Flagellin-adjuvanted trivalent mucosal vaccine targeting key periodontopathic bacteria

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Abstract

Periodontal disease (PD) is caused by microbial dysbiosis and accompanying adverse inflammatory responses. Due to its high incidence and association with various systemic diseases, disease-modifying treatments that modulate dysbiosis serve promising therapeutic approaches. In this study, to simulate the pathophysiological situation, we established a ‘temporary ligature plus oral infection model’ that incorporates temporary silk ligature and oral infection with the cocktail of live Tannerella forsythia (Tf), Pophyromonas gingivalis (Pg), and Fusobacterium nucleatum (Fn) in mice and tested the efficacy of a new trivalent mucosal vaccine. It has been reported that Tf, a red complex pathogen, amplifies periodontitis severity by interacting with periodontopathic bacteria such as Pg and Fn. Here we developed a recombinant mucosal vaccine targeting a surface-associated protein BspA of Tf by genetically combining truncated BspA with built-in adjuvant flagellin (FlaB). To simultaneously induce Tf-, Pg-, and Fn-specific immune responses, it was formulated as a trivalent mucosal vaccine containing Tf-FlaB-tBspA (BtB), Pg-Hgp44-FlaB (HB), and Fn-FlaB-tFomA (BtA). Intranasal immunization with the trivalent mucosal vaccine (BtB+HB+BtA) prevented alveolar bone loss and gingival pro-inflammatory cytokine production. Vaccinated mice exhibited significant induction of Tf-tBspA-, Pg-Hgp44-, and Fn-tFomA-specific IgG and IgA responses in the serum and saliva, respectively. The anti-sera and anti-saliva efficiently inhibited epithelial cell invasion by Tf and Pg and interfered with biofilm formation by Fn. In summary, the flagellin-adjuvanted trivalent mucosal vaccine-mediated immunomodulation would serve as a promising choice for clinically managing dysbiotic bacteria-induced periodontitis.

INTRODUCTION

Periodontal disease (PD) is a prevalent oral health condition, affecting 20–50% of the global population and imposing a significant economic burden and public health problem. In addition to tooth loss, the correlation between periodontitis and systemic diseases became evident. Related systemic diseases include but are not limited to, cancer, cardiovascular disease, diabetes, respiratory tract infections, adverse pregnancy outcomes, and Alzheimer’s disease. Considering that the dysbiosis of oral microorganisms is tightly related to PD, dysbiosis-modifying treatments rebalancing the oral microbiome are thought to be promising therapeutic approaches not only in controlling periodontal conditions but also in preventing or delaying various systemic diseases associated with PD. In this regard, the vaccination strategy targeting dysbiosis-causing bacteria may serve as a physiological means for the long-term control of periodontal disease.

PD is associated with a heterogeneous microbial community within the subgingival plaque, which initiates and sustains inflammation, resulting in progressive destruction of dental tissue. Cooperative interaction among PD-related pathobionts, which are adept at evading host immune responses, contributes to the persistence of dysbiotic microbial communities. Therefore, effective strategies to control PD should prioritize the reduction of periodontopathic bacteria and modulation of the host inflammatory response at the same time. An effective vaccine should disrupt the synergistic
periodontopathic interactions by selectively suppressing key dysbiotic microorganisms in the dental plaque. In addition, considering that multiple pathogenic bacteria are contributing to the destructive inflammation in periodontitis, the preventive and/or therapeutic vaccine should aim at multiple targets in the polymicrobial community.

Given that periodontal disease manifests within the oral mucosa, efficacious periodontal vaccines should be capable of eliciting protective immune responses in the oral mucosae. Injected vaccines are generally poor inducers of mucosal immunity and are, therefore, less effective against infections at mucosal sites. To induce protective immune responses in mucosal sites, mucosal vaccines eliciting secretory antibodies and cellular immune responses at the portal of entry are actively developed.

Mucosally administered antigens are generally less immunogenic and inclined to induce tolerance. In this context, induction of mucosal immunity through vaccination is a rather difficult task, and potent mucosal adjuvants, vectors, or other special delivery systems are required. Bacterial flagellin, a cognate ligand for Toll-like receptor 5 (TLR5) on the cell surface and the NAIP5/NLRC4 inflammasome in the cytosolic compartment has potent mucosal immune modulatory activities.

Flagellin has shown potent adjuvant activities in numerous mucosal vaccine formulations, both as a component of mixtures and fusion proteins.

The appropriate animal model system reproducing human clinical situations is essential for the clinical translation of vaccines. In PD studies, many different animal models have been devised to imitate human pathophysiology. Experimental periodontal infections were generated through oral gavage, silk ligature, ligature soaked in bacteria, or a combination of these methods. Since the oral microbiome is distinctively different between humans and mice, a very high dose of human periodontopathic bacteria should be gavaged in a sticky formulation into the oral cavity to reproduce human PD in mice. But, the high dose periodontopathic bacterial inoculation may not be enough to induce human-like PD in mice depending upon the causative pathogens. The ligature-induced PD models serve as useful in vivo experimental systems to explore both pathogenic mechanisms and therapeutic efficacies. The silk ligature would be colonized by gavaged pathobionts for an extended period of time, which will more likely reproduce human PD than oral gavage alone. In the present study, we established a temporary ligature-oral gavage experimental PD model using specific-pathogen-free (SPF) mice. Porphyromonas gingivalis (Pg), a member of the red bacterial complex, plays a central role in chronic periodontitis and contributes significantly to the pathogenicity of the bacterial community during the progression of the disease. The Gram-negative bacterium Fusobacterium nucleatum (Fn), classified as the “orange complex”, primarily acts as a bridging colonizer that promotes the transition between early and late colonizers during plaque formation. Tannerella forsythia (Tf), a component of the red complex bacteria, has been observed in a chronic and aggressive form of periodontitis. Moreover, under particular conditions, Tf exerts a significant synergistic impact on the severity of periodontal disease. Among Tf's virulence factors, BspA plays a crucial role in binding to fibronectin and fibrinogen, causing coaggregation with various organisms and consequently contributing to alveolar bone loss. In
previous studies, we have shown that flagellin potentiates secretory IgA responses against mucosally administered \( Pg \) Hgp44 and \( Fn \) FomA antigens\(^{39,40} \). For the application in human subjects, the protection against \( Pg \) and \( Fn \) would not be enough to cover the wide range of dysbiosis, which had driven us to design a clinical-grade anti-PD mucosal vaccine formulation covering above mentioned PD keystone pathobionts.

