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LpxR and PagL expression in live attenuated auxotrophic *Pseudomonas aeruginosa* vaccines modulates lipid A reactivity *in vitro* while preserving immunogenicity

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Introduction: *Pseudomonas aeruginosa* is a Gram-negative opportunistic pathogen responsible for severe respiratory tract infections. We previously developed a live attenuated auxotrophic vaccine candidate, PAO1 Δ murl, which conferred protection in murine infection models but exhibited significant reactivity when administered intranasally. To reduce the toxicity of PAO1 Δ murl without compromising its protective efficacy, we engineered strains with a modified lipid A structure, as lipid A is one of the main toxic components of whole-cell vaccines.

Methods: Two lipid A-modifying enzymes, LpxR and PagL were overproduced in PAO1 Δ murl derivatives. The resulting lipopolysaccharide (LPS) was analyzed by MALDI-TOF mass spectrometry. *In vitro* assays with HEK293-Blue reporter cells expressing murine and human Toll-like receptor 4 (TLR4) were used to assess LPS-associated toxicity, while *in vivo* reactivity and protective efficacy were evaluated in a murine acute pneumonia model.

Results: LPS extracted from the wild-type strain showed heterogeneous lipid A structures with varying degrees of acylation, and a predominant penta-acylated species. Expression of LpxR led to enrichment in tetra-acylated species, while PagL expression reduced the heterogeneity observed in the wild type. Both mutant strains showed decreased TLR4 activation *in vitro* as compared to the wild type. In mice, lipid A-modified derivatives retained protective efficacy; however, no reduction in reactivity was observed.

Discussion: Lipid A modifications mediated by LpxR and PagL attenuated TLR4 signaling *in vitro* but were insufficient to reduce *in vivo* reactivity. Additional

modifications or targeting of other toxic components may be required. This strategy may serve as an initial basis for optimizing live attenuated *P. aeruginosa* vaccines, although additional approaches will likely be necessary to achieve substantial improvements.

KEYWORDS

Pseudomonas aeruginosa, lipopolysaccharide, lipid A modifying-enzymes, live vaccines, D-glutamate auxotrophy, humoral immunity, protective efficacy, reactogenicity

1 Introduction

Pseudomonas aeruginosa is an opportunistic pathogen responsible for a wide range of infections, particularly in immunocompromised individuals or those with skin or mucosal lesions (Killough et al., 2022). In 2019, this pathogen was ranked among the top five bacterial pathogens associated with global mortality, mainly due to lower respiratory tract and bloodstream infections (Collaborators, G. B. D. A. R., 2022). In addition, due to its notable resistance to antimicrobials (Mulani et al., 2019; De Oliveira et al., 2020), the World Health Organization has classified carbapenem-resistant *P. aeruginosa* as a “high priority” (Priority 2) pathogen (Jesudason, 2024).

Given the limited effectiveness of antibiotics against multidrug-resistant *P. aeruginosa* and the slow development of new antimicrobials, vaccines represent a promising preventive strategy, particularly for high-risk individuals (Killough et al., 2022). Various vaccine formulations have been proposed, yet only a limited number have advanced to clinical trials, and none have received regulatory approval to date (Santamarina-Fernandez et al., 2025). We previously evaluated an attenuated D-glutamate auxotrophic, PAO1 Δ murI, which proved safe and effective in a murine sepsis model (Cabral et al., 2017). Intranasal (IN) administration of this strain elicited systemic and mucosal antibodies, activated IL-17-producing CD4⁺ T cells, and significantly improved survival rates in mice with acute pulmonary infections caused by cytotoxin-producing *P. aeruginosa* strains (Cabral et al., 2017, 2020). However, high-dose IN vaccination was associated with adverse effects, likely due to lipopolysaccharide (LPS) endotoxin activity (Cabral et al., 2020; Fuentes-Valverde et al., 2022).

One of the most relevant virulence factors in *P. aeruginosa* respiratory infections is the LPS, the primary structural component of the outer membrane in Gram-negative bacteria. LPS consist of three main regions: lipid A, the oligosaccharide core, and the O-antigen. It acts as a physical barrier, stimulates the production of reactive oxygen species, and triggers pulmonary inflammation. Additionally, LPS contributes to antibiotic resistance and tissue damage through the endotoxin activity of lipid A (Huszczynski et al., 2020). The canonical lipid A is composed of a β (1',6)-linked glucosamine disaccharide, with phosphate groups at the 1 and 4' positions, fatty acids at positions 2 and 2' linked via amide bonds, and fatty acids at positions 3 and 3' linked via ester bonds.

Secondary acyl chains can also be linked to the primary ones. However, the number, length and pattern of acyl chains, as well as additional chemical modifications, vary among bacterial species. Some strains can further modify the lipid A structure in response to environmental cues producing lipid A-modifying enzymes (Simpson and Trent, 2019).

In *P. aeruginosa*, lipid A exhibits as a heterogeneous mixture of penta- to hepta-acylated species. Environmental isolates or laboratory-adapted strains are enriched in penta-acylated bis-phosphorylated species, which consist of a diglucosamine backbone with one 3-hydroxydecanoic acid (3OH-C10) and two 3-hydroxydodecanoic acid (3OH-C12) as primary fatty acids, and secondary acyl chains such as 2OH-C12 and dodecanoic acid (C12) chain and two phosphate residues (Figure 1A). In contrast, clinical isolates from cystic fibrosis patients often synthesize a hexa-acylated lipid A due to the addition of a palmitate chain mediated by the outer membrane enzyme PagP (Ernst et al., 1999). A less frequent hepta-acylated lipid A variant has also been identified in cystic fibrosis patients, which retains 3OH-C10 at position 3, presumably due to the loss of activity of the acyltransferase PagL (Cigana et al., 2009; Geurtsen et al., 2005). PagL is known to hydrolyze the ester bond at position 3 of lipid A (Figure 1B). This modification of lipid A is associated with increased resistance to β -lactam and polymyxin B antibiotics, though not to aminoglycosides, and reduced recognition by the Toll-like Receptor 4 (TLR4) (Ernst et al., 2006; Kawasaki et al., 2004; Ernst et al., 2007; Zhang et al., 2017). Other modifications have also been identified in *P. aeruginosa* isolates from cystic fibrosis patients, such as alterations to the phosphate groups through the incorporation of 4-amino-4-deoxy-L-arabinopyranose (Ara4N) by ArnT or phosphoethanolamine (pEtN) at positions 1 and 4' by EptA (Moskowitz et al., 2004; Nowicki et al., 2015; Simpson and Trent, 2019). In contrast, *P. aeruginosa* lacks LpxR, a 3'-O-deacylase identified in *Salmonella* Typhimurium that modulate lipid A structure by removing the primary acyl chain at the 3' position, thereby reducing activation of the human TLR4/MD2 complex (Reynolds et al., 2006) (Figure 1C).

LPS activates the immune system by triggering the production of proinflammatory interleukins. Once released from the bacterial surface/membrane, LPS monomers are transferred to the TLR4/MD-2 complex on immune cell surfaces through two accessory proteins: LPS-binding protein and CD14. Recognition by TLR4/

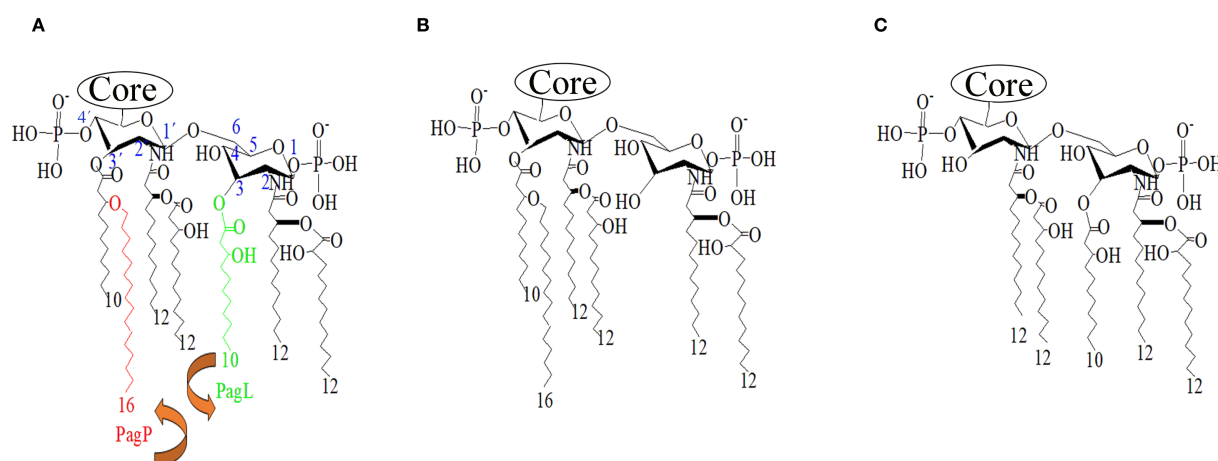


FIGURE 1

Lipid A structures of *P. aeruginosa*. (A) Typical lipid A produced by *P. aeruginosa* strain PAO1. Acyl chains targeted by the deacylases PagL (green) and the acyltransferase PagP (red). The position of the residues on the glucosamine backbone is indicated in blue. (B) Predicted structure after overexpression of PagL. (C) Predicted structure after overproduction of *S. Typhimurium* LpxR. Structures were generated using ChemDraw software.

