lab) at a concentration of 5 μg/mL. Subsequently, 50 μL of either serum, fecal, or intestinal extract, serving as the primary antibody, was added at dilutions of 1:250 for serum and 1:50 for fecal/intestinal extract, followed by overnight incubation at 4°C. The wells were then incubated with either anti-mouse IgG (whole molecule) peroxidase-conjugated antibody produced in rabbits (Sigma, A9044) at a dilution of 1:40,000 or anti-mouse IgA (α-chain specific) peroxidase-conjugated antibody produced in goats (Sigma, A4789) at a dilution of 1:10,000 for 2 hours. All samples were analyzed in triplicate, and absorbance at 450 nm (OD_{450}) was measured using a CLARIOstar plate reader. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test in GraphPad Prism software (USA).

RESULTS

Construction of vector pHT2315

The target gene $hla_{H35LH48L}$, with an expected size of 993 bp, was amplified from pHT2328 via PCR and subsequently fused with the C-terminal yhcR-encoding sequence of pHT1796, resulting in the construction of pHT2315. The structure of vector pHT2315 is illustrated in **Figure 1A**. Following transformation, *E. coli* colonies harboring the recombinant plasmids were selected on LB agar plates containing ampicillin, and colony PCR was performed. The colony PCR results revealed a band at 1151 bp (**Figure 1B**), confirming the presence of the fusion gene $hla_{H35LH48L}$-yhcR in pHT2315. The sequence of $hla_{H35LH48L}$-yhcR was further verified by DNA sequencing (*data not shown*).

Generation of recombinant BsHT2315 integrated yhcR-$hla_{H35LH48L}$ fragment

The constructed vector pHT2315 was successfully delivered into *Bacillus subtilis* HT800F through the process of natural transformation. During the transformation, DNA uptake occurred in some bacterial cells, leading to the integration of the heterologous fusion gene $hla_{H35LH48L}$-yhcR into the *B. subtilis* chromosome at the homologous amyE locus. Bacterial cells that did not incorporate the target fusion gene were eliminated through a preliminary screening on chloramphenicol LB agar plates.

PCR analysis was performed on the newly generated strain to confirm the integration of the $hla_{H35LH48L}$-yhcR fusion gene into the *B. subtilis* chromosome via a double-crossover recombination event. This analysis employed three specific primer pairs with distinct functions: one pair verified the presence of the gene of interest, while the other two targeted the integration sites at the 3′ and 5′ ends of amyE, respectively (**Figure 1C**). Gel electrophoresis results showed visible bands of varying sizes for all colonies with the three primer pairs (**Figure 1D**), matching the predicted sizes as outlined in **Table 2**, thereby confirming the successful creation of the integrated strain. The newly engineered strain was designated BsHT2315.

Display of $Hla_{H35LH48L}$ on *B. subtilis* vegetative cell surface

In this experiment, the vegetative cells of BsHT1796 and BsHT2315 strains were lysed after resuspension in a lysis buffer containing lysozyme. Proteins released from both strains, along with the control (purified Hla produced by our lab), were loaded onto an

