



**UNIVERSITÀ  
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1240**

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Coordinatore: Prof. Francesco Iannelli

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*Candidato/a*

Valentina Caradonna

Università degli Studi di Siena

*Firma digitale del/della candidato/a*

*Supervisore*

Dott.ssa Elena Pettini

Università degli Studi di Siena

VALENTINA CARADONNA

26/01/2026 14:03:14

*Valentina Caradonna*

*Co-supervisore*

Dott.ssa Francesca Mancini

GSK Vaccines Institute for Global Health

Anno accademico di conseguimento del titolo di Dottore di ricerca

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*Commissione giudicatrice*

Reiner Siebert

Claudia Sala

Lorenzo Franchi

Flavia Artese

Francesco Santoro

Francesco Iannelli

*Supplente*

German Ott

Arianna Di Napoli

Teresa Marafioti

Stefano Lazzi

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## GENERAL ABSTRACT

In the present thesis, insights into the global epidemiology of *Shigella*, together with evaluations of the humoral and cellular immunogenicity and cross-reactive potential of the quadrivalent GMMA-based vaccine altSonflex1-2-3, currently in phase II development, were investigated to address the challenge of shigellosis. A review of *Shigella* serotypes distribution revealed its high burden among children under five in low- and middle-income countries, complicated by extensive O-antigen variability and rising antimicrobial resistance. This analysis underscored the urgent need for innovative vaccine strategies targeting the predominant *S. sonnei* and *S. flexneri* serotypes in these regions. Preclinical studies in mice, rats, and rabbits were conducted to examine the humoral immune responses elicited by altSonflex1-2-3 against its target strains and provide translational insights. Vaccination induced robust O-antigen-specific IgG responses and functional bactericidal activity, with distinct dose-response patterns revealing interspecies differences. Notably, the immune profiles in rats closely mirrored those of human adults, suggesting high predictive value for clinical outcomes. To further evaluate vaccine-induced functionality and potential broad protection against strains not targeted by the vaccine, a high-throughput luminescence-based serum bactericidal assay was characterized for five heterologous *S. flexneri* serotypes. This assay demonstrated excellent repeatability, precision, and specificity with human sera and effectively assessed both homologous and heterologous antibody responses. Subsequent serological studies using preclinical animal sera and clinical sera from European adults enrolled in a Phase I trial, confirmed that altSonflex1-2-3 was able to generate cross-reactive antibodies capable of binding and killing non-vaccine *S. flexneri* strains. These findings were supported by phylogenomic analyses, which verified that the heterologous strains represent circulating isolates, and by antigenic cartography, which provided a clear visualization of cross-reactivity. For the first time in the present thesis, the O-antigen-specific cellular response elicited by altSonflex1-2-3 was investigated. Multiparametric flow cytometric analyses revealed that the vaccine stimulated both immediate and long-lasting immunity through rapid induction of germinal centers, activation of T follicular helper cells, and generation of durable memory B cells and long-lived plasma cells, alongside a predominantly Th1-polarized CD4<sup>+</sup> T cell response, with activation sustained up to 22 weeks post-boost. Collectively, these integrated findings establish altSonflex1-2-3 as a promising candidate for broadly protective *Shigella* vaccination, laying a strong foundation for its continued clinical development and future global impact.

## **CHAPTER 1. General introduction to *Shigella***

### **1. Global burden of diarrhoeal diseases**

Infectious diseases continue to pose one of the greatest challenges to global health, particularly in low- and middle-income countries (LMICs), despite considerable advances in antibiotics, vaccines, and sanitation technologies. Among these, diarrhoeal diseases represent a critical threat to child health. In 2021, diarrhoea was responsible for approximately 9% of all deaths in children under five years of age, equating to roughly 444,000 deaths annually in this age group (1, 2). Children in LMICs typically experience multiple episodes of diarrhoea each year, particularly during their first two years of life, a period critical to growth (3). Repeated exposure to enteric pathogens during early life is closely associated with growth faltering, undernutrition, impaired cognitive development, and subclinical inflammatory conditions, such as Environmental Enteric Dysfunction (EED). EED disrupts the intestinal lining, reduces nutrient absorption, and increases vulnerability to other infections (4, 5). These biological impacts reinforce long-standing socioeconomic inequalities by limiting educational attainment and reducing long-term productivity and earning potential (6, 7). Despite improvements in global health metrics during the last decade, the burden of diarrhoeal illness remains unacceptably high.

Geographically, the burden of diarrhoeal diseases is heaviest in sub-Saharan Africa and South Asia, where infrastructural limitations in water, sanitation, and hygiene (WASH) significantly amplify the risk of enteric infections (8). In these settings, inadequate access to safe water and inappropriate waste disposal perpetuates pathogen transmission cycles, especially in densely populated and low-income communities. According to the 2022 WHO/UNICEF Joint Monitoring Programme for Water Supply, Sanitation and Hygiene, 1.7 billion people globally lacked access to safely managed drinking water, while 3.4 billion lacked access to safely managed sanitation services, conditions directly linked to elevated diarrhoeal morbidity and mortality (9). These figures highlight systemic infrastructural gaps that contribute not only to sustained transmission of diarrhoeal pathogens but also to long-term health and development deficits. Moreover, environmental and socio-political factors such as climate change, rapid urbanization, and mass population displacement due to conflict or natural disasters further exacerbate the transmission of diarrhoeal pathogens by increasing vulnerability (10, 11). These overlapping risk factors underscore the complexity of diarrhoea control efforts in resource-limited settings.

### **1.1. Emerging role of *Shigella***

Data from the Global Enteric Multicenter Study (GEMS) and global networks such as Enterics for Global Health (EFGH) consistently identify *Shigella* as one of the leading bacterial causes of moderate-to-severe diarrhoea in children under five across diverse LMIC settings (12, 13).

Recent analyses from the Global Burden of Disease project reinforce the pathogen hierarchy: while rotavirus remains the leading global cause of diarrhoea-related mortality in young children, *Shigella* has emerged as the second most prominent aetiological agent (14). Trends suggest that *Shigella* may soon overtake rotavirus as the predominant cause of paediatric diarrhoeal hospitalizations and deaths in several regions (15). This shifting epidemiological landscape underlines the urgency of prioritizing *Shigella* within global enteric disease control strategies, including vaccine development efforts aimed at reducing the burden in the most affected populations.

## **2. *Shigella* biology, transmission, and pathogenesis**

*Shigella* is a genus of facultative anaerobic, non-motile, non-spore-forming, rod-shaped Gram-negative bacteria in the Enterobacteriaceae family (16). First identified by Kiyoshi Shiga in 1897 during a severe dysentery epidemic in Japan, the genus is now classified into four human-restricted species: *S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei* (17). Each species, except for *S. sonnei* that only comprises one serotype, is characterized by a pronounced serotype diversity: *S. dysenteriae* comprises 14 serotypes, *S. boydii* nineteen, and *S. flexneri* more than twenty (historically fifteen, with multiple subvariants)(16). Serotyping is based primarily on differences in the structure of the O-antigen (OAg), a component of the lipopolysaccharide (LPS) layer on the bacterial surface that plays a crucial role in immune recognition (18) (Liu et al., 2008). These structural differences contribute to the antigenic variability observed among *Shigella* species (19). A more comprehensive examination of OAg structure is provided in Chapter 2.

Transmission of *Shigella* occurs primarily via the fecal–oral route, most often through ingestion of contaminated food or water or direct person-to-person contact (20). The pathogen is remarkably resilient: it can survive passage through the acidic environment of the stomach and persist on fomites, utensils, surfaces, and even on mechanical vectors such as flies (16, 17, 21). Its infectious dose is exceptionally low, often estimated at only 10–100 organisms (22), making it one of the

most contagious bacterial enteropathogens. In recent years, *Shigella* has also been increasingly recognized as a sexually transmitted pathogen, particularly among men who have sex with men (MSM) (23-26). Another group at elevated risk are people experiencing homelessness (PEH), who are vulnerable to outbreaks due to crowded living conditions, limited access to hygiene facilities, and overlapping risk factors such as substance use and poor nutrition (27, 28). Both MSM and PEH populations have been disproportionately affected by extensively drug-resistant (XDR) strains, including in high-income countries (26, 29). Additionally, international travelers to and from endemic regions represent an important transmission bridge, with returning travelers implicated in introducing *Shigella* into non-endemic areas (30, 31).

Once ingested, *Shigella* exploits M cells in the Peyer's patches to cross the intestinal epithelium, where it is quickly engulfed by resident macrophages. Rather than being destroyed, the bacteria induce pyroptotic cell death through activation of caspase-1, resulting in the release of pro-inflammatory cytokines like IL-1 $\beta$  and IL-18 and enabling bacterial escape into the subepithelial space (32, 33). From there, *Shigella* accesses the basolateral membrane of intestinal epithelial cells and invades using a Type III Secretion System (T3SS), which injects effector proteins that manipulate host cell pathways to promote bacterial entry and cytosolic survival (34). Once in the cytosol, *Shigella* evades autophagic degradation and innate pattern recognition by masking or degrading host signaling intermediates such as NF- $\kappa$ B components, suppressing early inflammatory responses (35).

This intracellular lifestyle enables *Shigella* to replicate while evading the immune system. Using actin-based motility mediated by the surface protein IcsA, the bacteria move within and between adjacent cells, facilitating direct cell-to-cell spread (36). This mechanism prevents exposure to circulating antibodies and complement, allowing the bacteria to persist despite generating intense local inflammation. Moreover, intracellular spread delays the recruitment of antigen-specific T cells and other adaptive immune components, giving *Shigella* a critical window to establish infection (37).

The molecular basis of *Shigella* virulence is orchestrated by a large (~220 kb) virulence plasmid known as pINV, which encodes the T3SS and several key effectors such as IpaB, IpaC, IpaD, and IpgD (38). These proteins manipulate the host actin cytoskeleton and disrupt epithelial tight junctions, facilitating bacterial invasion and cytoplasmic escape (34, 39). Another important

effector molecule is IcsA (or VirG) that drives actin polymerization and permits the bacteria, once internalized, to replicate and spread to neighboring cells. Recent work has also identified host factors critical to dissemination, such as PIK3C3 kinase, which mediates formation of vacuolar protrusions and double-membrane vacuoles required for spread of *S. flexneri* (40).

Clinically, shigellosis manifests after 1 to 4 days incubation period with fever, abdominal cramping, tenesmus, and mucoid or bloody diarrhoea, hallmarks of colonic inflammation and ulceration (20). While most cases resolve within 5–7 days, severe forms are common in children under five, the elderly, and immunocompromised individuals (20). Complications include dehydration, febrile seizures, rectal prolapse, toxic megacolon, bowel perforation, and bacteremia, which may be fatal if not managed appropriately (41).

Treatment of shigellosis primarily focuses on maintaining hydration, either orally or intravenously depending on the severity of diarrhoea and fluid loss (16). Antibiotic therapy is recommended for severe cases, immunocompromised patients, or those at risk of complications, as it shortens disease duration, reduces transmission, and prevents systemic spread. First-line antibiotics traditionally include fluoroquinolones such as ciprofloxacin for adults, while macrolides such as azithromycin are increasingly used in children or in regions with fluoroquinolone resistance (16, 42, 43).

### **2.1. Antibiotic resistance in *Shigella* spp.**

*Shigella* exhibits extraordinary genomic plasticity, largely enabled by horizontal gene transfer mechanisms such as plasmids, integrons, and insertion sequences. These mobile genetic elements facilitate the rapid acquisition of antimicrobial resistance (AMR) genes, allowing *Shigella* to develop multidrug-resistant (MDR) and XDR phenotypes (44–47). Genetically, the escalation of AMR in *Shigella* is driven by a combination of plasmid-borne genes and chromosomal mutations. The ease of horizontal gene transfer between *Shigella* and other Enterobacteriaceae enables rapid resistance dissemination within and across species boundaries (44, 48, 49). Due to the growing resistance threat, the World Health Organization now classifies fluoroquinolone-resistant *Shigella* as a priority AMR pathogen, emphasizing the urgent need for novel therapeutic and vaccine interventions (50). Fluoroquinolone resistance is mainly caused by mutations in *gyrA* and *parC* and by plasmid-mediated quinolone resistance genes (*qnr* and *aac(6′)-Ib-cr*) (51). Resistance is not only directed against fluoroquinolone but also to macrolides like azithromycin, and in this case is often mediated by genes encoding *mphA* methylases, *ermB* esterases, and efflux pumps, frequently

carried on conjugative R-plasmids (52). A recent multicenter meta-analysis also confirmed the global prevalence of  $\beta$ -lactamase genes which confer resistance to ceftriaxone and ampicillin (53).

In regions such as sub-Saharan Africa and South Asia, where shigellosis burden is highest, resistance rates among clinical *Shigella* isolates routinely range from 40% to 60%, indicating widespread loss of efficacy for many standard treatments (48, 54). Alarming, high-income countries have not been spared. In the United States, XDR *S. sonnei* infections defined by resistance to all five first-line oral antibiotics (ciprofloxacin, azithromycin, ceftriaxone, trimethoprim-sulfamethoxazole, and ampicillin), increased from 0% in 2015 to approximately 5% by 2022 (55). In 2018 and 2022, nearly 50% of *S. flexneri* isolates in Ontario were MDR, while XDR isolates represented 6.6%, climbing to 9.1% in early 2023 (56). A genetically related cluster of XDR *S. sonnei*, first detected during a 2021–2022 outbreak in the United Kingdom, was subsequently reported across multiple European countries, including Austria, Belgium, Denmark, France, Germany, Ireland, Italy, Norway, and Spain, suggesting cross-border transmission. These isolates consistently exhibited resistance to ciprofloxacin, azithromycin, ceftriaxone, TMP-SMX, and ampicillin (57).

The primary driver of *Shigella* AMR is widespread misuse and overuse of antibiotics, particularly in LMICs where antibiotics are often purchased without prescriptions (58), and clinical microbiology infrastructure is limited (59). As a result, empirical treatment is commonly initiated without pathogen identification or susceptibility testing.

Considering the rapid emergence of resistance to each newly introduced antibiotic class and the difficulties associated with developing novel and effective drugs, relying solely on research into resistance mechanisms and new antibiotic development is inadequate (60). Vaccination has profoundly improved human health and offers long-term protection, with a considerably lower likelihood of driving resistance compared to antibiotics (61). Ultimately, due to the narrowing antibiotic pipeline, vaccines are now considered essential to reduce *Shigella* disease burden and limit AMR spread. Protecting at-risk populations (young children in LMICs, MSM, PEH, the elderly, and the immunocompromised) depends on the successful development and deployment of broadly protective, affordable vaccines.

### 3. Immunity to *Shigella* and challenges in vaccine development

Understanding the immune response to *Shigella* infection is essential for designing effective vaccines and establishing reliable measures of protection. *Shigella* employs sophisticated evasion strategies, which allow it to avoid early innate and adaptive recognition, delay immune activation, and persist within the colonic epithelium, as better described in Paragraph 2.

Epidemiologic and immunologic evidences indicate that naturally acquired immunity reduces susceptibility to reinfection in both adults and children (62-65). In endemic regions, disease incidence diminishes in older children and adults, and pathogen-specific host defenses, lacking in early infancy, progressively increase with age (63, 66). The length of exposure and boosting of immunity through repeated infections is more strongly associated with lower disease incidence (67). This naturally acquired protective immunity tends to be directed to the OAg (67-70). Indeed, serum IgG to LPS or OAg have been repeatedly associated with reduced risk of disease in natural infection and vaccination (71, 72). Early human challenge studies showed that higher baseline serum IgG to the OAg of *S. sonnei* or *S. flexneri* was associated with lower risk of culture-confirmed disease (62, 73). More recent community-based data in Israeli children and adolescents confirmed that elevated pre-existing anti-LPS IgG correlates with reduced disease incidence (74). Subjects from endemic regions typically develop broader and more functional antibody repertoires, with responses to both OAg and conserved proteins (75).

In early life, protection derives from maternal antibodies transferred transplacentally and via breast milk (65). Cord blood from infants in endemic areas contains IgG reactive to *S. flexneri* and *S. sonnei*, often recognising both OAg and conserved proteins (65, 76). However, maternal antibody protection is transient: IgG titres decline rapidly within the first months of life, and functional activity in cord blood is consistently lower than in maternal serum (76, 77). Longitudinal studies in Kenyan and Malian cohorts show that endogenous OAg-specific IgG and IgA develop gradually between 6 months and 2 years, with improving but still suboptimal functional capacity compared to adults (78, 79).

Controlled human infection model (CHIM) trials of OAg-conjugate vaccines (e.g., Flexyn2a) and GMMA-based candidates (e.g., 1790GAHB) have confirmed that vaccination induces robust anti-LPS IgG and complement-mediated killing, but these parameters do not universally correlate with

protection (80, 81). CHIM data with *S. sonnei* identified  $\alpha 4\beta 7^+$  IgG/IgA-secreting memory B cells, indicative of gut mucosal immunity, as a possible correlate of protection (CoP) (82).

Given the absence of a single validated CoP, *Shigella* vaccine trials increasingly integrate multiple immunological assays. ELISA for serum anti-LPS/OAg IgG remains the gold-standard primary humoral endpoint due to its reproducibility and cross-study comparability (83-85). Functional readouts such as serum bactericidal assays (SBA) and luminescence-based SBA (L-SBA) quantify complement-mediated killing and are routinely used to assess the immune response against *Shigella* (83). High SBA titres have been linked to partial protection against *Shigella* disease, suggesting that SBA may serve as a functional correlate of immunity and emphasizing the protective role of antibodies during *Shigella* infection (82, 86). Opsonophagocytic killing assays (OPA/OPKA) and adhesion/invasion inhibition assays (AIA) provide additional insights (87, 88). Another integrative approach is systems serology, profiling antibody subclass distribution, Fc receptor interactions, glycosylation, and effector functions such as antibody-dependent neutrophil phagocytosis (ADNP) and complement deposition (ADCD), and is emerging as a promising tool to define immune signatures predictive of protection (89, 90).

These findings point towards a model in which protection is mediated by a composite immune signature integrating systemic and mucosal humoral immunity, functional antibody quality, and targeted cellular responses. The identification of such signatures across diverse populations and vaccine platforms will be critical to guide the development of broadly protective *Shigella* vaccines.

#### **4. GMMA as vaccine platform**

A comprehensive overview of current *Shigella* vaccine strategies, including live-attenuated, inactivated, subunit, and conjugate platforms, is provided in Chapter 2. Among these, Generalized Modules for Membrane Antigens (GMMA) represent a particularly promising and innovative approach. GMMA are engineered outer membrane vesicles (OMVs) derived from Gram-negative bacteria and are designed to retain native antigenic properties while maximizing scalability and immunogenicity (91). OMVs are naturally secreted by bacteria such as *Shigella*, *Neisseria meningitidis*, and *Salmonella* during growth and environmental stress (92). These spherical particles, ranging in size from ~20–250 nm, consist of a phospholipid bilayer embedded with LPS and various outer membrane proteins (OMPs), mirroring the composition of the bacterial outer membrane (93).

Historically, OMV-based vaccines faced significant production challenges, primarily due to low vesicle yield and complex purification processes that hindered scalability (94). Early strategies, such as detergent extraction of OMVs (dOMVs), were implemented to enhance vesicle recovery (95). These methods used deoxycholate to release membrane fragments, but at the cost of depleting essential immune-stimulatory components, including LPS and membrane-anchored lipoproteins, thereby reducing the antigenic and adjuvant integrity of the final product (96, 97). The GMMA platform was developed to address these limitations through targeted bacterial engineering. Specifically, the Tol–Pal system, a conserved envelope-spanning protein complex involved in maintaining outer membrane integrity and curvature, was identified as a key regulator of vesicle release (98). Genetic deletion of *tolR* disrupts this system, impairing the constriction of the outer membrane and leading to constitutive hypervesiculation, or “hyperblebbing” (91). These mutations enhance natural vesicle production by 10–100 fold without compromising the inclusion of native antigens, allowing for high-yield, scalable manufacturing (91, 99). Importantly, the resulting GMMA particles retain the complete outer membrane architecture, including LPS, OMPs, and periplasmic cargo, thereby providing an antigenically rich and physiologically relevant mimic of the parent bacterium. One of the hallmarks of GMMA is their ability to engage pattern recognition receptors (PRRs), notably Toll-like receptors (TLRs), which are pivotal in initiating innate immune responses (100, 101). The LPS component of GMMA is recognized primarily by the TLR4–MD-2–CD14 complex, triggering downstream activation of the NF- $\kappa$ B signaling pathway and production of proinflammatory cytokines (100, 102). This TLR engagement underlies the self-adjuvant nature of GMMA. To fine-tune this innate immune activation, a second key genetic modification was introduced: alteration of the lipid A portion of LPS. In its native hexa-acylated form, lipid A exhibits high agonist activity at TLR4. However, GMMA strains are engineered to express a pentacylated lipid A structure, typically via targeted deletion or modification of late-stage acyltransferase genes (e.g., *htrB* or *msbB*) (102). This structural change reduces binding affinity for the TLR4/MD-2 complex and modifies downstream signaling, resulting in a more controlled and tolerable immune response (103, 104). Together, these two core modifications, hypervesiculation and lipid A refinement, define the GMMA platform’s unique ability to combine high production yields with built-in immune stimulation, making them suitable for safe use across populations, including infants and vulnerable groups. Manufacturing is streamlined using tangential flow filtration, which concentrates vesicles and removes

contaminants (99). For this reason, compared to other traditional vaccine platforms, GMMA are rapid to produce, low-cost, and well-suited for deployment in low-resource settings (105).

From an immunological standpoint, GMMA offer several key advantages. Their optimal nanoparticle size (~50–100 nm) enhances uptake by antigen-presenting cells such as dendritic cells and macrophages (106, 107).

#### **4.1. Development of the 4-component altSonflex1-2-3 vaccine**

The development of altSonflex1-2-3 reflects the culmination of preclinical insights, epidemiological evidence, and design refinement aimed at overcoming the limitations of earlier *Shigella* vaccine candidates. It builds directly on the foundational work of 1790GAHB, a monocomponent GMMA-based vaccine developed by the GSK Vaccines Institute for Global Health (GVGH) targeting *S. sonnei* (108). 1790GAHB demonstrated excellent safety and immunogenicity in Phase I studies among European adults, eliciting strong anti-OAg IgG titers and durable responses upon boosting (80). However, a pivotal 2024 Phase IIb CHIM trial revealed that 1790GAHB did not meet its primary efficacy endpoint. Although the vaccine reduced disease severity and elicited functional serum responses, protection against moderate-to-severe shigellosis was not statistically significant (109).

This led to the rational design of altSonflex1-2-3, a multivalent GMMA-based vaccine comprising four components: *S. sonnei*, *S. flexneri* 1b, 2a, and 3a. This formulation was guided by extensive epidemiological data, including findings from the GEMS, which identified these serotypes as responsible for approximately 80% of global *Shigella* cases in young children (110). Also, early studies aimed at optimizing vaccine composition identified a minimal set of GMMA components capable of inducing broadly cross-bactericidal antibody responses against a wide range of *S. flexneri* serotypes (111). This strategy enabled the design of a formulation with broad functional coverage while keeping vaccine complexity manageable (106, 111). Preclinical studies in mice and rabbits confirmed that all four components individually elicited strong IgG responses and serum bactericidal activity, and that the multivalent combination did not result in antigenic interference (112).

Following successful preclinical evaluation, altSonflex1-2-3 progressed to an ongoing 2-staged, phase 1/2 randomized clinical trial (ClinicalTrials.gov, NCT05073003; EU Clinical Trials

Register, 2021-000891-12). The Phase I was conducted in healthy European adults aged 18–50 years. This first-in-human, observer-blind trial assessed safety, tolerability, and immunogenicity of altSonflex1-2-3. Participants received a total OAg content of 60 µg (corresponding to 15 µg per serotype, respectively) in two intramuscular doses (113). The results demonstrated a strong safety profile, with adverse events limited to localized reactions (e.g., pain at the injection site) and no serious vaccine-related events reported (113). From an immunological standpoint, altSonflex1-2-3 induced functional serotype-specific immune responses, allowing further clinical development in the target population (113).

The study is progressing to the Phase II conducted in Kenya, designed to assess safety, reactogenicity, and immunogenicity in the vaccine’s target infant population. The trial followed an age de-escalation approach, beginning with African adults, then moving sequentially to children aged 24–59 months, and finally to infants aged 9 months, the group at highest risk of severe *Shigella* disease. In each cohort, three antigen dose levels—low (Dose A), medium (Dose B), and high (Dose C)—were tested to identify the optimal dose for infants. Infants in the dose-finding stage received three vaccine doses, while an additional cohort was enrolled to evaluate a two-dose schedule (ClinicalTrials.gov, NCT06663436).

In June 2025, GSK entered a strategic licensing agreement with Bharat Biotech to advance altSonflex1-2-3. Bharat Biotech will lead late-stage development, the design of Phase III clinical trial, regulatory filings, and large-scale manufacturing, targeting access in LMICs where *Shigella* remains a major health burden (114).