MD-2 initiates an intracellular signaling cascade via either MyD88 (myeloid differentiation primary response 88) or TRIF (TIR-domain-containing adaptor-inducing interferon- β). The MyD88-dependent pathway induces pro-inflammatory cytokines, whereas the TRIF-dependent pathway promotes interferon-regulated cytokines with weaker inflammatory activity (Simpson and Trent, 2019). The lipid A structure strongly influences TLR4 activation. Particularly, the number of acyl chains is a critical determinant (Needham et al., 2013). Hexa-acylated lipid A is the most potent agonist of human TLR4 (hTLR4), whereas penta-acylated form are approximately 100 times less active, and the tetra-acylated forms, act as antagonist (Park et al., 2009).

Modulating the expression of lipid A biosynthesis or modifying enzymes by genetic engineering has been successfully used to reduce the toxicity of whole-cell *Bordetella* vaccines in humans and animals (Arenas et al., 2020; Perez-Ortega et al., 2021). In this study, we investigated whether increasing the production of antagonist or low-activity lipid A species in the *P. aeruginosa* PAO1 $\Delta murI$, through heterologous expression of LpxR and PagL, could attenuate LPS-mediated reactivity by reducing TLR4-driven inflammatory signaling.

2 Materials and methods

2.1 Bacterial strains, growth conditions and plasmids

The bacterial strains and plasmids used in this study are shown in Table 1. All strains were cultured in Luria-Bertani (LB) broth (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl) with aeration at 37°C or on LB plates with 2% agar. For plasmid selection in *Escherichia coli*, apramycin (Sigma-Aldrich, Inc.) or ampicillin (Sigma-Aldrich, Inc.) were added at a concentration of 50 and 100 $\mu\text{g/ml}$, respectively. *P. aeruginosa* PAO1 $\Delta murI$ was cultivated

in LB supplemented with 10 mM D-glutamate (Sigma-Aldrich, Inc.). For gene expression and plasmid maintenance in PAO1 $\Delta murI$, 1 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG; Thermo Scientific) and apramycin at 200 $\mu\text{g/ml}$ were added to the medium supplemented with 8 mM D-glutamate.

2.2 DNA manipulation

All primers used in this study are listed in Table 2. Plasmid DNA from *E. coli* was obtained with the High Pure Plasmid Isolation Kit (Roche Diagnostics, S.L.). PCR amplifications were performed using Expand High Fidelity PCR System (Roche Diagnostics, S.L.) and GoTaq Flexi DNA polymerase (Promega Biotech Ibérica, S.L.). The restriction enzymes FastDigest[®], FastAP Alkaline Phosphatase (Thermo-Fisher Scientific) and T4 DNA ligase (Promega Biotech Ibérica, S.L.) were used as recommended by the supplier. To construct plasmid pMMB67EH-PagL_{pa}-Apra, an apramycin resistance cassette was amplified from plasmid pIJ773 using primers Apra Cas PuvI F and Apra Cas PuvI R, digested with restriction enzymes, and cloned into plasmid pMMB67EH-PagL_{pa}. The resulting plasmid derivative was called pMMB67EH-PagL_{pa}-Apra. For the generation of pMMB67EH-LpxR_{pa}-Apra plasmid, the *lpxR* gene was amplified from *S. Typhimurium* LT2 genomic DNA, digested with appropriated restriction enzymes, and cloned into plasmid pMMB67EH-PagL_{pa}. Then, the apramycin resistance cassette was inserted as described above. Both plasmid constructs were verified by Sanger sequencing using primers pMMB F and pMMB R, which anneal to the flanking regions of the inserted gene.

2.3 Transformation by electroporation

To prepare electrocompetent cells of *E. coli* or *P. aeruginosa*, a 1:100 dilution of an overnight culture was inoculated into fresh LB

TABLE 1 Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant features	Reference
Strains		
<i>P. aeruginosa</i>		
PAO1	Reference strain. Isolate from a patient with a burn wound infection.	CECT 4122; (Stover et al., 2000)
PAO1 Δ murI	PAO1 derivative, Δ PA4662. Auxotroph for D-glutamate.	(Cabral et al., 2017)
PA14	Hypervirulent strain isolated from a patient with a burn wound infection.	(Lee et al., 2006)
PAO1 Δ murI plpxR _{ST}	PAO1 Δ murI derivative with the plasmid pMMB67EH-LpxR _{ST} , which overexpresses the deacylase LpxR from <i>Salmonella</i> Typhimurium. D-glutamate auxotroph. Apr ^R .	This study
PAO1 Δ murI ppagL	PAO1 Δ murI derivative with the plasmid pMMB67EH-PagL _{Pa} , which overexpresses the deacylase PagL from <i>P. aeruginosa</i> . D-glutamate auxotroph. Apr ^R .	This study
<i>E. coli</i> and <i>Salmonella</i>		
DH5 α	Δ lacZ Δ M15 Δ (lacZYA-argF)U169 recA1 endA1 hsdR17(rK ⁺ mK ⁺) supE44 thi-1 gyrA96 relA1	Lab collection (Hanahan, 1983)
<i>Salmonella enterica</i> subsp <i>enterica</i> LT2	Serovar Typhimurium, O:4,[5],12:H:i:1,2	(McClelland et al., 2001)
Plasmids		
pIJ773	pBluescript II SK(+) derivative containing the cassette <i>aac</i> (3)-IV – <i>oriT</i> (RK2) and flanking FRT sites; Apr ^R , Amp ^R	(Gust et al., 2003; Huang et al., 2014)
pMMB67EH-PagL _{Pa}	pMMB67EH derivative harboring the <i>pagL</i> gene from <i>P. aeruginosa</i> cloned under the inducible <i>tac</i> promoter. Amp ^R .	(Geurtsen et al., 2006)
pMMB67EH-PagL _{Pa} -Apra	pMMB67EH-PagL _{Pa} derivative containing the <i>aac</i> (3)-IV gen of pIJ773. Apr ^R .	This study
pMMB67EH-LpxR _{ST} -Apra	pMMB67EH derivative harboring <i>lpxR</i> from <i>S. Typhimurium</i> cloned under the inducible <i>tac</i> promoter and the <i>aac</i> (3)-IV gen of pIJ773. Apr ^R .	This study

Amp^R, ampicillin-resistant; Apr^R, apramycin-resistant; CECT, Spanish Type Culture Collection.

medium with the necessary supplements. The culture was incubated at 37°C with continuous shaking until an optical density at 600 nm (OD₆₀₀) of 0.7 was reached. The bacterial cells were harvested by centrifugation (3,900 × g, 20 min, 4°C), washed three times with cold 10% glycerol, and gradually concentrated in the same solution. The final suspension, concentrated 130-fold, was aliquoted into volumes of 50–100 μ L, which were used immediately or stored at –80°C for later use (Lofblom et al., 2007). Electroporation was performed following the procedure described by Dower et al. (1988) (Dower et al., 1988), with slight modifications. For *E. coli*, 50 μ L of electrocompetent cells were transformed with 2–4 μ L (approximately 1 μ g) of plasmid DNA obtained from a minipreparation, which had been microdialyzed to remove residual salts. The mixture of competent cells and DNA was incubated on ice for 5 min and then transferred to a 0.1 cm gap electroporation cuvette (Bio-Rad). A Gene Pulser Xcell system (Bio-Rad, USA) was used for electroporation with the following parameters: 25 μ F, 200 Ω , and 1,800 V. For *P. aeruginosa*, the procedure was similar, but used a larger volume of electrocompetent cells (200 μ L) mixed with the plasmid DNA in a 0.2 cm gap cuvette (Bio-Rad), and a voltage of 2,500 V was applied. In both cases, immediately after the pulse, 1 mL of SOC medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) supplemented as required was added, and the cell suspension was

incubated at 37°C with gentle shaking for 1 hour. After incubation, potential transformants were plated on LB agar supplemented with 8 mM D-glutamate and the appropriate selection antibiotic.

2.4 Growth and viability curves

To generate growth and viability curves for *P. aeruginosa* PAO1, D-glutamate auxotroph PAO1 Δ murI and its derivatives expressing enzymatically modified LPS (namely, PAO1 Δ murI plpxR_{ST} and PAO1 Δ murI ppagL), a single colony of each strain was inoculated into 5 mL of LB medium supplemented with either 8 mM D-glutamate and 200 μ g/mL apramycin (LGA medium), when were required. Cultures were incubated with shaking at 37°C for 16 hours. Subsequently, a 1:50 dilution was made into 100 mL of fresh medium, and 1 mM IPTG was added to induce gene expression. These cultures were then incubated at 37°C with shaking (180 rpm) for 8 hours. Samples were taken every hour to measure culture turbidity by determining the OD₆₀₀ with a spectrophotometer. To assess viability, the drop-plating technique was used (Sieuwerts et al., 2008). Briefly, 1:10 serial dilutions of the different aliquots were prepared, and 5 μ L of each dilution was plated in triplicate on supplemented solid medium. Plates were incubated at 37°C for 24 hours, and spots with a colony forming units (CFU) count of 30 or more were recorded.

TABLE 2 List of primers used in this study.

