

Development of a PapG–AcmA Surface-Displayed Antigen on *Lactobacillus reuteri* as a Vaccine Strategy against Uropathogenic *Escherichia coli*

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Research Article

Keywords: Uropathogenic *Escherichia coli* (UPEC), PapG Adhesin, *Lactobacillus reuteri*, Fusion Protein Vaccine, Immune Response

Posted Date: June 19th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-9986822/v1>

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Archives of Microbiology on September 15th, 2026. See the published version at <https://doi.org/10.1007/s00203-026-05214-9>.

Abstract

The prevalence of urinary tract infections (UTI) is very high in different societies. The uropathogenic strains of *Escherichia coli* strain commonly is identified as the foremost contributor to UTI incidence and causes more than 85% of cystitis and acute pyelonephritis. One way to prevent acute urinary tract infection is to vaccinate against these infections. Given that the attachment step is essential for establishing colonization and initiating infection, one of the most effective strategies is to eliminate bacteria binding. The subunit of papG from P-type P *Escherichia coli* is an important factor in bacterial colonization in the urinary tract. In this project, a chimera sequence was designed using papG (accession No. YP 543821.1) and AcmA (accession No. NC 0117511), subcloned in PET21a expression vector and transferred into *E. coli* origami (DE3) expression host. The bioengineered chimeric protein variant was isolated and its identity verified through Western blot analysis. *Lactobacillus reuteri* was adjacent to the recombinant papG.AcmA protein and binding was confirmed by ELISA. Seventy experimental mice were allocated into five groups (14 mice per group) and were underwent a triple-dose vaccination regimen with alum-based PapG given every two weeks, PapG formulated with Freund's adjuvant, PapG-AcmA/*L. reuteri*, or PBS/*L. reuteri*. *L. Reuteri* and PBS to study the effect of compounds in stimulating the humoral and cellular immune response. The findings indicated that bacteria displaying PapG may serve as a promising competitive inhibitor against UPEC.

1. Introduction

Infections of the urinary tract (UTIs), among the most prevalent bacterial infectious diseases, are primarily attributed to uropathogenic *Escherichia coli* (UPEC) and may present as symptomatic or asymptomatic across all age groups [1, 2]. UPEC strain reservoir is a fecal flora that spreads to the mucous membrane of UTIs, as it climbs to the bladder and connects to the epithelial region of the area. After binding, the bacteria proliferates and causes the infection localized cystitis [3, 4]. If the bacteria has an upward movement and reaches the kidney, it can lead to pyelonephritis. Since the adhesion step is critical for bacterial colonization and subsequent infection, one potential strategy involves inhibiting this binding process [5, 6]. The papG in P-pili of UPEC, consisting of 355 amino acids, is an important factor in the bacterial colonization in epithelial cells of the intestine, vagina, and urethra [7–9]. PapG is positioned at the distal tip of the fimbriae and mediates the attachment of the uropathogen to its specific host receptors which might be on surfactant-like particles released by the human intestine [10]. Studies have shown that 91% of *Escherichia coli* obtained from Pyelonephritis patients have P fimbriae [11]. Antibiotics have been widely used to treat UTIs caused by UPEC, however, increasing application of antibiotics has many side effects, including disrupting normal flora and bacterial resistance in different organs during treatment [12]. Therefore, treatment with normal and protective flora bacteria, such as lactobacilli and probiotics, is introduced to treat UTIs [13, 14]. Lactobacilli provides a bactericidal barrier against pathogens that can colonize in the bladder and climb into the urethra [15]. Lactobacillus also significantly affect the production of antibodies and immune cell responses, thus they can play a role in resistance to diseases [16, 17]. The gradual application of these bacteria over other mechanisms may inhibit uropathogens to move toward the bladder, due to the interference of lactobacilli with the expression of pathogenic virulence, as well as the competition in the replacement [15, 18]. Although several clinical trials evaluating vaccine candidates against urinary tract infections have been conducted in humans [19, 20], none have yielded satisfactory outcomes. Consequently, developing a vaccine that targets UPEC adhesion may offer a promising approach for controlling UTIs. In this study, we therefore aimed to display surface-expressed PapG protein in *Lactobacillus* species. to study whether on the one hand, the compound increases the protein binding on bacterial surface, and on the other hand, the lactobacillus strain acts as a competitive strain against strains. In the following, a study was conducted to evaluate the protein in the mouse model to develop a vaccine candidate against urinary system infection.

2. Material and Methods

2.1. Construct design

In silico analyses were employed to examine the receptor-binding region of the PapG antigen displayed on erythrocyte membranes. Therefore, the binding sequence in eight uropathogenic *E. coli* strains was extracted (<https://www.expasy.org/>)

and studied using concurrent alignment of multiple data sets by CLUSTALW (Thompson et al., 1997). The sequence encoding amino acids 639–895 of Acetylmuramidase (AcmA) was conjugated with the carboxyl-terminal domain of the target gene via a hydrophobic linker consisting of five repeats of the EAAAK motif. NdeI and BamHI restriction sites were incorporated at the amino- and carboxy-terminal regions of the construct, respectively. The resulting sequence was artificially synthesized and inserted into the pEXA vector by Bioneer Co. (Korea). The PapG-AcmA chimera was then digested with NdeI and BamHI (Fermentas, Germany) and ligated into the similarly digested pET21a vector using T4 DNA ligase (Fermentas, Germany) to generate the pET21a-PapG-AcmA plasmid. pET21a/PapG vector was used to express PapG protein[21]. *E. coli* BL21 (DE3) was transformed using Electroporation was performed using a Bio Rad micro-pulser at 1,200 V in a 0.1 cm cuvette. The presence of the pET-hm plasmid was verified through restriction enzyme digestion. and sequencing (Macrogen Institute).

2.2. Expression and Purification

Recombinant PapG and PapG-AcmA proteins were generated using a bacterial expression platform based on genetically engineered *Escherichia coli*. Expression constructs encoding the target proteins were introduced into the host strain, and cultures were propagated under conditions that supported high metabolic activity. Induction of recombinant protein synthesis was achieved through chemical activation of the expression system, while parallel non-induced cultures were processed to evaluate background expression. Recovery of recombinant proteins was performed under denaturing conditions using an affinity-based enrichment approach exploiting histidine-dependent metal binding. This strategy enabled selective isolation of His-tagged products from complex cellular extracts. The presence of the PapG-AcmA fusion protein was confirmed by immunochemical analysis employing tag-specific antibodies, and overall protein integrity was evaluated using electrophoretic separation coupled with dye-based visualization. To ensure suitability of the purified preparations for downstream immunological analyses, residual endotoxin levels were assessed using a standardized chromogenic detection assay. Endotoxin concentrations were consistently maintained within acceptable limits for experimental immunology applications, in accordance with established guidelines [22, 23].