In this study, we designed a recombinant mucosal vaccine that specifically targets the surface-associated protein \( Tf \) BspA by genetically conjugating truncated BspA (tBspA) to built-in adjuvant flagellin (FlaB). We then evaluated the induced protective immune responses of a trivalent vaccine consisting of \( Tf \)-FlaB-tBspA (BtB), \( Pg \)-Hgp44-FlaB (HB), and \( Fn \)-FlaB-tFomA (BtA) in a murine ‘temporary ligature plus oral infection model’.

**RESULTS**

**Establishment of a temporary ligature plus oral infection (LigR+OI) model**

To evaluate the efficacy of the periodontal vaccine targeting three major PD-related pathobionts, we looked for the optimal animal model that could testify to the protective efficacy. The model should ensure cooperative pathogenesis by the combination of three PD pathobionts. We thought silk ligature would provide the bed for the cooperative infection of multiple pathobionts. First, we tried to combine ligature-induced periodontitis (LIP) with oral bacterial infection (OI) of \( Tf \), \( Pg \), and \( Fn \). The second maxillary molar (M2) of the BALB/c mice was ligated by 5-0 surgical silk. After ligature placement, mice were orally infected with a mixed suspension containing the three bacterial species (\( Tf \), \( Pg \), and \( Fn \)). Two more rounds of oral bacterial infections were conducted on the 3\(^{rd} \) and 5\(^{th} \) day (Supplementary Fig. 1a). To assess the extent of periodontitis resulting from mixed ligature-oral infection, a comparison of alveolar bone loss was conducted between two groups: the ligature placement group (Lig) and the mixed ligature-oral infection (Lig+OI) group, employing micro-CT analysis. As shown in Supplementary Fig. 1b, consistent alveolar bone loss was seen on days 6, 9, 12, and 15 after ligature placement. However, no significant additional alveolar bone loss was observed in the Lig+OI group compared to the Lig group at any time point (Lig vs Lig+OI, \( P > 0.05 \)). These findings imply that the induction of periodontitis via ligature placement (LIP) alone prompts considerable periodontal damage, which suggests that mouse microbiota could contribute to PD generation. Ligature maintenance by itself was sufficient to generate significant periodontal pathology, thereby rendering it challenging to assess periodontal inflammation and alveolar bone loss attributed to \( Tf \), \( Pg \), and \( Fn \).

Next, we modified the above LIP procedure by temporarily maintaining silk, which would delineate the contribution of \( Tf \), \( Pg \), and \( Fn \)-mediated inflammation to alveolar bone loss. Briefly, the M2 ligated BALB/c mice were orally infected with mixed bacterial suspensions three times in 2-day intervals. The silk ligature was then removed, followed by two more oral bacterial infections on days 6 and 7 (LigR+OI) (Supplementary Fig. 2a). To evaluate alveolar bone loss induced by LigR+OI, micro-CT analysis was performed on days 15 and 24. Notably, at 9 days (day 15) after ligature removal, LigR+OI mice
showed significant alveolar bone loss compared with the LigR group (LigR vs LigR+OI, $P < 0.05$), demonstrating that additional two infections further exacerbated the periodontitis induced by the ligature plus three-time oral infections. By day 18 after ligature removal, the LigR group showed recovery of alveolar bone loss, reaching levels similar to naive mice (naive vs. LigR, $P > 0.05$) (Supplementary Fig. 2b). These findings highlight the relevance of the temporary ligature-oral infection model (LigR+OI) to specifically assess periodontitis induced by exogenous bacteria at day 15.

**Development of a Tanneraella forsythia BspA-specific mucosal vaccine with flagellin as a built-in adjuvant**

To develop a vaccine antigen targeting $Tf$, we selected a surface-associated protein BspA. Computational analysis was then employed to design immunogenic vaccine antigens. We chose the C terminus of BspA (truncated BspA, tBspA) based upon potential as a B cell epitope and the likelihood of exposure on the cell surface as was done previously (Supplementary Fig. 3a) $^{17,41}$. Subsequently, we engineered a series of eight fusion proteins to generate an optimal built-in-adjuvanted mucosal vaccine formulation. These proteins are composed of various linker peptides that connect the tBspA antigen and the FlaB adjuvant (Supplementary Fig. 3b) $^{17}$. Through pilot studies, we identified FlaB-L2-tBspA (BtB) as the candidate vaccine based on its stability and ability to induce strong TLR5 stimulation (Supplementary Fig. 3c). As shown in Supplementary Fig. 3d, BtB exhibited superior tBspA-specific serum-IgG and saliva-IgA responses compared to the mixture formulation of FlaB and tBspA. Conclusively, we came up with an optimal built-in adjuvanted anti-$Tf$ vaccine candidate that will be co-formulated with previously reported anti-$Fn$/ $Pg$ mucosal vaccine $^{17}$.

**A trivalent mucosal vaccine (BtB+HB+BtA) prevents alveolar bone loss induced by mixed bacterial infection in the temporary ligature plus oral infection model**

To evaluate the effectiveness of the trivalent mucosal vaccine on $Tf$, $Pg$, and $Fn$-mediated alveolar bone loss, we performed a series of experiments combining intranasal vaccination and LigR+OI challenge. BALB/c mice were intranasally immunized with a trivalent vaccine formulated with $Tf$-FlaB-tBspA (BtB), $Pg$-Hgp44-FlaB (HB), and $Fn$-FlaB-tFomA (BtA). Two weeks after the last immunization, we challenged vaccinated animals with the temporary ligature-oral infection regimen (Fig. 1a). Nine days after the ligature removal, we assessed the effectiveness of induced immune responses by measuring the extent of alveolar bone loss. Mice vaccinated with the trivalent vaccine (Vax+LigR+OI group) were effectively protected from alveolar bone loss caused by the infection with a mixture of $Tf$, $Pg$, and $Fn$. This was evidenced by assessments of CEJ-ABC (LigR+OI vs. Vax+LigR+OI, $P < 0.05$) and bone volume density (BV/TV) (LigR+OI vs. Vax+LigR+OI, $P < 0.01$) (Fig. 1b, c). The vaccine efficacy is discernible in histologic H&E staining (Fig. 1d), shown by attenuated degradation of periodontal tissue around the second molar. Moreover, vaccination consistently resulted in a significant reduction of myeloperoxidase-positive neutrophil infiltration (Supplementary Fig. 4). These findings clearly demonstrate that the trivalent mucosal vaccine (BtB+HB+BtA) prevents alveolar bone loss caused by mixed PD pathobiont infection.
The trivalent mucosal vaccine (BtB+HB+BtA) inhibits PD-related gene expression induced by a mixed PD pathobiont infection