Primers	Sequence	UPL probe
PCR, sequencing and/or cloning		
pMMB F	AATCATCGGCTCGTATAATGT	–
pMMB R	CCTGGCAGTTCCTACTCT	–
LpxR Fw-NdeI	GCGCGCCATATGAACAAA TACAGCTATTG	–
LpxR Rev-BamHI	GCGCGCGGATCCTCAGAAAA AGAACGTTATAT	–
Apra Cas PuvI F	CGATCGATCGATTCCGGGGA TCCGTCGACC	–
Apra Cas PuvI R	CGATCGATCGTGTAGGCTGGAG CTGCTTC	–
RT-qPCR		
<i>eptA</i> #156_left	GCCGGTACCACCTACTTCAT	#156
<i>eptA</i> #156_right	GGTTGGTCTGCATGACGTTG	#156
<i>pagL</i> #124_left	CCTGAACCTCGAAGACCGCA	#124
<i>pagL</i> #124_right	AATAGTGGATCGCCGAACG	#124
<i>pagP</i> #26_left	ATCTCATCCTCAGCCTGCTG	#26
<i>pagP</i> #26_right	CTGCAGGTACCAGAAGTCGC	#26
<i>lpxRst</i> #124_left	ATTCTCCATCAGGCGCCAAT	#124
<i>lpxRst</i> #124_right	TCCGGTATGAAGAAAGGCGC	#124
<i>rpoS</i> #63_left	AAGAAGGGCCGGAGTTTGAC	#63
<i>rpoS</i> #63_right	ACGACTCGTCCAGCATGATG	#63
<i>apra</i> #96_left	CCATCCATTTCGCTTTGCGG	#96
<i>apra</i> #96_right	GCAGGGGCAATGGATCAGAG	#96

F, forward primer; R, reverse primer. *Restriction sites introduced into the sequence for cloning are indicated in bold. UPL, TaqMan probes from the Universal Probe Library (Roche, Germany).

2.5 Lipid A extraction and structural analysis

Lipid A was extracted using the MBT Lipid Xtract Kit (Bruker, Germany) from *P. aeruginosa* PAO1 Δ *murI* strain and its derivatives expressing deacylases, following the manufacturer’s instructions. Bacteria were grown on LB agar supplemented with 8 mM D-glutamate or LGA agar with 1 mM IPTG at 37°C for 24 hours. Then, 1- μ L inoculation loop of bacteria was hydrolyzed for lipid extraction as previously described (Hernandez-Garcia et al., 2024). Mass spectra of lipid A were acquired using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) in a MALDI Biotyper[®] Sirius System (Bruker Daltonics, Inc.). Spectra were recorded in negative linear mode over a mass range of 500-3,000 *m/z*. The structural analysis was then performed using Clover MSDS software (Clover Bioanalytical Software, Granada, Spain).

2.6 RNA extraction and gene expression analysis

Total RNA was extracted from logarithmic-phase cultures using the High Pure RNA Isolation Kit (Roche Diagnostics, Germany) and adjusted to a concentration of 50 ng/ μ L. The relative expression levels of genes involved in lipid A modification (*eptA*, *lpxR*, *pagL* and *pagP*) were evaluated using two-step reverse transcription quantitative PCR (RT-qPCR). In the first step, reverse transcription of 200 ng of RNA was performed using random hexamer primers (60 μ M) and the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Germany). In the second step, 2 μ L of the resulting cDNA was used in a 20 μ L quantitative PCR reaction carried out on the LightCycler[®] 480 Instrument II. Amplification was conducted using the LightCycler[®] 480 Probes Master Kit (Roche Diagnostics, Germany), according to the supplier’s instructions. Gene-specific primers and optimal TaqMan probes from the Universal Probe Library (Roche, Germany) were used (Table 2). These primers were designed using the software provided by the University of the Sunshine Coast (Australia), accessible at <https://primers.neoformit.com/>. The relative gene expression was calculated using the Livak method (Livak and Schmittgen, 2001) and was normalized to the endogenous control gene *rpoS* of *P. aeruginosa* and the *aac(3)-IV* apramycin resistance gene encoded on the pMMB67EH-derived plasmids. All RNA extractions and RT-qPCR assays were performed in duplicate across four independent experiments.

2.7 TLR4 stimulation assays

HEK293-Blue TLR4 cells co-expressing either human (hTLR4) or murine (mTLR4) TLR4 complex and an NF- κ B-inducible Secreted Embryonic Alkaline Phosphatase (SEAP) reporter (Invivogen) were cultured as recommended by the supplier. For TLR4 activation, 2.5×10^4 cells per well were incubated with serial dilutions of either heat-killed bacterial cells for 17 hours at 37°C, 5% CO₂ in a 96-well plate. Then, 20 μ L of the supernatant from the stimulated cells was collected and incubated with 180 μ L of the QUANTI-Blue[™] reagent for 3 hours, and absorbance was measured at 630 nm, according to the manufacturer’s instructions (InvivoGen).

2.8 Animal experiments

Animal experiments were conducted in compliance with European Union guidelines (Directive 2010/63/EU) and current national legislation (RD 53/2013) on the protection of animals used for scientific purposes. All procedures received prior approval from the Animal Experimentation Ethics Committee of CHUAC (Project Reference Number 15002/2018/007). BALB/c mice (females, 7–10 weeks old; males for subcutaneous (SC) immunization), were housed in pathogen-free facilities at the Training Technology

Center of the *Xerencia de Xestión Integrada A Coruña* (CTF-XXIAC), and were provided with pelleted rodent chow and water *ad libitum*.

For mouse inoculations, bacterial cultures were incubated in the appropriate medium at 37°C with shaking for 16 hours. After this period, cultures were diluted 1:50 in fresh medium and allowed to grow to an OD₆₀₀ of 0.7. Bacteria were harvested by centrifugation (5,000 × g, 15 min, 4°C), washed three times with sterile saline solution (0.9% NaCl), and then resuspended in saline solution to the required volume to achieve the concentration needed for *in vivo* administration. An aliquot of this suspension was plated on the appropriate culture medium and incubated overnight at 37°C. The following day, the number of viable bacteria administered per mouse was determined (Supplementary Table S1). For immunogenicity assessments of various vaccine candidates in a murine model, different administration routes were used: IN, intradermal (ID), intramuscular (IM) and SC. In all cases, mice were anesthetized with sevoflurane before each inoculation. Immunizations schedules were optimized according to the route of administration, as previously reported (Cabral et al., 2020): for the IN route, two doses were administered with a 14-day interval; for the ID and SC routes, a three-dose was applied with weekly intervals; in the combined IN and IM regimen, two IN doses were given 14 days apart, along with three weekly IM doses; with both routes aligned during the first and third weeks. Blood (around of 50 µL) was collected by puncturing the submandibular vein and allowed to clot for 15 min. The samples were then centrifuged (2,800 × g, 15 min, 4°C) to remove cellular components, and the resulting serum was stored at -80°C until use. Vaginal fluid lavage (VFL) samples were obtained by flushing with 50 µL of sterile saline. These samples were centrifuged (800 × g, 15 min, 4°C), and the supernatant was transferred to a new tube containing 5 µL of a 10× protease inhibitor cocktail (Sigma-Aldrich), and then stored at -80°C until use.

2.9 Acute lung infection model

To induce acute lung infection in BALB/c mice, animals were anesthetized via inhalation with sevoflurane and administered the hypervirulent *P. aeruginosa* PA14 strain (1 × 10⁶ CFU) via IN. To minimize expulsion of the infectious material and to promote the development of acute pneumonia, mice remained under anesthesia for an additional 2 min. The monitoring protocol involved daily observation and individual tracking of each mouse after each procedure. Parameters included body weight loss and clinical symptoms, such as inactivity or lack of response to stimuli, hunching, piloerection, tip-toe walking, lack of grooming, stereotypies, slow or labored breathing with nasal discharge, among others. Each indicator was assigned a score as follows: 0 indicated normality; 1, a slight deviation from normal; 2, a moderate deviation; and 3, a significant deviation. This scoring system allowed for the quantification of procedure severity. A humane endpoint was applied for mice with a severity score of 14 or higher, or experiencing irreversible body weight loss exceeding

25%, in accordance with previous guidelines (Hawkins et al., 2011; Morton and Griffiths, 1985). Euthanasia was conducted by administering a barbiturate overdose via IP injection of 100 µL thiopental (50 mg/mL, Braun).

2.10 ELISA

The levels of serum IgG and IgA in VLF were quantitatively determined with a whole-bacterial cell ELISA in accordance with the previously described protocol (Cabral et al., 2020; Fuentes-Valverde et al., 2022). Each well was coated with formalin-inactivated bacteria (1 × 10⁷ CFU) in 100 µL of 100 mM carbonate-bicarbonate buffer, pH 9.6, and incubated overnight at 4°C. Formalin-killed cells were prepared beforehand by incubation with 1% (v/v) paraformaldehyde at 37 °C under agitation for 2 hours and washing with sterile PBS. The horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Sigma-Aldrich) or IgA (Bethyl Laboratories) diluted 1:5,000 in DMEM supplemented with 10% fetal bovine serum were used as the secondary antibodies.