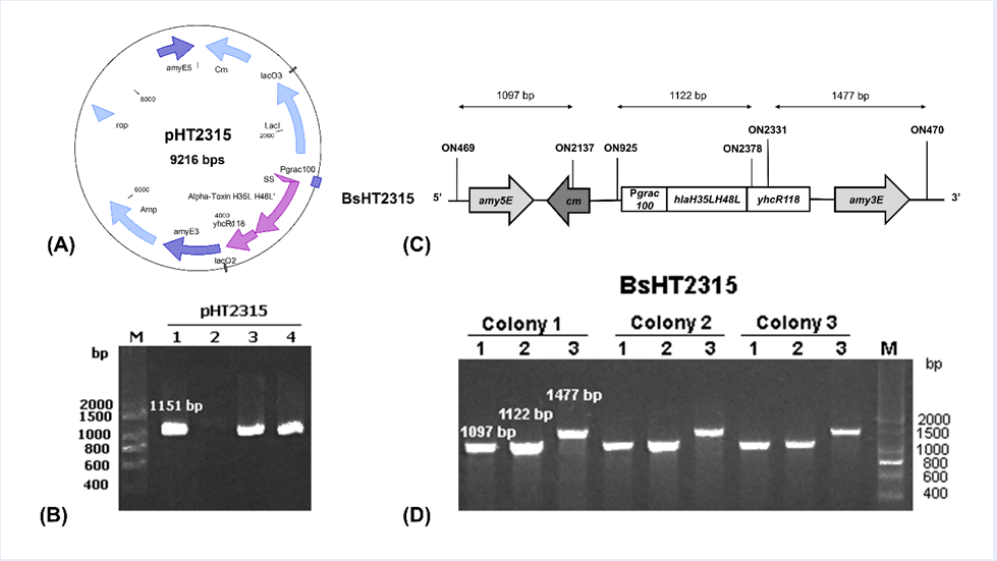

Figure 1: **Generation of BsHT2315 (A) Illustration map of the vector pHT2315; (B) Colony PCR result of pHT2315 generation; (C) Binding sites of primers for chromosomal integration verification of BsHT2315; (D) PCR result for verifying integration of BsHT2315. Abbreviations: M**: DNA ladder; **bp**: base pairs; **PCR:** Polymerase chain reaction

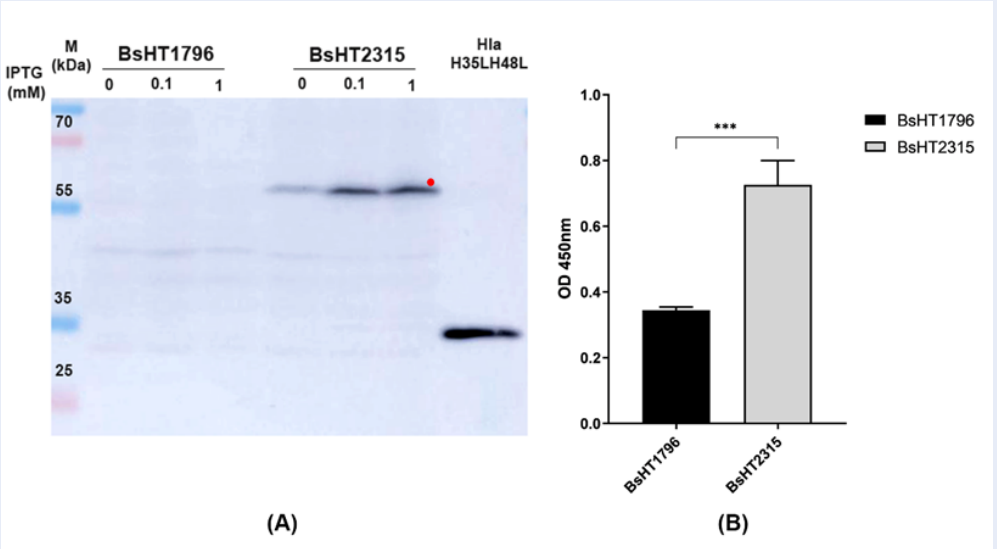

Figure 2: **Confirmation of the expression of Hla$_{H35LH48L}$ on the cell surface of BsHT2315 (A) Western blot results of BsHT1796 and BsHT2315 (B) bactELISA results of BsHT1796 and BsHT2315. Abbreviations**: **M**: PageRuler^{TM} Prestained Protein Ladder (Thermo); **Hla$_{H35LH48L}$**: purified alpha-toxin; ***: $p < 0.001$; **bactELISA:** Bacterial-enzyme-linked immunosorbent assay

SDS-PAGE gel, separated, and transferred to a nitrocellulose membrane. Western blot analysis was then conducted using a primary antibody raised in mice against Hla and a secondary rabbit anti-mouse IgG conjugated with HRP. Finally, the interaction between the ECL substrate and the HRP enzyme produced a chemiluminescent signal, visualizing the proteins on the membrane.