## **5. Objectives of the PhD project**

The aim of the PhD project reported in this thesis was to further characterize the immune responses elicited by the multicomponent GMMA-based vaccine altSonflex1-2-3. In particular, the project focused on two main objectives:

1. to investigate the ability of altSonflex1-2-3 to elicit cross-functional antibody responses against *S. flexneri* strains other than those included in the vaccine;
2. to evaluate the induction, magnitude and persistence of the OAg-specific cellular immune response induced by the altSonflex1-2-3 vaccine.

To contextualize these objectives, Chapter 2 provides an updated overview of *S. sonnei* and *S. flexneri* distribution, underscoring the urgency of developing a broadly protective vaccine. By highlighting the most frequently circulating serotypes and their geographic patterns, this chapter supports the design rationale behind a multicomponent vaccine with potential cross-protective capabilities. In Chapter 3, the immunogenicity of altSonflex1-2-3 in various preclinical models (mice, rats, and rabbits) was investigated to identify which species most accurately predict the humoral response observed in humans. This work was essential for optimizing animal models for downstream immunological evaluations.

Chapters 4 and 5 represent the core of the functional antibody analysis. Chapter 4 describes the development and characterization of a high-throughput luminescence-based serum bactericidal assay (L-SBA), tailored for assessing human sera against a panel of *S. flexneri* serotypes. Chapter 5 then builds on this method to evaluate the cross-functional potential of vaccine-elicited antibodies. Using both L-SBA and a flow cytometry-based binding assay, it was assessed whether the vaccine could induce antibodies capable of binding to and killing heterologous *Shigella* strains in both preclinical and clinical samples. This chapter compares results between animal models and early-phase human trials, offering insights into the translational performance of the vaccine. To determine whether the *Shigella* strains included in these immunological assessments were representative of the global strain diversity, Chapter 6 introduces a phylogenetic analysis. Here, the heterologous bacterial strains used in the SBA assessment of crossreactivity were compared with a broader genomic dataset, spanning isolates from Global Enteric Multicenter Study (GEMS). This work proved that the selected strains are representative of the genetic diversity currently circulating worldwide. Chapter 6 also contains antigenic maps constructed based on the SBA cross-reactivity results, which confirmed and facilitated the visualization of cross-reactivity between homologous and heterologous strains and differences between animal models.

Chapter 7 focuses on cellular immunity, presenting for the first time, a comprehensive characterization of the OAg-specific B- and T-cell responses induced by altSonflex1-2-3 vaccine in mice. Both short-term and long-term cellular responses were analyzed providing novel evidences that the altSonflex1-2-3 vaccine induces an OAg-specific memory B cell response that is sustained over time. Collectively, the work presented in this thesis contributes to a deeper

understanding of how GMMA-based vaccines elicit broad and functional immune responses supporting the development of an effective *Shigella* vaccine.

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## CHAPTER 2. *Shigella sonnei* and *Shigella flexneri*: epidemiology supporting development of cross-protective vaccines

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Valentina Caradonna<sup>1</sup>, Agnes Hunyady<sup>2</sup>, Usman Nakakana<sup>2,3</sup>, Miren Iturriza<sup>2</sup>, Francesca Micoli<sup>2</sup>, Omar Rossi<sup>2</sup>, Francesca Mancini<sup>2</sup>

<sup>1</sup>*University of Siena, Siena, Italy;* <sup>2</sup>*GSK Global Health Vaccines R&D (GVGH), Siena, Italy;*

<sup>3</sup>*Present address: Bill and Melinda Gates Foundation, Seattle, WA, USA.*

### Abstract

Shigellosis represents a major global health challenge, significantly contributing to mortality among children under five, particularly in sub-Saharan Africa and South Asia. Advances in molecular techniques have underscored the extensive global impact of *Shigella* and the spread of multi- and extensively drug resistant strains poses an increasing threat. Currently, no widely available vaccine exists for *Shigella*. Developing such a vaccine is promising, yet challenging due to the diversity of *Shigella* serotypes mediated by the immune dominance of the O-antigen. This review analyses the distribution of *Shigella* serotypes in both endemic and non-endemic regions and examines the impact of O-antigen diversity on cross-reactivity. Various vaccine strategies, with a particular focus on the O-antigen as a key antigenic target, will be presented, highlighting the need for an effective vaccine especially due to the increased antimicrobial resistance associated to this pathogen. Overall, this review aims to provide a thorough understanding of different aspects relevant to the development of effective control strategies against *Shigella* and its global burden.

Keywords: *Shigella*, epidemiology, cross-reactivity, vaccines.

### 1. Introduction

Diarrhoeal diseases constitute an important global health problem, causing more than 1 million deaths annually, particularly in sub-Saharan Africa and South Asia (1) (Figure 1). In 2016, *Shigella* was the second leading cause of diarrhoeal deaths after rotavirus (2). Recent molecular techniques

have revealed a severe underestimation of the global burden of *Shigella* infections (3). Multi- and extensively drug-resistant (MDR and XDR) strains are becoming increasingly prevalent both in endemic (4) and non-endemic countries (5-8). However, there still is no widely available vaccine against *Shigella*.

Shigellosis occurs when epithelial cells in the colon, ileum, and rectum are invaded (9). The infection leads to inflammation, diarrhoea, and frequently bloody diarrhoea, depending on the serogroup (10), along with fever and abdominal cramps (9). *Shigella* invades the host intestine through M cells located above the lymphoid follicles, where it is engulfed by macrophages. It quickly escapes from the macrophages by initiating pyroptosis, releasing the bacteria at the basal surface of the epithelial cells. It then enters epithelial cells via macropinocytosis. Once inside the cytosol, *Shigella* replicates and uses actin-based motility to evade host defenses and spread to adjacent cells (11). *Shigella* species are typically classified as waterborne but can also be foodborne pathogens, as the most common transmission vehicles are water and contaminated food, even though they are essentially found in stool of infected people or primates (12).

Risk factors for *Shigella* infection include poor water, sanitation, and hygiene (WASH) standards (13), and the disease is indeed prevalent in areas with inadequate sanitation and limited healthcare access. *Shigella* has the potential to cause large outbreaks across all age demographics, but the burden especially affects vulnerable groups such as children, in whom infection can lead to a cycle of malnutrition and diarrhoeal disease (14), resulting in significant long-term complications such as growth faltering and cognitive impairment (15). While traditionally associated with low- and middle-income countries (LMICs), *Shigella* infections also pose a growing concern in high-income countries (HICs), particularly due to the emergence of antimicrobial resistance (AMR)(16). In HICs, certain populations, such as men who have sex with men (MSM), are disproportionately affected (17).

The complexity of fighting diarrhoeal diseases is due to their multifactorial nature, and while improved sanitation significantly reduces diarrhoea incidence, sustained progress necessitates additional public health measures like appropriate surveillance systems and immunization programs (18).

Protection through vaccination against *Shigella* is achievable, as early studies on natural exposure demonstrated that exposed subjects had serotype-specific IgG and IgA antibody responses that

offered protection from reinfection (19, 20). A threshold of 1:1600 IgG anti-*S. sonnei* lipopolysaccharide (LPS) titer in Israeli adults was associated with a 74% vaccine efficacy (VE). In children aged 3-4 years, a threshold of 4.5 Enzyme-linked immunosorbent assay (ELISA) Units corresponded to a predicted VE of 63%, and for children aged 2-4 years, the same threshold predicted a VE of 49% (21). Studies have shown that the bactericidal activity of antibodies in sera is associated with reduced clinical disease and may predict vaccine efficacy against *Shigella* infection (22). Findings also suggest that specific immune markers, such as Serum Bactericidal Assay (SBA) titers and anti-LPS IgG are associated with a lower risk of shigellosis and could potentially serve as correlates of risk (23). Controlled human infection models have further confirmed the plausibility of achieving immunity through vaccination (24).

Serotype specificity is linked to the O-antigen (OAg), a component of the outer membrane LPS of *Shigella*, which is the primary target of the host's humoral immune response. Several vaccines in development aim to induce an immune response that can recognise and neutralise *Shigella* bacteria based on their OAg.

However, developing a broadly protective vaccine against *Shigella* is challenging due to the diversity of *Shigella* serotypes, each characterised by a different OAg structure. The *Shigella* genus comprises four principal serogroups: *Shigella dysenteriae* (group A), *Shigella boydii* (group B), *Shigella flexneri* (group C), and *Shigella sonnei* (group D), with more than 50 serotypes. Among them, the predominant and widespread species are *S. flexneri* (more than 15 serotypes) and *S. sonnei* (1 serotype) (25).

Despite the diversity of serotypes, cross-reactivity could potentially be leveraged to develop a vaccine that provides protection against multiple serotypes. If antibodies induced against the OAg of one *Shigella* serotype can cross-react with the OAg of another serotype, a vaccine incorporating the former OAg could potentially provide protection against both serotypes (26).

In this review, we will explore the distribution of *S. sonnei* and *flexneri* in both endemic and non-endemic countries, unraveling the factors that influence the variation in prevalence of different serotypes. We will delve into the implications of OAg diversity on cross-reactivity between serotypes and the potential for cross-protection. The urgent need for a vaccine, especially in the face of rising antimicrobial resistance, will be discussed by examining various vaccine strategies for *Shigella*, with an emphasis on the OAg as antigenic target. We aim to provide a thorough

understanding of different aspects relevant to the development of effective control strategies against *Shigella*.

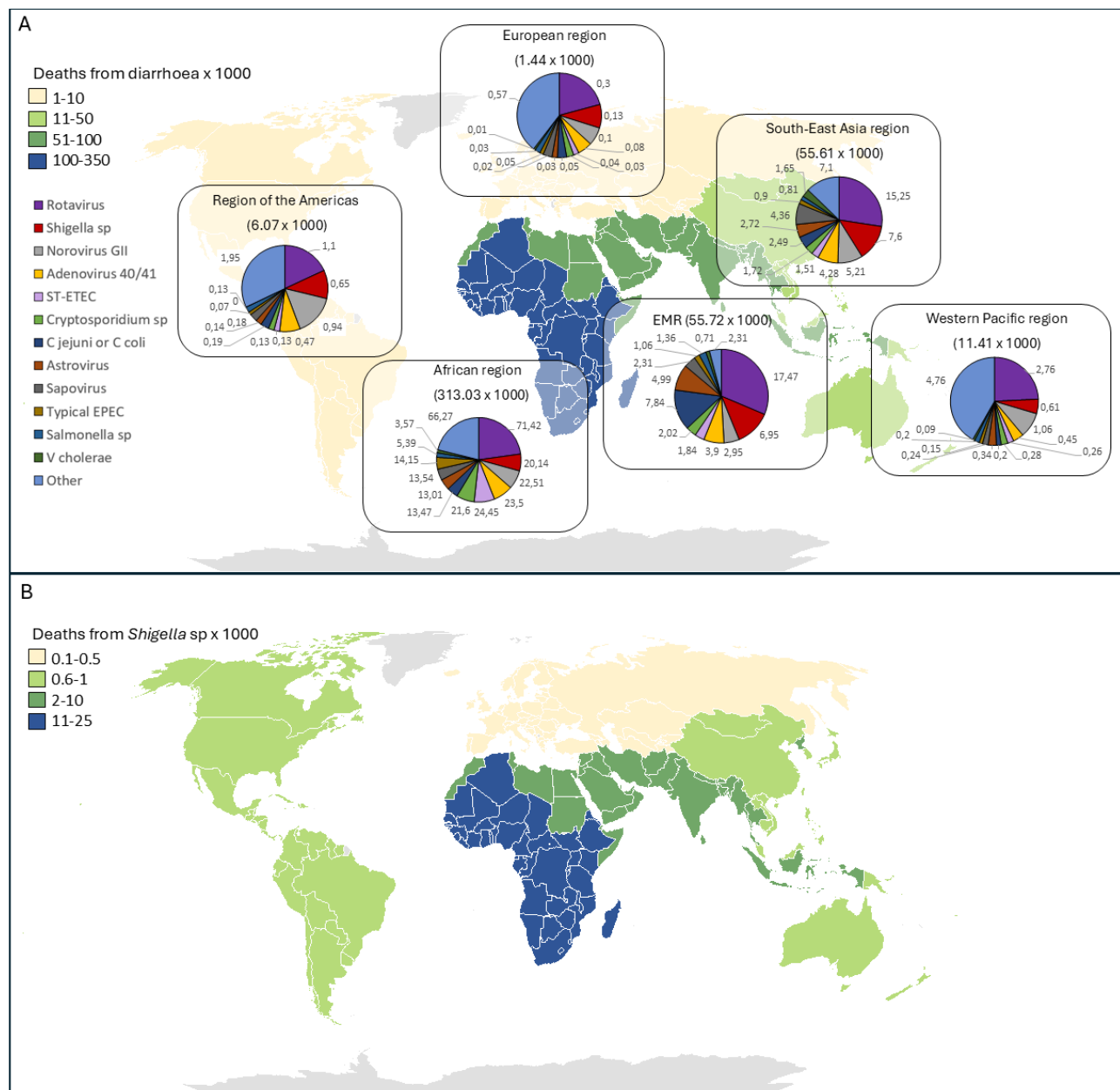


Figure 1. Comparative analysis of global mortality in children under 5 years of age: A) Regional distribution and percentage breakdown of deaths caused by diarrhoeal diseases; B) Mortality rates attributed to *Shigella* sp. infections. Data sourced from Black et al., Lancet (2024) (27), based on year 2021 statistics. ST-ETEC = heat-stable, toxin-producing, enterotoxigenic *Escherichia coli*; EPEC = enteropathogenic *E. coli*.

## 2. Distribution of *Shigella*

The prevalence of *S. flexneri* and *S. sonnei* species can vary across regions due to multiple factors including hygiene practices, sanitation, and healthcare infrastructure. Numerous large studies conducted in countries of the Asian continent undergoing economic transition, illustrate where the spread of *S. flexneri* has been progressively replaced by *S. sonnei* as health conditions have improved (28-31). Concurrently, there has been an increase in antibiotic-resistant strains of *S. sonnei*, possibly contributing to its raised prevalence (32). Generally, literature indicates that *S. sonnei* tends to be more prevalent in industrialized countries or regions with better sanitation and hygiene practices, while *S. flexneri* is often more prevalent in developing countries or regions with poor sanitation and hygiene facilities.

However, many developing countries face challenges such as under-resourced healthcare systems, that may struggle to diagnose, monitor, and control infectious diseases such as shigellosis, resulting in an inaccurate understanding of *Shigella* prevalence (33). The World Health Organization (WHO) has established treatment guidelines for *Shigella* that do not require routine diagnosis for treatment (34). This approach is primarily because *Shigella* infections often present with distinctive symptoms, such as dysentery, allowing for empirical treatment. The goal is to quickly initiate treatment and control the infection, thereby reducing the risk of complications and the spread of the disease.

The underestimation of *Shigella* cases is further exacerbated by nonspecific symptoms that can overlap with other gastrointestinal illnesses. Consequently, many cases go undiagnosed or misdiagnosed, hindering the compilation of accurate epidemiological data. Addressing this knowledge gap requires collaborative efforts to implement effective surveillance systems and increase awareness about *Shigella* in both developed and developing regions.

Indeed, efforts like the Global Enteric Multicenter Study (GEMS) (35), the Vaccine Impact on Diarrhoea in Africa (VIDA)(4), and Enterics for Global Health (EFGH) *Shigella* surveillance study, have played pivotal roles in enhancing our understanding of *Shigella* distribution in endemic countries. While large-scale initiatives like GEMS, VIDA, and EFGH provide valuable insights, smaller studies can offer region-specific details and variations in *Shigella* serotypes and strains. This chapter presents an overview of the studies conducted in the last 20 years, organised by geographical region, to highlight regional variations in *Shigella* prevalence and strains (Table 1).

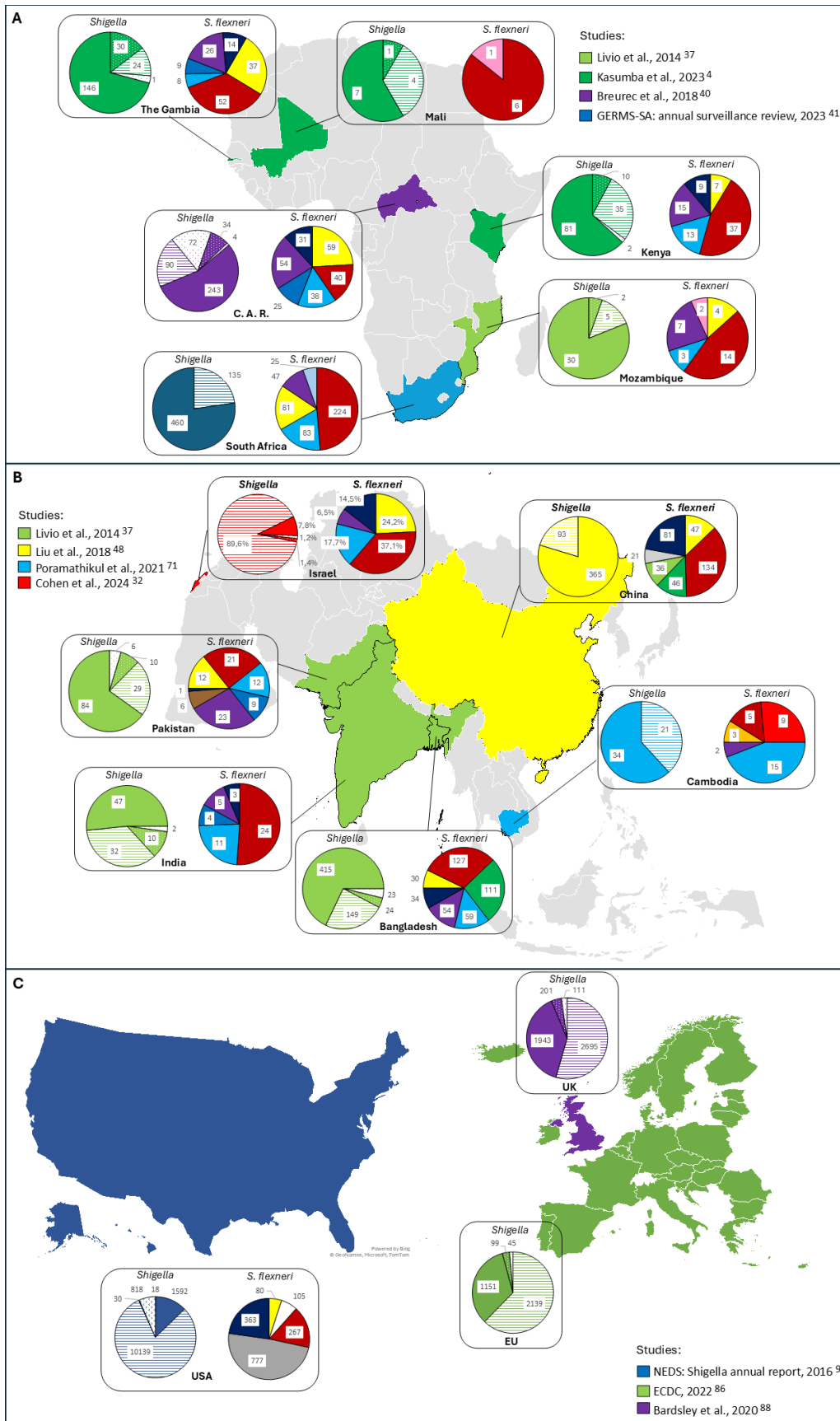


Figure 2. Distribution of *Shigella* sp. and *S. flexneri* serotypes across selected regions in the African continent (A), Asian continent (B), and in the USA, European Union and UK (C) based on analysed study data. Number of respective isolates indicated in each pie chart. Color coding of countries is based on data source.

### 2.1. *Shigella* in shigellosis-endemic countries

In delving into the examination of the prevalence of *Shigella*, it becomes evident that a substantial proportion of research and data is centred around Sub-Saharan Africa and Asia. These regions, being hotspots for *Shigella*, draw heightened attention due to their unique challenges, encompassing factors like inadequate sanitation, dense urban populations, and distinct socioeconomic dynamics. Thus, the concentration of studies on Sub-Saharan Africa serves as a focal point in unravelling the complexities of *Shigella* distribution and impact on a global scale. In addition to the extensive research focused on Sub-Saharan Africa and Asia, it is crucial to acknowledge the presence of *Shigella* in South America, which also experiences endemic levels of this pathogen.

The most impactful contribution to the epidemiology of *Shigella* in endemic countries came from the GEMS case-control study that monitored moderate-to-severe diarrhoea, elucidating its causes, for 3 years (from Dec, 2007 to Mar, 2011) in seven sites both in Africa and in Asia (Bangladesh, Pakistan, India, The Gambia, Mali, Kenya, and Mozambique) (36). The subsequent study from Livio et al. focused the attention on *Shigella* isolates gathered in the GEMS study in all the seven countries (35). This analysis confirmed the prevalence of *S. flexneri* and *S. sonnei* that accounted respectively for 65.9% and 23.7% of the cases of shigellosis in children below 60 months of age. Also, a better understanding of the distribution of the serotypes of *S. flexneri* was possible in this context: of 1130 *Shigella* isolates of children presenting with moderate to severe diarrhoea, 20.2% were diagnosed with *S. flexneri* 2a, 11% with *S. flexneri* 6, the 10.9% with *S. flexneri* 2b, the 9.4% with *S. flexneri* 3a and 7.5% with *S. flexneri* 1b, confirming these serotypes as the most widespread (35).

**Africa:** A recent VIDA investigation evaluated the burden of *Shigella* in children below 59 months of age, focusing on three Sub-Saharan African sites analysed in the GEMS study (The Gambia, Mali, and Kenya) spanning from 2015 to 2018, yielded substantial insights into the prevailing patterns of *Shigella* distribution in Africa (4). Particularly noteworthy were findings regarding the percentage of prevalence of the most prominent serotypes, principally including *S. flexneri* 2a

(40.6%), 1b (18.8%), 6 (17.5%), 3a (9.0%), and 4a (5.1%) (4). Additional data concerning serotype distribution are available from various African countries. For instance, research by Breurec et al. in the Central African Republic revealed that five *S. flexneri* serotypes comprised 54.9% of all isolates: 6 (14.4%), 1b (13.3%), 2a (9.0%), 3a (8.6%), and 4a (5.6%) within that specific region from 2002 to 2013 (37). Similarly, in South Africa, the National Institute for Communicable Diseases' GERMS-SA annual surveillance review for 2023 identified *S. flexneri* 2a as the predominant serotype (224 *S. flexneri* 2a isolates/705 serotyped isolates; 32%). This was followed by *S. sonnei* (135/705, 19%), *S. flexneri* 3a (83/705, 12%), and *S. flexneri* 1b (81/705; 11%) (38). In a smaller-scale study conducted in Malawi in children below 5 years of age by Stenhouse et al. from 2012 to 2015, only three *S. flexneri* serotypes were identified: *S. flexneri* 3a, 4a, and 6 (39).

**Asia:** In a multicenter study conducted between 2000 and 2004, encompassing a collective cohort of over 600,000 patients spanning Bangladesh, China, Pakistan, Indonesia, Vietnam, and Thailand, *S. flexneri* emerged as the predominant *Shigella* species (68%) across all sites except Thailand, where *S. sonnei* prevailed (85%). Among the isolated *S. flexneri* serotypes, the majority comprised *S. flexneri* 2a, 3a, 1a, 1b, 2b, 1c, 6, and X, with considerable variation in their distribution across distinct locations (40). The latest significant studies examining *Shigella* distribution in the Asian continent have primarily centered on Bangladesh, specifically in the urban center of Dhaka and the rural Matlab subunit within the Chandpur District. Findings from these investigations confirm a higher prevalence of *S. sonnei* in urban regions and *S. flexneri* in rural regions (32, 41). Similarly, data collected from the rural community of Mirzapur between 2007 and 2010 also corroborate *S. flexneri* as the most widespread species, particularly noting the presence of *S. flexneri* 1b, 2a, 2b, 3a, and 6 (42). Substantial contributions to the understanding of *Shigella* species prevalence in Asia have emanated from the GEMS study spanning from 2007 to 2011 and insights from Livio et al. (35). According to these findings, *S. flexneri* emerged as the prevailing species in the three Asian countries examined during the GEMS study (67.9% in Bangladesh, 65.1% in Pakistan, and 51.7% in India), with *S. flexneri* 2a being the most abundant serotype across Bangladesh, and India (20.8% in Bangladesh and 26.4% in India) (35). Notably, two separate studies conducted in India (Kolkata) identified *S. flexneri* 2a and 3a as the predominant serotypes, followed by *S. flexneri* 4 and 1b (43, 44). Additionally, a study spanning from 2008 to 2014 in China reaffirmed the dominance of *S. flexneri* 2a out of all the other *S. flexneri* isolates (36.7%), followed by 1b (12.9%), 2b (12.6%), and X (9.9%) (45).