2.11 Statistical analysis

Statistical analyses were performed using GraphPad Prism software package (version 6.01). Means were compared using Student's *t* test with Welch's correction and the multiple *t*-test applied the Holm-Sidak method was used for the comparison of body weight changes. For curve comparison, data from TLR4 stimulation assays were analyzed for statistical significance using two-way ANOVA (Dunnett's correction for multiple comparison). The Kaplan-Meier survival analysis was assessed with the Mantel-Cox log-rank test. For comparisons between two groups, the nonparametric and unpaired Mann-Whitney *U* test was applied, and Kruskal-Wallis test for comparisons of three or more groups. *P* values of < 0.05 were considered statistically significant.

3 Results

3.1 Production of heterologous LPS-modifying enzymes in *P. aeruginosa* PAO1 Δ murl

LPS is a relevant immunostimulatory factor to the protective efficacy of attenuated vaccines, as it activates the host immune system. However, its strong biological activity can also lead to toxicity, resulting in reactogenic responses. Therefore, achieving an optimal balance between immune activation and tolerable reactogenicity is essential for the development of safe while effective whole-cell vaccines. We decided to engineer a novel vaccine strain that produce enriched lipid A forms with either fewer compared to the canonical toxic hexa-acylated lipid A, as these modifications result in poor activation of TLR4/MD-2 (Maeshima and Fernandez, 2013). To this end, we overexpressed

PagL to ensure an enrichment of penta-acylated lipid A species by removing the 3OH-C10 acyl chain from position 3 (Ernst et al., 2006; Rutten et al., 2006) (Figure 1B). In addition, we produced exogenous LpxR from *S. Typhimurium*, which is expected to remove the acyloxy acyl residue from the 3' position of lipid A (Reynolds et al., 2006) (Figure 1C). Both *lpxR_{ST}* and *pagL* genes were cloned into the broad host-range expression vector pMMB67EH under the IPTG-inducible promoter. The resulting plasmids were introduced into the PAO1 Δ *murI* strain, generating the derivatives PAO1 Δ *murI* *plpxR_{ST}* and PAO1 Δ *murI* *ppagL*.

The expression levels of *lpxR_{ST}* and *pagL* in both mutant derivatives and the parent strain were tested by RT-qPCR (Figure 2A). *rpoS* was used as housekeeping gene, and *aac(3)-IV* (apramycin resistance cassette) as a plasmid expression control. In the PAO1 Δ *murI* *plpxR_{ST}* strain, *lpxR* expression was enhanced about 30-fold compared to PAO1 Δ *murI* and PAO1 Δ *murI* *ppagL*. Similarly, *pagL* expression was upregulated by about 5-fold in the PAO1 Δ *murI* *ppagL* strain relative to the other strains. We also examined the expression of other lipid A modifying enzymes in *P. aeruginosa*, such as *eptA* and *pagP*. Notably, a significant upregulation of *eptA* was observed in both the PAO1 Δ *murI* *plpxR_{ST}* and PAO1 Δ *murI* *ppagL* strains. Furthermore, *pagP* was upregulated about two-fold in PAO1 Δ *murI* *ppagL* strain. In contrast, a slight reduction in *pagP* and *pagL* expression was detected in the PAO1 Δ *murI* *plpxR_{ST}* strain. Together, these

results confirm the successful expression of the targeted genes, and show that their expression influences that of other LPS-modifying enzymes. Next, we evaluate whether the activity of these enzymes on lipid A affected the growth and viability of the bacterial strains. To do this, we compared their growth kinetics in LGA medium supplemented with IPTG. Compared to the PAO1 Δ *murI*, both mutant derivatives exhibited a slight delay in the exponential growth phase relative to the parent strain (Figure 2B, left panel), which was associated with a modest but significant decrease in the number of viable bacteria (Figure 2B, right panel).

3.2 Analysis of recombinant lipid A structures

Lipid A was extracted from exponentially growing cultures of *P. aeruginosa* PAO1 Δ *murI* and their mutant derivatives grown in the presence of IPTG and antibiotics, and analyzed using MALDI-TOF in negative linear mode. An ample range of peaks from *m/z* 1200 to 1800 was detected across all strains, but their number and relative abundance varied considerably (Figure 3A). In *P. aeruginosa* PAO1 Δ *murI*, the most predominant lipid A species appeared at *m/z* 1404.2. This peak may correspond to a penta-acylated, bis-phosphorylated lipid A with one 3OH-C10 and two 3OH-C12 primary acyl chains, one C10:O and one C12:O secondary acyl

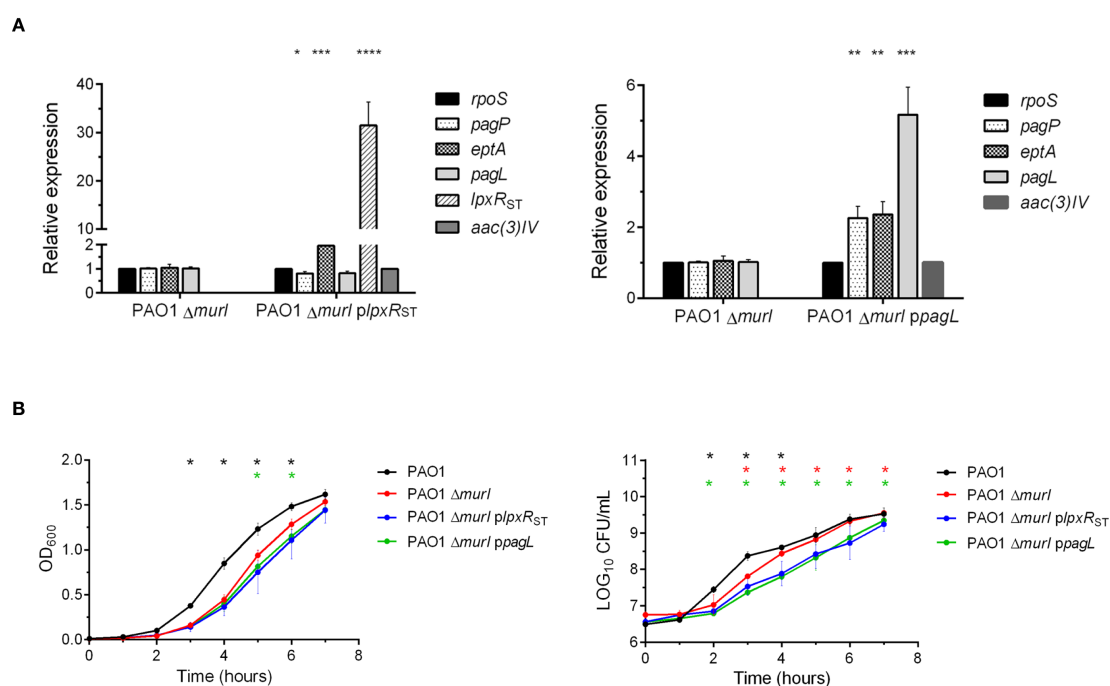


FIGURE 2

Gene expression and growth characteristics of LPS-modified *P. aeruginosa* strains. (A) Relative expression levels of genes involved in lipid A modification (*pagP*, *eptA*, *pagL* and *lpxR*) in the derivative strains PAO1 Δ *murI* *plpxR_{ST}* and PAO1 Δ *murI* *ppagL* were analyzed by RT-qPCR and compared to the reference strain PAO1 Δ *murI*. Gene expression was quantified using the Livak method ($2^{-\Delta\Delta C_t}$) and normalized to the housekeeping gene *rpoS* of *P. aeruginosa* and the *aac(3)-IV* gene present in the pMMB67EH-derived plasmids. Statistical significance was determined by unpaired t-test with Welch's correction: **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001. (B). Growth and viability of *P. aeruginosa* PAO1, PAO1 Δ *murI* and its LPS-modified derivatives PAO1 Δ *murI* *plpxR_{ST}* and PAO1 Δ *murI* *ppagL*. Cells were grown in LB medium supplemented with D-glutamate plus apramycin and IPTG, when required. Left panel: Growth was monitored by measuring culture turbidity (OD at 600 nm) at different times. Right panel: Culture viability (Log₁₀ CFU per mL). Data represent the mean $\pm$ SD. **p* < 0.01, unpaired t-test, compared to the parent strain PAO1 Δ *murI*.