The development and progression of PD are dominantly influenced by the host's inflammatory responses to periodontal pathogens. Effective suppression of the overgrowth of pathobionts will result in the alleviation of inflammatory responses that should lead to periodontal tissue damage and alveolar bone loss. To determine whether the trivalent vaccine suppressed periodontal inflammation induced by polymicrobial infection (Tf+Pg+Fn) in the LiR+OI model, we assessed the mRNA expression profile in gingival tissue using qRT-PCR in a separate experimental setting. Initially, we determined the timing of evaluation in the temporary ligature-oral infection model specifically targeting gingival tissue inflammation by measuring the transcription of IL-1β, IL-6, TNF-α, and MMP9 genes. Briefly, mice underwent ligature accompanying oral infections with the mixture of live Tf, Pg, and Fn. Subsequently, the ligatures were removed after one day, followed by an additional bacterial challenge. The expression of PD-related genes was evaluated at 12, 24, and 48 hours after the ligature removal (Supplementary Fig. 5a). Upon comparing the gene expression levels between ligature removal (LigR) and ligature removal combined with oral infection (LigR+OI), notably elevated IL-1β, IL-6, and MMP9 gene expressions were detected in the LigR+OI group at 24 hours following ligature removal (Supplementary Fig. 5b). To evaluate the efficacy of a trivalent mucosal vaccine (BtB+HB+BtA) in suppressing inflammation in LigR+OI animals, BALB/c mice were intranasally immunized with a trivalent vaccine (BtB+HB+BtA) challenged with live bacterial infection following the protocol outlined in Supplementary Fig. 5a. Briefly, two weeks after the final immunization, the mice underwent a ligature placement/removal procedure along with oral infections. Twenty-four hours after the ligature removal, we evaluated gene expression levels in the gingival tissue using qRT-PCR. As shown in Fig. 1e, the trivalent mucosal vaccine (BtB+HB+BtA) suppressed the expression of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α), chemokine (CLCX2), extracellular matrix-degrading enzymes (MMP-3 and MMP-9), and RANKL.

Intranasal immunization of the trivalent mucosal vaccine(BtB+HB+BtA)induced antigen-specific antibody responses in both mucosal and systemic immune compartments

We, to substantiate the protective efficacy, assessed antigen-specific antibodies following intranasal immunization with monovalent (BtB, HB, or BtA), divalent (BtB+HB, BtB+BtA, or HB+BtA), and trivalent (BtB+HB+BtA) vaccines. As shown in Fig. 2, each component of the mucosal vaccine induced significant antigen-specific IgG and secretory IgA in serum and saliva, respectively. Immunofluorescence staining for confocal microscopy was conducted to ascertain whether the anti-sera recognize the native forms of the respective antigens (BspA, Hgp44, and FomA) on the surface of live Tf, Pg, and Fn cells. In contrast to pre-immune sera, which did not detect antigenic epitopes on the surface of all three bacteria, the anti-sera generated by trivalent vaccine (anti-BtB+HB+BtA) successfully identified corresponding antigens expressed on the surface of Tf, Pg, and Fn (Fig. 3). The monovalent (BtB, HB, or BtA) and divalent vaccines (BtB+HB, BtB+BtA) demonstrated similar staining to the trivalent vaccine (Fig. 2 and Supplementary Fig. 6). These findings corroborate that antibodies raised by the immunization with a trivalent vaccine specifically
recognized infecting pathobionts and subsequently suppressed destructive inflammatory responses in vivo.

**Anti-sera and anti-saliva elicited by the intranasal immunization of trivalent vaccine (BtB+HB+BtA) inhibited host-bacteria interactions**

The adhesion and invasion of periodontal pathogens to periodontal tissue is a crucial step for the establishment of destructive infection. Protective antibodies targeting surface-expressed adhesion-related antigens of periodontal pathogens would inhibit the infection from the adhesion/invasion stages \(^{42}\). We performed invasion assays employing anti-sera and anti-saliva, using flow cytometry and confocal microscopy. Purified IgG from anti-sera and anti-saliva were used to evaluate the neutralizing efficacy against BspA-mediated invasion of Tf into epithelial KB cells. Notably, flow cytometry analysis demonstrated that the purified IgG and anti-saliva generated by the trivalent vaccine (BtB+HB+BtA) successfully inhibited Tf invasion (Fig. 4a, b). The outcome was consistent with the data obtained from confocal microscopy (Fig. 4c, d). Additionally, both purified IgG from anti-sera and anti-saliva derived from monovalent (BtB) and divalent (BtB+HB, BtB+BtA) vaccination were also effective in inhibiting the Tf invasion (Supplementary Fig. 7). In contrast, non-specific antibodies in serum and saliva (anti-HB+BtA and anti-PBS) did not exert any inhibitory effect (Fig. 4). These results indicate the specific inhibitory action of immune sera or saliva against BspA would effectively inhibit the adhesion and invasion of Tf oral epithelia.

To assess whether the anti-sera and anti-saliva from the trivalent vaccine can neutralize the activities of Hgp44, inhibiting the invasion of Pg into KB cells, an invasion assay was also conducted. Non-specific antibodies (anti-BtB+BtA and anti-PBS) were utilized as negative controls. The outcomes demonstrated a significant reduction in the percentage of Pg invasion into KB cells with both IgG purified from anti-sera (Fig. 5a, c) and anti-saliva (Fig. 5b, d) elicited by BtB+HB+BtA immunization. Conversely, treatment with either purified IgG from serum or saliva from BtB+BtA- or PBS-administered animals showed no inhibition. These results suggest the specific inhibitory effect of immune sera or saliva against Hgp44 also contributed to the suppression of Pg-mediated inflammation.