*Table 1. Studies that gathered information about Shigella distribution in the African and Asian continents during the last 20 years. Symptoms are reported as: D = diarrhoea, AD = acute diarrhoea, PD = persistent diarrhoea/dysentery, WD = watery diarrhoea, BD = bloody diarrhoea, MD = mucoid diarrhoea, LS = loose stools, MSD = moderate-to-severe diarrhoea, ND = non-diarrhoeal or asymptomatic. AR = Antimicrobial Resistance; NR = not reported.*

| Study area                          | Ref. | Years of data collection | Category (symptoms)           | Methods   | Total <i>Shigella</i> (n) | <i>S. flexneri</i> (n) | <i>S. flexneri</i> (%) | Sample size | Identified serotypes in order of prevalence      | AR information |
|-------------------------------------|------|--------------------------|-------------------------------|-----------|---------------------------|------------------------|------------------------|-------------|--------------------------------------------------|----------------|
| <b>Africa</b>                       |      |                          |                               |           |                           |                        |                        |             |                                                  |                |
| <b>Multi-region studies</b>         |      |                          |                               |           |                           |                        |                        |             |                                                  |                |
| Kenya, Mali, Mozambique, The Gambia | (35) | 2007-2011                | children <5y (MSD)            | culture   | 299                       | 199                    | 66.6                   | 1130        | 2a, 1b, 6, 3a, 4a, 7a, X, Y, 1a                  | no             |
| South Africa, Tanzania              | (25) | 2009-2012                | children <2y (WD, BD, LS, ND) | molecular | 316                       | 257                    | 81.3                   | 1202        | 6, 2a, X, 3a, 3b, 1b, 1a, 4b, 5a, 4a, 1d, 5b, 7a | no             |
| <b>Burkina Faso</b>                 |      |                          |                               |           |                           |                        |                        |             |                                                  |                |
| Burkina Faso, Ouagadougou           | (46) | 2009-2010                | children <5y (AD, PD, WD, LS) | culture   | 10                        | 6                      | 60.0                   | 309         | NR                                               | yes            |
| <b>Central African Republic</b>     |      |                          |                               |           |                           |                        |                        |             |                                                  |                |
| Central African Republic, Bangui    | (47) | 2007                     | children (BD, WS, MD)         | culture   | 15                        | 15                     | 100.0                  | 156         | NR                                               | yes            |
| Central African Republic, Bangui    |      | 2002-2013                | all ages (D)                  | culture   | 443                       | 243                    | 54.9                   | 443         | 6, 1b, 2a, 3a, 4a, 1c                            | yes            |
| Central African Republic, Bangui    | (48) | 2011-2012                | all ages (NR)                 | culture   | 182                       | 7                      | 3.8                    | 2500        | NR                                               | yes            |
| <b>Ethiopia</b>                     |      |                          |                               |           |                           |                        |                        |             |                                                  |                |
| Ethiopia, Alamura                   | (49) | 2019                     | children <14y (BD, WD, MD)    | culture   | 20                        | NR                     | NR                     | 263         | NR                                               | yes            |
| Ethiopia, Ambo                      | (50) | 2014                     | children <5y (WD, BD, MD, LS) | molecular | 6                         | 3                      | 50.0                   | 239         | NR                                               | yes            |

|                                                                                               |      |           |                                     |               |     |     |       |      |                  |     |
|-----------------------------------------------------------------------------------------------|------|-----------|-------------------------------------|---------------|-----|-----|-------|------|------------------|-----|
| Ethiopia,<br>Bahir-Dar                                                                        | (51) | 2011-2012 | children <5y<br>(AD, WB,<br>BD, MD) | culture       | 40  | 18  | 45.0  | 422  | NR               | yes |
| <b>Ghana</b>                                                                                  |      |           |                                     |               |     |     |       |      |                  |     |
| Ghana,<br>Accra                                                                               | (52) | 2004-2005 | all ages (D)                        | culture       | 24  | 17  | 70.8  | 594  | 2, 1, 6, 3, 7, 4 | yes |
| <b>Kenya</b>                                                                                  |      |           |                                     |               |     |     |       |      |                  |     |
| Kenya,<br>Kericho<br>and<br>Kisumu                                                            | (53) | 2009-2011 | all ages (AD,<br>BD)                | culture       | 41  | 16  | 39.0  | 864  | NR               | yes |
| Kenya,<br>Kisii<br>Provincial,<br>Homa Bay<br>District,<br>and Migori<br>District<br>Hospital | (54) | 2011-2014 | children <5y<br>(WD, PD,<br>LS)     | culture       | 63  | 21  | 33.3  | 1247 | NR               | yes |
| Kenya,<br>Turkana,<br>County                                                                  | (55) | 2019      | children <5y<br>(LS, WS, MS,<br>BS) | culture       | 20  | 7   | 35.0  | 196  | NR               | yes |
| Kenya,<br>Murang'a<br>County                                                                  | (56) | 2017      | children <5y<br>(LS)                | culture       | 14  | NR  | NR    | 163  | NR               | yes |
| Kenya,<br>Nairobi                                                                             | (57) | 2007-2010 | all ages (D,<br>AD, PD)             | culture       | 262 | 166 | 63.4  | 1096 | NR               | yes |
| Kenya,<br>Nairobi                                                                             | (58) | N/A       | children <5y<br>(MD, BD,<br>WD)     | molecula<br>r | 14  | 6   | 42.9  | 354  | NR               | yes |
| Kenya,<br>Nyanza<br>province                                                                  | (59) | 2011-2014 | children <15y<br>(AD)               | culture       | 75  | 27  | 36.0  | 292  | NR               | yes |
| <b>Madagascar</b>                                                                             |      |           |                                     |               |     |     |       |      |                  |     |
| 14 sites in<br>Madagascar                                                                     | (60) | 2008-2009 | children <5y<br>(AD, LS, MS,<br>BS) | culture       | 38  | 28  | 73.7  | 2692 | NR               | no  |
| <b>Malawi</b>                                                                                 |      |           |                                     |               |     |     |       |      |                  |     |
| Malawi,<br>Blantyre                                                                           | (39) | 2012-2015 | children <5y<br>(AD)                | molecula<br>r | 8   | 5   | 50.0  | 1417 | 3a, 6, 4av       | yes |
| Malawi,<br>Lilongwe                                                                           | (61) | 2015-2016 | all ages (AD)                       | molecula<br>r | 34  | 34  | 100.0 | 243  | NR               | yes |
| <b>Nigeria</b>                                                                                |      |           |                                     |               |     |     |       |      |                  |     |
| Nigeria,<br>Ago-Iwoye;<br>Ijebu-Igbo                                                          | (62) | 2008      | children <<br>10y (NR)              | culture       | 21  | 16  | 90.4  | 271  | NR               | yes |
| Nigeria,<br>Ile-Ife                                                                           |      | 2003-2006 | children ≤5y<br>(AD)                | culture       | 102 | 45  | 44.1  | 300  | NR               | yes |

|                                                                           |      |           |                                     |           |      |      |      |        |                                                                  |     |
|---------------------------------------------------------------------------|------|-----------|-------------------------------------|-----------|------|------|------|--------|------------------------------------------------------------------|-----|
| Nigeria,<br>Maiduguri                                                     | (63) | 2013      | all ages (D)                        | culture   | 32   | 15   | 46.9 | 400    | NR                                                               | yes |
| <b><i>Somalia</i></b>                                                     |      |           |                                     |           |      |      |      |        |                                                                  |     |
| Somalia,<br>Mogadishu                                                     | (64) | 2019      | children <5y<br>(D)                 | culture   | 37   | 26   | 70.3 | 180    | NR                                                               | yes |
| <b><i>South Africa</i></b>                                                |      |           |                                     |           |      |      |      |        |                                                                  |     |
| 9 regions in<br>South Africa                                              | (38) | 2023      | all ages (NR)                       | culture   | 595  | 460  | 77.3 | 952    | 2a, 3a, 1b, 6, 4                                                 | no  |
| <b>Asia</b>                                                               |      |           |                                     |           |      |      |      |        |                                                                  |     |
| <b><i>Multi-region studies</i></b>                                        |      |           |                                     |           |      |      |      |        |                                                                  |     |
| Bangladesh<br>, India,<br>Nepal,<br>Pakistan                              | (25) | 2009-2012 | children <2y<br>(WD, BD,<br>LS, ND) | molecular | 621  | 387  | 62.3 | 1202   | 6, 2a, 3a, 1a, 4a,<br>X, 3b, 1b, 7a,<br>2b, 5a, 4b, 1d,<br>5b 5b | no  |
| Bangladesh<br>, India,<br>Pakistan                                        | (35) | NR        | children <5y<br>(MSD)               | culture   | 831  | 546  | 65.7 | 1130   | 2a, 6, 2b, 3a,<br>1b, 4a, 7a, X, Y,<br>5b, 1a, 3b                | no  |
| Bangladesh<br>, China,<br>Pakistan,<br>Indonesia,<br>Vietnam,<br>Thailand | (40) | 2000-2004 | all ages (WD,<br>MS, LS)            | culture   | 2927 | 1976 | 67.5 | 56958  | 2a, 3a, 1a, 1b,<br>2b, 1c, 6, X                                  | yes |
| <b><i>Bangladesh</i></b>                                                  |      |           |                                     |           |      |      |      |        |                                                                  |     |
| Bangladesh<br>, Dhaka                                                     |      | 2011      | all ages (NR)                       | culture   | 650  | 306  | 47.1 | 10827  | NR                                                               | yes |
| Bangladesh<br>, Dhaka                                                     |      | 2001-2020 | children <5y<br>(NR)                | culture   | 883  | 463  | 52.4 | 26,804 | NR                                                               | yes |
| Bangladesh<br>, Kushtia                                                   |      | 2016-2018 | all ages (PD)                       | molecular | 12   | 8    | 66.7 | 150    | 2a                                                               | yes |
| Bangladesh<br>, Matlab                                                    |      | 2001-2020 | children <5y<br>(NR)                | culture   | 1263 | 945  | 74.8 | 15,684 | NR                                                               | Yes |
| Bangladesh<br>, Mirzapur                                                  | (42) | 2007-2010 | children <5y<br>(MSD)               | culture   | 101  | 67   | 66.3 | 192    | 2a/6, 2b, 3a, 1b                                                 | no  |
| <b><i>Cambodia</i></b>                                                    |      |           |                                     |           |      |      |      |        |                                                                  |     |
| Cambodia,<br>Battambang                                                   | (65) | 2014-2015 | all ages (AD)                       | culture   | 11   | 9    | 81.8 | 212    | 3a, 2a, 2v                                                       | yes |
| Cambodia,<br>Battambang                                                   | (66) | 2014-2019 | all ages (AD,<br>ND)                | culture   | 55   | 34   | 61.8 | 1985   | 3a, 2v, 2a, 1a, 6                                                | yes |
| <b><i>China</i></b>                                                       |      |           |                                     |           |      |      |      |        |                                                                  |     |
| China,<br>Beijing                                                         | (29) | 2009-2010 | all ages (D)                        | culture   | 55   | 5    | 9.1  | 62     | 1b, 2a                                                           | no  |

|                                           |      |           |                                  |         |     |     |      |      |                                                              |     |
|-------------------------------------------|------|-----------|----------------------------------|---------|-----|-----|------|------|--------------------------------------------------------------|-----|
| China,<br>Xinjiang                        |      | 2008-2014 | all ages (D)                     | culture | 458 | 365 | 79.7 | 458  | 2a, 1b, 2b, Xv,<br>6, X, 1a, Y, 4s,<br>4a, 3a, 2c, 3b,<br>4b | yes |
| <b>India</b>                              |      |           |                                  |         |     |     |      |      |                                                              |     |
| India,<br>Andaman<br>& Nicobar<br>Islands | (67) | 2006-2011 | children <15y<br>(MD, BD)        | culture | 50  | 34  | 68.0 | 412  | NR                                                           | yes |
| India,<br>Bengal<br>islands               | (67) | 2006-2011 | children <15y<br>(MD, BD)        | culture | 88  | 55  | 62.5 | 943  | NR                                                           | yes |
| India,<br>Kolkata                         | (44) | 2007-2010 | all ages (AD)                    | culture | 212 | 160 | 75.5 | 3262 | 2a, 3a                                                       | yes |
| India,<br>Kolkata                         | (43) | 2011-2019 | All ages<br>(MD, WD,<br>BD)      | culture | 535 | 407 | 76.1 | 9276 | 2a, 3a, 4a, 1b                                               | yes |
| India,<br>North<br>Karnataka              | (68) | 2006-2017 | all ages (AD)                    | culture | 189 | 90  | 47.6 | 4578 | NR                                                           | yes |
| <b>Iran</b>                               |      |           |                                  |         |     |     |      |      |                                                              |     |
| Iran                                      | (69) | 2013-2014 | children <14y<br>(AD)            | culture | 85  | 36  | 42.4 | 676  | NR                                                           | yes |
| Iran,<br>Abadan                           | (70) | 2011-2013 | children ≤15y<br>(PD)            | culture | 36  | 19  | 52.8 | 705  | NR                                                           | yes |
| Iran, Ahvaz                               | (71) | 2008-2010 | children <12y<br>(D)             | culture | 175 | 87  | 49.7 | 4380 | 2, 1, 6, 4, 3                                                | yes |
| Iran,<br>Ardabil                          | (72) | 2019-2020 | children (D)                     | culture | 113 | 5   | 10.2 | 1280 | NR                                                           | no  |
| Iran,<br>Kerman                           | (73) | 2013-2014 | all ages (AD)                    | culture | 56  | 31  | 55.4 | 624  | NR                                                           | no  |
| Iran,<br>Mashhad                          | (74) | 2018-2019 | children <14y<br>(BD, WD,<br>MD) | culture | 94  | 23  | 24.5 | 233  | NR                                                           | yes |
| Iran, Tehran                              |      | 2016      | all ages (AD)                    | culture | 26  | 11  | 42.3 | 300  | NR                                                           | yes |
| <b>Nepal</b>                              |      |           |                                  |         |     |     |      |      |                                                              |     |
| Nepal,<br>Sukraraj                        | (75) | 2014      | all ages (NR)                    | culture | 29  | 17  | 58.6 | 650  | NR                                                           | yes |
| <b>Pakistan</b>                           |      |           |                                  |         |     |     |      |      |                                                              |     |
| Pakistan,<br>Khyber<br>Pakhtunkw<br>a     | (76) | 2016-2017 | children <18y<br>(NR)            | culture | 160 | 155 | 96.9 | 2014 | NR                                                           | no  |
| <b>Turkey</b>                             |      |           |                                  |         |     |     |      |      |                                                              |     |
| Turkey,<br>Ankara                         |      | 2014      | children <18y<br>(AD)            | culture | 77  | 8   | 10.4 | 113  | NR                                                           | yes |

**South America:** Laboratory data encompassing 19 countries of Latin America were gathered through the Latin American Network for Antimicrobial Resistance Surveillance spanning from 2000 to 2015. The predominant isolated species included *S. flexneri* (49%) and *S. sonnei* (28%), notably highlighting an increase in resistance against ciprofloxacin (77). A recent retrospective substudy of the Malnutrition and Enteric Disease (MAL-ED) observational birth cohort study, which focused on the epidemiology of *Shigella* species and serotypes in children, examined the distribution of *Shigella* in Fortaleza, Brazil, and Loreto, Peru, from 2009 to 2012. The findings indicated a notable difference in the prevalence of *Shigella* species between the two sites. In Peru, *S. flexneri* was the most prevalent among the other *Shigella* species, accounting for 80.1% of cases, whereas in Brazil, it represented only 31.8% of cases (25). The most common *S. flexneri* serotype in Brazil was *S. flexneri* 2a, comprising 22.7% of cases, while in Peru, *S. flexneri* 6 was the most prevalent serotype at 30.1%, followed by *S. flexneri* 2a at 25.6%.

Regarding studies focusing on endemic regions, particularly those that contain substantial sample sizes, an undeniable trend emerges: the pervasive presence of *S. flexneri* as the primary *Shigella* species in most cases. This consistent dominance underscores the significance of *S. flexneri* in the *Shigella* infections distribution across diverse geographical settings. Among the serotypes observed, *S. flexneri* 2a has always been found and indicated as a recurrent and widely distributed strain, highlighting its remarkable prevalence and adaptability. Moreover, the recurring identification of serotypes such as *S. flexneri* 1b, 3a, and 6 in numerous studies further emphasizes their significant role in *Shigella* epidemiology. Their presence serves as a critical indicator of their widespread dissemination and reinforces the importance of including antigens from these serotypes in vaccine formulations, as endorsed by WHO guidelines (78).

Many areas facing *Shigella* lack robust tracking systems and data, leading to an underestimation of its true impact. Changes in public health strategies and evolving antibiotic resistance patterns contribute to fluctuations in the distribution of *Shigella* species at different places and different times, add complexity, and highlight the need for adaptable surveillance strategies. To support this claim of epidemiologic transition, it is beneficial to delve deeper into the differences between the GEMS study and VIDA study. The latter was specifically designed to provide a more comprehensive understanding of the epidemiology of *Shigella* species over time and following

introduction of rotavirus vaccine utilizing the same protocol as the one utilized for the GEMS study.

## **2.2. *Shigella* in shigellosis non-endemic countries**

While the development of vaccines against *Shigella* has rightly been focused on LMICs in Sub-Saharan Africa and Asia, due to the significant disease burden, it is important to broaden our perspective. Reports on *Shigella* in non-endemic settings are relatively scarce, yet there is an emerging trend indicating a significant disease burden in these regions as well. This is particularly noteworthy as *Shigella* is routinely notifiable in these non-endemic countries, presenting an opportunity for more comprehensive surveillance and understanding.

Through the contribution of national surveillance systems and studies carried out in non-endemic regions to analyse epidemics or isolate cases of shigellosis, there is evidence in Europe, the United States, and other industrialized regions. *Shigella*, though less prevalent, presents its own set of implications, especially considering outbreaks in certain settings (i.e MSM) and outbreaks caused by antimicrobial-resistant pathogens.

**Europe:** All European Union/European Economic Area countries annually submit epidemiological surveillance data to the European Antimicrobial Resistance Surveillance Network. The most recent available report on shigellosis cases recorded during surveillance pertains to the year 2022, encompassing 30 countries and documenting 18,426 instances of shigellosis from 2018 to 2022 (79). France, Spain, and the Netherlands emerged as the nations with the highest caseloads in 2022, accounting for 31%, 14%, and 10% of all cases, respectively, with a considerable proportion of cases linked to travel. Furthermore, *S. flexneri* 2a, 1b, and 3a were identified as the most prevalent serotypes, while *S. sonnei* was the predominant species (79).

**United Kingdom:** Since 2015, Public Health England (PHE) has integrated whole-genome sequencing into their surveillance program in England and Wales to monitor *Shigella* cases. Data collected from 2015 to 2018 by the Gastrointestinal Bacterial Reference Unit (GBRU) of PHE revealed a prevalence of *S. sonnei* (54.4%) totalling 2695 cases, and *S. flexneri* (39.2%) totalling 1943 cases, with *S. flexneri* 2a (1113/1943, 57.2%), 1b (254/1943, 13.1%), and 3a (206/1943, 10.6%) emerging as the dominant *S. flexneri* serotypes (80). England witnessed several outbreaks in recent years attributed to *S. sonnei* and *S. flexneri* 2a affecting principally MSM (7, 81, 82).

XDR *S. sonnei* and XDR *S. flexneri* 2a exhibited similarities in plasmid-mediated antimicrobial resistance and epidemiology, hinting at a phenomenon of horizontal antimicrobial resistance transfer (7). Previous outbreaks in England and Wales affecting MSM were also linked to *S. flexneri* 3a. Interestingly, in the work of Charles et al. (83), an in-depth analysis was conducted using samples retrieved from laboratory-confirmed *Shigella* cases in England during the period from 2016 to 2023. This analysis revealed shifts in serotype distribution. *S. flexneri* 2a serotype dominated prior to the implementation of lockdown restrictions for the COVID-19 pandemic. *S. flexneri* 1b, 2a, and 3a serotypes drove increases by late 2019 and persisted during the pandemic, primarily among adult males. Post-lockdown, *S. flexneri* 1b and 2a were the most prevalent, with a sharp rise of *S. flexneri* 2a observed in early 2023, further confirming that *S. flexneri* 2a did not decrease but instead increased in prevalence in the post-lockdown period.

**United States of America:** The National Enteric Disease Surveillance report, compiled by the National Center for Emerging and Zoonotic Infectious Diseases, issues annual assessments of shigellosis monitoring in the USA. The latest report, pertaining to the year 2016, documented 12597 cases of shigellosis across 52 states and regional public health laboratories. *S. sonnei* emerged as the predominant species (80.5%), followed by *S. flexneri* (12.6%), with the highest incidence observed among children aged 1 to 4 years. While the distribution of *S. flexneri* serotypes is only available for a subset of states, evidence suggests the presence of various serotypes, including *S. flexneri* 2a/2 unspecified, 1b/1 unspecified, 3a/3 unspecified, 4a/4 unspecified, and *S. flexneri* 6, in order of prevalence. Furthermore, a study conducted across 10 states focusing on individuals above 9 years of age also reported a higher prevalence of *S. sonnei* relative to *S. flexneri* cases (84). Notably, cases of XDR or MDR *Shigella* strains causing outbreaks have been reported in the USA. A recent investigation screening stool samples collected from 2017 to 2020 in San Diego, California, revealed a slightly higher frequency of *S. flexneri* (55%) compared to *S. sonnei* (45%) identifications and associated with resistance to a wide range of antibiotics (85). Another study analysed samples collected in Minnesota from 2012 to 2015, comprising culture-confirmed cases of shigellosis by the Minnesota Department of Health Public Health Laboratory. Among the 882 infections identified, 85% were attributed to *S. sonnei* and 14% to *S. flexneri*, with half of the infections occurring in children (86). Moreover, an outbreak of shigellosis in New York, analysed alongside sporadic samples collected over 26 months from 2013 to 2015, revealed that

nearly all infections were caused by either *S. sonnei* (65%) or *S. flexneri* (34%), both exhibiting high rates of antimicrobial resistance to ampicillin and trimethoprim/sulfamethoxazole (87).

**Other:** A multinational study included the United Kingdom (UK), Australia, Belgium, France, and the USA, involving 943 *Shigella* isolates. This investigation unveiled an internationally linked outbreak of XDR *S. sonnei* strains, indicating a recent common ancestor. Shigellosis has been emerging as a growing cause of gastroenteritis in Australia, with notable outbreaks in remote First Nations communities and among MSM in urban centers. From 2001 to 2019, 18,363 shigellosis cases were documented in the National Notifiable Diseases Surveillance System. Among the reported cases included in the analysis (18327), 42% were attributed to *S. sonnei* and 29% to *S. flexneri* (88). In Australian metropolitan areas, shigellosis predominantly affects returning travellers from endemic regions and MSM. A study conducted in Victoria between 2016 and 2018 identified two significant MSM-associated lineages: one of *S. sonnei* and another of *S. flexneri* 2a (89). Analysis of isolates from 2013 to 2023 revealed that *S. sonnei* was the most frequently cultured species (61.9%), followed by *S. flexneri* (33.5%). The incidence of shigellosis among men rose until 2020, decreased during the initial period of the COVID-19 pandemic, and surged again in 2022-2023. There was a significant rise in MDR *Shigella* species, particularly *S. sonnei*, with cases increasing from 2022 to 2023. While *S. flexneri* maintained susceptibility to at least one oral antibiotic, *S. sonnei* showed escalating resistance to third-generation cephalosporins, azithromycin, and ciprofloxacin, indicating a troubling trend towards MDR and XDR strains (90). The emergence of XDR and MDR *Shigella* strains, particularly in non-endemic settings such as the UK, Europe, the USA, and others, is a stark indicator of a global trend towards increasing antimicrobial resistance. These findings lend credence to the hypothesis of a global phenomenon of antimicrobial resistance facilitated by the horizontal transfer of genes, a mechanism highlighted by Baker et al. (91). Both endemic and non-endemic regions are experiencing these trends, albeit with variations in the specific strains and resistance patterns.

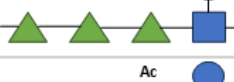
In non-endemic and industrialized countries, *S. sonnei* often emerges as the predominant species, aligning with previous observations. *S. flexneri* appeared consistently in 100% of the examined studies, often with a frequency comparable to that of *S. sonnei*, particularly evident in extensive surveillance programs. Similarly, to endemic countries, when reported, the prevailing serotype of *S. flexneri* was always identified as *S. flexneri* 2a. Other prevalent serotypes included *S. flexneri*

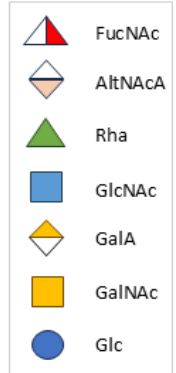
1b and 3a, with *S. flexneri* 6 less frequently encountered in industrialized nations compared to endemic regions.

### **3. O-antigen mediated cross-reactivity**

Cross-reactivity in *S. flexneri* refers to the phenomenon where antibodies generated against one serotype can recognize and interact with antigens from other serotypes (26). The primary source of cross-reactivity among *S. flexneri* serotypes lies in shared epitopes within the OAg (Caradonna et al., 2025, submitted). Although each *S. flexneri* serotype possesses a distinct OAg structure, certain epitopes are conserved or shared among multiple serotypes (92). All *S. flexneri* serotypes, except *S. flexneri* 6 – which genomically clusters closer to *S. boydii* and more distantly to the other *S. flexneri* species (93)- share a common sugar repeat backbone comprising one N-acetylglucosamine and three rhamnose repeats (94-99), as illustrated in Table 2.