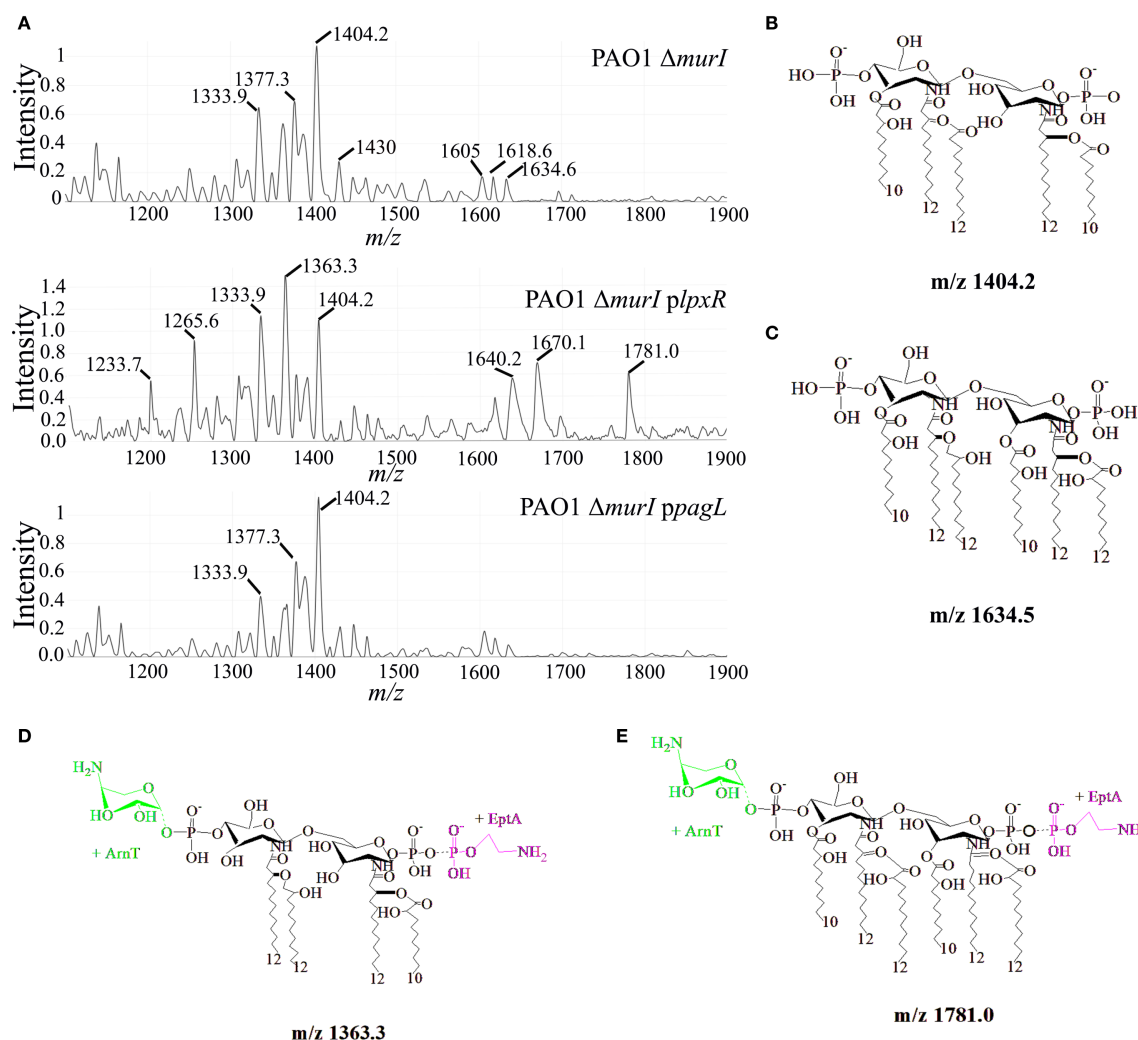


FIGURE 3

Structural analysis of lipid A in *P. aeruginosa* PAO1 $\Delta murI$, PAO1 $\Delta murI$ *plpXR*_{ST}, and PAO1 $\Delta murI$ *ppagL*. (A) Purified lipid A was analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), and negative-ion mode spectra are shown. (B–E) Proposed structures corresponding to selected m/z peaks annotated in the spectra shown in panel (A). Structures were generated using ChemDraw software.

chains (Figure 3B) (Hernández-García et al., 2024). This result partially differs from that reported by Ernst et al. (2006), who identified the major lipid A species of PAO1 lipid A at m/z 1447 (Ernst et al., 2006). This discrepancy may be explained by the substitution of a C12 secondary acyl chain at position 2 with a C10 acyl chain in our vaccine strain. Incorporation of the C12 secondary acyl chain at this position is catalyzed by LpxL2 (also known as HtrB2), which, however, showed high specificity for C12 substrates in PAO1 (Hittle et al., 2015). Recent studies have reported lipid A species at m/z 1403 in clinical *P. aeruginosa* isolates harboring mutations in *lpxL2*, *acrB2* or *cbrA* (Hernández-García et al., 2024), suggesting that our vaccine strain may harbor additional mutations beyond *murI*. In addition, the presence of this prominent species reflects the strong activity of PagL, and the lack of activity of dioxygenase enzymes: LpxO1 and LpxO2. Additionally, a broad set of low-abundance peaks between m/z 1430.9 and 1534.3 were observed. These likely correspond to penta-acylated lipid A

species with two C12:O secondary acyl chains, possibly differing in their hydroxylation status (e.g. m/z 1430.9, 1447.6, 1463.4, respectively). Of note that peaks corresponding with lipid A species harboring also C12 secondary acyl chains at 2 position were also detected in clinical isolates harboring mutations in *lpxL2*, *acrB2* or *cbrA* (Hernández-García et al., 2024), suggesting a reduced of specificity of LpxL2. Some of these peaks may carry additional modifications, such as Ara4N or pEtN, presumably at the 1 and/or 4' phosphate groups, resulting in peaks at m/z 1564, 1578, respectively. In addition, four abundant peaks with slightly lower m/z values (ranging from 1333.9 to 1377.3) were detected, presumably corresponding to dephosphorylated variants of the major penta-acylated structure (e.g., m/z 1377.3), either tetra-acylated species lacking both secondary acyl chains and AraN4 groups (e.g., m/z 1333.9). Furthermore, a minor group of peaks at m/z 1605, 1618.6 and 1634.6 were detected, and likely corresponds to hexa-acylated lipid A species. For example, the m/z 1634.6 peak

may represent a bis-phosphorylated hexa-acylated species with four hydroxylated acyl chains, while the reduced mass observed for the peaks at 1618 and 1605 suggest loss of hydroxyl groups (Figure 3C).

The expression of *lpxR_{ST}* in *P. aeruginosa* PAO1 Δ *murI* resulted in a substantial increase in the abundance of tetra-acylated lipid A species (m/z 1233.7–1363.3). The most prominent among these was the peak at m/z 1363.3, which likely corresponds to a tetra-acylated lipid A form with two primaries 3OH-C12 acyl-chains at 2 and 2' positions, two secondary acyl chains and an Ara4N and pEtN groups (Figure 3D). The synthesis of this species may reflect the combined activity of LpxR, PagL and EptA (Figure 2A). The remaining peaks may reflect structural variants of this tetra-acylated form, resulting from the loss or substitution of polar groups at positions 1 and 4' (e.g., m/z 1265.6). Curiously, two additional peaks of moderate abundance appeared at m/z 1670.1 and 1781.0, presumably corresponding with novel hexa-acylated forms with pEtN and lacking one phosphate group (m/z 1670.1), or additionally modified with Ara4N (m/z 1781) (Figure 3E). The emergence of these species may reflect the absence of PagL activity and the upregulation of pEtN modification pathways, in agreement with results from gene expression analysis (Figure 2A). The expression of *pagL* in *P. aeruginosa* PAO1 Δ *murI* led to an increased relative abundance of the peak at m/z 1404.2, and a subsequent reduction of hexa-acylated forms, which may in part be attributed to enhanced activity of PagL. Together, MS spectrometry analysis shows that LpxR and PagL expression in *P. aeruginosa* PAO1 Δ *murI* varies the heterogeneity and abundance of lipid A species which may have implications in their biological activity.

3.3 Stimulation assays of TLR4

We then investigated whether the altered LPS profiles in the mutant derivatives affected TLR4 signaling. To this end, NF- κ B SEAP reporter HEK293 cells expressing the murine TLR4/MD-2/CD14 complex were stimulated with heat-inactivated whole-cell bacteria preparations of PAO1 Δ *murI* and its derivatives. TLR4 activation was evaluated by measuring SEAP reporter gene

expression. Interestingly, both mutant strains stimulated approximately 100-fold lower mTLR4 activation compared to the parental strain (Figure 4A). This reduction is likely due to increased proportion of tetra-acylated lipid A species, known as TLR4 antagonist, in PAO1 Δ *murI* *plpxR_{ST}*, and a decrease in highly immunostimulatory hexa-acylated species in PAO1 Δ *murI* *ppagL*, resulting in an overall reduction in whole-cell bacterial activity. Because human and murine TLR4 respond differently to various LPS structures and given that our vaccine is intended for human use, we next assessed hTLR4 activation. PAO1 Δ *murI* *plpxR_{ST}* stimulated hTLR4 approximately 100-fold less than the parental strain, while PAO1 Δ *murI* *ppagL* showed a milder reduction between 10 and 100-fold depending on the dose (Figure 4B). Together, these data suggest that both recombinant strains exhibit reduced TLR4-mediated activity, indicating a potential decrease in LPS-associated toxicity compared to the wild-type vaccine strain.

3.4 Assessment of vaccine efficacy in experimental animals

In spite LPS cause reactogenic reactions in attenuated vaccines, it plays a relevant role in the immunogenic response. Therefore, reducing LPS activity could potentially compromise the efficacy of our vaccine strain. Moreover, a reduction in the TLR4 activation may not suffice to reduce the vaccine toxicity *in vivo*. To address both concerns, we evaluated the protective capacity and toxic activity of the novel recombinant strains in a murine model.