**Anti-sera and anti-saliva elicited by the intranasal immunization of trivalent vaccine (BtB+HB+BtA) inhibited *F. nucleatum*-mediated biofilm formation**

*Fn* readily forms co-aggregates with various oral bacteria by mediating FomA \(^{43,44}\). To determine the functional inhibitory impact of antibodies derived from trivalent vaccination on *Fn*-induced biofilm formation, we conducted a colorimetric biofilm inhibition assay. Antibodies in both sera and saliva, elicited by intranasal vaccination with the BtB+HB+BtA vaccine, exhibited a dose-dependent inhibition of Fn biofilm formation (Fig. 6a, b). Conversely, these inhibitory effects were absent in the groups treated with non-specific antibodies (anti-BtB+HB, anti-PBS). These findings suggest that antibodies induced in both systemic and mucosal compartments functionally inhibited FomA-mediated biofilm formation by *Fn*.
DISCUSSION

In this study, we propose a mucosal anti-PD built-in adjuvanted vaccine targeting biofilm bridging colonizer *Fn* and two "red complex pathogens" *Pg* and *Tf*, which could effectively counteract against the founders of severe periodontal diseases. While proving the efficacy of the vaccine, we established an experimental system that could be widely used for PD protection studies, employing temporary silk ligature and oral gavage with live bacteria. Complex interactions of oral microbiota and pathobionts contribute to the dysbiosis leading to PD, and among large numbers of PD-related pathobionts, the three bacteria mentioned above play pivotal roles in the pathogenesis of PD. *Fn* links early colonizers, such as streptococci, and later-stage colonizers, including the red complex species, such as *Pg*, *Tf*, and *Treponema denticola*. *Fn* and *Tf* develop a synergistic partnership in inducing inflammation under PD conditions through interspecies sensing and metabolite exchange.

*Fn* scavenges reactive oxygen species in the subgingival plaque, providing a favorable growth environment for strictly anaerobic pathogens such as *Pg*. We generated a trivalent mucosal vaccine that specifically targets the surface-associated virulence factors of *Tf* (BspA), *Pg* (Hgp44), and *Fn* (FomA) through genetically fusing built-in mucosal adjuvant FlaB. In a previous study, we observed that the FlaB built-in adjuvanted HB and BtA mixture vaccine induced excellent protective immune responses in oral secretions without interfering with each other. For clinical application, covering only one red complex pathogen (*Pg*) would not be sufficient to have effects on diverse types of PDs. In the present study, we show that the trivalent mucosal vaccine (BtB + HB + BtA) effectively prevented alveolar bone loss and down-regulated pro-inflammatory cytokine production in the experimental model, realizing polymicrobial dysbiosis with temporary silk ligature plus oral infection with key pathobionts.

The temporary ligature plus oral infection model combines a ligature placement/removal in the maxillary molar of mice and oral gavage with live *Tf*, *Pg*, and *Fn*. Initial simultaneous application of the general ligature placement and oral infection resulted in rapid and severe destructive alveolar bone loss (Supplementary Fig. 1). Considering that ligature placement may potentially induce microbial accumulation and microulceration within the sulcular epithelium, thereby facilitating the invasion of the oral microbiome into connective tissues, the extensive alveolar bone loss observed in the initial trial appeared to be influenced by both existing host microbiota and orally challenged pathogens (*Tf*, *Pg*, and *Fn*). Therefore, it was difficult to distinguish between PD caused by the host oral microbiota and the heterologous human key pathogens. Through many rounds of pilot experiments, we found that temporary ligature plus 2 additional oral gavage provided a clinical setting that would be optimal for testing the efficacy of vaccination. As shown in Supplementary Fig. 2, 9 days after the ligature removal and two more rounds of oral infection, we could distinguish the three bacteria-induced PD from ligature-mediated PD (LigR vs LigR + OI, *P* < 0.05). Eighteen days after the ligature removal, ligation-mediated alveolar bone loss was recovered to the control mice level (Naïve vs LigR, *P* > 0.05). This finding corresponds to previous reports showing that spontaneous regeneration of alveolar bone can be observed after 15 days of ligature removal. Following this temporary ligature plus oral infection model, we could clearly address the trivalent vaccine's protective efficacy (Fig. 1b-d). Considering the close
association of \(Tf, Pg,\) and \(Fn\) with peri-implantitis\(^{55}\), we believe this modified ligation and oral infection model could be applied to the study of peri-implantitis or other periodontal conditions that accompany tissue injury plus polymicrobial infection.

The interplay between immune cells and oral microbial dysbiosis results in pathological alveolar bone resorption as the consequence of inflammatory responses to dysbiotic microorganisms\(^{56}\). We also investigated whether the trivalent mucosal vaccine could modulate inflammation-related gene expressions. Cytokine gene expression profiles in periodontal tissue have been reported to undergo changes during PD initiation\(^{23}\). We established separate temporary ligature plus oral infection models to address expression profiles of PD-related proinflammatory cytokines, and genes associated with tissue damage (Supplementary Fig. 5). Our experimental results address that the trivalent mucosal vaccine was effective in down-regulating PD-related gene expressions such as pro-inflammatory cytokines (IL-1\(\beta\), IL-6 and TNF\(\alpha\)), matrix-degrading enzyme MMP9, and RANKL in gingivae treated with live \(Tf, Pg,\) and \(Fn\) mixture (Fig. 1e). The role of periodontal immune responses in maintaining alveolar bone homeostasis during periodontitis has been emphasized\(^{57}\). Clinical and preclinical studies have demonstrated microorganisms within dental plaque promote osteoclast formation and subsequent alveolar bone loss by upregulating RANKL\(^{58,59}\). Our findings offer further mechanistic insights into the impact of mucosal vaccination on PD-associated alveolar bone loss.