Table 2. OAg structure of *S. sonnei* and *S. flexneri* serotypes. *S. flexneri* is classified into the various serotypes based on specific chemical modifications such as glucosylation and O-acetylation on the common backbone. These modifications result in the type (I–VI) and group (3(4), 6, 7(8), 9, 10) specificities that are used to define the various serotypes. FucNAc = N-Acetyl-L-Fucosamine; AltNAcA= N-acetyl-alturonic acid; Rha = Rhamnose; GlcNAc = N-Acetylglucosamine ; GalA = Galacturonic acid ; GalNAc = N-Acetylgalactosamine; Glc = glucose. % = degree of O-acetylation.

| Serotype              | O-antigen structure                                                                                   | References                                    | Type/Group             |
|-----------------------|-------------------------------------------------------------------------------------------------------|-----------------------------------------------|------------------------|
| <i>S. sonnei</i>      |                      | Gamian & Romanowska (1982) <sup>108</sup>     |                        |
| <i>S. flexneri</i> 1a | 65%/25% Ac           | Perepelov et al. (2009) <sup>109</sup>        | Type I/Group 3(4)      |
| <i>S. flexneri</i> 1b | 70%/15% Ac  80% Ac   | Perepelov et al. (2009) <sup>109</sup>        | Type I/Group 6         |
| <i>S. flexneri</i> 2a | 60%/25% Ac  Ac 60%   | Perepelov et al. (2009) <sup>109</sup>        | Type II/ group 3(4)    |
| <i>S. flexneri</i> 2b |                      | Kenne et al. (1978) <sup>110</sup>            | Type II/Group 7(8)     |
| <i>S. flexneri</i> 3a |  Ac Ac 40%         | Perepelov et al. (2012) <sup>115</sup>        | Type III/ 6, 7(8)      |
| <i>S. flexneri</i> 3b |  Ac                | Kenne et al. (1978) <sup>110</sup>            | Type III/Group 3(4), 6 |
| <i>S. flexneri</i> 4a |                    | Kenne et al. (1978) <sup>110</sup>            | Type IV/Group 3(4)     |
| <i>S. flexneri</i> 4b |  Ac                | Perepelov et al. (2009) <sup>109</sup>        | Type IV/Group 6        |
| <i>S. flexneri</i> 5a | 35%/25% Ac         | Perepelov et al. (2012) <sup>114</sup>        | Type V/Group 3(4)      |
| <i>S. flexneri</i> 5b |                    | Kenne et al. (1978) <sup>110</sup>            | Type V/Group 7(8)      |
| <i>S. flexneri</i> 6  | 60%/30% Ac         | Katzenellenbogen et al, (1976) <sup>111</sup> | Type VI                |
| <i>S. flexneri</i> X  |                    | Sun et al. (2012) <sup>114</sup>              | Group 7, (8)           |
| <i>S. flexneri</i> Y  | 30%/20% Ac  Ac 40% | Perepelov et al. (2012) <sup>115</sup>        | Group 3, (4)           |



The genetic basis for these antigenic variations lies in the genes responsible for the biosynthesis and modification of the OAg. These genes are typically located on the bacterial chromosome or on plasmids (100). Horizontal gene transfer, mutations, and genetic recombination contribute to the diversity of *S. flexneri* serotypes by introducing new genetic variations that alter the antigenic properties of the Oag (92, 101, 102). While each serotype may exhibit unique variations in OAg modifications such as glycosylation and O-acetylation (103), certain regions of the genome encoding enzymes involved in OAg synthesis may share sequence homology, resulting in structurally similar Oag (104).

For a broadly protective vaccine, leveraging the similarities between the OAg structures of different serotypes can be a strategic approach. By identifying common antigenic determinants shared among multiple serotypes, a vaccine can be designed to elicit an immune response that provides cross-protection against a wide range of serotypes (78).

However, it is important to note that the cross-reactivity observed in serological assays may not solely be attributed to type or group similarities between OAg structures (105). Instead, conformational similarity of epitopes, the exposure of specific substituents within the three-dimensional structure, and the flexibility of OAg structures likely play a more critical role in mediating cross-reactivity. These factors may influence how the immune system recognizes and responds to OAg, ultimately affecting the ability of a vaccine to provide broad protection (106-108). The phenomenon of cross-reactivity between serotypes of *S. flexneri* has been observed in both pre-clinical and clinical contexts, supporting the claim of cross-protection (105, 109-112)(Caradonna et al., 2024, submitted).

In one of the earliest studies, Simmons (1958) investigated how OAg-mediated cross-reactivity works among different *S. flexneri* serotypes. This was done by observing the agglutination reactions between whole bacteria from *S. flexneri* 1a, 2a, 3, 4a, 5, 6, X, and Y serotypes and various antisera from the same serotypes. The results showed that the 2a strain could cross-react with antisera from *S. flexneri* 1a and Y and the *S. flexneri* 3 strain could cross-react with antisera from *S. flexneri* 1a, X, and Y.

After these very early observations of a possible cross-reactivity between *S. flexneri* strains, a study from Noriega et al. postulated that the *Shigella* antisera cross-reactivity could be mainly mediated by group specificities (3(4), 7(8), 9, and 10). In the study, attenuated *S. flexneri* 2a and *S. flexneri*

3a strains were used in a single formulation to immunize guinea pigs. The researchers measured geometric mean titers (GMT) of LPS-specific IgG and IgA against various *S. flexneri* serotypes, including 1a, 1b, 2a, 2b, 3a, 3b, 3c, 4a, 4b, 4c, 5a, 5b, 6, X, and Y. Results from ELISA testing showed high IgG titers against the vaccine serotypes, with significant cross-reactivity observed particularly against *S. flexneri* 1b and 1a. For IgA, a broader cross-reactivity was observed, with responses also against *S. flexneri* 2b, 4b, 5b and Y. In a subsequent experiment, the immunized animals were challenged with wild-type *S. flexneri* bacteria of various serotypes (*S. flexneri* 1a, 1b, 2a, 2b, 3a, 3b, 3c, 4a, 4b, 4c, 5a, 5b, 6, X, and Y), demonstrating cross-protection against all serotypes tested, except for *S. flexneri* 6 (26).

One of the pioneering studies demonstrating this concept was conducted by Van De Verg et al., which revealed different patterns of cross-reactivity between serotypes in humans vaccinated with a hybrid *E.coli* K12-*S. flexneri* 2a live-attenuated vaccine and humans and non-human primates challenged with *S. flexneri* 2a. To measure IgG and IgA titers, ELISA was conducted using a broad panel of LPS from *S. flexneri* 1a, 1b, 2a, 2b, 3a, 3b, 4a, 4b, 5a, X, and Y strains. Both the vaccinated or challenged human patients, gave a high percentage of IgG cross-reactivity against *S. flexneri* 5a, X, and Y serotypes. In terms of IgA titers, vaccinated subjects exhibited modest responses across all tested strains, with higher titers against serotypes X, Y, 5a, 2b and 1a. For challenged human subjects, these individuals demonstrated elevated IgA titers for serotypes Y, 5a, 1b and 1a, with modest responses also against 4a, 3b, 3a and 2b that showed low or no IgG titers (109). In challenged non-human primates, high IgG titers were observed against all tested strains, and particularly strong IgA responses against Y, 5a, 4b, 4a, 3a and 1b (109).

One of the studies on *Shigella* cross-reactivity showed evidence of cross-reactivity between *S. flexneri* strains when evaluating a *S. flexneri* 2a OAg conjugate vaccine. The study showed unexpected cross-reactive IgG antibodies against *S. flexneri* 6 LPS in adult, 4-7 years old and 1-4 years old patients but failed to replicate this cross-reactivity in mice, responding almost exclusively to the type 2a conjugate. The study suggests that the O-acetylated disaccharide shared by *S. flexneri* 2a and 6 OAg is likely responsible for the cross-reactivity observed in humans (110). However, a precedent work identified that the O-acetylation of the rhamnose III sugar at positions 3 or 4 is common to all known *S. flexneri* 6 and some of the *S. flexneri* 2a isolates (100).

These observations are underscoring the complexity of cross-reactive immune responses and the differences that can be observed in these responses between different animal species make difficult to predict results in humans.

Cross-reactivity between *S. flexneri* serotypes was also studied for the rational design of a broad-coverage *Shigella* vaccine based on Generalized Modules for Membrane Antigens (GMMA) technology. GMMA from 14 *S. flexneri* subtypes were used to immunize mice and rabbits and to generate antisera against *S. sonnei* and *S. flexneri*. It was found that some immunogens induced broadly cross-reactive antibodies, while others had narrower specificity. For example, *S. flexneri* 1b GMMA antisera were able to bind to and very efficiently kill *in vitro* *S. flexneri* 1b, 2a, 3a, 3b, 4a and 6. Also, *S. flexneri* 3a GMMA-raised antibodies that showed the ability to efficiently kill *in vitro* *S. flexneri* 1b, 2b, 3b, 4a, and 5b strains. On the other hand, GMMA from *S. flexneri* 2a, 4b, X and Y, and to a lesser extent *S. flexneri* 6, generated antisera that reacted with relatively few bacterial isolates. Results from this study confirmed that the observed cross-reactivity cannot always be attributed to group specificities. Some examples of this are antisera raised against *S. flexneri* 1b and 1c GMMA that showed a particularly high binding to *S. flexneri* 2b, that have no group specificities in common (105).

The cross-reactivity across various serotypes and species using multivalent Outer Membrane Vesicles (OMV)-based vaccines has been corroborated by additional studies. For example, the presence of IgG subclasses and secretory IgA specific to both LPS and outer membrane proteins (OMP), and bactericidal activity were detected against the four strains included in a vaccine composed of antigens from *S. sonnei* and *S. flexneri* 2a, 3a, and 6. A OMP- and LPS-specific immune response was observed as well against *S. dysenteriae* type 1 and *S. boydii* type 4. Furthermore, the protection of the vaccine was assessed by challenging vaccinated mice with the homologous strains present in the vaccine and *S. dysenteriae* type 1 and *S. boydii* type 2, yielding promising results (113).

#### **4. Vaccine strategies for *Shigella*: O-antigen as an antigenic target for broad protection**

Shigellosis requires a vaccine that can elicit a broadly reactive immune response, particularly targeting the most prevalent species such as *S. flexneri* and *S. sonnei* (35). According to WHO

guidelines on preferred product characteristics for *Shigella* vaccines, a vaccine incorporating OAg structures from *S. sonnei* and various *S. flexneri* serotypes, specifically *S. flexneri* 2a, 3a, 6 and 1b, could directly protect against 72% of circulating, widespread, and clinically significant *Shigella* strains. Additionally, through cross-protection mechanisms, such a vaccine could provide coverage for approximately 89% of circulating strains (78). This estimate is based on the finding that five serotypes/subserotypes (*S. flexneri* 2a, 6, 3a, 2b, and 1b) account for 89% of *S. flexneri* isolates in the study by Livio et al. (35). Additionally, the inclusion of these serotypes can provide indirect protection against other less common serotypes due to the common structure of the OAg (35).

Efforts to develop an effective *Shigella* vaccine have primarily concentrated on stimulating antibody responses, particularly IgG against the *Shigella* OAg since these antibodies have been proposed as a correlate of protection for *Shigella* and play a vital role in defence mechanisms by facilitating opsonization, phagocytosis, and intracellular cytotoxicity (19). Table 3 summarises OAg based vaccines currently in clinic.

Table 3. *Shigella* OAg based vaccines currently in clinical trials.

| Candidate (developer)                                           | Serotypes included in the vaccine                                                      | Platform                              | Phase of development | Population | Locations              |
|-----------------------------------------------------------------|----------------------------------------------------------------------------------------|---------------------------------------|----------------------|------------|------------------------|
| ZF0901 (Beijing Zhifei Lvzhu Biopharmaceutical)                 | <i>S. sonnei</i> , <i>S. flexneri</i> 2a                                               | Chemical conjugate                    | Phase III            | Infants    | China and Bangladesh   |
| <i>Shigella</i> 4V2 (LimmaTech AG/Valneva)                      | <i>S. sonnei</i> , <i>S. flexneri</i> 2a, <i>S. flexneri</i> 3a, <i>S. flexneri</i> 6  | Bioconjugate                          | Phase II             | Infants    | Kenya                  |
| SF2a-TT15 (Institut Pasteur)                                    | <i>S. flexneri</i> 2a                                                                  | Synthetic glycoconjugate              | Phase II             | Infants    | Kenya                  |
| altSonflex1-2-3 (GSK Vaccines Institute for Global Health/BBLI) | <i>S. sonnei</i> , <i>S. flexneri</i> 1b, <i>S. flexneri</i> 2a, <i>S. flexneri</i> 3a | GMMA                                  | Phase II             | Infants    | Kenya                  |
| InvaplexAR-Detox (WRAIR)                                        | <i>S. flexneri</i> 2a                                                                  | Lipopolysaccharide complex to protein | Phase I              | Adults     | Netherlands and Zambia |

The most advanced vaccine is a bivalent OAg glycoconjugate developed by Beijing Zhifei Lvzhu Biopharmaceutical, targeting *S. flexneri* 2a and *S. sonnei*. It has progressed through phase II trials and is currently in phase III clinical trials in China (114).

LimmaTech Biologics AG has developed a vaccine composed of bioconjugates targeting *S. sonnei* and *S. flexneri* 2a, 3a, and 6 (115). The cross-reactivity of this quadrivalent (4V) *Shigella*

bioconjugate vaccine and the corresponding monovalent formulations, against serotypes that are not targeted by the vaccine, was assessed in rabbits. Sera from rabbits immunized with the 4V vaccine non adjuvanted showed bactericidal activity against *S. flexneri* 2a, *S. sonnei*, and *S. flexneri* 3a isolates, but not against *S. flexneri* 6 isolates. In contrast, serum from rabbits immunized with the adjuvanted 4V vaccine exhibited bactericidal activity against all targeted serotypes and additional non-targeted ones (*S. flexneri* 2b, 4a, 4b). Particularly high bactericidal titers were detected against *S. flexneri* 2b, with moderate activity against *S. flexneri* 4a and 4b (112). A phase 1/2 dose-finding study in Kenya showed that the vaccine is safe and immunogenic in 9-month-old infants (results presented at BactiVac 2023 and ASM Microbe 2024). A Ph2b CHIM study is ongoing and a study to expand safety and immunogenicity data in 9-month-old Infants is planned.

The Institut Pasteur has utilized the synthetic approach to generate a conjugate made of *S. flexneri* 2a OAg chains terminally linked to tetanus toxoid (116). Based on very promising results in a phase 2 in Kenya in 9-month-old infants (results presented at ASM Microbe 2024 and VED 2025), a 4-component formulation based on the same technology, including *S. sonnei* and *S. flexneri* 2a, 3a, and 6, is in preclinical development (117).

OMVs present another avenue, combining both OAg and proteins capable of inducing cross-reactive immune responses. In particular, altSonflex1-2-3, based on GMMA technology (118) is progressing in clinic. Results obtained with a mono-component version (1790GAHB), targeting *S. sonnei* only (119-121) paved the way to altSonflex1-2-3, consisting of GMMA from *S. flexneri* 1b, 2a, 3a, and *S. sonnei*, currently undergoing phase 2 clinical trials (111). Studies conducted with altSonflex1-2-3 showed OAg mediated cross-reactivity by SBA against *S. flexneri* 4a, 5b, 6, X, and Y heterologous serotypes in different animal models (111). As GMMA mimic the structure and composition of a bacterial outer membrane, they include soluble periplasmic proteins, lipoproteins and outer membrane proteins (Omps). However, their contribution to triggering bactericidal antibodies against *S. sonnei* and *S. flexneri* is very limited compared to OAg, which serves as the primary factor driving this functional activity (105, 122, 123). The ability of the vaccine to induce functional cross-reactive antibodies against the same panel of heterologous *Shigella* strains has been confirmed with adults sera, even if with different patterns and specificities compared to animal data.

Alternative strategies that leverage the immunogenicity of *Shigella* OAg include whole-cell killed (124, 125) and live-attenuated vaccines featuring genetically weakened *Shigella* strains (126-128). One method focuses on expressing *Shigella* OAg on live-attenuated vectors from the *Shigella* genus or similar pathogens (129, 130).

Other than the OAg, conserved components of the *Shigella* cell envelope, such as Ipa proteins and Omps, have been explored as potential vaccine targets. Ipa proteins, critical for *Shigella* invasion of host cells, have shown efficacy in preclinical studies when targeted alone or in combination with *Shigella* OAg (131-133). Moreover, Ipa proteins exhibit a large degree of conservation among different *Shigella* serotypes, making them attractive candidates for broad-spectrum vaccines. Indeed, a *S. flexneri* 2a OAg-IpaB vaccine administered in mice showed 56% protection against heterologous *S. sonnei* challenge. In the same study, a group of mice was vaccinated with *S. flexneri* 2a OAg-mutant diphtheria toxoid cross-reacting material 197 (CRM) and showed lack of protection (11% survival), indicating that the cross-protection against *S. sonnei* was conferred by the IpaB protein in the case of OAg-IpaB vaccine.

The Invaplex (AR Detox) vaccine contain a mixture of invasion plasmid antigens and LPS extracted from genetically attenuated *S. flexneri* 2a . Initially, the vaccine comprised native product isolated from wild type *Shigella*, but it evolved into a formulation composed of purified LPS and recombinant IpaB and IpaC (133). Evidence showed that this vaccine, currently under clinical development, could also offer protection against *S. flexneri* 3a and 6, since a bactericidal antibody response was observed against these two heterologous serotypes, even if it was attributed to the presence of IpaB and IpaC antigens (133).

Likewise, Omps, like OmpA and OmpC have garnered attention due to their role in bacterial adhesion and invasion (134-136). Additionally, the Omp IcsP has been identified as a common antigen among different *Shigella* serotypes, suggesting its potential for vaccine development (137).

The synergy between the immune responses elicited by *Shigella* OAg and proteins presents a promising approach for vaccine development aimed at conferring protection against various *Shigella* serotypes. While OAg typically induce a robust immune response upon vaccination, proteins can broaden the vaccine's protective efficacy due to their conservation across serotypes. By incorporating these elements into a vaccine formulation, synergistic responses could be achieved.

## 5. Conclusion

This review highlights the significant global health challenge posed by *Shigella* infections, emphasizing the need for enhanced surveillance and refined methodologies to better understand its distribution and develop effective vaccine strategies. Indeed, the EFGH multicounty surveillance study has been recently performed, providing critical data on *Shigella* incidence, disease severity, serotype distribution and antimicrobial resistance, laying essential groundwork for future vaccine trials (138). While regional epidemiological studies and surveillance initiatives offer invaluable insights into the prevalence and distribution of *Shigella* species, their utility is inherently limited by underestimation challenges and the lack of advanced techniques for precise serotype identification, particularly in endemic regions. Advanced molecular techniques have played a pivotal role in identifying *Shigella* in developed non-endemic countries, whereas culture-based methods remain predominant in endemic areas. Additionally, the overuse of antibiotics in LMICs is not only posing significant diagnostic challenges but also driving the spread of antimicrobial-resistant strains. Also, attenuated clinical signs in most industrialized nations, due to prompt medical care, complicate the diagnostic process, highlighting the need for improved diagnostic capabilities.

The review underscores the importance of developing a broadly protective vaccine, focusing on OAg as key targets for inducing robust immunity against multiple *Shigella* serotypes. While animal models offer insights into cross-reactivity, integrating these findings with human clinical trial data is essential for a comprehensive understanding of vaccine performance. Therefore, while the structural similarities of OAg are key considerations in designing a broadly protective vaccine, it is crucial to conduct comprehensive studies to understand the full spectrum of immune responses elicited by the vaccines. Researchers are exploring various vaccine platforms, including live-attenuated and subunit vaccines, with OAg-based formulations showing promise for cross-protection. Indeed, while cross-protection mediated by proteins might appear simpler due to conserved epitopes, OAg-based vaccines achieve cross-reactivity by leveraging structural similarities between OAg variants, which is a more complex but highly effective approach for addressing pathogen diversity. The importance of developing a vaccine that could protect against the majority of the *Shigella* strains causing a burden in endemic countries is paramount. Additionally, a vaccine targeting resistant strains would be a significant breakthrough, particularly

for the traveller market, to prevent the global dissemination of MDR and XDR *Shigella* strains (139).

Despite these advancements, gaps remain in our understanding of *Shigella* cross-reactivity. Addressing these gaps requires the development of refined methodologies to generate comprehensive data on the serotype coverage of current vaccines. A deeper understanding of cross-reactivity, including the mechanisms beyond group-specificities that may drive this phenomenon, and the potential of antibodies elicited to cross-react with heterologous OAg is essential. Furthermore, current approaches for evaluating cross-reactivity are limited, emphasizing the need for advanced high-throughput assays to facilitate a more robust analysis of sera against multiple serotypes. The implementation and optimization of these tools have the potential to greatly enhance our knowledge and accelerate progress in vaccine development.

In summary, concerted efforts are needed to combat *Shigella* infections by leveraging advances in vaccine development and epidemiological surveillance. By addressing these issues, we can reduce the morbidity and mortality associated with *Shigella* infections and improve global public health outcomes.

## **Author contributions**

All authors contributed to the conception and writing of the article and approve the final version of the article.

## **Conflict of interest**

V. Caradonna is a student at the University of Siena and participates in a post-graduate studentship program at GSK. U. Nakakana was a Senior Project Physician in GSK. All other authors are employed by GSK. The authors declare no other financial or non-financial relationships or activities.

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### CHAPTER 3. Immunogenicity evaluation of altSonflex1-2-3 Shigella vaccine across mice, rats, and rabbits to inform human translational insights

*Manuscript under revision*

Valentina Caradonna<sup>1</sup>, Renzo Alfini<sup>2</sup>, Marika Pinto<sup>2</sup>, Roberta Di Benedetto<sup>2</sup>, Carlo Giannelli<sup>2</sup>, Miren Iturriza<sup>2</sup>, Omar Rossi<sup>2</sup>, Francesca Micoli<sup>2</sup>, Francesca Mancini<sup>2</sup>.

<sup>1</sup>University of Siena, Siena, Italy; <sup>2</sup>GSK Global Health Vaccines R&D (GVGH), Siena, Italy

#### Abstract

*Shigella*, a leading cause of bacillary dysentery, represents a significant global health challenge, particularly in low- and middle-income countries. Shigellosis predominantly affects children under the age of five and is associated with high morbidity and mortality rates. To address this burden, a GMMA-based vaccine, altSonflex1-2-3, incorporating *S. sonnei* and *S. flexneri* 1b, 2a, and 3a O-antigens, has been developed. This study aimed to evaluate and compare the immunogenicity of the altSonflex1-2-3 vaccine in mice, rats, and rabbits.

Significant increase in O-antigen specific IgG response was observed in all animal models after one single injection, that further increased post-second vaccination in mice and rats for all antigens and tested doses. In rabbits, booster effects were observed for all antigens, except for *S. flexneri* O-antigen at the highest dose and *S. sonnei* O-antigen at intermediate and high dose. This study showed how each species exhibited its own unique dose-response pattern against *S. flexneri* 1b and 3a. Instead *S. flexneri* 2a consistently showed a positive dose-response in every model examined. A hook effect was observed for *S. sonnei* IgG across all models, with responses peaking at medium doses and decreasing at higher doses. This trend was most pronounced in mice and less evident in rats. Across all antigens, mice and rabbits exhibited more homogeneous immune responses to the 4 antigens, while rats showed numerically higher response to *S. sonnei* and *S. flexneri* 2a compared to *S. flexneri* 1b and 3a. Interestingly, this pattern in rats aligns more closely with responses recently observed in European adults. The vaccine has now advanced to Phase 2 clinical trials in the target population of 9-month-old infants, where different doses of the vaccine are being tested. Immune data collected will allow to further evaluate which preclinical model can better predict humoral response elicited in different age group populations. Expanding studies of

this kind across different platforms and pathogens could provide valuable insights into the optimal animal models for supporting rapid vaccine design and development prior to clinical trials.

## 1. Introduction

*Shigella*, a genus of Gram-negative bacteria, is the causative agent of shigellosis, a severe form of dysentery that primarily affects children under five years in low- and middle- income countries (1). The disease is characterized by symptoms such as diarrhoea, fever, and stomach cramps, and can lead to significant morbidity and mortality (2-5). Several studies conducted during the most recent years showed how the burden has been also complicated by the emergence of antimicrobial-resistant strains of *Shigella sonnei* and *Shigella flexneri*, causing outbreaks also in high-income countries where it is diffused in populations like MSM (6-12). For this reason *Shigella* was inserted in the WHO Bacterial Priority Pathogens List (13).

The development of effective vaccines against *Shigella* is a priority for public health organizations worldwide, with several candidates currently in clinical development (14).