To evaluate the immunogenic capacity of PAO1 Δ *murI* *plpxR_{ST}* and PAO1 Δ *murI* *ppagL* relative to the parent strain, mice were immunized with each of the three strains, with PBS as a control, via different routes: IN, a combination of IN and IM, ID, and SC (Supplementary Table S1). The combined IN and IM routes was designed to balance mucosal immunity with overall protection by using lower, and therefore potentially safer, doses via the IN route (Cabral et al., 2020). Blood and VFL samples were collected 7 days after the final vaccine dose (day 22, D22), and again between 4 to 6 weeks post-vaccination (D41–D55) (Figure 5A), and antibody titers

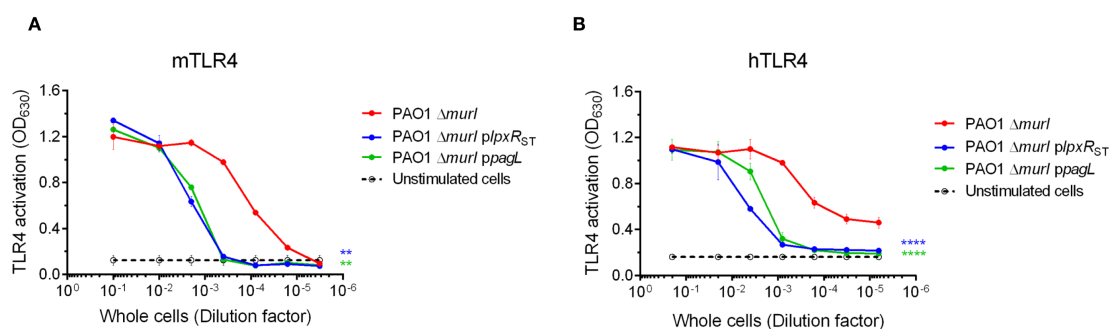


FIGURE 4

TLR4 activation by whole-cell preparations of PAO1 Δ *murI* and its derivatives with enzymatically modified lipid A by LpxR_{ST} or PagL. HEK293-Blue cells expressing mTLR4 (A) or hTLR4 (B) were cultured in microtiter plates and stimulated with 1:10 serial dilutions of heat-inactivated whole-cells (initial OD₆₀₀ = 0.1) for 17 hours at 37°C, 5% CO₂. Alkaline phosphatase activity in the culture supernatants was measured at OD₆₃₀. Results represent the mean $\pm$ SD of three independent experiments. ** p < 0.01, **** p < 0.0001 (two-way ANOVA with Dunnett's multiple comparisons test), relative to the parent strain.

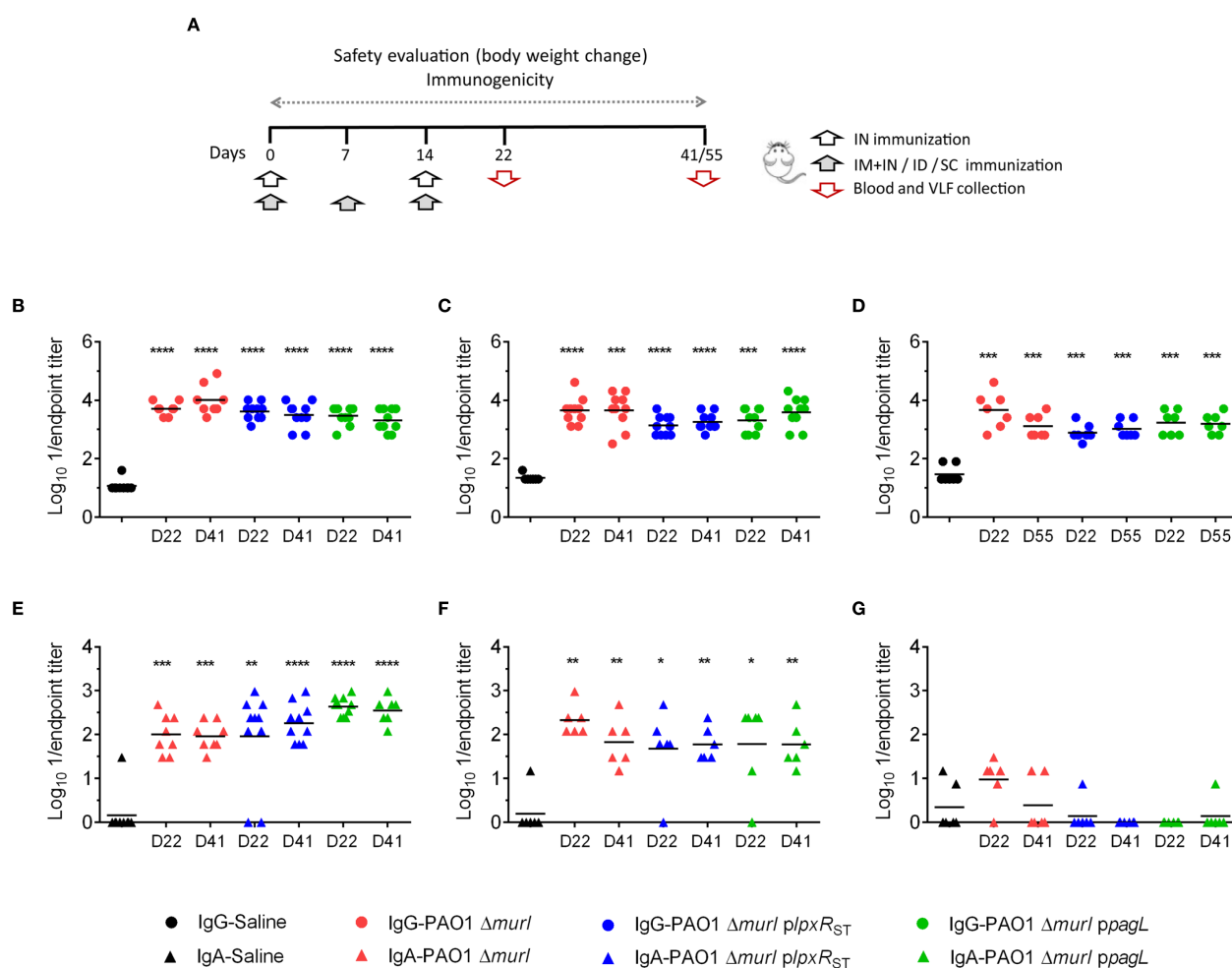


FIGURE 5

Systemic and mucosal antibody-mediated immune response against PAO1 in BALB/c mice after immunization with PAO1 $\Delta murI$ and its lipid A variants. (A) Immunization schedule based on the administration routes used. Determination of serum IgG (B–D) and vaginal wash IgA (E–G) titers against PAO1 by ELISA at 7 days (D22) and 4 to 6 weeks (D41–D55) after the final vaccine dose. Serum IgG titers (circles) following IN (B), ID (C), and SC (D) immunizations. Vaginal fluid lavage IgA titers (triangles) following IN (E), IN+IM (F), and ID (G) immunizations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (Mann-Whitney test), compared to the saline group.

were determined by whole-cell ELISA. Interestingly, all vaccine candidates elicited robust serum IgG responses across all administration routes tested (IN, ID and SC) (Figures 5B–D). However, elevated IgA levels were detected only in VFL samples from mice vaccinated via the IN route and the combined IN plus IM route (Figures 5E, F). Unexpectedly, IN administration of PAO1 $\Delta murI$ *ppagL* resulted in significantly higher IgA titers compared to PAO1 $\Delta murI$, both at D22 ($p = 0.0036$) and D41 ($p = 0.0059$) of the vaccination schedule (Figure 5E). No significant differences were detected in ID immunizations (Figure 5G).

To assess vaccine efficacy, animals were immunized via IN, IM or a combination of routes as described in Figure 5A. Then, mice were IN challenged with the virulent *P. aeruginosa* PAO1 strain. Survival rates of the vaccinated mice were compared to those of an unvaccinated control group. Protection varied according to the route of vaccine administration. Mice immunized IN showed the highest survival rate (87.5%), whereas those vaccinated subcutaneously exhibited lower survival, ranging from 30% to

50% (Figures 6A–D). No statistically significant differences in survival were observed among the groups vaccinated with the different recombinant strains. However, mice vaccinated with PAO1 $\Delta murI$ *ppagL* via IN plus IM or SC routes showed relatively higher survival compared to those vaccinated with PAO1 $\Delta murI$ *plpxR_{ST}* (Figures 6B, D). For example, at 72 hours post challenge, mice immunized with PAO1 $\Delta murI$ *ppagL* showed survival rates of 58–83%, whereas those immunized with PAO1 $\Delta murI$ *plpxR_{ST}* showed survival rates of 33–42%. Together, our data reflects that vaccine efficacy was not compromised by the overproduction of *LpxR_{ST}* or *PagL*.