2.3. PapG-AcmA binding condition on *Lactobacillus reuteri*

Preparation of cell-associated fractions was performed using stationary-phase cultures grown in MRS medium. Following biomass recovery, bacterial cells were processed to remove residual medium components and subsequently resuspended in a defined volume of fresh MRS broth. To facilitate surface association, the cell suspension was exposed to PapG or PapG–AcmA fusion proteins at a final concentration of 50 µg/mL and maintained at ambient temperature for a defined interaction period. After completion of the binding phase, cells were separated from unbound protein through sequential washing steps using phosphate-buffered saline (PBS; 58 mM Na₂HPO₄·2H₂O, 17 mM NaH₂PO₄, 68 mM NaCl; pH 7.2). The presence of surface-associated PapG was subsequently evaluated by enzyme-linked immunosorbent assay (ELISA) following a previously established analytical protocol [23].

2.4. Mice and immunization

Female BALB/c mice aged 6–8 weeks were used as the experimental model in this study. Animals were obtained from an accredited breeding facility and allocated into multiple experimental cohorts to enable parallel assessment of immunological responses and infection challenge outcomes. Group assignments were designed to ensure appropriate representation for immunoassays, challenge experiments, and negative control conditions, as summarized in Table 1.

For evaluation of systemic immune responses, mice received a recombinant PapG-AcmA formulation via subcutaneous administration according to a prime–boost schedule. Each immunization consisted of 50 µg of antigen formulated with complete Freund's adjuvant and was administered at defined intervals. Control animals received either antigen formulated with alum or phosphate-buffered saline using an identical dosing schedule. To assess mucosal immunization and protection, a separate cohort of mice was orally administered live *Lactobacillus reuteri* engineered to display PapG on the bacterial cell surface. Bacterial suspensions were prepared in sterile phosphate-buffered saline at a standardized cell density and delivered by oral gavage over multiple consecutive administrations. Blood samples were collected at predetermined time points for serological analysis, and sera were stored at – 20°C until further use. All animal handling and experimental procedures were

performed in accordance with institutional guidelines for the care and use of laboratory animals and were approved by the Animal Care and Use Committee of Islamic Azad University.

2.5. Lymphocyte proliferation assay

Antigen-dependent cellular immune responses were assessed by monitoring DNA synthesis in splenocyte populations following in vitro stimulation. Proliferation was quantified using a bromodeoxyuridine (BrdU) incorporation assay coupled to ELISA-based detection (Roche Molecular Biochemicals, Mannheim, Germany). Spleens were aseptically excised from immunized mice two weeks after the final booster immunization, and single-cell suspensions were prepared under cold conditions in phosphate-buffered saline supplemented with fetal bovine serum. Red blood cells were selectively eliminated, after which viable leukocyte populations were recovered and maintained in RPMI 1640 medium formulated to support lymphocyte viability and metabolic activity.

For assessment of proliferative capacity, equal volumes of splenocyte suspensions were distributed into 96-well culture plates and challenged with PapG antigen at a defined concentration. Phytohemagglutinin-A was included as a positive mitogenic stimulus, whereas unstimulated cultures and medium-only wells were used to define baseline and background responses. Following an extended incubation period sufficient to permit antigen-driven cell cycle progression, BrdU was supplied to the cultures to label newly synthesized DNA. Cellular material was subsequently immobilized, and excess medium was removed prior to signal development.

Incorporated BrdU was detected using a specific monoclonal antibody and visualized by an enzymatic colorimetric reaction employing tetramethylbenzidine (TMB) substrate under light-restricted conditions. Signal intensity was quantified spectrophotometrically, and proliferative responses were expressed as a stimulation index (SI), calculated as the ratio of absorbance values obtained from antigen-stimulated cultures relative to unstimulated controls [24].

2.6. Quantitative detection of cytokines in vivo

Levels of the cytokines tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and interleukin-10 (IL-10) were quantified by enzyme-linked immunosorbent assay (ELISA). Two weeks following the final immunization, splenocytes were isolated and seeded at a density of 3×10^6 cells per well in 24-well culture plates. Cells were stimulated with PapG-AcmA protein at a final concentration of 10 $\mu\text{g/mL}$ and incubated at 37°C under a humidified atmosphere containing 5% CO₂.

After 72 hours of stimulation, culture supernatants were harvested and analyzed for cytokine content using commercially available ELISA kits (Mabtech, Sweden) in strict accordance with the manufacturer's protocols. Cytokine concentrations were calculated from standard curves and are reported as picograms per milliliter (pg/mL).

2.7. Determination of IgG1, IgG2a subclasses

Antigen-specific humoral immunity against PapG was characterized through a plate-based immunoassay designed to detect serum-derived antibodies. To establish antigen capture, PapG protein was immobilized onto high-affinity microplate surfaces under controlled conditions, after which nonspecific interactions were minimized using a protein-based blocking strategy. Serum samples collected from immunized mice were analyzed across a broad dilution range to assess antibody reactivity profiles. Following exposure of the immobilized antigen to diluted sera, antigen-bound antibodies were identified using enzyme-linked secondary detection reagents specific for murine immunoglobulins. Signal generation was achieved through a colorimetric reaction, and antibody reactivity was quantified by measuring absorbance values using a microplate-based detection system. To further resolve the nature of the antibody response, IgG1 and IgG2a subclasses were evaluated independently using isotype-specific secondary antibodies. Subclass analyses were performed at a fixed serum dilution to allow direct comparison of IgG1- and IgG2a-associated reactivity, in accordance with standardized detection procedures recommended by the reagent manufacturer.

2.8. Murine urinary tract infection model

Fresh overnight bacteria, isolated from a from a paulonyphritis patient, were grown in shaking fresh LB broth for 2-day period at 37°C, pelleted, and resuspended in LB broth before challenge. 1×10^8 cfu/ml bacteria was inoculated ascendantly into bladders. Mice were divided into five groups (heads/group) and euthanized after 6, 12, 24, 48 and 72 h followed by harvesting aseptically bladder and kidney. Homogenized tissues were prepared in three different dilutions of 10^{-1} , 10^{-2} and 10^{-3} , cultured on agar and incubated for 18h at 37 °C. Bacterial numbers were determined by colony counts and expressed as CFU/ml.

2.9. Statistical analysis

All experiments were performed in triplicate, and the data were reported as means $\pm$ SD. All statistical analyses were carried out by the T-Test, one way annova test and SPSS v18 software. In all of the cases, P values < 0.05 were considered to be statistically significant.