One promising approach in the fight against *Shigella* involves the use of Generalized Modules for Membrane Antigens (GMMA). GMMA are outer membrane vesicles derived from genetically modified bacteria that present multiple antigens in their native conformation, potentially eliciting a robust immune response (15, 16). In the case of *Shigella*, GMMA are used as a delivery system for the O-antigen (OAg), a component of the lipopolysaccharide on the bacterial surface, which plays a crucial role in immune response against *Shigella* (17, 18). The OAg is also crucial because it defines the diversity within the *Shigella* genus, which is formed by four species and more than 50 serotypes distinguished based on the OAg structure (19, 20). The altSonflex-1-2-3 vaccine targets four *Shigella* serotypes (*S. sonnei*, *S. flexneri* 1b, 2a and 3a), aiming to provide broad protection against this diverse pathogen (21). The vaccine is currently undergoing phase 2 clinical trials (NCT05073003 and NCT06663436) and resulted to be safe and immunogenic in European adults (NCT05073003) (22).

Animal models are essential for the evaluation of immune responses to vaccines besides the assessment of safety and efficacy (23, 24). Mice are commonly used in vaccine research due to their well-characterized immune system (23). Also, the ease of genetic manipulation of mice had led to the availability of numerous well characterized outbred strains which permits to evaluate

variations in responses due to genetic differences (25). They are particularly useful for initial screening of vaccine candidates, studying the mechanisms of immune responses, and evaluating the efficacy of different adjuvants or comparing different platforms for vaccine design. Mice were selected due to their established use in preliminary immunogenic studies and the ability to give strong immune responses at very low doses that also correspond with their smaller body size and weight (26, 27). Maintaining mice is also cost-effective. This species can also rapidly reach the age stage that is needed for immunological studies (young adults), making them a convenient species for studies that require rapid generation of data. However, mouse strains typically exhibit lower levels of complement compared to humans or to other rodents like rats (28), which can limit their predictive value for human immune responses. Other rodents for which there are also several records for the use for the same purposes are rats. Rats offer certain advantages such as their larger size which allows for easier handling and sample collection. More importantly, activated T cells in humans and rats express major histocompatibility complex class II, and CD8 and CD4 expression is observed on macrophages in these species, a feature not shared by mice (29). Rats are used to study the immunogenicity, safety, and pharmacokinetics of vaccine candidates and therapeutics (30), providing valuable data that can help bridging the gap between preclinical findings and human clinical responses. Rats were utilized for the presence of a complex immune system, taking into account their intermediate body weight to ensure precise dosing (29). Finally, rabbits have been used for immunogenicity studies since they possess a sophisticated adaptive immune system with genes more similar to humans than those of rodents (31, 32, 33). Rabbit antibody repertoire, characterized by high affinity and specificity, has been instrumental in generating both polyclonal and monoclonal antibodies for diagnostic and therapeutic applications (34, 35). Moreover, rabbits can typically receive the full human dose of a vaccine (36, 37), and their body weight is relatively similar to that of infants, unlike other species where lower doses must be administered, complicating allometric calculations (38). Rabbits larger body size allows for the administration of the full human dose and rabbit was the model that was chosen for providing safety and toxicology data on GMMA-based vaccines against *Shigella* (21, 39). Each species offers unique insights into the immune response and their predictive value for human outcomes can vary. For this reason, assessing vaccines across multiple animal models enhances our understanding of their potential in humans.

When the immunogenicity of altSonflex1-2-3 was first assessed in preclinical experiments, no negative immunointerference was observed in mice and rabbits, with similar responses against all four GMMA (21). However, the results in terms of immunogenicity obtained in European adult patients, showed a response to *S. flexneri* 1b and 3a GMMA that was numerically lower compared to the one induced by *S. sonnei* and *S. flexneri* 2a GMMA (22). To address these discrepancies and gain a deeper understanding of the vaccine's behavior in animals, we conducted studies using three animal models: mice, rats, and rabbits, testing the vaccine at increasing doses and administered intramuscularly rather than intraperitoneally to more accurately replicate the human route of immunization.

This study aimed to assess the immunogenicity of the altSonflex1-2-3 vaccine across different animal models by evaluating the impact of varying doses and the effect of a second vaccination on the elicited immune response.

## **2. Methods**

### **2.1. Strains**

*S. sonnei* 53G was obtained from Walter Reed Army Institute of Research, Washington, D.C., USA. The *S. sonnei*  $\Delta virG::cat$  strain with stabilized OAg used in FACS and SBA was generated by Caboni *et al.*(40). *S. flexneri* strains (*S. flexneri* 1b NCTC5, *S. flexneri* 2a NVGH3134, *S. flexneri* 3a NCTC9989) were purchased from Public Health England, London, UK. Frozen 20% glycerol stocks were prepared from lyophilized cultures and stored at  $-80^{\circ}\text{C}$ .

### **2.2. Vaccine formulation**

altSonflex1-2-3 was produced and characterized according to the methods previously described (21). Briefly, altSonflex1-2-3 was formulated by adsorbing at 1:1:1:1 weight ratio of OAg GMMA from *S. sonnei*, *S. flexneri* serotypes 1b, 2a, and 3a in a solution containing 154 mM NaCl and 10 mM  $\text{NaH}_2\text{PO}_4$  at pH 6.5 and Alhydrogel at a concentration of 0.7 mg/ml ( $\text{Al}^{3+}$ ). Further dilutions for immunogenicity studies were performed with Alhydrogel diluent (0.7 mg/ml  $\text{Al}^{3+}$  in NaCl 154 mM  $\text{NaH}_2\text{PO}_4$  10 mM pH 6.5).

### **2.1. Immunogenicity studies**

altSonflex1-2-3 vaccine at different doses was used to immunize mice, rats and rabbits. GSK is committed to the Replacement, Reduction and Refinement of animal studies (3Rs). Non-animal

models and alternative technologies are part of our strategy and employed where possible. When animals are required, the application of robust study design principles and peer review minimizes animal use, reduces harm, and improves benefit in studies. Animal studies were performed in GSK Animal Resources center in Siena, Italy (mice and rabbits) and in Charles River Laboratories in Lyon, France (rats) in compliance with the relevant guidelines (European directive 2010/63/UE) and the GSK Policies on the Care, Welfare and Treatment of Animals.

Ten CD1 mice per group (female, 4 to 6 weeks old) or eight Sprague Dawley rats (female, 200-300g) per group or six New Zealand white rabbits (female, 2.3-2.7 kg) per group were immunized at day 0 and 28. All animals were immunized intramuscularly with either 50µl (mice), 200µl (rats) or 500µl (rabbits) of altSonflex1-2-3.

In the Phase 1 clinical trial of altSonflex1-2-3 conducted in European adults, the established human dose involved an injection containing 15 µg of OAg of each serotype, which corresponds to 60 µg of total OAg (22). For preclinical evaluation, varying doses of total OAg were administered to the different animal models. In mice, the tested doses per serotype were 0.06 µg, 0.6 µg, and 6 µg OAg, corresponding to approximately 1/1000th, 1/100th, and 1/10th of the human dose, respectively. Immunization in rats utilized doses of 0.24 µg, 2.4 µg, and 24 µg OAg, which equate to approximately 1/250th, 1/25th, and 1/2.5th of the human dose. In rabbits, the administered doses were 0.6 µg, 6 µg, and 60 µg OAg, representing approximately 1/100th, 1/10th, and 1-fold the human dose, respectively.

Serum was collected on days -1 (baseline), 27 (post-dose 1) and 42 (post-dose 2) and tested in immunoassays.

## **2.2. Enzyme-linked immunosorbent assay (ELISA)**

Sera from animals were analyzed using ELISA plates coated with the following antigens: *S. sonnei* LPS at 0.5 µg/ml in phosphate-buffered saline (PBS), *S. flexneri* 1b OAg at 2 µg/ml in carbonate buffer, *S. flexneri* 2a OAg at 5 µg/ml in carbonate buffer, and *S. flexneri* 3a OAg at 1 µg/ml in PBS. The plates were then blocked with 5% PBS milk for 1 hour at 25°C and afterwards incubated with sera diluted at 1:100, 1:4000, and 1:160000 in PBS-Tween 0.05% with 0.1% bovine serum albumin (BSA) for mice and rat sera and in 5% milk in PBS for rabbit sera. Following this, IgG detection was carried out using an enzyme-labeled secondary antibody (anti-species specific IgG-

alkaline phosphatase) in PBS-Tween 0.05% with 0.1% BSA at different concentrations depending on the antigen used and for mice, rabbits and rats, respectively. The presence of anti-*S. sonnei* LPS/*S. flexneri* 1b, 2a, 3a OAg antibodies was identified by adding a substrate solution, resulting in a yellow color, which was measured by absorbance at 405 nm minus the absorbance at 490 nm. The samples were compared to calibrated specie-specific reference standard sera, with results expressed in ELISA units/mL relative to the reference serum. One ELISA unit is defined as the reciprocal of the dilution of the reference serum that produces an OD of one in the assay.

### **2.3. Luminescence Serum Bactericidal Assay (L-SBA)**

Serum samples were evaluated for bactericidal activity against altSonflex1-2-3 *Shigella* serotypes by adapting previously published conditions (41, 42). All tested samples were heat inactivated (HI) prior to testing in L-SBA at 56 °C for 30 min to remove endogenous complement activity. In brief, bacterial strains were cultured to log-phase ( $OD_{600} = 0.25 \pm 0.02$ ) and then diluted 1:1000 in PBS. Heat-inactivated sera were diluted in PBS when tested against *S. sonnei* and *S. flexneri* 2a, 3a strains and in LB when tested against *S. flexneri* 1b strain. Serial dilutions of sera from mice, rats, rabbits were added to 96-well plates, followed by the addition of Baby Rabbit Complement (BRC, Cederlane). The concentration of BRC utilized in each assay was different depending on the strain utilized: 30% for *S. flexneri* 1b, 20% for *S. sonnei* and *S. flexneri* 3a, 7.5% for *S. flexneri* 2a. The plates were incubated at 37°C for 3 hours. The viability of the surviving bacteria was assessed using BacTiter-Glo reagent (Promega) to measure ATP production, with luminescence detected via a Synergy HT luminometer (Biotek). The bactericidal activity was quantified by determining the IC<sub>50</sub> value, which is defined as the reciprocal serum dilution that leads to a 50% reduction in luminescence after 180 minutes of incubation ( $T_{180}$ ) relative to the luminescence measured at the start of the assay ( $T_0$ ). For titers below the minimum measurable signal, a value of equal to half of the lowest dilution tested was assigned. Curve fitting and IC<sub>50</sub> calculations were performed using GraphPad Prism 9 software.

### **2.4. Statistical Analyses**

Mann–Whitney two-tailed test was used to compare the total IgG immune response and bactericidal activity elicited by two different groups and Wilcoxon test was used to compare total IgG immune response between post-dose 1 and post-dose 2 total IgG immune responses for each group.

Dose dependent responses have been evaluated by comparing groups of mice receiving the different doses of GMMA using Spearman rank correlation. The dose response relationship has been considered significant if the P value of the Spearman rank correlation was  $\leq 0.05$  and the correlation coefficient (Spearman r) was  $> 0$ .

Within-animal geometric mean ratios (GMRs) over the baseline values were also calculated for each animal group.

Pearson correlation was used to calculate r coefficients of the correlation between total IgG measured by ELISA and bactericidal activity measured by SBA in the three animal models.

### **3. Results**

#### **Immunogenicity induced by altSonflex 1-2-3 in different animal models**

##### **3.1.1. Effect of two immunizations of altSonflex1-2-3 at different doses in mice**

When tested in mice, in the range of OAg doses between 0.06-6 mg, altSonflex1-2-3 elicited significant anti-OAg-specific IgG response already post-dose 1, with further increase post-dose 2. Specifically, for all the tested OAg, a significant increase in IgG was observed post-dose 2 compared to post-dose 1 across all three antigen concentrations ( $P \leq 0.01$ , \*\*).

To verify dose-dependent responses, Spearman rank correlation was used and the results are summarized in Table 1 and detailed in Supplementary materials (Tables 1-6). Twenty-eight days after 1<sup>st</sup> injection, a significant correlation was found for *S. flexneri* 2a OAg (Spearman's rank correlation 0.4860,  $P \leq 0.01$ , \*\*) and *S. flexneri* 3a OAg (Spearman's rank correlation 0.5691,  $P \leq 0.01$ , \*\*), but not for *S. sonnei* and *S. flexneri* 1b OAg (Table 1). Post-dose 2, a significant increase in IgG at the increase of the total OAg dose in the vaccine formulation was observed only for *S. flexneri* 2a (Spearman's rank correlation 0.6650,  $P \leq 0.0001$ , \*\*\*\*). Notably, for *S. sonnei*, the 6  $\mu$ g dose of total OAg elicited significantly lower IgG compared to the 0.6  $\mu$ g dose ( $P \leq 0.01$ , \*\*).

Sera collected post-dose 2 were also assessed for serum bactericidal activity. No dose response was observed, except for *S. flexneri* 2a, for which a positive correlation between the increase of the OAg dose and bactericidal activity was observed (Spearman's rank correlation 0.6698,  $P \leq 0.0001$ , \*\*\*\*)(Figure 2 and Table 1).

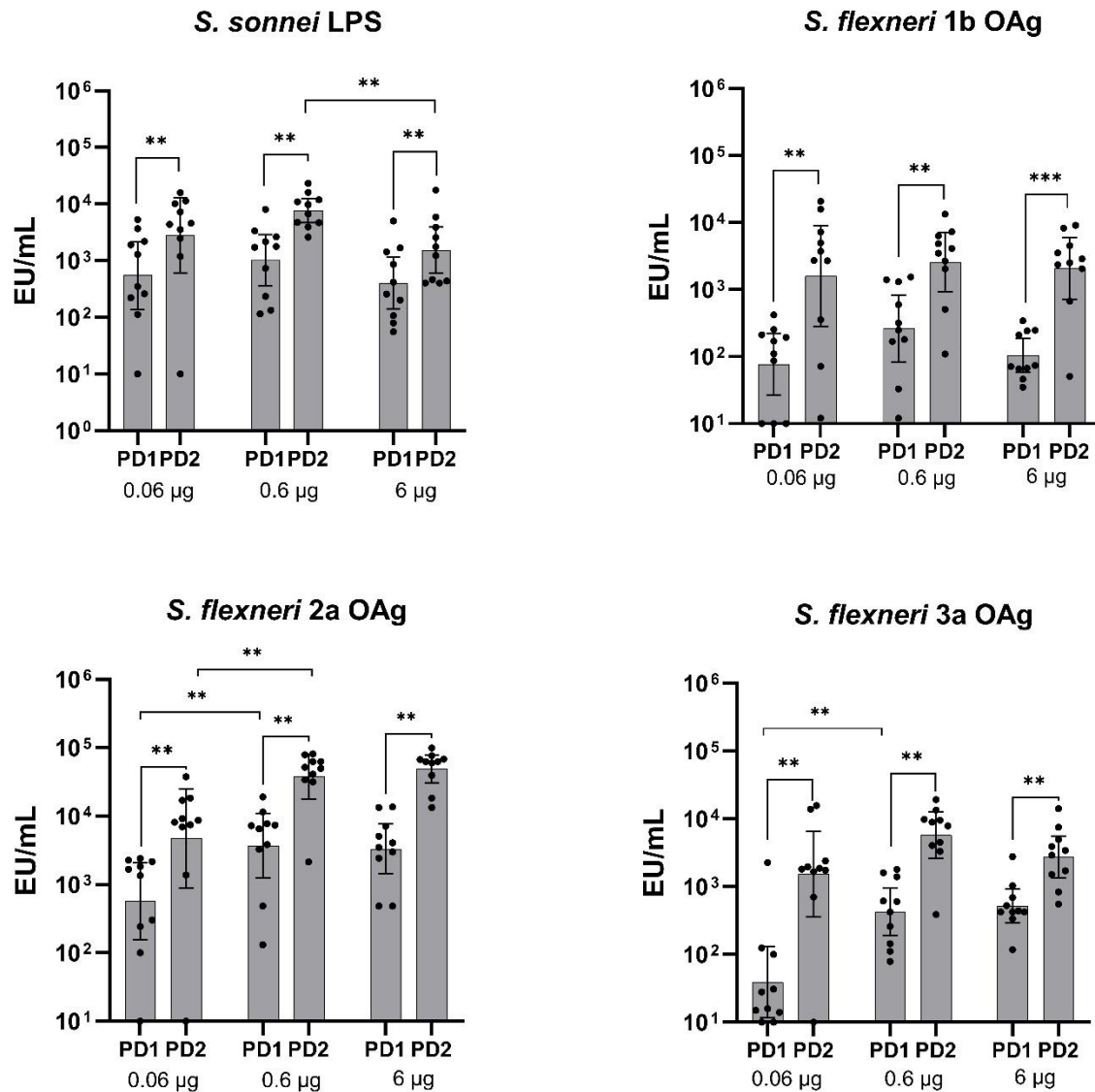


Figure 2. OAg-specific IgG immune response (EU/mL). Groups of 10 mice were immunized IM at days 0 and 28, and sera were analyzed by ELISA at day 27 (post-dose 1: PD1) and at day 42 (post-dose 2 = PD2) in mice vaccinated with three different doses of altSonflex1-2-3 (0.06, 0.6 or 6 µg of tot. OAg). Geometric mean (bars) and 95% CI (error bars) is reported for all groups together with individual values (dots). The Mann–Whitney test was used for assessing statistical differences between groups and the Wilcoxon test was used for assessing statistical differences between PD1 and PD2 results for each group (\*\*  $P \leq 0.01$ ).

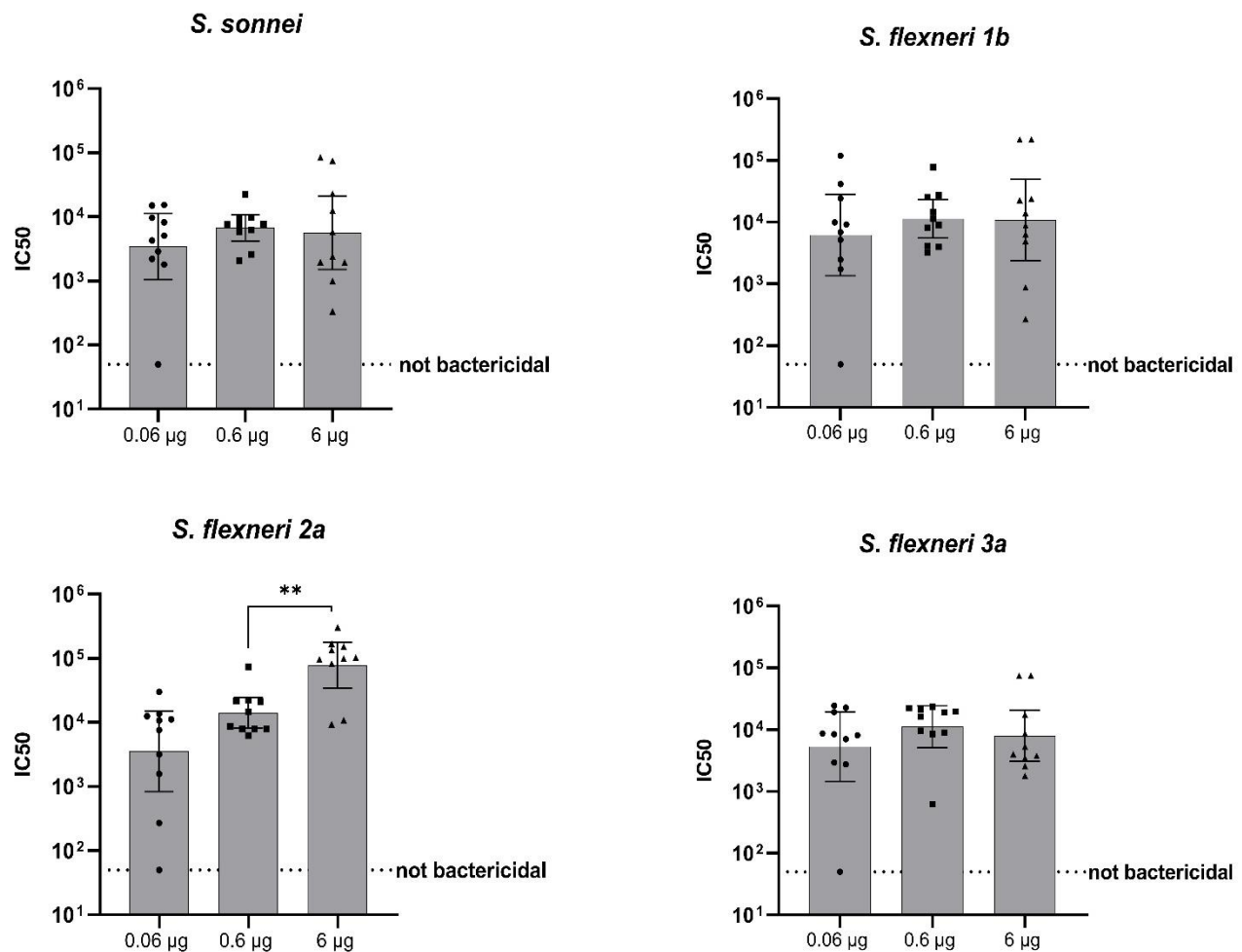


Figure 3. Bactericidal activity of antibodies ( $IC_{50}$ ). Groups of 10 mice were immunized IM at days 0 and 28, and sera were analyzed by SBA at day 42 (post-dose 2) in mice vaccinated with three different doses of altSonflex1-2-3 (0.06, 0.6 or 6  $\mu$ g of tot. OAg). Bactericidal activity was determined as the dilution necessary to obtain 50% CFU reduction at  $T_{180}$  compared with  $T_0$ . Geometric mean (bars) and 95% CI (error bars) is reported for all groups together with individual values (dots). Baseline values (day -1) are indicated by the dotted line. The Mann–Whitney test was used for assessing statistical differences between groups ( \*\*  $P \leq 0.01$  ).

### 3.1.2. Effect of two immunizations of altSonflex1-2-3 at different doses in rats

When tested in rats, in the range of OAg doses between 0.24-24 mg, altSonflex1-2-3 elicited a strong immune response in terms of OAg-specific IgG against all the four antigens, already after the first immunization. IgG levels further increased post-dose 2 (Figure 3). In particular, a

significant increase in OAg-specific IgG post-dose 2 was observed at all the antigen doses tested against all of the four *S. sonnei* and *S. flexneri* OAg, with P-values of  $P \leq 0.01$  (\*\*).

A dose-dependent OAg-specific total IgG response was observed post dose 1 against *S. flexneri* 1b OAg (Spearman's rank correlation 0.7007,  $P \leq 0.001$ , \*\*\*) and *S. flexneri* 2a OAg (Spearman's rank correlation 0.5234,  $P \leq 0.01$ , \*\*), but not against *S. sonnei* and *S. flexneri* 3a OAg (Table 1). Following the second vaccination, a significant positive correlation between total OAg dose and IgG levels was found exclusively for *S. flexneri* 2a (Spearman's rank correlation 0.4497,  $P \leq 0.05$ , \*).

For the SBA titers post dose 2, instead, no dose-response correlation was found against any of the four strains tested (Figure 4 and Table 1).