As shown in **Figure 2A**, compared to the BsHT1796 strain, which lacks the hla$_{H35LH48L}$ protein-encoding gene, BsHT2315 under inducible conditions with 0.1 and 1 mM IPTG displayed visible bands of relatively equal intensity. Meanwhile, the BsHT2315 sample without IPTG induction also exhibited a band, but of lighter intensity. The difference in band thickness corresponding to the inducer concentration indicated that expression of the target fusion protein was regulated by the correct Pgrac212 promoter and lacI operon system in the presence of the inducer. Additionally, the larger molecular size of the fusion protein produced by the strain BsHT2315, compared to the purified Hla$_{H35LH48L}$, is likely due to the contribution of the yhcR C-terminal region, which increased the molecular weight.

To confirm that Hla$_{H35LH48L}$ was anchored on the *B. subtilis* cell wall after being linked to YhcR, a bactELISA assay was performed. In this assay, only samples induced with 0.1 mM IPTG were evaluated. As antigens, *B. subtilis* vegetative cells were directly coated onto a microplate. Cells with target proteins anchored on the cell surface would subsequently bind to anti-Hla and secondary antibodies. The coated cells remained stable during a procedure that allowed them to retain their shape, clearly demonstrating the display of the fusion protein on the cell wall. **Figure 2B** demonstrated a significant two-fold increase in the signal intensity for Hla$_{H35LH48L}$ on the cell wall of BsHT2315 compared to the control (0.7260 $\pm$ 0.0074 *vs.* 0.3447 $\pm$ 0.0097, p = 0.0009). This indicated successful surface display of the target protein on the cell wall of *B. subtilis*.

IgG and IgA response by oral administration

We confirmed the successful surface display of alpha toxin on *B. subtilis* cells. To assess the immunogenicity of cell-surface displayed Hla$_{H35LH48L}$ and its oral delivery efficacy, Swiss mice were orally immunized with three doses of *B. subtilis* vegetative cells on days 0, 14, and 28. IgG and IgA antibody levels were evaluated in serum, fecal, and small intestinal samples.

ELISA results in **Figure 3B** revealed a significant increase in anti-Hla$_{H35LH48L}$ intestinal IgA levels in mice immunized with BsHT2315 cells on day 42 (OD450 = 0.135 $\pm$ 0.004, p = 0.0005), which was two-fold higher than those immunized with BsHT1796 (OD450 = 0.066 $\pm$ 0.008). Similarly, a significant difference (p = 0.0012) was observed between BsHT2315 and PBS groups (OD450 = 0.076 $\pm$ 0.017). As expected, no significant differences were observed between the two control groups (BsHT1796 and PBS), as BsHT1796 lacks the target protein sequence. There was no significant increase in fecal IgA levels in the BsHT2315 group compared to the control (data not shown). In addition, serum IgG levels of BsHT2315 exhibited a slight increase on days 21 (0.194 $\pm$ 0.030, p = 0.0239), 35 (0.164 $\pm$ 0.019, p = 0.8436), and 42 (0.203 $\pm$ 0.045, p = 0.0151) compared to the control group BsHT1796. However, no significant differences were observed between dosage regimens throughout the immunization process (**Figure 3A**).

These results suggest that *B. subtilis* cells displaying Hla$_{H35LH48L}$ on their surface via YhcR were successfully delivered to the mouse gut and elicited a local immune response at mucosal sites following oral administration.

DISCUSSION

This research focused on generating a *B. subtilis* strain that displayed a staphylococcal alpha-toxin variant on its cell surface. Here, we successfully engineered plasmid pHT2315, containing the fusion gene hla$_{H35LH48L}$-yhcR. The constructed vector was designed to incorporate the gene of interest into the *B. subtilis* chromosome via double-crossover homologous recombination, ensuring stability and retention of the target gene even in the absence of selective pressure[20,21]. This design could be advantageous for large-scale production, as the use of antibiotics is often a concern in biotechnological manufacturing. In addition, the host strain *B. subtilis* HT800F is a multiple-protease-deficient strain designed to prevent protein degradation and maintain stability in recombinant gene expression. Unlike other commonly used *B. subtilis* host cells, such as WB800 and WB800N, HT800F is free from repetitive DNA sequences, further enhancing its ability to maintain stable expression of target proteins[22]. Furthermore, the newly integrated strain BsHT2315 would express Hla$_{H35LH48L}$ under the control of the strong promoter Pgrac100, which enables robust protein expression in *B. subtilis* while suppressing background expression in *E. coli*[23].