3. Results

3.1. Construct design

The chimeric PapG–AcmA sequence was generated by fusing 198 amino acids of PapG (residues 480–620) to 85 amino acids of AcmA (residues 639–895) using hydrophobic linkers, followed by addition of a C-terminal 6×His tag (Fig. 1A). Bioinformatic assessment predicted high structural stability with preserved surface exposure of the antigenic domains.

3.2. Expression, purification, and confirmation of chimeric protein

Successful construction of the chimeric gene was verified by restriction enzyme digestion analysis, which produced the expected fragment pattern (Fig. 1B). Expression analysis demonstrated the appearance of a distinct recombinant protein band with an apparent molecular weight of approximately 34 kDa in *Escherichia coli* cultures following IPTG induction. The target protein was efficiently recovered by nickel–nitrilotriacetic acid (Ni-NTA) affinity purification, as evidenced by SDS–PAGE analysis performed on a 12% polyacrylamide gel (Fig. 1C). The molecular identity of the expressed product was further validated by immunoblotting using anti–His-tag antibodies, confirming the presence of the His-tagged PapG and PapG-AcmA constructs (Fig. 1D). Assessment of endotoxin contamination using the Limulus Amebocyte Lysate (LAL) assay indicated that endotoxin levels in the purified recombinant proteins were consistently below 0.07 EU/mL, meeting acceptable thresholds for downstream immunological applications.

3.3. Detection of PapG Surface Expression on *Lactobacillus reuteri*

Surface localization of the PapG protein on *Lactobacillus reuteri* was verified by ELISA using an anti–His-tag antibody, exploiting the presence of a C-terminal 6×His tag on the recombinant PapG construct. ELISA measurements demonstrated a markedly higher absorbance signal in bacteria displaying PapG on their surface compared with untreated control cells, confirming successful surface presentation of the antigen. In addition, longitudinal analysis of optical density (OD) values following storage at 4°C for 1, 2, 3, 4, and 60 days indicated sustained signal intensity over time. These findings collectively confirm the stability of the surface-displayed PapG protein on *L. reuteri* throughout the evaluated incubation period (Fig. 3).

3.4. Assessment of Lymphocyte Proliferation

Analysis of splenocyte proliferative activity revealed distinct differences among the immunization strategies. Enhanced lymphocyte proliferation was observed in mice receiving PapG formulated with alum, PapG combined with Freund's adjuvant, and PapG-AcmA/*Lactobacillus reuteri* when compared with baseline control responses, indicating effective antigen-driven cellular activation. A comparative evaluation of proliferation profiles demonstrated that formulations containing classical adjuvants (alum or Freund's) elicited significantly stronger lymphocyte expansion than the PapG-AcmA/*L. reuteri* preparation ($p = 0.0011$). Consistent with this observation, stimulation index analysis identified the PapG/alum formulation as the most

potent inducer of lymphocyte proliferation, displaying a significantly higher stimulation index relative to other experimental groups ($p < 0.0008$) (Fig. 3).

3.5. Detection of cytokines

Cytokine profiling of splenocyte cultures demonstrated a clear immunomodulatory effect following administration of the different PapG-based formulations. Compared with control samples, stimulation of cells derived from mice immunized with PapG/Freund's, PapG/alum, and PapG-AcmA/*Lactobacillus reuteri* resulted in elevated secretion of IL-4, IFN- γ , and TNF- α . Among the evaluated cytokines, IL-4 production was most pronounced in splenocytes obtained from the PapG-AcmA/*L. reuteri* group, showing a highly significant increase ($p < 0.0001$). In contrast, IL-4 levels induced by PapG/alum and PapG/Freund's were comparable, with no statistically significant difference observed between these two adjuvant formulations. A similar pattern was noted for IFN- γ release, as both PapG/alum- and PapG/Freund's-immunized groups exhibited equivalent levels of this cytokine ($p = 0.7069$). Analysis of TNF- α secretion revealed a distinct response profile, with the highest concentrations detected in the PapG/alum group, which differed significantly from the control condition ($p < 0.0228$) (Fig. 6).

3.6. Determination of IgG1, IgG2a subclasses

Analysis of humoral immune responses revealed a clear enhancement of antigen-specific IgG production following immunization with PapG formulated with different adjuvant systems. Elevated total IgG levels were detected in mice receiving PapG/alum, PapG/Freund's adjuvant, as well as in the group administered *Lactobacillus reuteri*, with all three immunization strategies showing statistically significant increases relative to baseline control values ($p < 0.05$). Comparison of antibody titration profiles demonstrated that PapG combined with alum or Freund's adjuvant generated more robust IgG responses over a broader range of serum dilutions, with detectable reactivity maintained up to 1:600 and 1:800 dilutions, respectively. In contrast, the PapG/*L. reuteri* group exhibited a comparatively lower maximal antibody titer. Despite these differences in response magnitude, no statistically meaningful variation was observed between the alum- and Freund's-adjuvanted formulations with respect to overall IgG induction (Fig. 5).

Evaluation of antigen-specific IgG1 and IgG2a subclasses revealed that immunization with PapG formulated with alum, PapG combined with Freund's adjuvant, and *Lactobacillus reuteri* resulted in elevated levels of both IgG1 and IgG2a relative to the control group. Notably, the PapG/alum and PapG/Freund's formulations elicited stronger IgG1 and IgG2a responses than those observed in the *L. reuteri*-immunized group. Further analysis of IgG2a responses demonstrated a statistically significant increase in this isotype in mice immunized with PapG/alum and PapG/Freund's compared with controls ($p = 0.0374$). In contrast, no significant difference in IgG2a titers was detected between the PapG/alum and PapG/Freund's groups ($p = 0.8604$), indicating comparable efficacy of these two adjuvant formulations in inducing IgG2a-mediated responses (Fig. 6).

3.7. Murine urinary tract infection model

The results indicated that the highest bacterial loads were recovered from the bladder and the kidneys of the mice after 48 h of inoculation, which in the bladder and the kidney is equivalent to 10^7 CFU and 10^5 CFU, respectively. The strain studied is an ascending UTI model and involves the upper parts of the urinary tract. Therefore, for half an hour after direct inoculation of the bacteria into the bladder, the kidneys and bladders of 10 naive mice were cultured, and after counting the number of isolated bacteria the result was confirmed by the fact that the bacteria were only located in the bladder and no bacteria were isolated from the kidneys, which confirms the correctness of the strain. The count of colonies in both bladder and kidney showed that the mice in the different groups tended to reduce the bacteria, with an average of 10^2 to 10^5 fold CFU reduction compared to the control group (Table 2). Mice injected with papG/Alum, papG/Alum and papG.AcmA / *L. reuteri* showed the best performance in reducing the amount of bacteria, respectively, with reduced indexes statistically lower than the control

group. Also, the papG/Alum group statistically significantly decreased the colonization of bacteria compared to the other two groups (Table 3, Fig. 7).