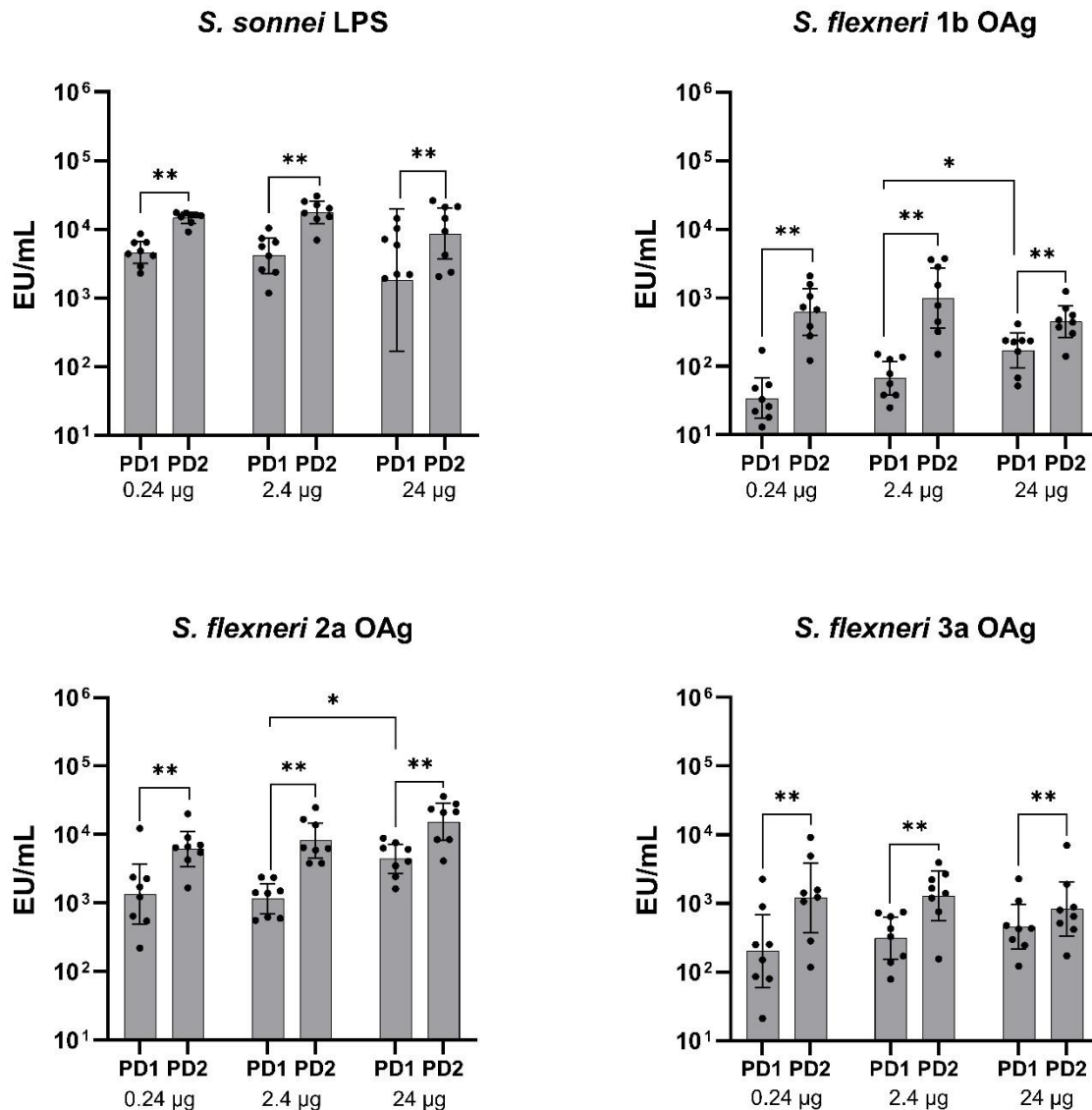


Figure 4. OAg-specific IgG immune response (EU/mL). Groups of 8 rats were immunized IM at days 0 and 28, and sera were analyzed by ELISA at day 27 (post-dose 1: PD1) and at day 42 (post-dose 2 = PD2) in rats vaccinated with three different doses of altSonflex1-2-3 (0.24, 2.4 or 24 µg of tot. OAg). Geometric mean (bars) and 95% CI (error bars) is reported for all groups together with individual values (dots). The Mann–Whitney test was used for assessing statistical differences

between groups and the Wilcoxon test was used for assessing statistical differences between PD1 and PD2 results for each group (\*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ ).

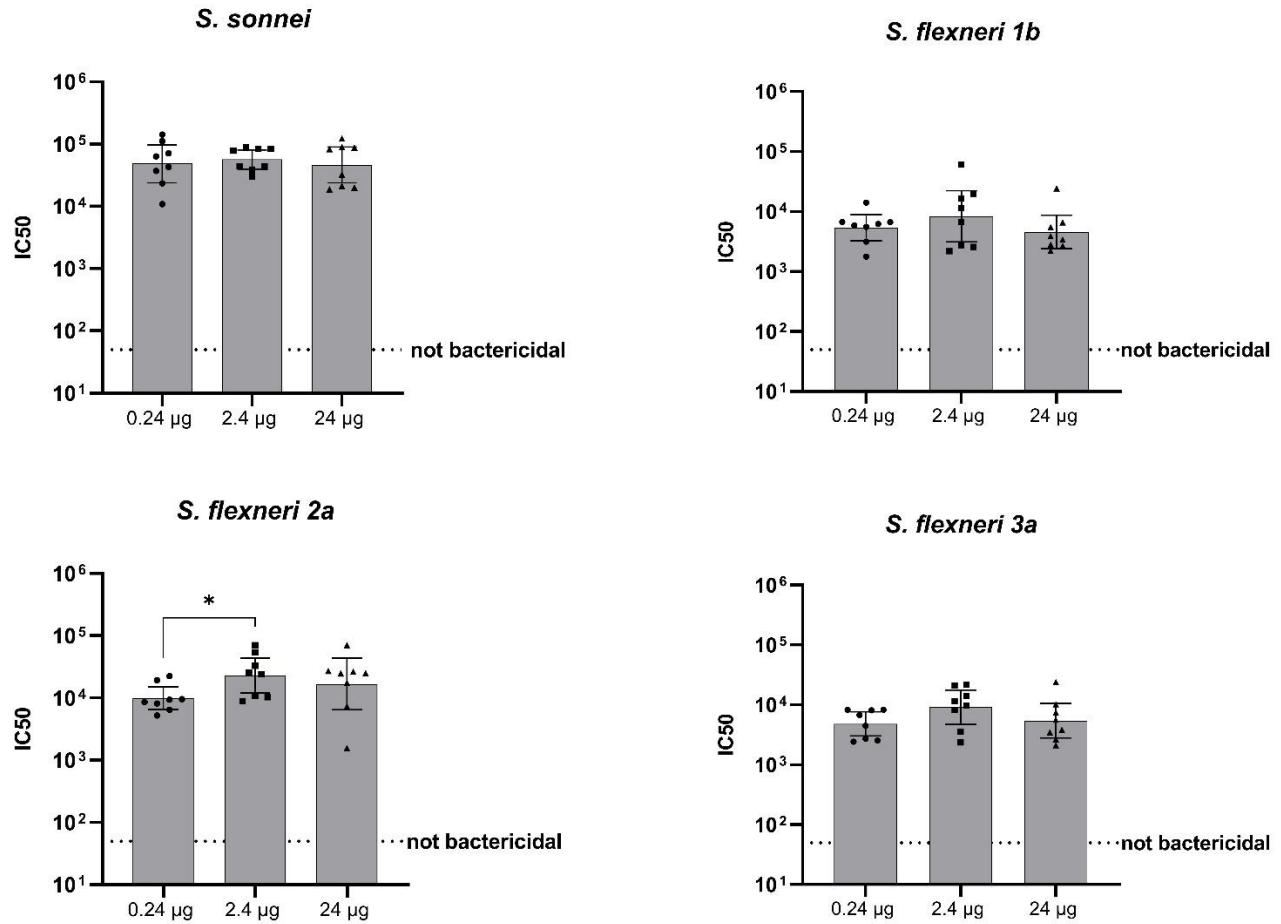


Figure 5. Bactericidal activity of antibodies (IC<sub>50</sub>). Groups of 8 rats were immunized IM at days 0 and 28, and sera were analyzed by SBA at day 42 (post-dose 2) in rats vaccinated with three different doses of altSonflex1-2-3 (0.24, 2.4 or 24 µg of tot. OAg). Bactericidal activity was determined as the dilution necessary to obtain 50% CFU reduction at T<sub>180</sub> compared with T<sub>0</sub>. Geometric mean (bars) and 95% CI (error bars) is reported for all groups together with individual values (dots). Baseline values (day -1) are indicated by the dotted line. The Mann–Whitney test was used for assessing statistical differences between groups (\*  $P \leq 0.05$ ).

### 3.1.3. Effect of two immunizations of altSonflex1-2-3 at different doses in rabbits

In rabbits, in the range of OAg doses tested between 0.6-60 mg, the first immunization elicited a robust IgG response against all four target antigens (Figure 5). A booster effect was observed following the second dose, with IgG titers significantly increasing for all the OAg but not at all the three antigen concentrations tested. Specifically, for *S. flexneri* 1b OAg, a significant elevation in IgG levels post-dose 2 was achieved for the 0.6  $\mu$ g ( $P \leq 0.01$ , \*\*) and 6  $\mu$ g ( $P \leq 0.05$ , \*) doses. A similar pattern was noted for *S. flexneri* 2a and 3a OAg, where both the 0.6  $\mu$ g and 6  $\mu$ g dose resulted in a significant increase ( $P \leq 0.05$ , \*), but not the 60  $\mu$ g of total OAg dose. IgG response specific to *S. sonnei* OAg significantly increased post-dose 2 at the lower total OAg concentration tested of 0.6  $\mu$ g ( $P \leq 0.05$ , \*), for the two higher concentrations of 6 and 60  $\mu$ g of total OAg dose, IgG titers remained comparable to the response observed post-dose 1.

Post-dose 1, a dose-dependent total IgG response was observed only against *S. flexneri* 2a OAg, with a Spearman's rank correlation of 0.6574 ( $P \leq 0.001$ , \*\*), and against *S. flexneri* 3a OAg with a correlation of 0.7003 ( $P \leq 0.01$ , \*\*). However, no dose response was observed for *S. sonnei* and *S. flexneri* 1b OAg (Table 1). Following the second vaccination, a significant positive correlation between the total OAg dose and IgG levels was detected exclusively for *S. flexneri* 3a (Spearman's rank correlation 0.7263,  $P \leq 0.01$ , \*\*).

In terms of SBA, a dose-response correlation was evident against the three *S. flexneri* strains tested. In particular, bactericidal activity increase correlated with dose increase in the case of SBA against *S. flexneri* 1b (Spearman's rank correlation (0.7081,  $P \leq 0.01$ , \*\*), *S. flexneri* 2a (Spearman's rank correlation (0.5847,  $P \leq 0.05$ , \*) and *S. flexneri* 3a (Spearman's rank correlation (0.7575,  $P \leq 0.001$ , \*\*\*) (Figure 6 and Table 1).

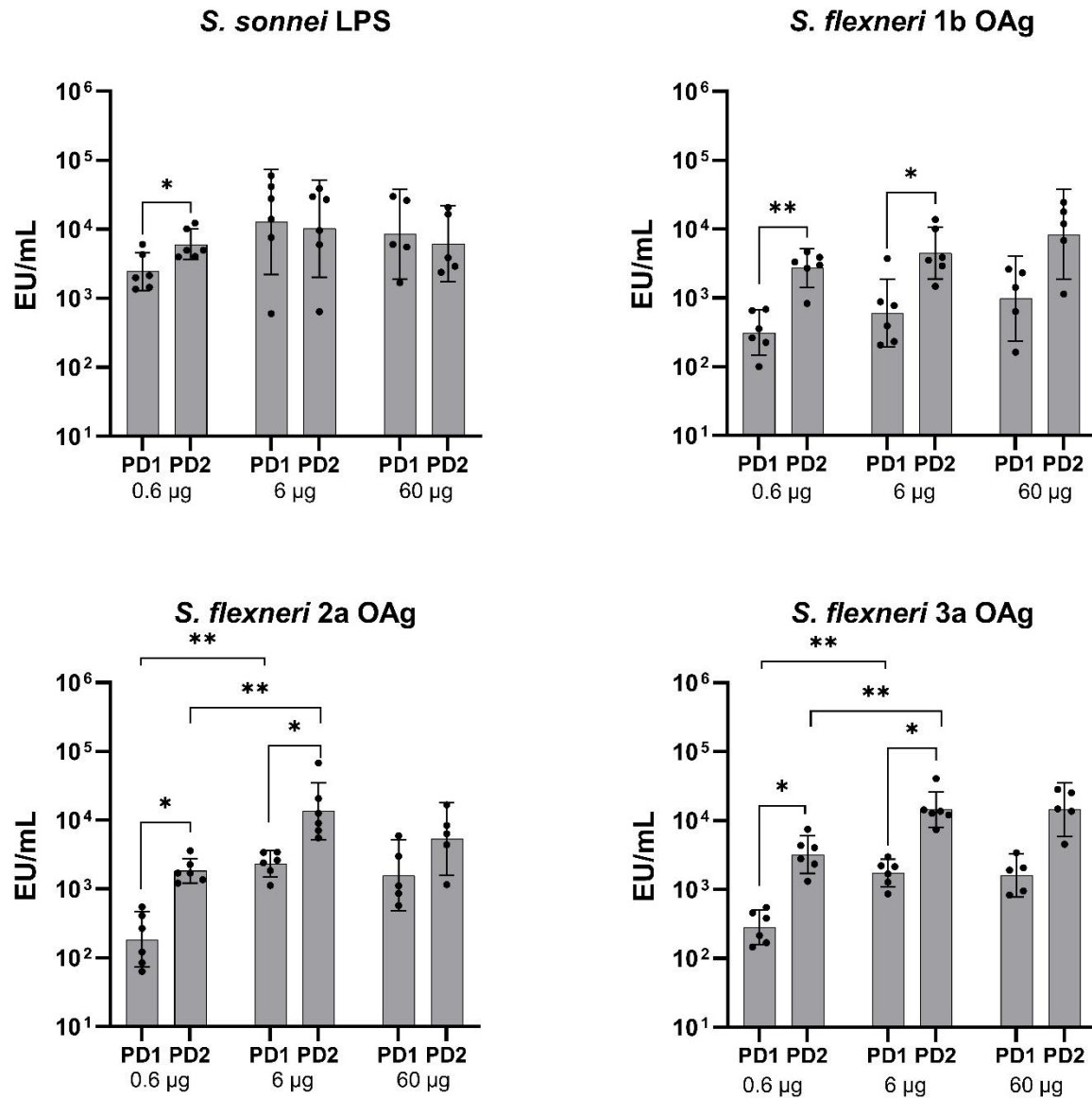


Figure 6. OAg-specific IgG immune response (EU/mL). Groups of 6 rabbits were immunized IM at days 0 and 28, and sera were analyzed by ELISA at day 27 (post-dose 1: PD1) and at day 42 (post-dose 2 = PD2) in rabbits vaccinated with three different doses of altSonflex1-2-3 (0.6, 6 or 60 µg of tot. OAg). Geometric mean (bars) and 95% CI (error bars) is reported for all groups together with individual values (dots). The Mann–Whitney test was used for assessing statistical differences between groups and the Wilcoxon test was used for assessing statistical differences between PD1 and PD2 results for each group (\*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ ).

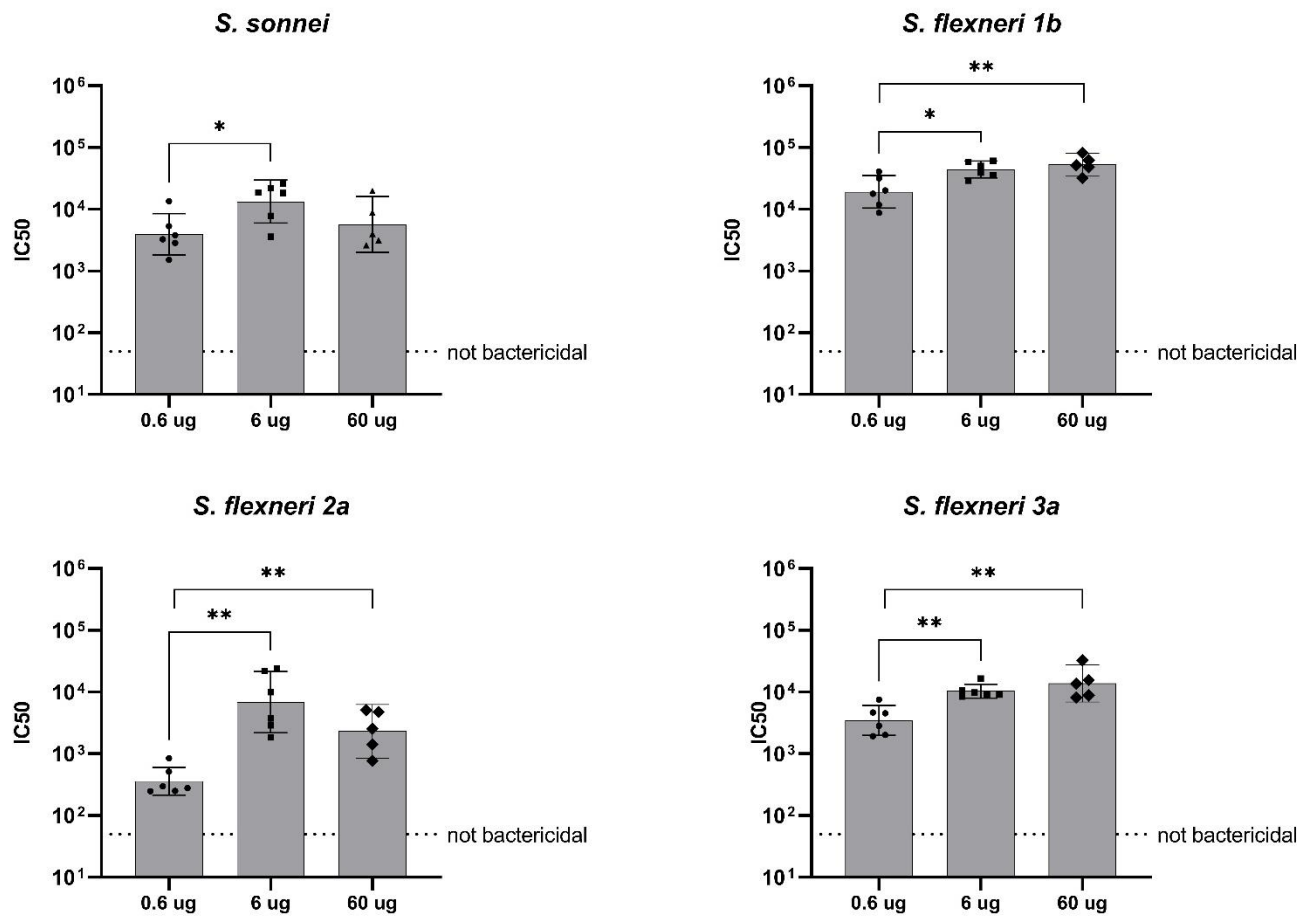


Figure 7. Bactericidal activity of antibodies (IC<sub>50</sub>). Groups of 6 rabbits were immunized IM at days 0 and 28, and sera were analyzed by SBA at day 42 (post-dose 2) in rabbits vaccinated with three different doses of altSonflex1-2-3 (0.6, 6 or 60 µg of tot. OAg). Bactericidal activity was determined as the dilution necessary to obtain 50% CFU reduction at T<sub>180</sub> compared with T<sub>0</sub>. Geometric mean (bars) and 95% CI (error bars) is reported for all groups together with individual values (dots). Baseline values (day -1) are indicated by the dotted line. The Mann–Whitney test was used for assessing statistical differences between groups (\*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ ).

Table 1. Evaluation of the OAg-specific IgG immune response and serum bactericidal activity at three different altSonflex1-2-3 doses in mice, rats, and rabbits. (a) boost effect observed after the second dose (PD2) for antigen-specific IgG: “+” indicates a boost effect, “-” indicates no boost effect. (b) correlation between dose and IgG response at PD1, (c) correlation between dose and IgG response at PD2, (d) correlation between dose and serum bactericidal activity at PD2, as assessed by Spearman correlation. Significance levels are indicated as follows: \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ , ns = not significant.

| Immune response                                                        |      | Mice |     |   | Rats |     |    | Rabbits |   |    |
|------------------------------------------------------------------------|------|------|-----|---|------|-----|----|---------|---|----|
| Dose ( $\mu\text{g tot. OAg}$ )                                        |      | 0.06 | 0.6 | 6 | 0.24 | 2.4 | 24 | 0.6     | 6 | 60 |
| Increase of IgG response after boost (PD2) <sup>a</sup>                | Sso  | +    | +   | - | +    | +   | -  | -       | - | -  |
|                                                                        | Sf1b | +    | +   | + | +    | +   | +  | +       | + | -  |
|                                                                        | Sf2a | +    | +   | + | +    | +   | +  | +       | + | -  |
|                                                                        | Sf3a | +    | +   | + | +    | +   | -  | +       | + | +  |
| IgG dose-response correlation PD1 <sup>b</sup>                         | Sso  | ns   |     |   | ns   |     |    | ns      |   |    |
|                                                                        | Sf1b | ns   |     |   | ***  |     |    | ns      |   |    |
|                                                                        | Sf2a | **   |     |   | **   |     |    | **      |   |    |
|                                                                        | Sf3a | **   |     |   | ns   |     |    | **      |   |    |
| IgG dose-response correlation PD2 <sup>c</sup>                         | Sso  | ns   |     |   | ns   |     |    | ns      |   |    |
|                                                                        | Sf1b | ns   |     |   | ns   |     |    | ns      |   |    |
|                                                                        | Sf2a | ***  |     |   | *    |     |    | ns      |   |    |
|                                                                        | Sf3a | ns   |     |   | ns   |     |    | **      |   |    |
| Serum bactericidal activity dose-response correlation PD2 <sup>d</sup> | Sso  | ns   |     |   | ns   |     |    | ns      |   |    |
|                                                                        | Sf1b | ns   |     |   | ns   |     |    | **      |   |    |
|                                                                        | Sf2a | ***  |     |   | ns   |     |    | *       |   |    |
|                                                                        | Sf3a | ns   |     |   | ns   |     |    | ***     |   |    |

### 3.1.4. Relative comparison of the immune response induced by the different components of altSonflex1-2-3

In order to compare the immune response elicited by the 4 components of the vaccine, antigen specific IgG and SBA responses were reported as GMR over baseline. In mice administered with 0.6  $\mu\text{g}$  of total OAg, the ELISA results showed a largely homogeneous IgG response across the four antigens, with the notable exception of *S. flexneri* 2a, which exhibited particularly high GMRs (925.8 at PD1 and 9501 at PD2) compared with *S. flexneri* 1b (65.8 PD1 and 647.2 PD2), *S. flexneri* 3a (107.4 PD1 and 1445.3 PD2), and *S. sonnei* (257.2 PD1 and 1929.8 PD2). Corresponding SBA values in mice followed a similar trend, with GMRs of 134.6 for *S. sonnei*, 226.6 for *S. flexneri* 1b, 282 for *S. flexneri* 2a, and 223.1 for *S. flexneri* 3a (Figure 7). In rats, ELISA measurements indicated higher IgG geometric mean titers (GMTs) for *S. sonnei* OAg (407.5 at PD1 and 1737.3

at PD2) and *S. flexneri* 2a OAg (170.4 at PD1 and 1192.5 at PD2) compared with those for *S. flexneri* 1b (11.4 PD1 and 169.6 PD2) and *S. flexneri* 3a (39.3 PD1 and 161.8 PD2). The SBA results in rats paralleled these findings, with GMRs of 1134.9 for *S. sonnei* and 460.1 for *S. flexneri* 2a, whereas values for *S. flexneri* 1b and *S. flexneri* 3a were lower (166.9 and 183.2, respectively). This pattern in rats mirrors closely the trends observed in European adults. In rabbits, the pattern of responses differed. ELISA data indicated that PD2, IgG titers were comparable for three antigens, *S. sonnei* OAg (GMR of 2553), *S. flexneri* 2a OAg (GMR of 3397.8), and *S. flexneri* 3a OAg (GMR of 3607.9), with a relatively lower response against *S. flexneri* 1b OAg (GMR of 1123). However, the SBA results in rabbits did not confirm the ELISA findings in terms of homogeneity of the immune response among the four vaccine strains, as bactericidal GMRs for *S. flexneri* 1b (GMR of 883.1) were higher than those for *S. sonnei* (GMR of 266.6), *S. flexneri* 2a (GMR of 137.1), and *S. flexneri* 3a (GMR of 206.9).

To facilitate the comparison with data from European adults immunized with altSonflex1-2-3 at a dose corresponding to 60 µg total OAg, the intermediate dose used in each of the animal models was selected (Figure 7). Data from the higher and lower doses used in the preclinical settings are shown in the Supplementary Material (Figures 1–2) to provide further insights into the dose-dependent effects in the three animal models of altSonflex1-2-3.