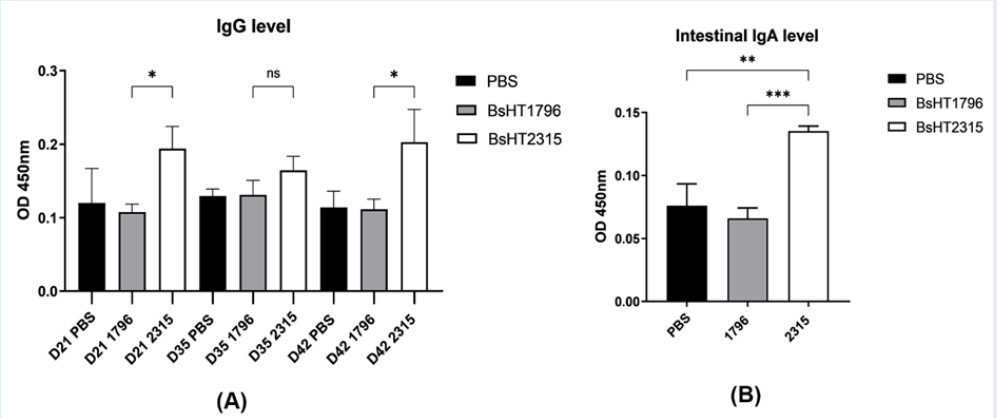

Figure 3: **Antibody level in mice orally immunized with PBS, BsHT1796 and BsHT2315 (A) serum IgG level on day 21, 35, 42; (B) Intestinal IgA levels on day 42. Abbreviations**: ns: p-value > 0.05; *: p < 0.05; **: p < 0.01; ***: p < 0.001; ****: p < 0.0001; **PBS:** Phosphate-buffered saline

In this study, surface anchoring was facilitated by the interaction between the YhcS sortase enzyme and the YhcR motif fused to the target alpha-toxin. YhcS, predominantly expressed during the stationary phase[6], recognized the YhcR sorting signal and catalyzed the covalent anchoring of the fusion protein $Hla_{H35LH48L}$-YhcR to the peptidoglycan layer of the cell wall. By measuring the intensity of each band in Western blot analysis, we concluded that BsHT2315 samples induced with 0.1 mM and 1 mM IPTG exhibited high signal intensity. A slightly visible band was detected in the uninduced BsHT2315 sample (0 mM IPTG). This could be attributed to several factors. Firstly, the bacterial *Lac* repressor may not bind to the operator site with 100% efficiency, allowing for some basal transcription[24]. Secondly, read-through transcription from upstream promoters might contribute to the low-level expression[25]. However, the leaky expression in the uninduced BsHT2315 sample had a negligible impact on our experimental outcomes. Additionally, *Lac* repressor leakiness does not impact vaccine safety, as the target antigen is a mutant alpha-toxin specifically designed for safe handling[15]. Furthermore, the study primarily examined bacteria that had undergone induction and were actively displaying antigen. Overall, the Western blot and bactELISA results demonstrated that BsHT2315 successfully expressed alpha-toxin and that YhcR effectively anchored it to the bacterial cell wall.