Table 1
Experimental groups and immunization/challenge design in BALB/c mice

Group No.	Group name	Immunization / Treatment	Adjuvant or vehicle	Route of administration	Purpose
1	PapG + Freund	PapG-AcmA recombinant protein (50 µg)	Complete Freund's adjuvant	Subcutaneous (days 0, 14, 28)	Positive control (systemic immunization)
2	PapG + Alum	PapG-AcmA recombinant protein (50 µg)	Alum	Subcutaneous (days 0, 14, 28)	Negative control (protein + weak adjuvant)
3	PBS	Phosphate-buffered saline	PBS	Subcutaneous (days 0, 14, 28)	Negative control
4	PapG-AcmA + <i>L. reuteri</i>	Live <i>Lactobacillus reuteri</i> displaying PapG antigen (5×10^9 cells/100 µl)	PBS	Oral gavage (days 0–4, 7)	Test group (oral mucosal immunization)
5	<i>L. reuteri</i> + PBS	Live <i>Lactobacillus reuteri</i> without PapG	PBS	Oral gavage (days 0–4, 7)	Negative control (vector only)

Table 2
Bacterial colony count in the bladder and the kidney of mice in each group

GROUP	PapG+Freund		PapG+Alum		PBS		PapG.AcmA + <i>L. reuteri</i>		<i>L. reuteri</i> + PBS	
	Bladder	Kidney	Bladder	Kidney	Bladder	Kidney	Bladder	Kidney	Bladder	Kidney
MOUSE 1	120000	6000	1000	0	50000000	100000	200000	8000	7500000	120000
MOUSE 2	32000	1800	35000	0	7500000	500000	176000	120000	510000	80000
MOUSE 3	65000	17000	1000	350	7000000	90000	230000	75000	8000000	120000
MOUSE 4	230000	27000	50000	240	420000	90000	160000	32000	30000000	150000
MOUSE 5	50000	5000	10000	80	9000000	80000	250000	9000	40000000	80000
MOUSE 6	16000	80	20000	0	10000000	120000	320000	6000	15000000	110000

Table 3
Mean and standard deviation from colony count in the bladder and theof mice in each group

	PAPG + F		PAPG + A		PBS		PAPG + L		L + PBS	
	Bladder	Kidney	Bladder	Kidney	Bladder	Kidney	Bladder	Kidney	Bladder	Kidney
Number of values	6	6	6	6	6	6	6	6	6	6
Minimum	80	80	0	0	80000	80000	6000	6000	80000	80000
25% Percentile	1370	1370	0	0	87500	87500	7500	7500	80000	80000
Median	5500	5500	40	40	95000	95000	10500	10500	115000	115000
75% Percentile	19500	19500	268	268	215000	215000	42750	42750	127500	127500
Maximum	27000	27000	350	350	500000	500000	75000	75000	150000	150000
Mean	9480	9480	112	112	163333	163333	23667	23667	110000	110000
Std. Deviation	10421	10421	149	149	165489	165489	26882	26882	26833	26833
Std. Error of Mean	4255	4255	61	61	67561	67561	10975	10975	10954	10954
Lower 95% CI of mean	-1457	-1457	-45.2	-45.2	-10337	-10337	-4545	-4545	81841	81841
Upper 95% CI of mean	20417	20417	269	269	337004	337004	51878	51878	138159	138159
Sum	56880	56880	670	670	980000	980000	142000	142000	660000	660000

4. Discussion

Uropathogenic *Escherichia coli* (UPEC) constitutes the major cause of urinary tract infections (UTIs) and remains a key public-health concern worldwide due to high morbidity and emerging antibiotic resistance. Infections usually begin in the bladder and may ascend to the kidneys, resulting in pyelonephritis or even bacteraemia. The development of intracellular bacterial communities (IBCs) within bladder epithelial cells complicates treatment and contributes to recurrent infections. These challenges highlight the necessity for effective vaccine strategies targeting critical adhesion molecules involved in UPEC colonization.

Among bacterial adhesins, PapG from P-fimbriae mediates attachment to uroepithelial receptors and plays a pivotal role during infection. Vaccines directed against adhesins such as FimH have shown promise in preventing bacterial binding and eliciting protective serum IgG responses. However, most previous vaccine studies have focused mainly on humoral immunity, overlooking the contribution of Th1-driven cellular responses that are essential for eliminating intracellular pathogens. Hence, an ideal anti-UPEC vaccine should induce both durable antibody (Th2) and cell-mediated (Th1) responses.

In this context, our study developed a recombinant PapG antigen anchored on the surface of *Lactobacillus reuteri* using the AcmA membrane domain as a surface display system. This probiotic-based approach offers notable advantages including biocompatibility, mucosal colonization capability, and intrinsic immunomodulatory effects. Expression, purification, and attachment tests confirmed successful surface display of the PapG-AcMA fusion protein, supporting its potential for mucosal vaccine applications. Immunological analysis demonstrated that formulations containing PapG antigen (PapG/Freund, PapG/Alum, and PapG-AcMA/*L. reuteri*) elicited substantial increases in total IgG, IgG1, IgG2a, interferon- γ , IL-4, and TNF- α .

compared with controls. The predominance of IgG2a together with elevated TNF- α and IFN- γ indicates strong stimulation of Th1-type cellular immunity, while the enhanced IgG1 and IL-4 responses reflect an effective Th2-humoral component. These balanced immune profiles suggest simultaneous activation of antibody-producing and cell-mediated arms, which are desirable for eliminating IBCs and preventing chronic infection. Our findings align with previous studies such as Habjanec et al., who reported similar dual Th1/Th2 responses using Freund's and Montanide adjuvants, and Robert et al., who demonstrated robust systemic and mucosal immunity against P-type fimbriae antigens.

The in-vivo challenge model further supported protective efficacy: mice immunized with PapG-based formulations exhibited notably reduced UPEC colonization in both bladder and kidney compared to non-immunized controls. The PapG/Alum formulation preferentially induced Th2 responses, whereas PapG/Freund skewed toward Th1 activation, consistent with their known adjuvant properties. Despite robust systemic responses, the *L. reuteri*/PapG-AcmA group showed a moderate reduction in bladder colonization, suggesting that mucosal delivery produced partial yet measurable protection. Overall, these outcomes confirm PapG as a critical target whose neutralization can prevent ascending infection and renal involvement, consistent with reports by Asadi-Karam et al. and Imani Fooladi et al., who observed reduced kidney colonization following fimbrial antigen immunization. Taken together, our observations indicate that PapG-AcmA-based vaccine candidates effectively stimulate both Th1 and Th2 pathways and mitigate bacterial colonization in urinary tissues. Among tested formulations, PapG/Alum and PapG/Freund displayed the most pronounced systemic and local immunity, while *L. reuteri* delivery provided the added advantage of probiotic compatibility. These findings announce a promising platform for mucosal vaccines targeting UPEC and other Gram-negative uropathogens.