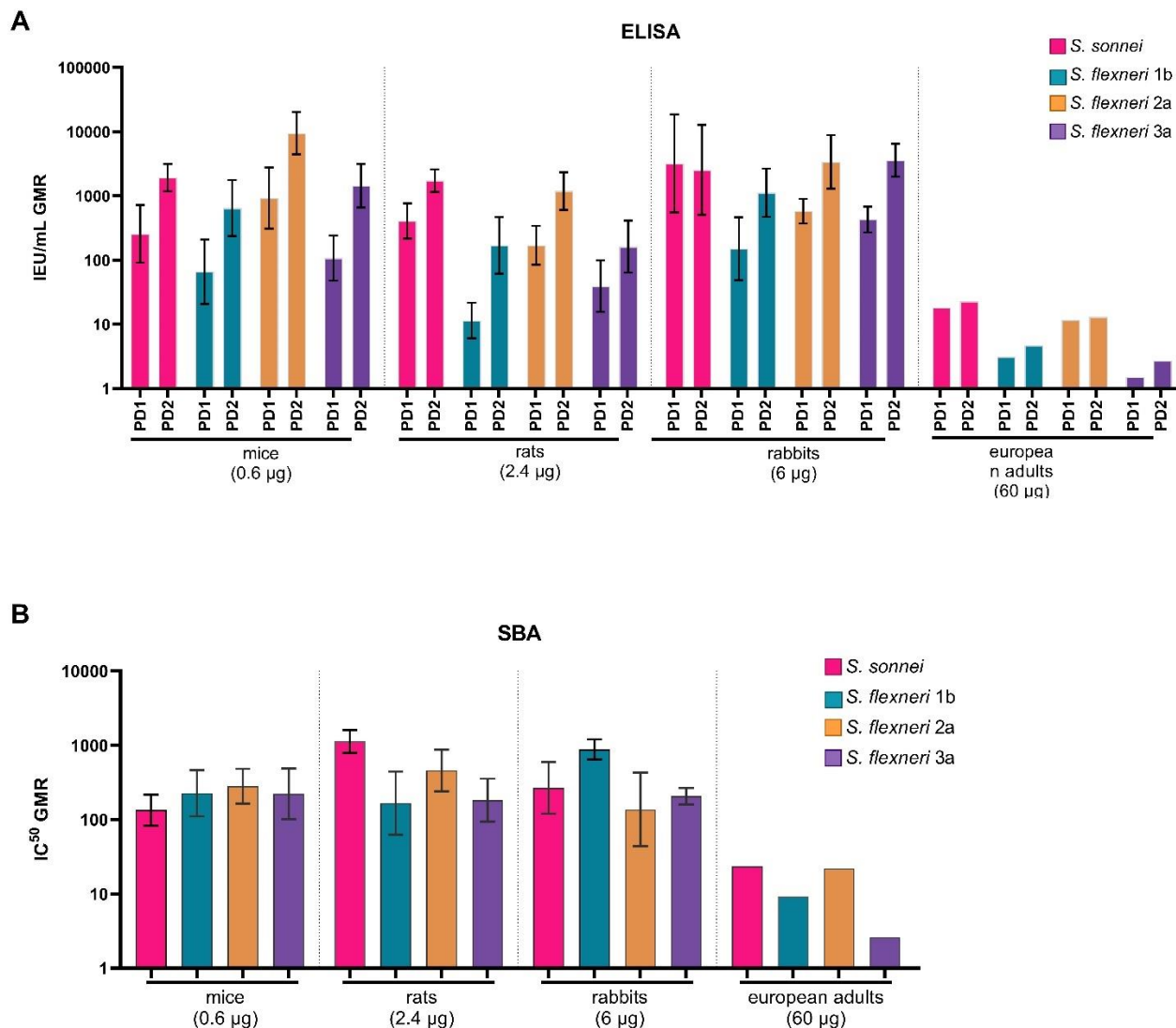


Figure 8. Comparison of humoral immune responses after vaccination with altSonflex1-2-3 between mice (0.6  $\mu$ g tot. OAg dose), rats (2.4  $\mu$ g tot. OAg dose), rabbits (6  $\mu$ g tot. OAg dose) and European adults (60  $\mu$ g tot. OAg dose) in terms of: a: *S. sonnei* and *S. flexneri* 1b, 2a and 3a OAg-specific IgG titers post-dose 1 (PD1) and post-dose 2 (PD2). Geometric mean ratio over the baseline values (bars) for all the groups and 95% CI (error bars) for the preclinical studies groups are reported; b:  $IC^{50}$  of bactericidal activity against *S. sonnei* and *S. flexneri* 1b, 2a and 3a strains. Geometric mean ratio over the baseline values (bars) for all the groups and 95% CI (error bars) for the preclinical studies groups are reported.

Interestingly, the overall analysis of the results obtained in the three species showed that SBA titres correlated differently based on the different species with the total IgG response (Table 2). In particular, in mice a strong correlation was found between anti-*S. flexneri* 3a OAg IgG response and bactericidal activity (Pearson  $r = 0.74$ ,  $P \leq 0.001$ ) and moderate correlation between *S. flexneri*

2a (Pearson  $r = 0.50$ ,  $P \leq 0.01$ ) OAg IgG responses and bactericidal activity. A weaker, albeit significant, correlation was observed for anti-*S. sonnei* OAg IgG (Pearson  $r = 0.37$ ,  $P \leq 0.05$ ) and no significant correlation was found for *S. flexneri* 1b. For what concerns the correlation in rats, only a moderate correlation between IgG responses and bactericidal activity against *S. sonnei* (Pearson  $r = 0.53$ ,  $P \leq 0.01$ ) was observed but weak correlation against *S. flexneri* 1b (Pearson  $r = 0.48$ ,  $P \leq 0.05$ ) or no significant correlation was found for *S. flexneri* 2a and 3a. In the rabbit model, a moderate correlation was found between IgG responses and bactericidal activity against *S. sonnei* (Pearson  $r = 0.56$ ,  $P \leq 0.05$ ), and strong correlations against *S. flexneri* 1b (Pearson  $r = 0.73$ ,  $P \leq 0.001$ ), *S. flexneri* 2a (Pearson  $r = 0.91$ ,  $P \leq 0.001$ ), and *S. flexneri* 3a (Pearson  $r = 0.93$ ,  $P \leq 0.001$ ).

*Table 2. Pearson correlation coefficients (Pearson r values) and p-values of the correlation among S. sonnei, S. flexneri 1b, 2a and 3a OAg-specific IgG responses and bactericidal activity in mice, rats and rabbits. The OAg-specific IgG response detected in all mice, rats and rabbits immunized with 3 different doses of altSonflex1-2-3 was correlated with serum bactericidal activity. Values close to 1 or -1 indicate a strong correlation while values close to 0 indicate a low correlation. n = 30 for mice, 24 for rats, 18 for rabbits.*

| Pearson Correlation Coefficients of IgG vs. Bactericidal Activity<br>(and p-Values of the Correlation) |              |               |              |
|--------------------------------------------------------------------------------------------------------|--------------|---------------|--------------|
|                                                                                                        | Mice         | Rats          | Rabbits      |
| <i>S. sonnei</i>                                                                                       | 0.37 (0.045) | 0.53 (0.008)  | 0.56 (0.020) |
| <i>S. flexneri</i> 1b                                                                                  | 0.34 (0.069) | 0.48 (0.017)  | 0.73 (0.001) |
| <i>S. flexneri</i> 2a                                                                                  | 0.74 (0.000) | -0.05 (0.835) | 0.91 (0.000) |
| <i>S. flexneri</i> 3a                                                                                  | 0.50 (0.005) | 0.36 (0.082)  | 0.93 (0.000) |

## 4. Discussion

Shigellosis remains one of the leading causes of diarrhoeal disease in low- and middle-income countries, particularly affecting young children (2). The growing concern over antimicrobial resistance in *Shigella* further exacerbates the public health challenge. Currently, no licensed vaccines exist against *Shigella*, though several candidates that target the OAg portion of lipopolysaccharides are in development (43). GVGH proposed GMMA as an innovative delivery system for the OAg (44) has led to the development of a 4-component vaccine candidate, altSonflex1-2-3. This vaccine includes GMMA from *S. sonnei* and three prevalent and epidemiologically relevant *S. flexneri* serotypes (1b, 2a, and 3a) and is currently undergoing a phase 2 clinical trial (21, 22).

In this study, we compared the *Shigella* 4-component GMMA-based vaccine candidate altSonflex1-2-3 in three different animal models (mouse, rat, and rabbit) for their ability to (i) boost immune responses after a second dose, (ii) elicit proportional IgG functional responses to the total OAg dose administered, and (iii) induce comparable immune responses across the four OAg components.

Different behaviors were observed among the three animal species and compared to humans in terms of boosting of the immune response after a second vaccination. In mice, there was a significant increase in IgG titers against all four OAg delivered by the vaccine after the second dose (Figure 1). Rats exhibited a similar response, with a noticeable increase in IgG levels post-dose 2 (Figure 3). Conversely, in rabbits, a boost effect was evident for *S. flexneri* OAg only at the lower and intermediate total OAg doses tested, and there was no significant change in IgG response against *S. sonnei* OAg between the first and second vaccination, except for the lower total OAg dose tested (Figure 5). In humans, although there was a slight numerical increase in IgG levels between post-dose 1 and post-dose 2, initial immunogenicity results from healthy European individuals indicated that altSonflex1-2-3 successfully elicited OAg-specific total IgG levels and bactericidal titers against the four vaccine components after a single immunization, and that the second immunization restored the immune response reached after the first one (22). Despite in animals a more pronounced boost effect was observed in most of the cases respect to what observed in humans, it is important to emphasize that a single immunization with altSonflex1-2-3 consistently induced already a strong primary immune response in all tested systems.

To evaluate the effect of different antigen doses, OAg-specific IgG and functional antibody activity were measured in animals following administration of three increasing doses of the vaccine (scaled by animal weight relative to the full human dose). Overall, the results indicate that significant dose-response correlations were antigen/model dependent. For example, in mice a significant dose-response was observed post-dose 1 for IgG against *S. flexneri* 2a and 3a OAg, with a corresponding trend in bactericidal activity against *S. flexneri* 2a OAg (Table 1). In rats, significant dose-response increases were recorded for *S. flexneri* 1b and 2a OAg post-dose 1, whereas after the second immunization a dose-response remained only for *S. flexneri* 2a and SBA data did not reveal consistent trends (Table 1). In rabbits, dose-response relationships were more apparent: OAg-specific IgG correlated with dose for *S. flexneri* 2a and 3a OAg after the first dose,

although after the second dose the effect persisted only for *S. flexneri* 3a. SBA responses in rabbits showed a clear dose-dependent increase against all three *S. flexneri* strains (Table 1). Overall, although not uniformly observed, these findings highlight species- and antigen-specific dose dependencies, with *S. flexneri* 2a emerging as the antigen most consistently showing a dose-response relationship across models. Importantly, across all three animal models the highest vaccine dose administered occasionally resulted in reduced OAg-specific IgG, most markedly against *S. sonnei* OAg, suggesting a ‘hook effect’. In the altSonflex1-2-3 Phase II trials, three different total OAg doses are being evaluated in infants, the target population. This will allow the comparison of preclinical results presented here with the immune responses observed in infants, thereby determining whether the dose-response behavior observed in animal models is recapitulated in humans. Previous insights from clinical studies using the *S. sonnei* 1790GAHB monocomponent vaccine in European adults showed that there was a significant correlation between dose and antibody response on day 85, i.e. 28 days post 3<sup>rd</sup> vaccination (Spearman rank  $\rho = 0.529$ ,  $P = 0.00013$ ). The response peaked with the 1.5  $\mu\text{g}$  OAg dose, and the antibody responses to the 1.5, 2.9 and 5.9  $\mu\text{g}$  doses were not significantly different (45). In African adults, antibody levels increased already post-first vaccination: 2.10- and 4.43-fold from baseline values in the 1.5 and 5.9 OAg  $\mu\text{g}$  groups, respectively, but no significant increase was observed after second injection (46).

All the analyses presented in this work were conducted using two principal immunological readouts: OAg-specific IgG measured by ELISA and functional activity measured by SBA. These same readouts have been adopted in the Phase I trial in European adults as the primary criteria to evaluate immunogenicity (22), and will also serve as the primary endpoints in the ongoing Phase II trials. Previous studies have demonstrated a correlation between anti-*S. sonnei* OAg IgG levels and bactericidal activity in subjects vaccinated with the *S. sonnei* (1790GAHB) monocomponent candidate vaccine (47). Similar findings were observed in mice immunized with the same candidate, where bactericidal activity was predominantly associated with the IgG1 and IgG3 subclasses (48). In the current study, we extended these observations by examining not only the response to *S. sonnei* but also to *S. flexneri* 1b, 2a, and 3a serotypes in the 4-component formulation. Furthermore, this analysis spanned three different animal models (mouse, rat, and rabbit) using a range of different doses of altSonflex1-2-3. Results obtained in this work show that while mice exhibit moderate correlations for certain serotypes, rabbits consistently demonstrate

strong correlations between IgG levels and bactericidal activity across multiple serotypes. Rats, however, display less consistent associations, highlighting also in this case clear species-dependent differences. Interestingly, in European adults who received altSonflex1-2-3 at a dose of 60 µg of total OAg, the results indicate a less pronounced association between OAg-specific IgG levels and bactericidal activity (22) compared to observations in mouse and rabbit models. In contrast, the responses observed in rats align more closely with the Phase I outcomes, further emphasizing the potential translational relevance of the rat model for this vaccine. The correlation between OAg-specific responses to the altSonflex1-2-3 vaccine antigens and bactericidal activity will be also investigated in subjects enrolled in the Phase II trial, this analysis will further help to determine which of the tested animal models best reflects the correlation observed in the vaccine target population.

Finally, in this work the homogeneity of the immune response among the four antigens was evaluated in the three animal models and the results were compared with clinical results from European adults from Phase I clinical trial. Indeed, from the Phase I clinical trial in which subjects were vaccinated with altSonflex1-2-3 at a dose of 60 µg of total OAg, a greater immune response was observed both in terms of OAg-specific IgG titers and bactericidal activity of the serum antibodies against *S. sonnei* and *S. flexneri* 2a, compared to *S. flexneri* 1b and *S. flexneri* 3a (22). Among animals, data revealed that the homogeneity of the antigen-specific responses varied among species. The rat model most closely reflected the clinical pattern with higher responses to *S. sonnei* and *S. flexneri* 2a, both post first and second vaccination (Figure 7). Indeed, rats have been extensively utilized in vaccine research to study immune responses (49-52). Supplementary Figures 1 and 2 indicate that the overall homogeneity of the response to the four antigens is also maintained at different doses of total OAg administered in each of the three animal models. The observed variations across the immune response to the four different serotypes may be due to factors such as negative immunointerference, where the high total OAg dose and the corresponding high protein content may have led to carrier suppression, particularly for *S. flexneri* 1b and 3a. This phenomenon was already observed for protein-based and glycoconjugate vaccines (53, 54). It is also important to highlight that some of the discrepancies observed between pre-clinical animal models and clinical human results can be also attributed to baseline differences in IgG titers. Animals exhibit no detectable baseline levels of *Shigella* OAg-specific IgG titers, whereas European adults in our study showed modest baseline titers, particularly against *S. flexneri* 1b and

*S. flexneri* 3a (22). Future clinical data in the target population could provide a more comprehensive comparison (22). Indeed, the immunogenic response in infants could represent differences compared to adults due to developmental variations in the immune system across age groups.

This study represents a rare exploration of the immune response elicited by a vaccine candidate across various animal models. Broadening research of this nature to include diverse platforms and pathogens has the potential to yield important information about the most suitable animal models to predict human results and for accelerating vaccine design and development ahead of clinical trials.

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## **Authors contributions**

Conceived and designed the experiments: all authors; Performed the experiments: V.C., F.Ma., O.R., R.A., M.P., R.D.B.; Analyzed the data: V.C., F.Ma., O.R., C.G., F.Mi.; Contributed to the writing of the manuscript: all authors. All authors have read and agreed to the published version of the manuscript and take accountability for all aspects of the work.

## **Data availability**

All data associated with this study are present in the paper. Requests for resources, data and reagents should be directed to the corresponding author.

## **Competing Interests**

V. Caradonna is a student at the University of Siena and participates in a post-graduate studentship program at GSK. All other authors are currently employed by the GSK group of companies. The authors declare no other financial or non-financial competing interests.

## Supplementary material

*Supplementary Table 1. Spearman correlation tabular results (Spearman  $r$  with their associated 2-sided 95% confidence intervals; two- tailed  $-value$ ) for OAg-specific IgG dose-response evaluation in mice.*

|                                | <i>S. sonnei</i><br>PD1 | <i>S. flexneri</i><br>1b PD1 | <i>S. flexneri</i><br>2a PD1 | <i>S. flexneri</i><br>3a PD1 | <i>S. sonnei</i><br>PD2 | <i>S. flexneri</i><br>1b PD2 | <i>S. flexneri</i><br>2a PD2 | <i>S. flexneri</i><br>3a PD2 |
|--------------------------------|-------------------------|------------------------------|------------------------------|------------------------------|-------------------------|------------------------------|------------------------------|------------------------------|
| <b>Spearman <math>r</math></b> | -0,1698                 | 0,01888                      | 0,4860                       | 0,5691                       | -0,2594                 | -0,07075                     | 0,6650                       | 0,07075                      |
| <b>95% confidence interval</b> | -0,5078 to 0,2135       | -0,3535 to 0,3861            | 0,1415 to 0,7255             | 0,2522 to 0,7757             | -0,5742 to 0,1222       | -0,4294 to 0,3072            | 0,3914 to 0,8306             | -0,3072 to 0,4294            |
| <b>P value (two-tailed)</b>    | 0,3697                  | 0,9211                       | 0,0065                       | 0,0010                       | 0,1663                  | 0,7103                       | <0,0001                      | 0,7103                       |
| <b>P value summary</b>         | ns                      | ns                           | **                           | **                           | ns                      | ns                           | ****                         | ns                           |

*Supplementary Table 2. Spearman correlation tabular results (Spearman  $r$  with their associated 2-sided 95% confidence intervals;  $P$ -value) for bactericidal activity of antibodies ( $IC^{50}$ ) dose-response evaluation in mice.*

|                                | <i>S. sonnei</i>  | <i>S. flexneri</i><br>1b | <i>S. flexneri</i><br>2a | <i>S. flexneri</i><br>3a |
|--------------------------------|-------------------|--------------------------|--------------------------|--------------------------|
| <b>Spearman <math>r</math></b> | 0,04245           | 0,08491                  | 0,6698                   | -0,02358                 |
| <b>95% confidence interval</b> | -0,3327 to 0,4060 | -0,2943 to 0,4410        | 0,3986 to 0,8332         | -0,3901 to 0,3494        |
| <b>P value (two-tailed)</b>    | 0,8237            | 0,6555                   | <0,0001                  | 0,9016                   |
| <b>P value summary</b>         | ns                | ns                       | ****                     | ns                       |

Supplementary Table 3. Spearman correlation tabular results (Spearman *r* with their associated 2-sided 95% confidence intervals; *P*-value) for OAg-specific IgG dose-response evaluation in rats.

|                                | <i>S. sonnei</i><br>D27 | <i>S. flexneri</i><br>1b D27 | <i>S. flexneri</i><br>2a D27 | <i>S. flexneri</i><br>3a D27 | <i>S. sonnei</i><br>D42 | <i>S. flexneri</i><br>1b D42 | <i>S. flexneri</i><br>2a D42 | <i>S. flexneri</i><br>3a D42 |
|--------------------------------|-------------------------|------------------------------|------------------------------|------------------------------|-------------------------|------------------------------|------------------------------|------------------------------|
| <b>Spearman r</b>              | -0,08847                | 0,7007                       | 0,5234                       | 0,2875                       | -0,08109                | -0,1548                      | 0,4497                       | -0,1843                      |
| <b>95% confidence interval</b> | -0,4846 to 0,3378       | 0,4039 to 0,8640             | 0,1398 to 0,7704             | -0,1435 to 0,6268            | -0,4789 to 0,3444       | -0,5345 to 0,2769            | 0,04395 to 0,7281            | -0,5558 to 0,2486            |
| <b>P value (two-tailed)</b>    | 0,6810                  | 0,0001                       | 0,0087                       | 0,1731                       | 0,7064                  | 0,4701                       | 0,0275                       | 0,3886                       |
| <b>P value summary</b>         | ns                      | ***                          | **                           | ns                           | ns                      | ns                           | *                            | ns                           |

Supplementary Table 4. Spearman correlation tabular results (Spearman *r* with their associated 2-sided 95% confidence intervals; *P*-value) for bactericidal activity of antibodies (IC<sub>50</sub>) dose-response evaluation in rats.

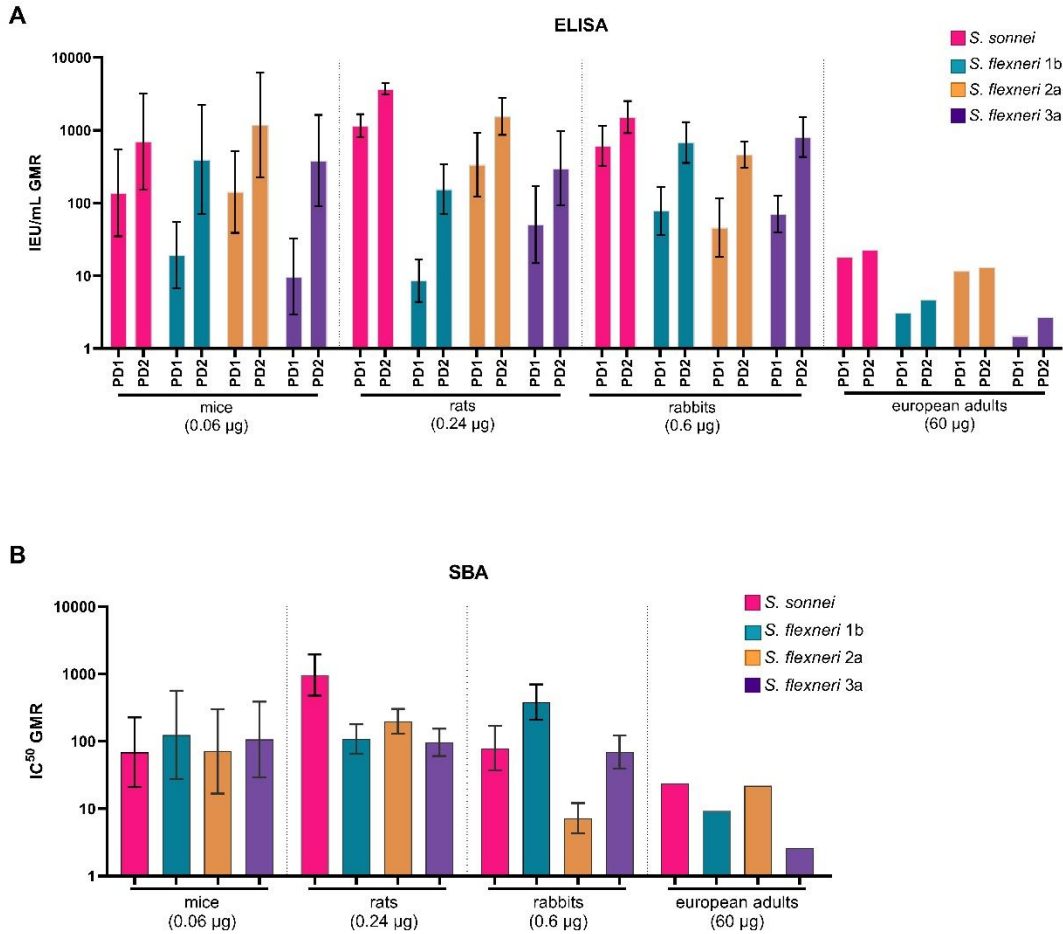
|                                | <i>S. sonnei</i>  | <i>S. flexneri</i><br>1b | <i>S. flexneri</i><br>2a | <i>S. flexneri</i><br>3a |
|--------------------------------|-------------------|--------------------------|--------------------------|--------------------------|
| <b>Spearman r</b>              | -0,007372         | -0,1474                  | 0,3907                   | 0,05162                  |
| <b>95% confidence interval</b> | -0,4200 to 0,4078 | -0,5291 to 0,2838        | -0,02769 to 0,6926       | -0,3702 to 0,4558        |
| <b>P value (two-tailed)</b>    | 0,9727            | 0,4918                   | 0,0591                   | 0,8107                   |
| <b>P value summary</b>         | ns                | ns                       | ns                       | ns                       |

Supplementary Table 5. Spearman correlation tabular results (Spearman *r* with their associated 2-sided 95% confidence intervals; *P*-value) for OAg-specific IgG dose-response evaluation in rabbits.