3.5 Safety assessment of vaccine candidates in experimental animals

The reactogenicity of the recombinant strains was evaluated in immunized animals (Figure 5A) by monitoring body weight and

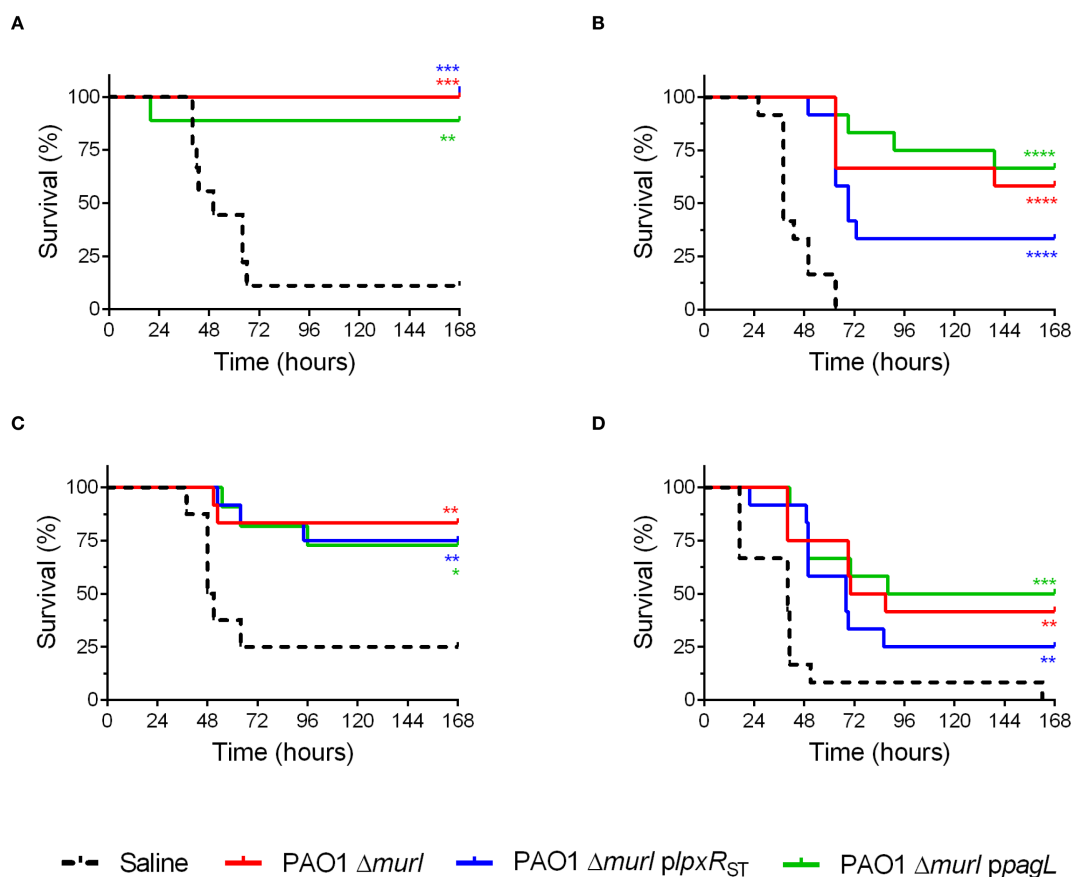


FIGURE 6

Protection against acute pneumonia after immunization with PAO1 $\Delta murl$ and its lipid A derivatives expressing the deacylases LpxR_{ST} or PagL. BALB/c mice ($n = 9-12$) were immunized via different delivery routes: IN (A), IN+IM (B), ID (C), and SC (D). Mice were IN challenged with PA14 (1×10^6 CFU) 4–6 weeks after last immunization. Survival rates of vaccinated versus sham-immunized mice are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (Log-rank Mantel-Cox test), compared to the saline control group.

clinical signs after immunization. Mice vaccinated via the IN route generally exhibited a significant decrease in body weight in some cases approaching a 20% reduction after the first vaccine dose, mainly PAO1 $\Delta murl$ $plpXR_{ST}$ strain (Figure 7A). These weight losses were transient, and most mice regained their initial weight within 7 days post-inoculation. In contrast, in the combined IN and IM immunization regimen, weight loss occurred only after IN administration (Figure 7B), while no adverse effects were detected after IM administration alone. Mice inoculated via ID and SC (Figures 7C, D) routes showed only minor weight fluctuations, with decreases under 5% relative to the initial group weight.

Mice vaccinated via the IN route generally displayed mild clinical signs, including a slightly unkempt appearance, piloerection, and in some cases, a hunched posture with reduced mobility. However, respiratory function remained unaffected, and no episodes of dyspnea were observed. No adverse effects were detected after IM administration alone, neither significant behavioral changes were observed. However, small skin lesions were observed at injection sites in mice receiving ID inoculations. Severity scores calculated across strains and time points revealed no notable differences between the recombinant strains and the wild type (Figure 7E).

4 Discussion

P. aeruginosa is a major respiratory pathogen, prompting efforts to develop effective antimicrobials and vaccines (Killough et al., 2022). Live attenuated vaccines, particularly auxotrophic strains, are promising due to their high immunogenicity and ability to elicit robust cellular responses. Deletion of the *murl* gene in *P. aeruginosa* PAO1 resulted in a D-glutamate auxotroph that exhibits strictly self-limiting growth *in vivo* and undergoes autolysis in its absence, thereby preventing infection while inducing protective and cross-reactive immune responses (Cabral et al., 2017). This intrinsic replication-limiting property clearly distinguishes our candidate from other attenuated strains that retain broader replicative capacity *in vivo*, and it provides a strong additional safety margin. However, high-dose IN administration caused significant weight loss in mice (Cabral et al., 2020; Fuentes-Valverde et al., 2022), likely due to the endotoxin-like activity of LPS. To reduce this reactogenicity, we engineered vaccine strains with modified lipid A by overexpressing the deacylases LpxR from *S. Typhimurium* and PagL from *P. aeruginosa*, aiming to reduce TLR4 activation. We anticipate comparable safety and efficacy profiles for the auxotrophic derivatives engineered to overexpress *pagL* and *lpxR*.

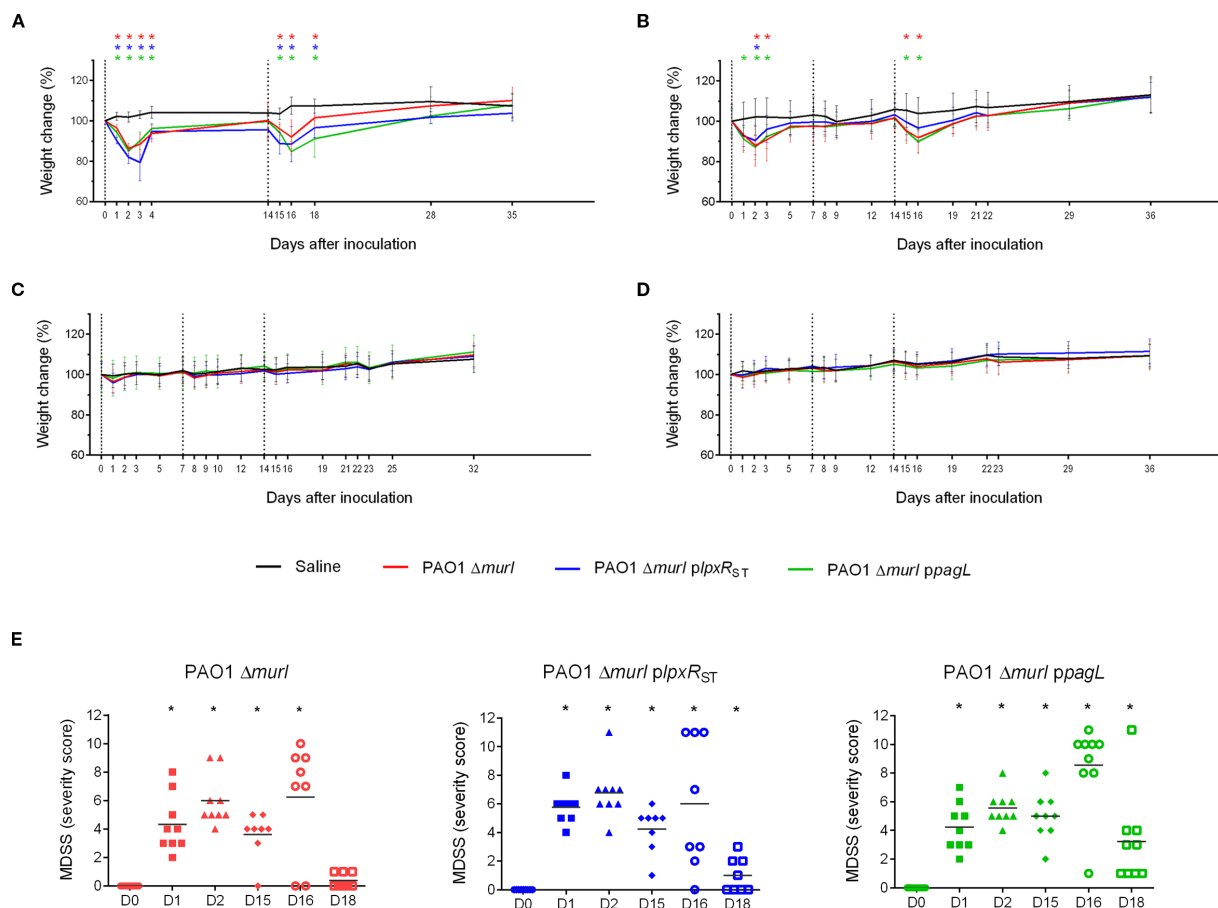


FIGURE 7

Vaccine safety assessment in BALB/c mice following administration of the PAO1 $\Delta murl$ strain or its lipid A variants expressing LpxR_{ST} or PagL. Effect of immunization on body weight following two IN doses (A), a combined IN and IM regimen (B), or three doses by ID (C) or SC (D) routes. The graph shows the percentage change in the average body weight of mice across experimental groups. Dotted lines indicate immunization days: day 0 (start of the experiment), day 7, and day 14. Data represent the mean $\pm$ SD. * $p < 0.05$, unpaired t -test, compared to the saline group. (E) Clinical evaluation following IN administration of the PAO1 $\Delta murl$ strain and its LPS-modified derivatives expressing LpxR_{ST} or PagL deacylases. Clinical assessments in BALB/c mice were performed using a scoring system considering parameters such as piloerection, grooming, mobility, respiratory pattern, dehydration, and body weight. D, day. * $p < 0.05$, unpaired t -test.