5. conclusion

This research has provide two ideal and promising compounds which produces high responses of Th1cell mediated immunity and Th2humoral responses as well as reducing the dose of the bacteria in the bladder and kidneys, and can be further studied on other animal models as a candidate for a new vaccine to treat Urinary tract infectioncaused by UPEC strains, which will be followed up in subsequent projects

Limitations and Perspectives

Although the present study provides compelling immunological and protective evidence, further optimization is required to enhance mucosal efficacy and ensure durable responses. Future research should evaluate dosage standardization, long-term immunity, and protective efficiency across diverse animal models, as well as explore combined antigenic strategies (PapG with FimH or OmpA) to increase coverage against heterogeneous UPEC strains.

Declarations

Acknowledgements

We appreciate the support of the Research Council of Aria Vaccine Alborz (AVA) company.

Author's Contributions

MeA contributed to conceptualization, methodology, validation, formal analysis, resources, data curation, writing—review & editing, supervision, project administration; MaA contributed to data curation, formal analysis, writing—review & editing; AA contributed to formal analysis, investigation, and writing—original draft; PH contributed to writing—review, formal analysis & editing.

Funding: not applicable

Data Availability Data will be made available on request.

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical Approval The protocol for the collection of rodents' samples was reviewed and approved by the Institutional Animal Care and Use Committee from Iranian veterinary organization by Ethics Committee. All methods were performed in accordance with the relevant guidelines and regulations.

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Figures

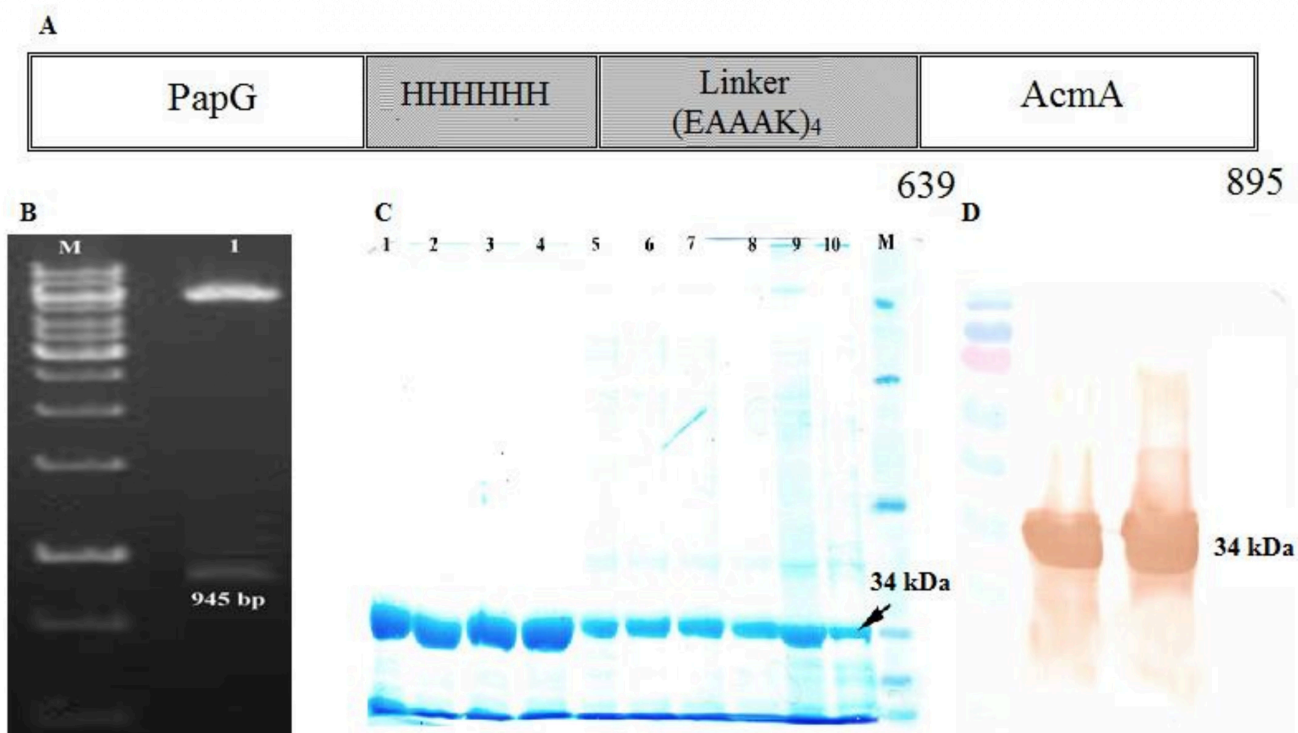


Figure 1

Designing construct, expression, and identification of recombinant construct. A) Schematic view of recombinant PapG-AcmA. B) 1 % Agarose gel electrophoresis analysis of digested pET21a- PapG-AcmA. Lane 1: molecular weight marker, lane 2: NdeI/BamHI digested construct. C) Different fractions from chromatography column. D) Confirmation of recombinant protein by Western blotting.