Mucosal vaccination effectively induces secretory IgA in saliva and IgG in gingival crevicular fluid (GCF), interfering with pathogen attachment and colonization at mucosal sites through the production of pathogen-neutralizing antibodies\(^{11,17,20,60}\). Mucosal vaccines, in contrast to parenteral vaccines, generate more effective protective immune reactions by stimulating secretory IgA responses and cell-mediated immunity within mucosal tissues, which serve as the main entry points for mucosal pathogens\(^{12}\). The use of appropriate mucosal adjuvants is crucial for eliciting optimal immune responses\(^{61}\). FlaB has been recognized as a potent adjuvant, especially in formulations fused with vaccine antigens that can enhance antigen-specific immune responses at mucosal sites\(^{16-19,62}\). Targeted suppression of keystone pathogens in the oral microbiota will lead to a resurgence of physiological commensals and contribute to establishing a healthy microbial homeostasis\(^{63,64}\). Another red complex periodontopathic bacterium, \(Tf,\) exhibits invasiveness toward oral epithelial cells and engages in intricate interactions with other bacteria to synergistic PD\(^{65}\). BspA of \(Tf\) plays a crucial role in host adhesion and invasion\(^{33}\). In this study, we developed an optimal vaccine antigen (tBspA) targeting \(Tf\)BspA by computational analysis and fused it with FlaB using different linkers (Supplementary Fig. 3). The tBspA did not contain leucine-rich repeat domains and predicted to harbor several B cell epitopes by BepiPred-2.0\(^{66}\). The construct showing the strongest TLR5 stimulating activity and best stability was selected for further studies (Supplementary Fig. 3c, d). Positioned as a keystone pathogen, \(Pg\) can subvert the host's immune response and drive dysbiosis, even with 10–15\% of subgingival plaque\(^{67}\). \(Fn\) promotes the formation and maturation of dental plaque\(^{68,69}\). Clinical relevance also showed a strong association of polymicrobial biofilm involving \(Tf, Pg,\) and \(Fn\) causing PD\(^{28,32,70-72}\). In this study, flagellin adjuvanted FlaB-tBspA (BtB) fusion protein
was combined with previously reported Hgp44-FlaB (HB) and FlaB-tFomA (BtA) to generate a trivalent vaccine formulation. Intranasal immunization with the trivalent mucosal vaccine (BtB + HB + BtA) induced antigen-specific serum IgG and salivary secretory IgA to each antigen without interfering with each other. The trivalent vaccine formulations resulted in similar levels of antigen-specific antibody responses with mono- or divalent vaccine (Fig. 2). In addition, the ability of the vaccine-induced antibodies to recognize native forms of antigens on live bacteria further strengthens the evidence of their potential to neutralize and inhibit the activities of these pathogens (Fig. 3). These antibodies demonstrated functional efficacy by inhibiting epithelial cell adhesion and invasion by Tf (Fig. 4) and Pg (Fig. 5) and interfering with biofilm formation by Fn (Fig. 6). The Fn-biofilm inhibition finding was further supported by the data showing that intranasal vaccination with the trivalent vaccine significantly inhibited Fn colonization in gingival tissue (Supplementary Fig. 8). However, we could not observe detectable colonization of Tf or Pg in the mouse oral cavity in the temporary ligature plus oral infection model (data not shown), which explains why temporary ligature was required to elicit osteoclastic inflammation.

**MATERIALS AND METHODS**

**Bacteria and culture conditions**

_Tannerella forsythia_ ATCC 43037 (Tf) was purchased from the America Type Culture Collection (ATCC, Massachusetts, USA). _Tf_ were grown in tryptic soy broth (BD, 211825) supplemented with 0.5% yeast extract (BD, 212750), 0.05% cysteine (Sigma, 168149), 10 µg/ml of hemin (Sigma, 51280), 5 µg/ml of menadione (Sigma, M5750), 10 µg/ml N-acetyl muramic acid (Sigma, A3007-100MG), 1 µg/ml of Vitamin K1 (Sigma, V3501-1G) at 37 °C under an anaerobic condition (85% N₂, 10% H₂, and 5% CO₂).

_Fusobacterium nucleatum subsp._ polymorphum ATCC 10953 (Fn) and _Porphyromonas gingivalis_ ATCC 33277 (Pg) were grown as described in a previous report. Bacteria were harvested at exponential growth by centrifugation (7,000 × g, 20 min, 4 °C). For live bacterial staining, 10⁹ CFU/ml bacteria were labeled with 10 µM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probe™, C1157) in 1 ml of PBS for 15 min at room temperature (RT) and then 1 ml of fetal bovine serum (FBS) (Gibco, 16000044) was added for a 10 min for blocking step at RT. Following two washes with phosphate-buffered saline (PBS), the bacteria were re-suspended in PBS.

**Animal and ethics statement**

All procedures using experimental animals were conducted following the guidelines of the Animal Care and Use Committee of Chonnam National University, with the protocol CNU IACUC-H-2022-44. Animals were maintained in compliance with the Animal Welfare Act legislated by the Korean Ministry of Agriculture, Food, and Rural Affairs guidelines.

**Periodontitis model induced by combination of ligature and oral bacterial gavage**
A PD model employing temporary ligature combined with bacterial gavage was established using SPF BALB/c mice. The ligature was done with a slight modification of previously published methods. Firstly, to suppress pre-existing oral microbiota, mice were fed with oral antibiotics (2 mg/ml of sulfamethoxazole and 0.4 mg/ml of trimethoprim) in drinking water for 3 days, followed by 3 days of antibiotic-free recovery. Then, mice were anesthetized by intraperitoneal injection of Zoletil® 50 (Virbac corporation, Carros, France) and Rompun ™ (Elanco Animal Health Korea Co.,Ltd.) and tied with a 5 − 0 surgical silk (FST, 18020-50) at the maxillary second molar (M2) tooth. Ligatures were applied on day 0, followed by three rounds of bacterial infections (Tf, Pg, and Fn) at 2-day intervals. For the first round of oral infection, mice were orally infected with a mixed suspension of the three bacterial species Tf, Pg, and Fn (1 x 10^9 CFUs each bacteria/100 µL) in 2% carboxymethylcellulose (Sigma, IGEPAL®CA-630) PBS after ligature placement. On the 6th day post ligature placement, the ligature was removed, and two rounds of oral bacterial infections were conducted at one-day intervals. To evaluate the periodontitis induced in the temporary ligature plus oral infection model, we performed micro-CT and histologic analysis by hematoxylin and eosin staining of the entire right maxilla.

**Intranasal immunization**

Seven-week-old female BALB/c mice were intranasally immunized with vaccine components three times in 2-week intervals under anesthesia, as previously described. The vaccines were diluted in PBS to a final volume of 10 µl/nostril (20 µl/mouse), resulting in immunization dosages of 6.1 µg of FlaB-L2-tBspA (BtB), 5.1 µg of BtA, 8 µg of HB, the mixture of 8 µg of HB and 5.1 µg of BtA (HB + BtA), the mixture of 6.1 µg of BtB and 5.1 µg of BtA (BtB + BtA), the triple combination of 6.1 µg of BtB, 5.1 µg of BtA, and 8 µg of HB (BtB + HB + BtA).