With regard to the potential increase in β -lactam and polymyxin B resistances associated with *pagL* overexpression, we believe the auxotrophic nature of our vaccine strain, together with the demonstrated lack of systemic persistence, substantially mitigates the associated risks.

LPS plays a crucial physiological role in stabilizing the outer membrane by forming ionic bridges with Ca^{2+} and Mg^{2+} cations (Knirel et al., 2006). During host-pathogen interaction, lipid A is delivered to the TLR4/MD-2 complex by CD14 and the LPS-binding protein. Within this complex, the acyl chains of lipid A establish hydrophobic interactions with aliphatic amino acids, while its phosphate and hydroxyl groups form hydrogen bonding with lysines (Park et al., 2009). The immunostimulatory potency of LPS depends on lipid A acylation, for example, the hexa-acylated lipid A form is approximately 100-fold more active than the penta-acylated form (Park et al., 2009). Our spectral analysis of vaccine strains with native LPS revealed considerable heterogeneity, including both penta- and hexa-acylated species.

Production of LpxR in PAO1 $\Delta murl$ produced an enrichment of tetra-acylated and hexa-acylated species decorated with Ara4N and pEtN groups. In *E. coli*, LpxR expression generated, among other species, a tetra-acylated form (R-lipid A) that was approximately 30-fold less active in stimulating hTLR4 than native hexa-acylated (U-lipid A), but 30- to 100-fold more active than the hepta-acylated variant (P-lipid A) produced through *pagP* expression (Kawasaki et al., 2012). Hence, the authors suggested that LpxR-mediated 3'-O-deacylation and PagP-dependent palmitoylation act synergistically in *S. Typhimurium* to modulate lipid A bioactivity. Thus, the alterations detected in our mutant may cause by a reduced PagL and PagP activity, together with upregulation of pEtN modification pathways in agreement with that detected in RT-qPCR assays. In contrast to *Enterobacteriaceae*, where PagP introduces a palmitate at the 2' position, *P. aeruginosa* PagP (PA1343) adds a secondary acyl chain at the 3' position of lipid A (Thaipisuttikul et al., 2014). This indicates potential competition between LpxR_{ST} and PagP for the same modification

site on the LPS molecule in *P. aeruginosa* (Figure 1). Moreover, heterologous expression of *lpxR_{ST}* in *P. aeruginosa* alters lipid A composition by increasing *eptA* expression, while slightly reducing *pagP* and *pagL* levels. This suggests a regulatory adjustment of these enzymes at reinforcing membrane charge and maintaining a balance of lipid A remodeling. Notably, stimulation with heat-killed PAO1 Δ *murI* *plpxR_{ST}* cells resulted in a 100-fold decrease in hTLR4 activation compared to native PAO1 (Figure 4B), highlighting its potential as a safer vaccine candidate.

In addition, we also tested if enhancement of PagL expression may reduce the number of active hexa-acylated species present in the wild type leading to reduced LPS activity (Figure 1B). Overexpression of *pagL* in *Bordetella pertussis* and *E. coli* reduces hTLR4 activation by generating modified LPS (Geurtsen et al., 2006; Kawasaki et al., 2004). In our *P. aeruginosa* vaccine strain, production of PagL revealed a low frequency of penta- and hexa-acylated variants as compared to the wild type (Figure 3), indicating altered acylation patterns. This change likely triggers membrane stress responses via the PhoPQ or PmrAB two component systems (Simpson and Trent, 2019), leading to *eptA* and *pagP* upregulation to restore membrane integrity. These modifications may interfere with TLR4/MD-2 recognition, particularly at the phosphate moieties, reducing TLR4 activation. Stimulation with heat-inactivated PAO1 Δ *murI* *ppagL* cells resulted in significantly lower mTLR4 and hTLR4 activation than wild-type PAO1 strain, suggesting again reduced reactogenicity of our vaccines. Interestingly, in *B. pertussis*, *pagL* overexpression reduce IL-6 levels in response to purified LPS, but increases activation with whole cells. This effect may result from a higher availability of free lipid A, as membrane-associated lipid A establishes fewer Van der Waals interactions, weakening its binding and promoting membrane evagination and enhanced outer membrane vesicles (OMV) production (Geurtsen et al., 2005). Similarly, studies have shown that PagL-mediated deacylation increases both the number and size of OMVs (Elhenawy et al., 2016). Furthermore, PhoPQ and PmrAB activation, under acidic pH or low Mg²⁺ concentration promotes OMV release enriched with hepta-acylated lipid A via PagP (Bonnington and Kuehn, 2016).

Identifying correlations between vaccine induced protection and biological indicators (biomarkers) is relevant for predicting efficacy and comparing formulations. In acute *P. aeruginosa* infection models, the humoral immune response plays a critical role in reducing pulmonary bacterial loads following IN administration of *P. aeruginosa* (Sen-Kilic et al., 2021). Previous work showed that immunization with PAO1 Δ *murI* induced serum IgG capable of recognizing heterologous *P. aeruginosa* strains, and passive antibody transfer conferred protection in mice (Cabral et al., 2017, 2020). In this study, although minor variations in administered CFU doses within the same order of magnitude, as well as differences in administration route and schedule, could potentially influence immune outcomes, our data show that all vaccine candidates (via IN, ID, SC) elicited comparable increases in serum IgG levels. Notably, IN immunization with PAO1 enhanced mucosal IgA levels in both bronchoalveolar and vaginal washes (Cabral et al.,

2020). A similar increase in vaginal IgA was observed with our enzyme-overexpression strains, suggesting that coordinated mucosal responses across different mucosal sites (Belyakov and Ahlers, 2009) are preserved in these mutants. Previous studies have also reported elevated total and pathogen-specific IgA during early CF infection (Collin et al., 2020), along with increased PagL expression in *P. aeruginosa* isolates from infants with CF (Ernst et al., 2006). However, PagL function can be lost during long-term airway adaptation, correlating with more severe disease (Ernst et al., 2007). In agreement with these observations, the PagL-overexpressing strain in our study induced slightly higher mucosal IgA despite reduced TLR4 activation. We hypothesize that attenuated inflammation may minimize tissue damage, facilitating dendritic cell interactions and promoting a cytokine milieu conducive to IgA class switching. Alternatively, PagL-modified lipid A may bias TLR4 signaling toward TRIF, as reported for monophosphoryl lipid A (Mata-Haro et al., 2007), thereby dampening pro-inflammatory cytokines and favoring a regulatory environment (IL-10, TGF- β) that supports IgA induction (Bagheri et al., 2019). Indeed, all mutants induced robust antibody responses, including IgG and IgA levels, and protected mice against acute lung infection caused by *P. aeruginosa*. However, protection outcomes varied noticeably by route of administration: IN immunization yielded the highest survival rates, whereas SC vaccination was associated with lower survival. Yet, our mutants failed to reduce vaccine reactogenicity *in vivo*, despite diminished TLR4 activation observed *in vitro*. This discrepancy suggests that additional immune pathways beyond TLR4 may contribute significantly to the inflammatory response, including the activation of other pattern recognition receptors, less effective lipid A modifications generated under host physiological conditions, or the involvement of non-LPS components. Our findings underscore a critical, route-dependent trade-off between vaccine efficacy and tolerability. While IN administration provided the strongest protection against a lung challenge, this benefit was counterbalanced by significant reactogenicity at the mucosal site. Conversely, systemic routes such as SC or ID injection were well tolerated, but conferred substantially weaker protection. This dichotomy represents a major obstacle for clinical translation. Achieving an optimal balance between safety and efficacy will likely require strategies such as detoxified adjuvants, prime-boost regimens that separate systemic priming from mucosal boosting, or novel formulations that minimize epithelial irritation while preserving mucosal immune engagement. Complementary to these approaches, future research should explore additional lipid A-modifying enzymes, extending the modifications generated in this study, as a means to develop safer and less reactogenic anti-*P. aeruginosa* vaccines.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by The Animal Experimentation Ethics Committee of CHUAC. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MM: Conceptualization, Formal Analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. VF-V: Formal Analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. PG: Conceptualization, Formal Analysis, Investigation, Visualization, Writing – review & editing. JAV: Conceptualization, Investigation, Writing – review & editing. JP-O: Formal Analysis, Investigation, Visualization, Writing – review & editing. RS-F: Formal Analysis, Investigation, Methodology, Writing – review & editing. AC: Formal Analysis, Investigation, Visualization, Writing – review & editing. MO: Formal Analysis, Funding acquisition, Visualization, Writing – review & editing. JA: Conceptualization, Formal Analysis, Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing. GB: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2025.1664169/full#supplementary-material>

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