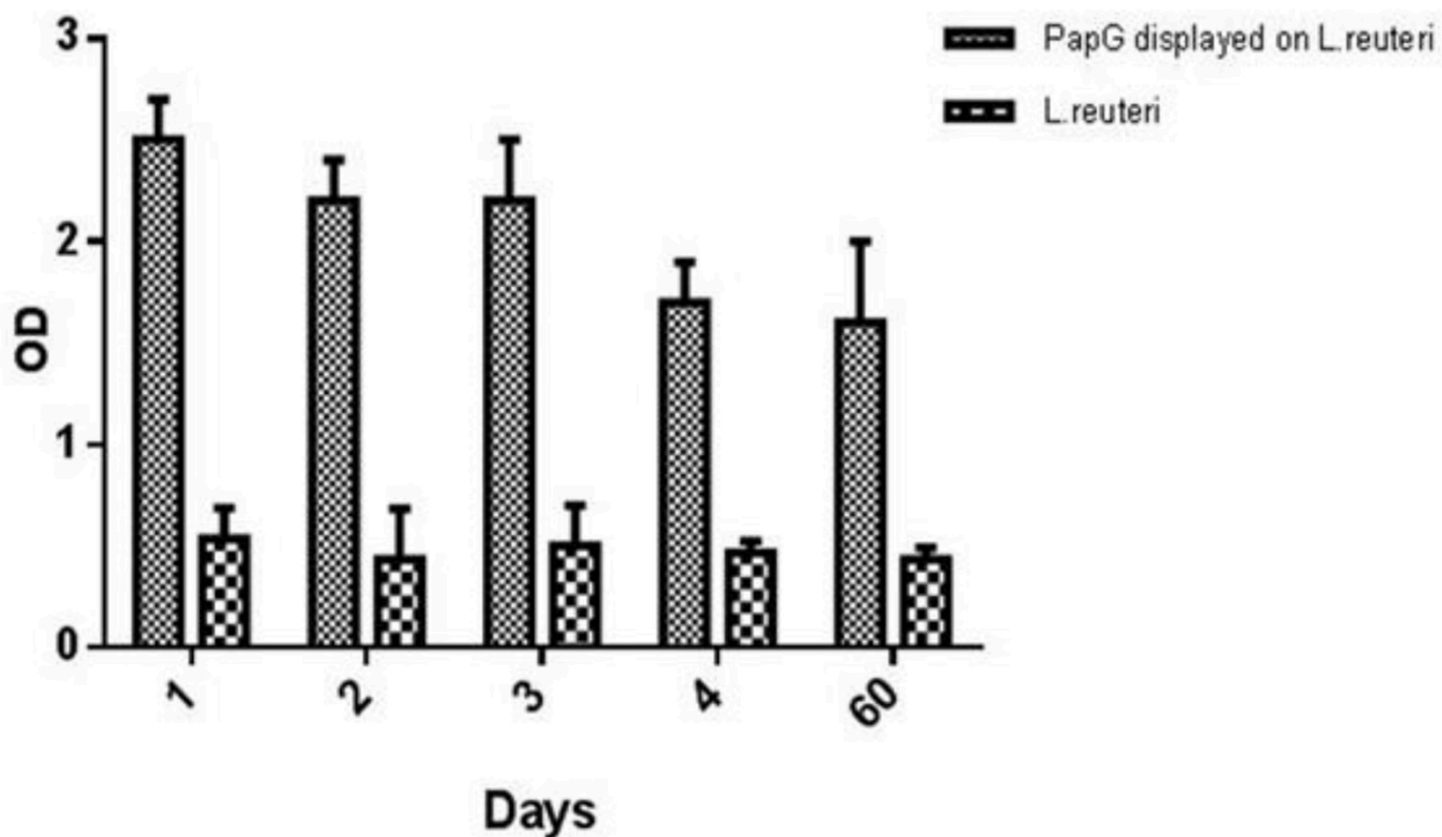


Figure 2

Surface Expression of Recombinant PapG Protein on *Lactobacillus reuteri*

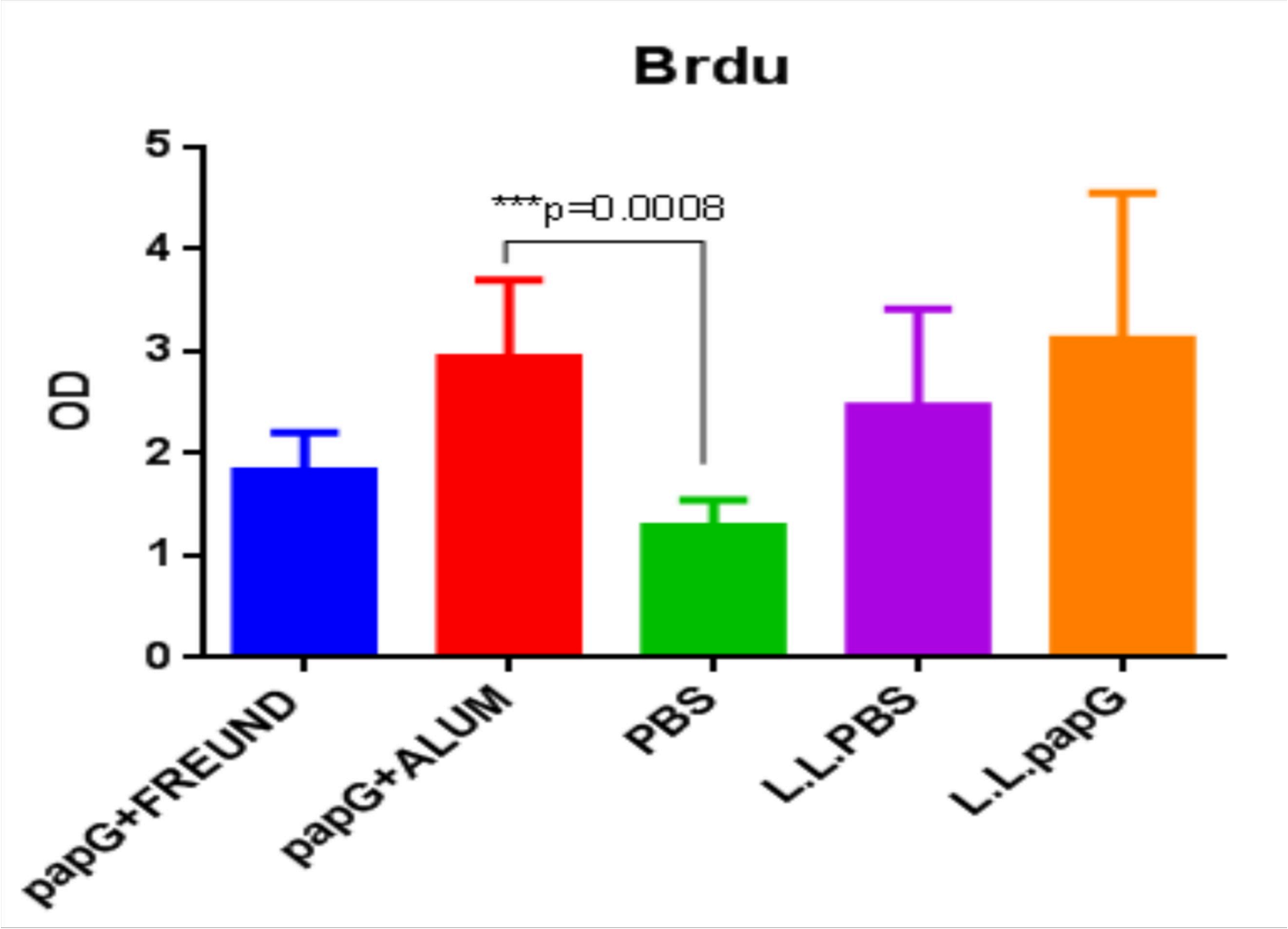


Figure 3

Lymphocyte proliferation according to stimulation index.