**Micro-computed tomography (micro-CT) analysis**

The entire right maxilla was excised, fixed overnight in 10% formaldehyde, and then preserved in 70% ethanol at 4 °C until micro-CT scanning. Micro-CT imaging was performed using the Skyscan 1172 CT system (Skyscan, Aartselaar, Belgium). The bone volume fraction (bone volume/tissue volume; BV/TV) was determined following the protocol previously described. The three-dimensional (3D) image from the buccal sides was constructed and analyzed using Mimics software 14.0 (Materialise NV, Leuven, Belgium). The linear distance was measured in millimeter (mm) from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) at 4 different sites of the buccal side in 3D images, including the disto-buccal site of the maxillary first molar (M1-2) and second maxillary molar (M2-1), the mesio-buccal of second maxillary molar (M2-2), and third maxillary molar (M3). Alveolar bone loss was determined by summing the distances of M2-1, M2-1, M2-2, and M3.

**Hematoxylin and eosin (H&E) staining**

The maxilla was fixed with 4% paraformaldehyde for 2 days and decalcified in 0.5 M ethylenediaminetetraacetic acid (EDTA) (LPS Solution, CBE002C) solution in PBS (pH 7.4) for 2 weeks at RT. The decalcified tissue was dehydrated and then embedded in paraffin. Serial sections with a thickness of 5 µm were sliced using a microtome, oriented in a bucco-palatal direction and parallel to this
plane. These sections included the distobuccal and palatal roots of the second molar, which were stained using Mayer's hematoxylin and eosin (Abcam, ab220365), after which they were observed and scanned through virtual microscopy using the Zeiss Axioscan 7 system (Germany).

**Quantitative RT-PCR (qRT-PCR)**

To analyze expression levels of periodontitis-related genes, total RNA was prepared from the gingival tissue, and qRT-PCR was conducted. Mice were intranasally immunized with the trivalent vaccine (BtB + HB + BtA) three times in two-week intervals. Two weeks after 3rd immunization, mice were treated with ligation plus mixed bacterial infection. One day later, ligatures were removed, followed by an additional bacteria challenge. Gingival tissue was collected at 24 hours post ligature removal or last infection. Gingival tissue was excised, and total RNA was extracted using TRizol (Invitrogen, 15596026). Subsequently, the isolated RNA samples were treated with RNase-free DNase I (Thermo Fisher Scientific, EN0521) to eliminate genomic DNA contamination. One microgram of extracted total RNA was used as a template to synthesize cDNA using Topscript™ RT DryMix dT18s (Enzynomics, RT200). Quantitative PCR was performed with Quantstudio™ 3 Real-Tim PCR machine (Applied Biosystem, Thermo Fisher, A28131) using SYBR Green qPCR PreMIX (Enzynomic, RT501M). The qRT-PCR data were generated from the cycle threshold (Ct) values normalized against the ribosomal protein L32 expression. Normalized fold-change was calculated following the 2^-ΔΔCt method. The primer sequences used in the experiment are listed in Supplementary Table 1.

**Measurement of antigen-specific antibody titers by enzyme-linked immunosorbent assay (ELISA)**

To assess antigen-specific antibody (Ab) titers, serum and saliva samples were obtained from immunized mice two weeks after the final immunization. ELISA assays were performed following established procedures 17. The ELISA plates (Corning Laboratories, 3690) were coated overnight at 4°C with the antigens tBspA, Hgp44, or tFomA at 1 µg/ml concentration in PBS. Subsequently, plates were washed with sterile distilled water (DW) to eliminate unbound antigens. To block non-specific binding, a blocking buffer [0.5% BSA (Sigma-Aldrich, A2153-50G), 1 mM EDTA (BIONEER, C-9007) in PBST (0.05% Tween-20 in PBS)] was applied at RT for 1 hour. Serially diluted sera or saliva in the blocking buffer were added to the plates and incubated for 2 hours at RT, and then the 5 washes were conducted using a Microplate washer (Agilent, BioTek 405 TS). Secondary antibodies, HRP-conjugated anti-mouse IgG (Southern Biotech, 103605) or IgA (Southern Biotech, 1040-05) antibodies, were used for detection. The signal was developed with the addition of 40 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (BD OptEIA, 555214), and the reaction was stopped by adding 40 µl of 1 N H$_2$SO$_4$. Optical density was measured at 450 nm using a microplate reader (Molecular Devices Corp., Menlo Park, CA). Antibody titers were expressed as the dilution's reciprocal log2 value, resulting in optical density values at 450 nm that were 2-fold higher than those of blank wells without serum.

*Anti-Tf, anti-Pg, and anti-Fn serum production*
To generate anti-\(Tf\), mice were immunized with the inactivated \(Tf\) following procedures described previous \(20\). Briefly, \(2 \times 10^9\) \(Tf\) was inactivated by the treatment with 0.3% (v/v) formalin (T&I, BPP-9004) overnight. The inactivated \(Tf\) was thoroughly washed with PBS and mixed with complete Freund's adjuvant (CFA) (Sigma, CAS9007-81-2) for vaccination. Six-week-old female BALB/c mice were immunized with the inactivated \(Tf\) cells three times at one-week intervals by subcutaneous injection. The anti-\(Pg\) and anti-\(Fn\) were produced following the procedures described in a previous study \(17\). The anti-\(Tf\), anti-\(Pg\), and anti-\(Fn\) sera were used as controls for microscopical observations.

**Immunostaining and confocal imaging**

To ascertain the ability of an antibody to recognize the specific antigen expressed in live bacteria \(Tf\), \(Pg\), and \(Fn\), immunostaining was conducted using confocal visualization, following previously described methods \(17\).

**Cell culture**

KB cells are a type of epithelial cell lines derived from HeLa cells that were obtained from ATCC and cultured in Eagles’ Minimum Essential Medium (EMEM) supplemented with 10% FBS and ampicillin at 37 \(^\circ\text{C}\) in an aerobic condition (5% \(\text{CO}_2\)). For infection experiments, cells were grown until they reached approximately 90% confluence.

**Bacterial invasion assay by flow cytometry**

To investigate the neutralizing efficacy of the anti-sera and anti-saliva, we performed a bacterial invasion assay using flow cytometry as previously studied \(74\). Serum and saliva samples were obtained from mice vaccinated with respective vaccines or PBS 2 weeks after the final immunization. The KB cells were plated at \(1.5 \times 10^5\) cells/well in 48-well plates (Costar, 3548) overnight. The \(1.5 \times 10^7\) CFSE-labeled bacteria were pre-incubated with IgG purified from anti-sera (equivalent to 19 \(\mu\)l of anti-sera/well or 9 \(\mu\)l anti-sera/well) by using the Melon™ Gel IgG Spin Purification Kit following the manufacturer's instructions (Thermo Scientific, 45206) or anti-saliva (equivalent to 1/4 or 1/8 dilution) for 1 hour at RT. KB monolayers were washed once with serum/antibiotic-free EMEM and subsequently infected at the multiplicity of infection (MOI) 1:100. The infection was carried out for 4 hours at 37 \(^\circ\text{C}\) under 5% \(\text{CO}_2\). Subsequently, the cell culture medium was aspirated, and the cells were subjected to an additional incubation in a serum-free medium containing gentamicin 300 \(\mu\)g/ml and metronidazole 200 \(\mu\)g/ml for 1 hour to kill any remaining extracellular bacteria. The cells were then washed three times with PBS and detached using 0.25% trypsin (Glibo, 25200072). To quench the fluorescence of any remaining extracellular CFSE-labeled bacteria, Trypan blue (Sigma, T8158) was added (0.2%) and analyzed by flow cytometry (BD Cantoll, USA).