The efficacy of oral vaccines can be compromised by various factors, including the harsh gastrointestinal environment, inefficient antigen presentation, and the risk of oral tolerance[26]. In this research, mice orally administered with BsHT2315 cells exhibited a two-fold increase in intestinal IgA levels compared to those administered with BsHT1796 or PBS. The presence of IgA in the intestines indicates that the vaccine has successfully interacted with the mucosal immune cells, triggering an immune response. However, fecal IgA levels may not show the same increase, suggesting that the immune response is localized to the intestinal mucosa and not fully reflected in the feces. Antigens delivered via the oral route interact with gut-associated lymphoid tissue (GALT), including Peyer's patches and M cells[27]. M cells transport antigens across the intestinal epithelium, where they are captured by antigen-presenting cells (APCs), such as dendritic cells (DCs) and macrophages[27]. While mucosal immunity primarily stimulates IgA production, some APCs migrate to systemic lymphoid organs, leading to systemic IgG induction[27,28]. In this study, the slight increase in IgG levels in mouse serum may be due to the limited immune stimulation typically observed with oral vaccines, suggesting that repeated or higher doses may be necessary. A greater antigen dose could enhance mucosa-associated lymphoid tissue (MALT) exposure to the target antigen, thereby strengthening systemic immune responses[29]. On the other hand, *B. subtilis* has been shown to be an effective adjuvant for oral vaccines[30]. A previous study reported that partial protection against lethal challenges was observed exclusively in mice orally immunized with recombinant *B. subtilis* strains, either as vegetative cells or spores, expressing intracellular *Escherichia coli* heat-labile toxin B subunit

(LTB)[31]. Therefore, further optimization with no adjuvant needed may be required to ensure an adequate antigen dose for effective immunization with BsHT2315.

Staphylococcus aureus remains a significant public health threat, and there is currently no approved vaccine[9]. Alpha-toxin is a major virulence factor contributing to various *Staphylococcus aureus* infections. To date, there have been few studies utilizing *B. subtilis* cell surfaces as a platform for antigen expression[31,32], and the expression of alpha-toxin on this platform is investigated for the first time in this study. We have developed a novel *B. subtilis* strain capable of displaying alpha-toxin on its cell surface and effectively delivering it as an antigen to mice. This represents an initial step toward further research into *B. subtilis* cell surface display systems for potential vaccine applications.

CONCLUSION

This study successfully constructed the vector pHT2315, harboring the target fusion gene $hla_{H35LH48L}$-*yhcR*, and developed a novel strain, BsHT2315, capable of displaying heterologous proteins on the bacterial cell wall using the sorting signal YhcR. Furthermore, a significant increase in intestinal IgA levels was observed, demonstrating the potential of *B. subtilis* vegetative cells to deliver antigens via the oral route and induce antibody production in the intestinal mucosa of mice.

ABBREVIATIONS

ANOVA (Analysis of variance), **APCs** (Antigen-presenting cells), ***B. subtilis*** (*Bacillus subtilis*), **bactELISA** (Bacterial-enzyme-linked immunosorbent assay), **CWAD** (Cell wall anchoring domain), **DCs** (Dendritic cells), ***E. coli*** (*Escherichia coli*), **ECL** (Enhanced chemiluminescence), **ELISA** (Enzyme-linked immunosorbent assay), **GALT** (Gut-associated lymphoid tissue), **GRAS** (Generally Recognized As Safe), **HRP** (Horseradish peroxidase), **IgA** (Immunoglobulin A), **IgG** (Immunoglobulin G), **IPTG** (Isopropyl β-D-1-thiogalactopyranoside), **LB** (Luria-Bertani medium), **LTB** (Heat-labile toxin B subunit), **MALT** (Mucosa-associated lymphoid tissue), **OD600** (Optical density at 600 nm), **PBS** (Phosphate-buffered saline), **PCR** (Polymerase chain reaction), **PMSF** (Phenylmethylsulfonyl fluoride), **RT** (Room temperature), ***S. aureus*** (*Staphylococcus aureus*), **SDS-PAGE** (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and **TMB** (3,3',5,5'-Tetramethylbenzidine).

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AUTHOR'S CONTRIBUTIONS

HDN designed the study. NN and AN analyzed the data and wrote the manuscript. HDN and TP revised the manuscript. NN carried out the cloning and strain generation. NN and LD carried out the sporulation. LD, NN, AN, TM carried out animal experiments. All authors read and approved the final manuscript.

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AVAILABILITY OF DATA AND MATERIALS

Data and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS APPROVAL

This study was approved by the ethics committee of the National Institute of Malariology - Parasitology - Entomology in Ho Chi Minh city, signed on March 01, 2020.

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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