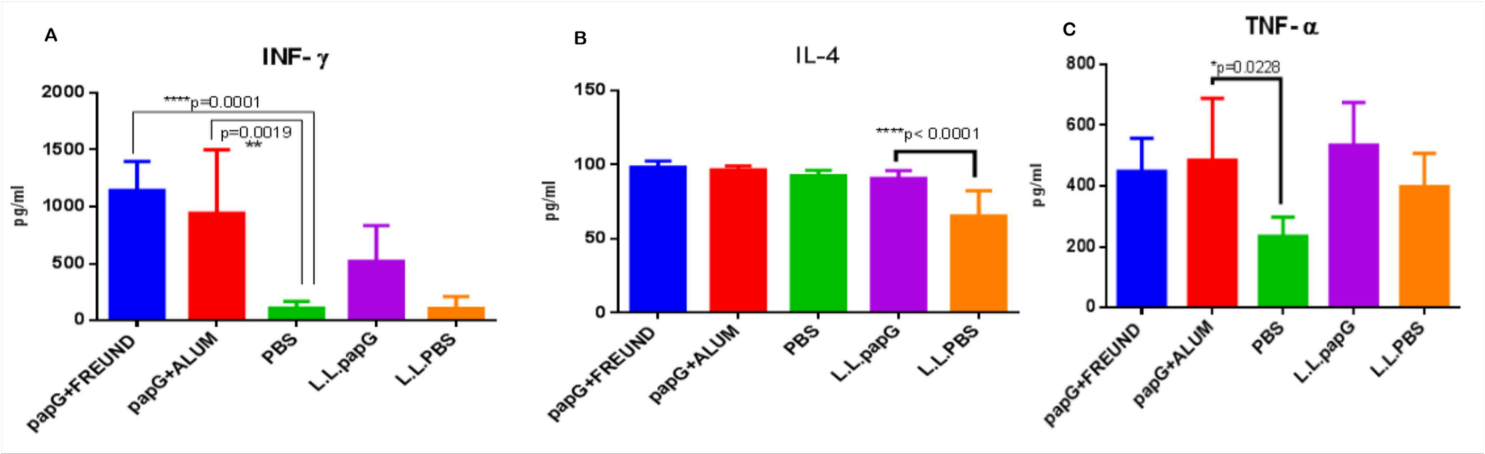


Figure 4

Interleukin-4, IFN- γ and TNF α cytokines in the experimental groups.

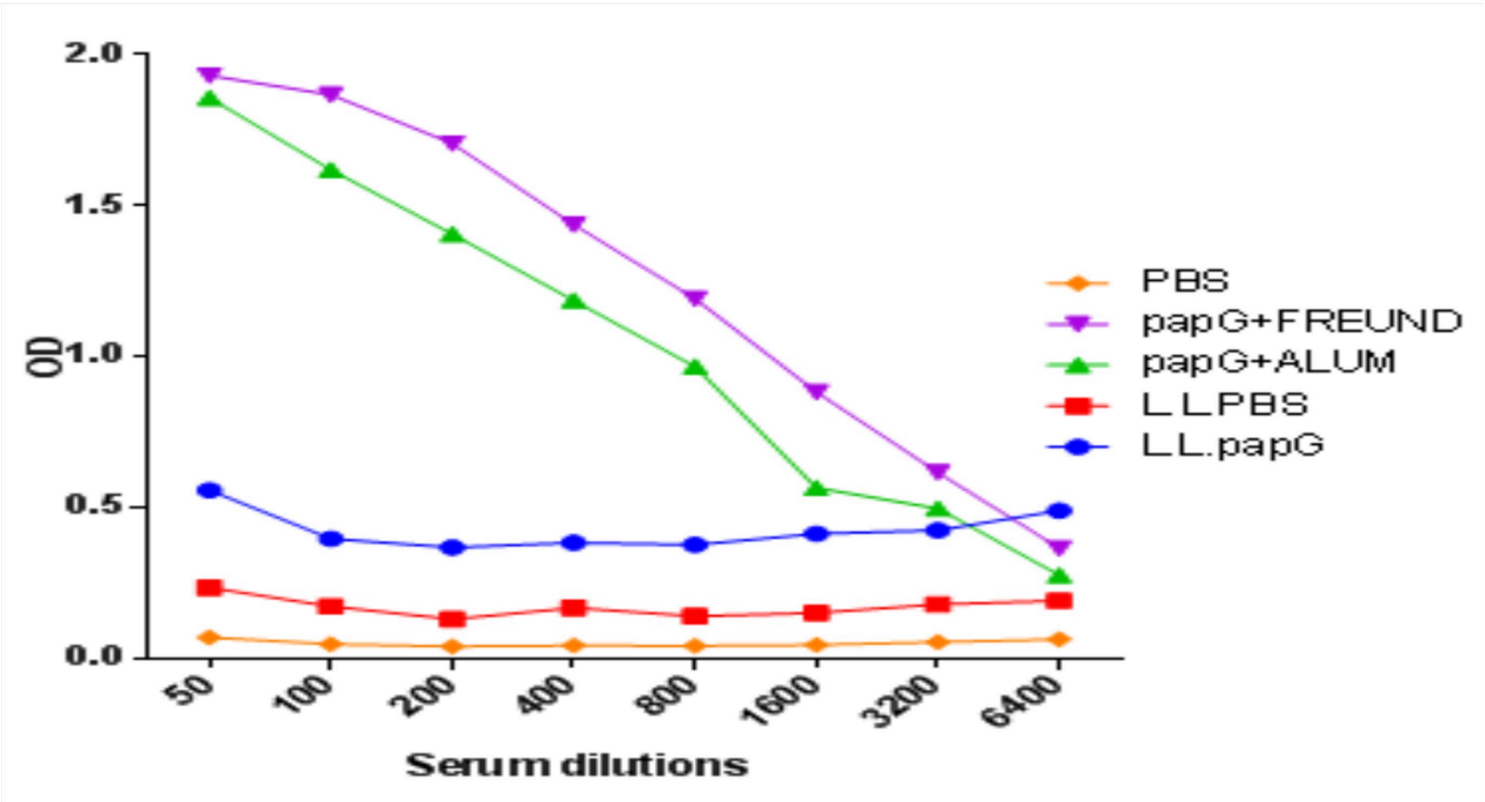


Figure 5
Total antibody responses after immunization course.