**Bacterial adhesion and invasion assay by confocal microscopy**
To examine bacterial adhesion and invasion by confocal microscopy, KB cells were seeded at a density of 1 x 10^5 cells/well in 4-well cell culture slides overnight (SPL, 30104). The cells were then infected with CFSE-labeled bacteria following the protocol mentioned above. After the bacterial infection, the medium was aspirated and thoroughly washed with PBS by gentle orbital shaking. The cells were then fixed with 3.2% paraformaldehyde for 10 min at RT, followed by another round of PBS washing. Then, the cells were blocked with PBS containing 1% BSA for 15 min. Afterward, cells were washed with PBS and stained for actin using 1x Rhodamine phalloidin (Thermofisher. R415) for 1 hour. Then, nucleic acids were stained with Hoechst 33342 (Invitrogen, H3570) at the dilution of 1:1000 for 15 min. Following three final washes with PBS, the slide was mounted using DPX Mounting (Sigma, 06522). The images were observed using a confocal microscope LSM510 (Carl Zeiss, Germany).

**Biofilm inhibition assay**

The inhibitory effect of anti-sera or anti-saliva on Fn-induced biofilm formation was determined following established methods. Freshly prepared Fn ATCC 10953 cells (1 x 10^9/well) were incubated with purified IgG from anti-sera (equivalent to 12 µl anti-sera/well or 6 µl anti-sera/well) or saliva (equivalent to 1/4 or 1/8 dilution) generated by vaccination with PBS (anti-PBS), a divalent (anti-BtB + HB), a trivalent (anti-BtB + HB + BtA) on high-binding 96-well plates (COSTAR, 3590) at RT for 3 hours, subsequently added Fn media up to 200 ul per well. The plates were further incubated in anaerobic conditions for 24 hours, followed by a gentle washing step using PBS. The wells were stained with 25 µl of 0.3% crystal violet for 15 minutes and then washed gently with PBS. The stained biofilm was extracted by adding 100 µl of 100% ethanol, and the optical density was quantified using a microplate reader (Molecular Devices Corp., Menlo Park, CA) at a wavelength of 595 nm.

**Statistical analyses**

The results are presented as the mean ± standard error of the mean (SEM) unless otherwise stated. A Mann-Whitney or an unpaired t-test was used to compare the two groups. Statistical analyses were performed using Prism 8.00 software for Windows (GraphPad Software, San Diego, CA). P values < 0.05 were accepted as statistically significant.

**Declarations**

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**DISCLOSURE STATEMENT**

No potential conflict of interest was reported by the author(s).
AUTHOR CONTRIBUTIONS

S.E.L., and J.H.R. conceptualized and designed the experiments of the study. V.L., S.P., S.H.H., L.Y.S. performed the experiments. V.L., S.P., K.R., J.T.K., J.K.K., S.E.L., J.H.R. analyzed and interpreted data. V.L., S.P., S.E.L., and J.H.R. drafted manuscripts. All authors contributed to the articles and approved the submitted version.

GRANT INFORMATION

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References


**Figures**

**Fig 1.**

a) Intranasal immunization

b) Measurement of EL-4BC in mm

- Naive
- LigR+OI
- Vax+LigR+OI

- Naive
- LigR+OI
- Vax+LigR+OI

- Naive
- LigR+OI
- Vax+LigR+OI

- Naive
- LigR+OI
- Vax+LigR+OI

- Naive
- LigR+OI
- Vax+LigR+OI

Figure 1
Intranasal immunization with the trivalent vaccine alleviates periodontitis caused by a mixed *Tf*, *Pg*, and *Fn* infection in a temporary ligature plus oral infection model. 

**a** Immunization and experimental schedule. Seven-week-old female BALB/c mice (*n* ≤ 13) were intranasally immunized with PBS or a mixture of 6.2 mg of BtB, 8 mg of HB, and 5.1 mg of BtA as the trivalent vaccine (BtB+HB+BtA) three times 2-week intervals. Two weeks after the final immunization, a ligature was tied to the second molar of the mouse and subsequently orally infected with a mixed *Tf*, *Pg*, and *Fn* (1 × 10^9 CFU/each in 100 μl/mouse) three times 2-day intervals. Two days after the third bacterial infection, the ligature was removed, followed by two additional rounds of bacterial infections daily. Eight days (day 15) after the fifth bacterial infection, mice were sacrificed, and micro-CT and hematoxylin and eosin (H&E) staining was performed. 

**b-d** The trivalent vaccine protects against alveolar bone loss in mice. 

- **Measurement of the distance from the cementoenamel junction to the alveolar bone crest (CEJ-ABC) and bone volume density (BV/TV).**

- **c** Representative images of sagittal 3-dimensional and bi-dimensional alveolar bone. The pink line indicates the distance CEJ-ABC on the buccal site.

- **d** H&E stains of periodontal tissue damage. Red arrows indicate the distance of the junctional epithelium.

**e** The trivalent vaccine inhibits bacterial-induced inflammation in the gingival tissue of mice. A presented schedule illustrates the assessment of mRNA gene expression patterns by qRT-PCR. Similarly, BALB/c mice (*n* ≤ 10) were intranasally immunized with PBS or the trivalent vaccine (BtB+HB+BtA) repeated 3 times at 2-week intervals. After the final immunization, ligatures were placed on day 0, followed by oral infection with mixed *Tf*, *Pg*, and *Fn* (1 × 10^9 CFU/each in 100 μl/mouse). The ligatures were removed a day later, followed by another round of oral infection. The mRNA gene expression patterns were assessed 1 day post-last oral infection. Naïve (control mice), LigR+OI (mice administered with PBS before ligature placement/removal plus oral bacterial infection), and Vax+LigR+OI [mice vaccinated with a trivalent vaccine (BtB+HB+BtA) before ligature placement/removal plus oral bacterial infection]. Results are presented as the mean ± SEM in each group. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. NS indicates non-significance.
The trivalent vaccine elicits both systemic and mucosal antibody responses. Seven-week-old female BALB/c mice were intranasally immunized 3 times at 2-week intervals with formulations: PBS, a monovalent vaccine (6.2 μg of BtB, 8 μg of HB, or 5.1 μg of BtA), a divalent vaccine [6.2 μg of BtB plus 8 μg of HB (BtB+HB), 6.2 μg of BtB plus 5.1 μg of BtA (BtB+BtA), or 8 μg of HB plus 5.1 μg of BtA (HB+BtA)], and a trivalent vaccine [6.2 μg of BtB plus 8 μg of HB plus 5.1 μg of BtA (BtB+BtA+HB)]. a-c Serum and
saliva samples were collected 2 weeks after the final immunization to measure tBspA-, Hgp44-, and tFomA specific antibody responses using ELISA. Results are presented as the mean ± SEM for each group. N=14, **P< 0.01, NS indicates non-significance, UD indicates under the detection limit.

Fig 3.

Figure 3
Anti-sera induced by the trivalent vaccine detects cognate antigens on the surface of live Tf, Pg, and Fn. Immunofluorescence was employed to detect the natural form of BspA, Hgp44, and FomA expressed on the surface of live Tf (a), Pg (b), and Fn (c), respectively. Freshly cultured bacteria were incubated with anti-sera derived from naïve mice (pre-immune sera), mice vaccinated with Tf (anti-Tf), Pg (anti-Pg), Fn (anti-Fn), divalent (anti-HB+BtA, anti-BtB+BtA, or anti-BtB+HB), or trivalent (anti-BtB+HB+BtA) vaccines. The specimens were visualized using confocal microscopy.

Fig 4.

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**Anti-sera induced by the trivalent vaccine detects cognate antigens on the surface of live Tf, Pg, and Fn.** Immunofluorescence was employed to detect the natural form of BspA, Hgp44, and FomA expressed on the surface of live Tf (a), Pg (b), and Fn (c), respectively. Freshly cultured bacteria were incubated with anti-sera derived from naïve mice (pre-immune sera), mice vaccinated with Tf (anti-Tf), Pg (anti-Pg), Fn (anti-Fn), divalent (anti-HB+BtA, anti-BtB+BtA, or anti-BtB+HB), or trivalent (anti-BtB+HB+BtA) vaccines. The specimens were visualized using confocal microscopy.

**Fig 4.**

**a.** Purified IgG from anti-serum

**b.** Anti-saliva

**c.** KB+ Tf-CFSE

**d.** DAPI

**Rhodamine phalloidin**

**Tf-CFSE**

**Merged**
Figure 4

The anti-sera and anti-saliva raised by the trivalent vaccine inhibit the adhesion and invasion of *Tf* into KB cells. The adhesion/invasion of *Tf* into KB cells was determined by using flow cytometry and confocal microscopy. CFSE-labelled *Tf* was pre-incubated with IgG purified from anti-sera (equivalent to 19 μl/well or 9 μl/well) or anti-saliva (equivalent to 1/4 or 1/8 dilution) obtained from mice immunized with PBS (anti-PBS), a divalent vaccine (anti-HB+BtA) or a trivalent vaccine (anti-BtB+BtA+HB) for 1 hour. Then KB cells were infected with CFSE-labelled *Tf* at an MOI of 1:100 for 4 hours. a b Cells were analyzed by flow cytometry after quenching the fluorescence of bacteria bound on the surface with trypan blue. c, d Representative confocal microscopic images illustrating the adhesion and invasion of *Tf* treated with purified IgG from anti-sera (equivalent to 19 μl/well) or anti-saliva (equivalent to 1/4 dilution), respectively. Data are represented as the mean ± SEM from three independent flow cytometry experiments. *** P < 0.001, **** P < 0.0001 and NS indicates non-significance.
Figure 5

The trivalent vaccine-derived anti-sera and anti-saliva inhibit the adhesion and invasion of *Pg* into KB cells. Inhibition of KB adhesion/invasion by *Pg* was determined by flow cytometry and confocal microscopy. CFSE-labelled *Pg* was pre-incubated with IgG purified from anti-sera (equivalent to 19 µl/well or 9 µl/well) or anti-saliva (equivalent to 1/4 or 1/8 dilution) derived from mice vaccinated with PBS (anti-PBS), a divalent vaccine (anti-BtB+BtA) or a trivalent vaccine (anti-BtB+BtA+HB) for 1 hour. KB cells were
infected with *Pg* at an MOI of 1:100 for 4 hours. **a, b** Cells were analyzed by flow cytometry after quenching the fluorescence of bacteria bound on the surface with trypan blue. **c, d** Representative confocal microscopic images illustrating the adhesion and invasion of *Tf* treated with purified IgG from anti-sera (equivalent to 19 μl/well) or anti-saliva (equivalent to 1/4 dilution), respectively. Data are represented as the mean ± SEM from three independent flow cytometry experiments. *** *P* < 0.001, **** *P* < 0.0001, and NS indicates non-significance.

**Fig 6.**
Figure 6

The anti-sera and anti-saliva produced by the trivalent vaccine prevent the formation of *Fn*-mediated biofilm. Inhibition of *Fn* biofilm formation. **a, b** The *Fn* (1 x 10^9 cells) was pre-incubated with IgG purified from anti-sera (equivalent to 12 μl/well or 6 μl/well) or anti-saliva (1/4 or 1/8) from mice vaccinated with PBS (anti-PBS), a divalent vaccine (anti-BtB+HB) or a trivalent vaccine (anti-BtB+BtA+HB) for 3 hours at RT, followed by overnight incubation at 37 °C under anaerobic conditions (85% N₂, 10% H₂, and 5% CO₂) overnight. Following a gentle wash with PBS, the wells were stained with 0.3% crystal violet for 15 min. The stained biofilm was extracted with 100% ethanol and diluted twofold with PBS, and the absorbance was measured at 595 nm. The right panels represent a microscopic observation of the crystal violet-stained biofilm. The presented data represent the mean ± SEM for each group, and the experiments were conducted with three replicates. *** P < 0.001, **** P < 0.0001, and NS indicates non-significance.

Supplementary Files

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