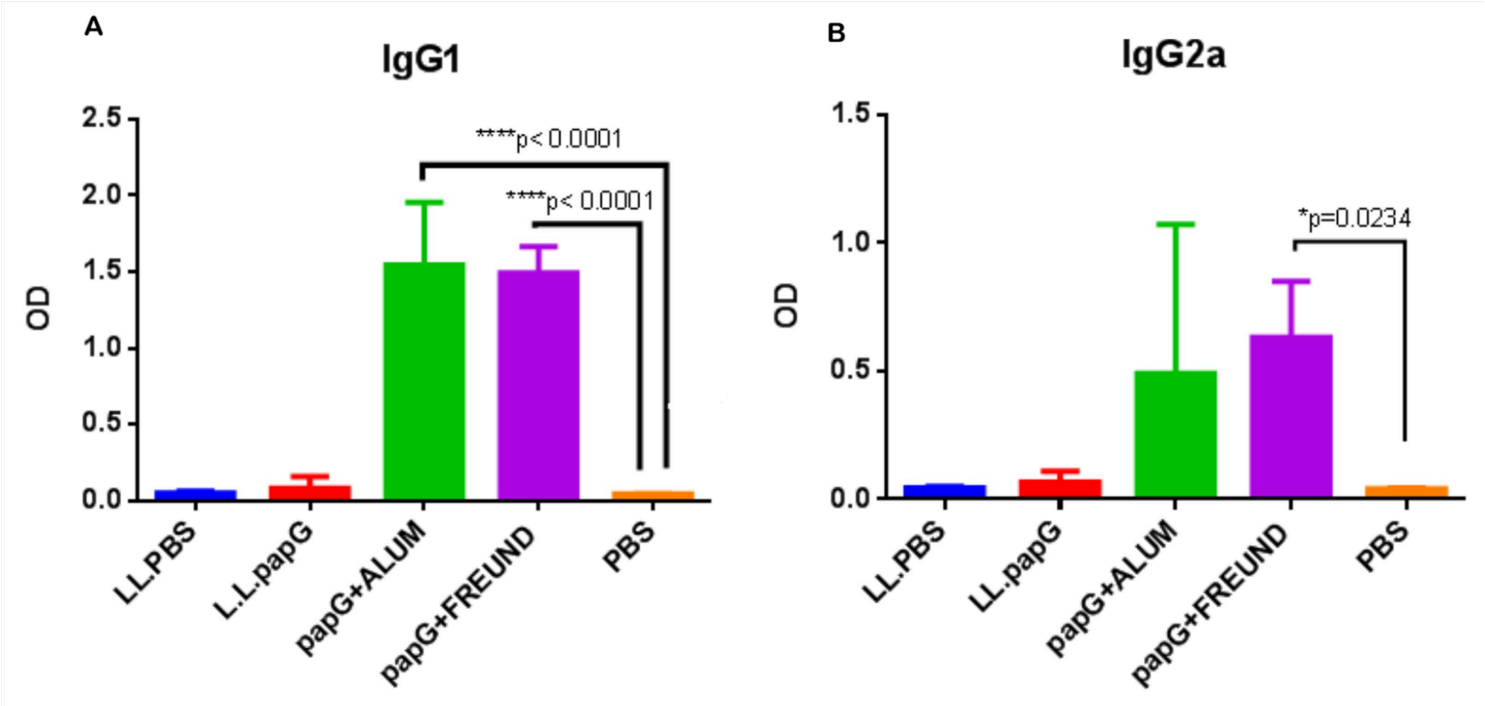


Figure 6
Levels of specific IgG1 and IgG2a in the experimental groups.

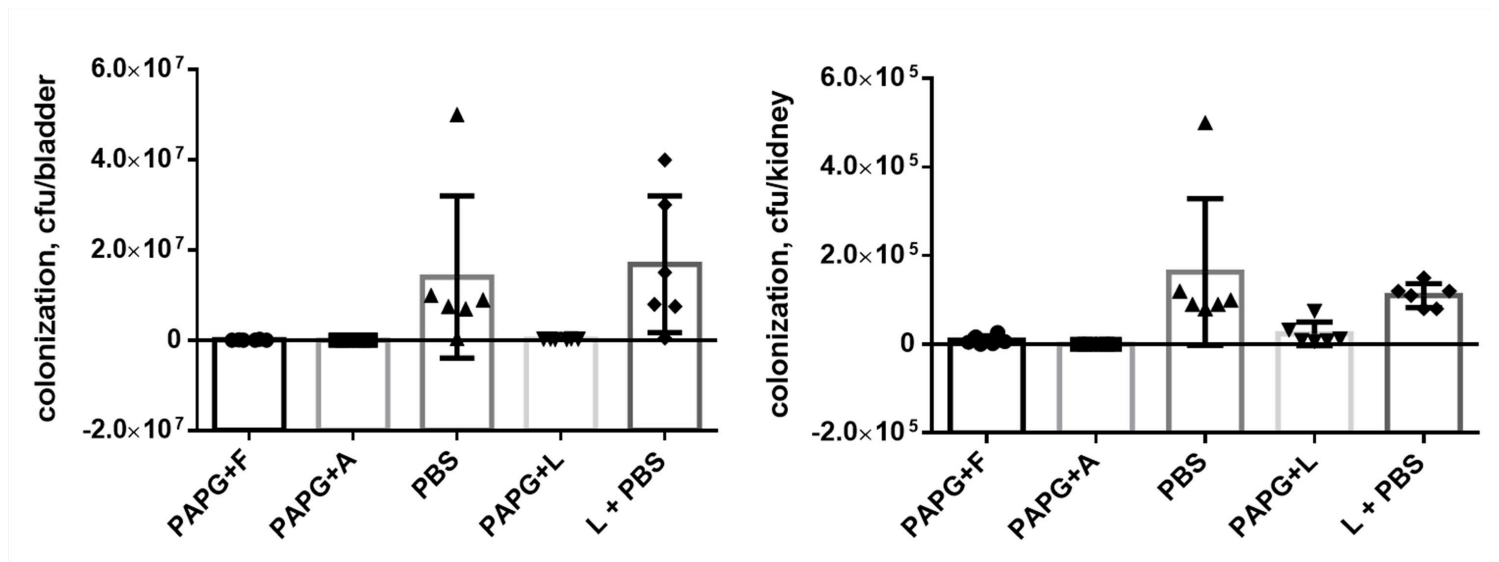


Figure 7

Colony count results in the bladder and the kidney of different mouse groups. The count of the colony of each mouse in each group is shown with different symbols, the median for each group is shown as a horizontal line between the symbols. Groups immunized with PapG/Freund's, PapG/alum, and PapG-AcmA/*L. reuteri* exhibited a statistically significant reduction in bacterial colonization of the bladder compared to the control group. Also, the papG/Alum group statistically significantly decreased the colonization of bacteria compared to the other two groups.