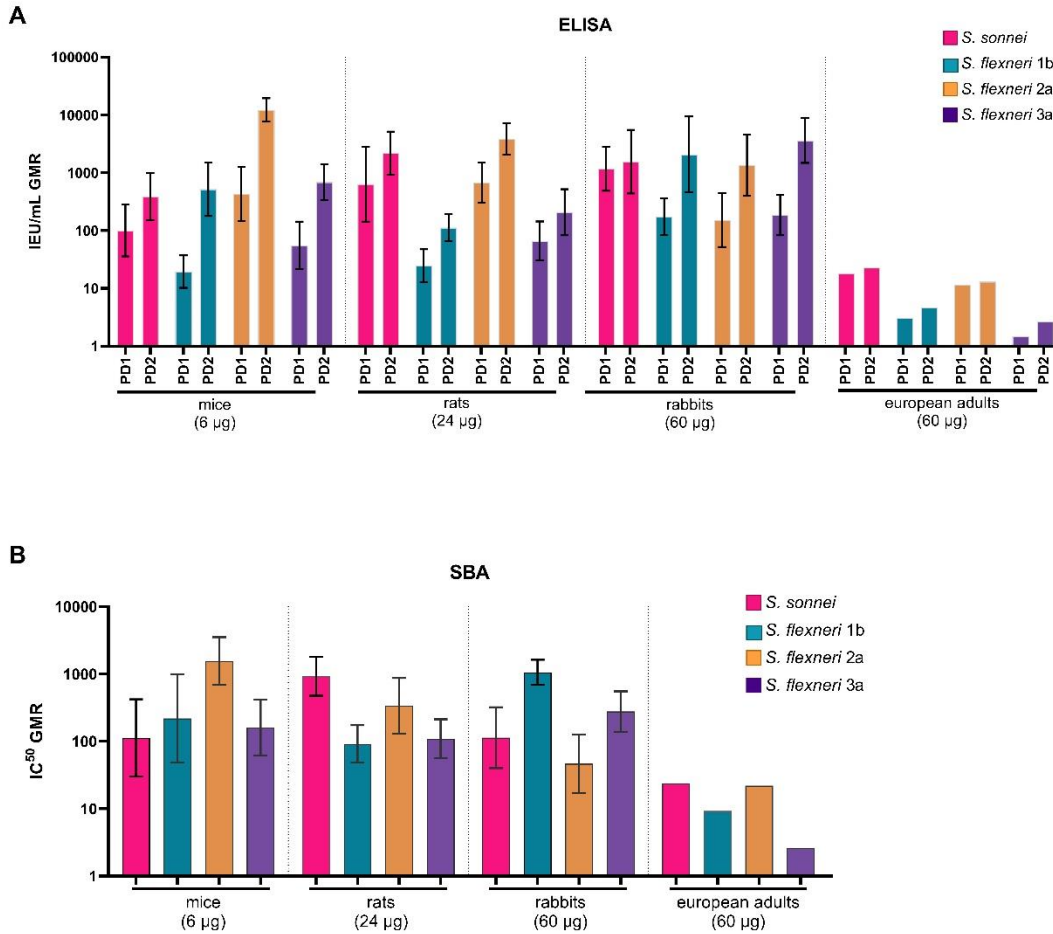
|                                | <i>S. sonnei</i><br>PD1 | <i>S. flexneri</i><br>1b PD1 | <i>S. flexneri</i><br>2a PD1 | <i>S. flexneri</i><br>3a PD1 | <i>S. sonnei</i><br>PD2 | <i>S. flexneri</i><br>1b PD2 | <i>S. flexneri</i><br>2a PD2 | <i>S. flexneri</i><br>3a PD2 |
|--------------------------------|-------------------------|------------------------------|------------------------------|------------------------------|-------------------------|------------------------------|------------------------------|------------------------------|
| <b>Spearman r</b>              | 0,3846                  | 0,3911                       | 0,6574                       | 0,7003                       | -0,05067                | 0,4781                       | 0,4352                       | 0,7263                       |
| <b>95% confidence interval</b> | -0,1331 to 0,7374       | -0,1256 to 0,7409            | 0,2439 to 0,8686             | 0,3172 to 0,8869             | -0,5299 to 0,4531       | -0,01877 to 0,7856           | -0,07283 to 0,7640           | 0,3640 to 0,8977             |
| <b>P value (two-tailed)</b>    | 0,1276                  | 0,1209                       | 0,0054                       | 0,0025                       | 0,8464                  | 0,0542                       | 0,0821                       | 0,0015                       |
| <b>P value summary</b>         | ns                      | ns                           | **                           | **                           | ns                      | ns                           | ns                           | **                           |

*Supplementary Table 6. Spearman correlation tabular results (Spearman r with their associated 2-sided 95% confidence intervals; P-value) for bactericidal activity of antibodies (IC50) dose-response evaluation in rabbits.*

|                                        | <b>S. sonnei</b>     | <b>S.<br/>flexneri<br/>1b</b> | <b>S.<br/>flexneri<br/>2a</b> | <b>S.<br/>flexneri<br/>3a</b> |
|----------------------------------------|----------------------|-------------------------------|-------------------------------|-------------------------------|
| <b>Spearman r</b>                      | 0,1936               | 0,7081                        | 0,5847                        | 0,7575                        |
| <b>95%<br/>confidence<br/>interval</b> | -0,3304 to<br>0,6263 | 0,3311 to<br>0,8901           | 0,1295 to<br>0,8363           | 0,4226 to<br>0,9103           |
| <b>P value<br/>(two-tailed)</b>        | 0,4527               | 0,0022                        | 0,0157                        | 0,0007                        |
| <b>P value<br/>summary</b>             | ns                   | **                            | *                             | ***                           |



*Supplementary Figure 1. Comparison of humoral immune responses after vaccination with altSonflex1-2-3 between mice (0.06 µg tot. OAg dose), rats (0.24 µg tot. OAg dose), rabbits (0.6 µg tot. OAg dose) and European adults in terms of: a: S. sonnei and S. flexneri 1b, 2a and 3a OAg-specific IgG titers post-dose 1 (PD1) and post-dose 2 (PD2). Geometric mean ratio over the baseline values (bars) for all the groups and 95% CI (error bars) for the preclinical studies groups are reported; b: IC<sub>50</sub> of bactericidal activity against S. sonnei and S. flexneri 1b, 2a and 3a strains. Geometric mean ratio over the baseline values (bars) for all the groups and 95% CI (error bars) for the preclinical studies groups are reported.*



Supplementary Figure 2. Comparison of humoral immune responses after vaccination with altSonflex1-2-3 between mice (6 µg tot. OAg dose), rats (24 µg tot. OAg dose), rabbits (60 µg tot. OAg dose) and European adults in terms of: a: *S. sonnei* and *S. flexneri* 1b, 2a and 3a OAg-specific IgG titers post-dose 1 (PD1) and post-dose 2 (PD2). Geometric mean ratio over the baseline values (bars) for all the groups and 95% CI (error bars) for the preclinical studies groups are reported; b: IC50 of bactericidal activity against *S. sonnei* and *S. flexneri* 1b, 2a and 3a strains. Geometric mean ratio over the baseline values (bars) for all the groups and 95% CI (error bars) for the preclinical studies groups are reported.

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**CHAPTER 4. High-throughput luminescence-based serum bactericidal assay optimization and characterization to assess human sera functionality against multiple *Shigella flexneri* serotypes**

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Article

# High-Throughput Luminescence-Based Serum Bactericidal Assay Optimization and Characterization to Assess Human Sera Functionality Against Multiple *Shigella flexneri* Serotypes

Valentina Caradonna <sup>1,2</sup> , Marika Pinto <sup>2</sup>, Renzo Alfini <sup>2</sup> , Carlo Giannelli <sup>2</sup> , Miren Iturriza <sup>2</sup>, Francesca Micoli <sup>2</sup>, Omar Rossi <sup>2</sup> and Francesca Mancini <sup>2,\*</sup>

<sup>1</sup> Laboratory of Molecular Microbiology and Biotechnology, Department of Medical Biotechnologies, University of Siena, 53100 Siena, Italy; v.caradonna@student.unisi.it

<sup>2</sup> GSK Vaccines Institute for Global Health (GVGH), 53100 Siena, Italy; renzo.x.alfini@gsk.com (R.A.); carlo.x.giannelli@gsk.com (C.G.)

\* Correspondence: francesca.x.mancini@gsk.com

**Abstract:** Shigellosis represents a significant global health concern particularly affecting children under 5 years in low- and middle-income countries (LMICs) and is associated with stunting and antimicrobial resistance. There is a critical need for an effective vaccine offering broad protection against the different *Shigella* serotypes. A correlate of protection has not yet been established but there is a general consensus about the relevant role of anti-O-Antigen-specific IgG and its functionality evaluated by the Serum Bactericidal Assay (SBA). This study aims to characterize a high-throughput luminescence-based SBA (L-SBA) against seven widespread *Shigella* serotypes. The assay was previously developed and characterized for *S. sonnei* and *S. flexneri* 1b, 2a, and 3a and has now been refined and extended to an additional five serotypes (*S. flexneri* 4a, 5b, 6, X, and Y). The characterization of the assay with human sera confirmed the repeatability, intermediate precision, and linearity of the assays; both homologous and heterologous specificity were verified as well; finally, limit of detection and quantification were established for all assays. Moreover, different sources of baby rabbit complement showed to have no impact on L-SBA output. The results obtained confirm the possibility of extending the L-SBA to multiple *Shigella* serotypes, thus enabling analysis of the functional response induced by natural exposure to *Shigella* in epidemiological studies and the ability of candidate vaccines to elicit cross-functional antibodies able to kill a broad panel of prevalent *Shigella* serotypes in a complement-mediated fashion.

**Keywords:** functional assay; SBA; human serum; vaccine; *Shigella*



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## 1. Introduction

*Shigella*, identified in the World Health Organization's list of antibiotic-resistant "priority pathogens" [1], is a bacterial genus consisting of four serogroups (*S. sonnei*, *S. flexneri*, *S. dysenteriae*, and *S. boydii*)

and over 50 serotypes [2,3]. This human pathogen gives rise to shigellosis, an acute infection of the small intestine transmitted through the oro-fecal route [4,5] that is a prevalent factor in cases of diarrhoea within low- and middle-income countries [6]. Recurrent infections result in impaired cognitive development, stunted growth, and a diminished life expectancy [7–10]. Shigellosis contributes significantly to global morbidity and mortality, particularly affecting children under 5 years of age [11], with recent data indicating 64,000 deaths in 2016 [12]. No vaccine is yet available against *Shigella*, and the challenge of shigellosis has been further complicated by the emergence of antibiotic-resistant strains [13–20]. The primary serotype accountable for shigellosis in developing nations is *S. flexneri*, with *S. sonnei* being the predominant cause of shigellosis in developed countries [21,22].

The O-Antigen (O-Ag) portion of the membrane lipopolysaccharides of the bacteria determines the differentiation between the different *Shigella* serotypes [23] and is acknowledged as the key antigen for developing protective immunity against shigellosis [24–26].

Indeed, the majority of the vaccines against *Shigella* that are currently being developed target the O-Ag. Findings from a Global Enteric Multicenter Study [27] suggested that a vaccine targeting four strains, including the most common serotypes *S. sonnei* and *S. flexneri* 2a, 3a, 6, or 1b, would directly protect against approximately 72% of circulating species and up to 89% when considering cross-protection [28]. Vaccine coverage may increase due to the cross-reactivity between different *Shigella flexneri* strains [29,30]. O-Ag-based *Shigella* vaccine candidates are either in Phase II or initiated Phase III clinical trials [31]. Among them, a four-component formulation based on Generalized Modules of Membrane Antigens (GMMA) has been proposed, targeting *S. sonnei* and *S. flexneri* 1b, 2a, and 3a serotypes [32–34], and is currently being tested in a Phase I/II clinical trial ([ClinicalTrials.gov](https://clinicaltrials.gov) number NCT05073003). Stage 1 of the clinical trial, performed in European adults, has been completed, showing the ability of the vaccine to induce good anti-O-Ag-specific IgG bactericidal against the serotypes included in the vaccine [35]. Other multivalent candidate vaccines advanced in clinical development include the quadricomponent *Shigella* bioconjugate vaccine, which carries the O-Ag of *S. flexneri* serotypes 2a, 3a, 6, and *S. sonnei* bioconjugated to the recombinant *Pseudomonas aeruginosa* exoprotein A carrier protein, currently in a Phase II study [36], and the bivalent O-Ag glycoconjugate targeting *S. sonnei* and *S. flexneri* 2a, currently in Phase III [37]. Another candidate vaccine against *Shigella flexneri* 2a is the monovalent carbohydrate-based synthetic conjugate developed by Institut Pasteur, which has demonstrated the ability to elicit a durable immune response in adults [38].

Besides the fact that a definitive correlate of protection against shigellosis has not been established, the measurement of antigen-specific IgG is traditionally used as the primary measure of vaccine immunogenicity [24,26]. Nevertheless, assessing the functionality of antibodies induced by the vaccine in vitro using the Serum Bactericidal Assay (SBA) might serve as an important predictor of efficacy against bacterial pathogens [39,40]. Specifically, concerning *Shigella*, there has been considerable speculation regarding the important role of SBA in providing a more comprehensive characterization of the functional immune response [41,42].

We have developed a high-throughput serum bactericidal assay based on the luminescence readout (L-SBA), which has been already applied to evaluate serum antibody functionality against *S. sonnei* in multiple clinical trials [43,44]. The assay has been also characterized against *S. flexneri* 2a, 1b, and 3a and has been utilized to analyze samples from the first stage of the GMMA four-component vaccine Phase I/II clinical trial, which was conducted in healthy European adults [35]. High bactericidal titers against *S. flexneri* 1b and 3a (numerically higher than those observed against *S. sonnei* and *S. flexneri* 2a) have been observed at baseline in these subjects.

Moreover, in order to assess potential vaccine coverage, it is important to evaluate the vaccine's capacity to provide broad bactericidal activity against a larger panel of *S. flexneri* serotypes not included in the vaccine [32,45]. Indeed, *S. flexneri* presents an extensive serotype diversity, determined by variation in the O-Ag structure [23], with over 19 known serotypes reported.

In addition to its use in evaluating vaccine coverage, the L-SBA could also be a valuable tool for public health surveillance in *Shigella*-endemic regions. By enabling the screening of sera for a broad range of *Shigella* serotypes, the assay could provide crucial insights into the serotype distribution in these populations. This information could then inform the development of targeted vaccination strategies.

In this work, we present the characterization of the L-SBA with human sera to other prevalent *S. flexneri* serotypes (*S. flexneri* 4a, 5b, 6, X, and Y) and the refinement of the assays against different *S. flexneri* 1b and 3a isolates compared to the ones tested previously [46]. The characterization comprises the repeatability, intermediate precision, linearity, specificity, and detection and quantification limits of the assay.

## 2. Results

### 2.1. Setup of L-SBA Conditions on Human Sera Against *S. flexneri* 4a, 5b, 6, X, and Y Strains

One of the aims of this study was to characterize the L-SBA to evaluate human serum bactericidal activity against *S. flexneri* 4a, 5b, 6, X, and Y strains. The assay conditions, such as the percentage of baby rabbit complement (BRC) and assay buffer established in the preclinical stage for *S. flexneri* 4a, 5b, 6, X, and Y strains [32,45], were applied to L-SBA to test the clinical samples and were found to be optimal for assessing human serum bactericidal activity based on the dose–response curve obtained by probing the human standard serum against each strain (Figure 1).

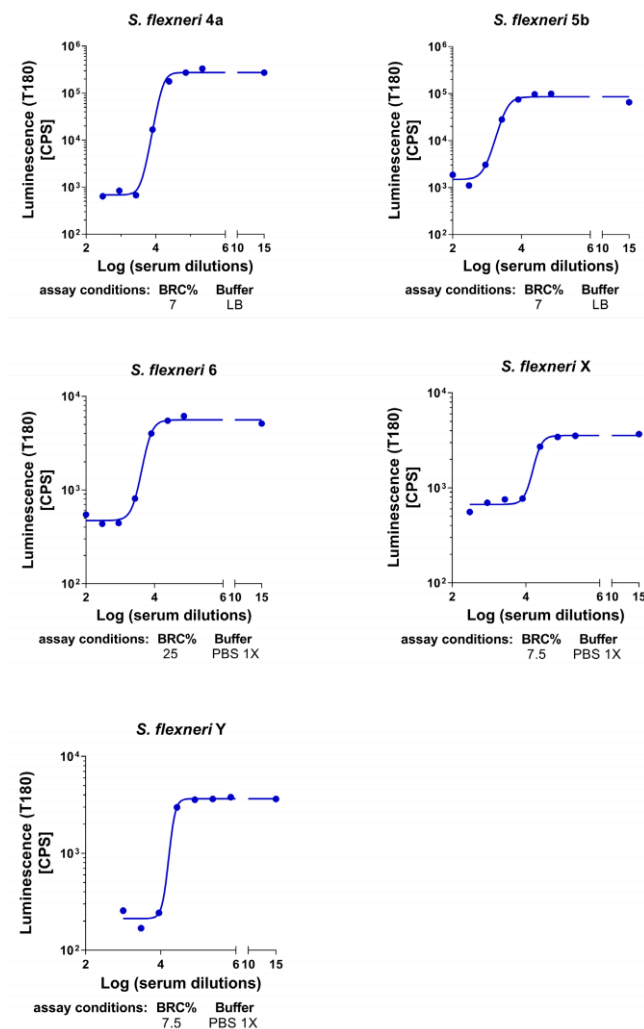


**Figure 1.** Human standard serum assessed in L-SBA against *S. flexneri* 4a, 5b, 6, X, and Y using the specified assay conditions. 4PL fitting of luminescence at T180 (measured as CPS, counts per second) vs. log-transformed serum dilution. BRC: baby rabbit complement; LB: Luria Bertani medium; PBS: phosphate-buffered saline.

### 2.2. Optimization of L-SBA on Human Sera Against *S. flexneri* 1b and 3a

The characterization of the assay against *S. flexneri* 1b and 3a was previously conducted [46] using *S. flexneri* 1b (Stansfield NTCT 5 strain) and *S. flexneri* 3a (6885 strain). However, in the SBA analysis of sera from the NCT05073003 clinical trial, high SBA titers were observed at baseline against *S. flexneri* 1b and 3a strains [35], and this was hypothesized to be due to the sensitivity of the strains used. Consequently, here, alternative *S. flexneri* 1b and 3a strains from Public Health England (PHE) were screened using pooled samples from individuals enrolled in the NCT05073003 clinical trial and collected at baseline (visit 1) or after dose 1 (visit 4). Among the tested *S. flexneri* 1b (NCTC9722, NCTC5, NCTC14120,

NCTC8517, and NCTC14154) and *S. flexneri* 3a (NCTC9724, NCTC9989, NCTC7, NCTC9782, and NCTC9783) strains, *S. flexneri* 1b NCTC5 and *S. flexneri* 3a NCTC9989 showed lower SBA titers at baseline. The optimal assay conditions were identified for the two new strains: the selected BRC percentage was 30% for *S. flexneri* 1b and 20% for *S. flexneri* 3a. Baseline titers were further assessed using sera from 20 subjects from the NCT05073003 clinical trial and *S. flexneri* 1b and 3a strains previously used or newly acquired from PHE. Results obtained from running SBA with the *S. flexneri* 1b (Stansfield NTCT 5 strain) and *S. flexneri* 3a (6885 strain) showed that a substantial proportion of subjects had elevated baseline titers (above 5000) against *S. flexneri* 1b (> 70%) and *S. flexneri* 3a (> 50%). In contrast, when the assay was performed using the new strains, less than 20% of tested subjects had elevated baseline titers (above 5000) against *S. flexneri* 1b and no subjects against *S. flexneri* 3a. Baseline SBA titers observed when running the L-SBA with the newly acquired strains were found to be comparable to those observed against *S. flexneri* 2a (Figure 2).

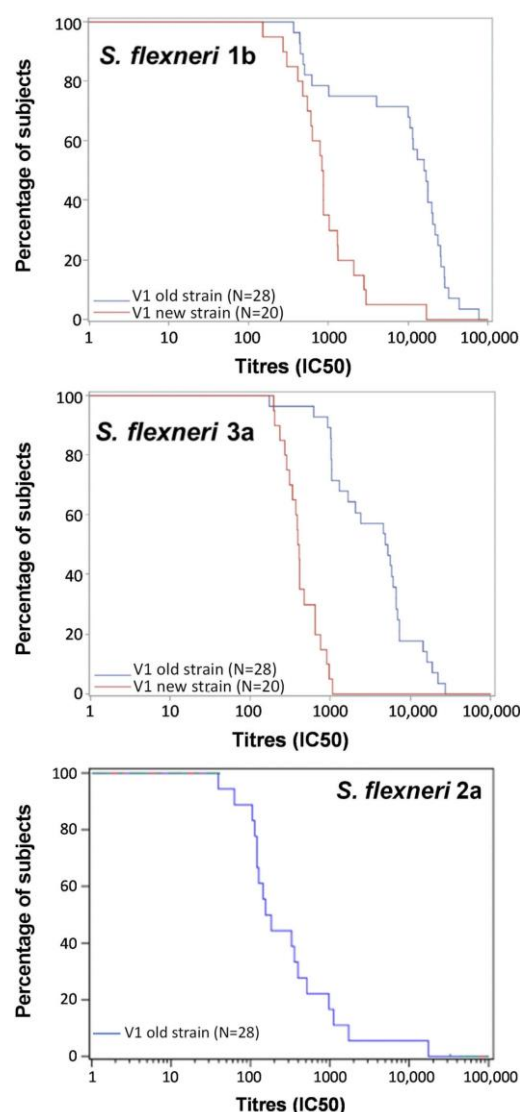


**Figure 2.** Reverse cumulative distribution plots of SBA titers observed at baseline against the newly retrieved *S. flexneri* 1b NTCT 5 strain and 3a NCTC9989 (new strains) compared to the previously used strains *S. flexneri* 1b NCTC5 Stansfield, *S. flexneri* 3a 6885 and *S. flexneri* 2a strain (old strains). SBA titers have been obtained analyzing sera from individuals enrolled in the NCT05073003 clinical trial. The blue line represents the assay conducted using the old strains, the red line represents the assay conducted using the new strains.

The same samples were then used to assess the impact of the use of a different source of BRC on L-SBA results. When Pel-Freez BRC was utilized instead of the BRC obtained from Cederlane used in all SBA experiments conducted so far, no significant differences were observed in IC<sub>50</sub> measured at visit 1 (baseline) and visit 4 (post-dose 1) against *S. flexneri* 1b, whereas IC<sub>50</sub> values changed for *S. flexneri* 3a but no impact on fold-change over baseline was observed (Figure 3).

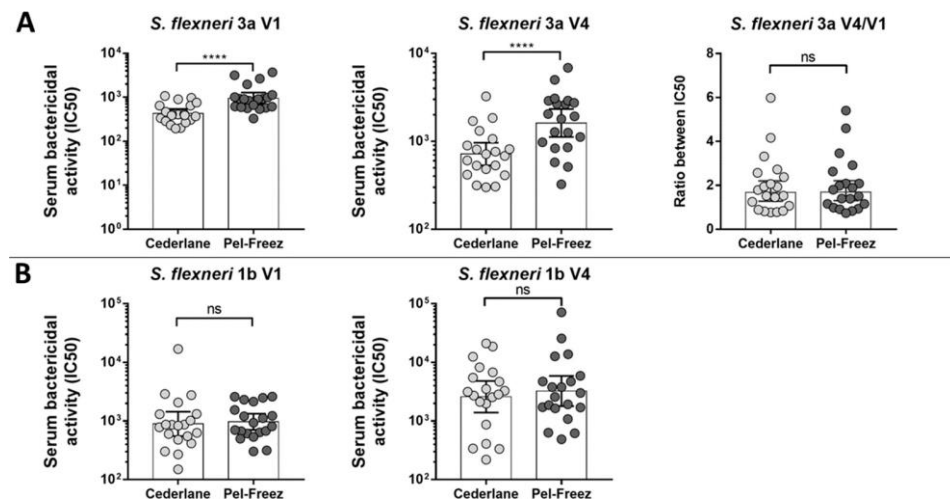


**Figure 3.** Comparison of bactericidal titers obtained in SBA using Cederlane or Pel-Freez baby rabbit complement for *S. flexneri* 3a (A) and *S. flexneri* 1b (B) both at visit 1 (V1) and at visit 4 (V4) and comparison between results obtained for samples at the two tested time points (V4/V1). ns,  $p > 0.05$ ; \*\*\*\*,  $p \leq 0.0001$ , Wilcoxon matched-pairs signed rank test.

### 2.3. L-SBA Characterization Against *S. flexneri* Serotypes

L-SBA assays performed using the newly selected *S. flexneri* 1b and 3a strains and *S. flexneri* 4a, 5b, 6, X and Y strains where comprehensively characterized.

#### 2.3.1. Precision

For all tested strains, precision was assessed as intermediate precision (CV% IP) and repeatability (CV% R) testing the same sera multiple times on three different days and by two operators (Figure 4).

The day and the operator, and their interaction, resulted as factors that did not significantly influence the assay's variability ( $p$ -values  $> 0.05$ ). The average LogIC<sub>50</sub> from all measurements, standard deviation, and coefficients of variation (CV%) values are reported in Table 1.

**Table 1.** Assay precision. Number of observations (n), average LogIC<sub>50</sub>, standard deviation (SD), CV% day, CV% operator, CV% day\*operator, CV% IP, and CV% R obtained in L-SBA against *S. flexneri* 4a, 5b, 6, X, and Y strains.

|                                | LogIC <sub>50</sub>   |                       |                       |                       |                      |                      |                      |
|--------------------------------|-----------------------|-----------------------|-----------------------|-----------------------|----------------------|----------------------|----------------------|
|                                | <i>S. flexneri</i> 1b | <i>S. flexneri</i> 3a | <i>S. flexneri</i> 4a | <i>S. flexneri</i> 5b | <i>S. flexneri</i> 6 | <i>S. flexneri</i> X | <i>S. flexneri</i> Y |
| n                              | 72                    | 72                    | 72                    | 72                    | 72                   | 72                   | 72                   |
| Average                        | 3.7                   | 4.4                   | 4.5                   | 3.7                   | 3.5                  | 4.2                  | 4.2                  |
| SD                             | 0.2                   | 0.1                   | 0.2                   | 0.1                   | 0.2                  | 0.1                  | 0.0                  |
| CV% day ( $p$ -value)          | 5.8 (0.191)           | 0.7 (0.418)           | 4.0 (0.181)           | 0.0 (0)               | 0.0 (0)              | 4.3 (0.16)           | 1.0 (0.253)          |
| CV% operator ( $p$ -value)     | 0.0 (0)               | 2.0 (0.289)           | 0.0 (0)               | 1.0 (0.409)           | 3.1 (0.341)          | 0.4 (0.313)          | 0.0 (0)              |
| CV% day*operator ( $p$ -value) | 3.0 (0.125)           | 1.6 (0.192)           | 1.6 (0.153)           | 2.4 (0.107)           | 4.4 (0.091)          | 0.0 (0)              | 0.8 (0.181)          |
| CV% R                          | 2.6                   | 2.2                   | 2.4                   | 3                     | 3.8                  | 1.6                  | 1.7                  |
| CV% IP                         | 7                     | 3.4                   | 4.9                   | 4                     | 6.6                  | 4.6                  | 2.1                  |

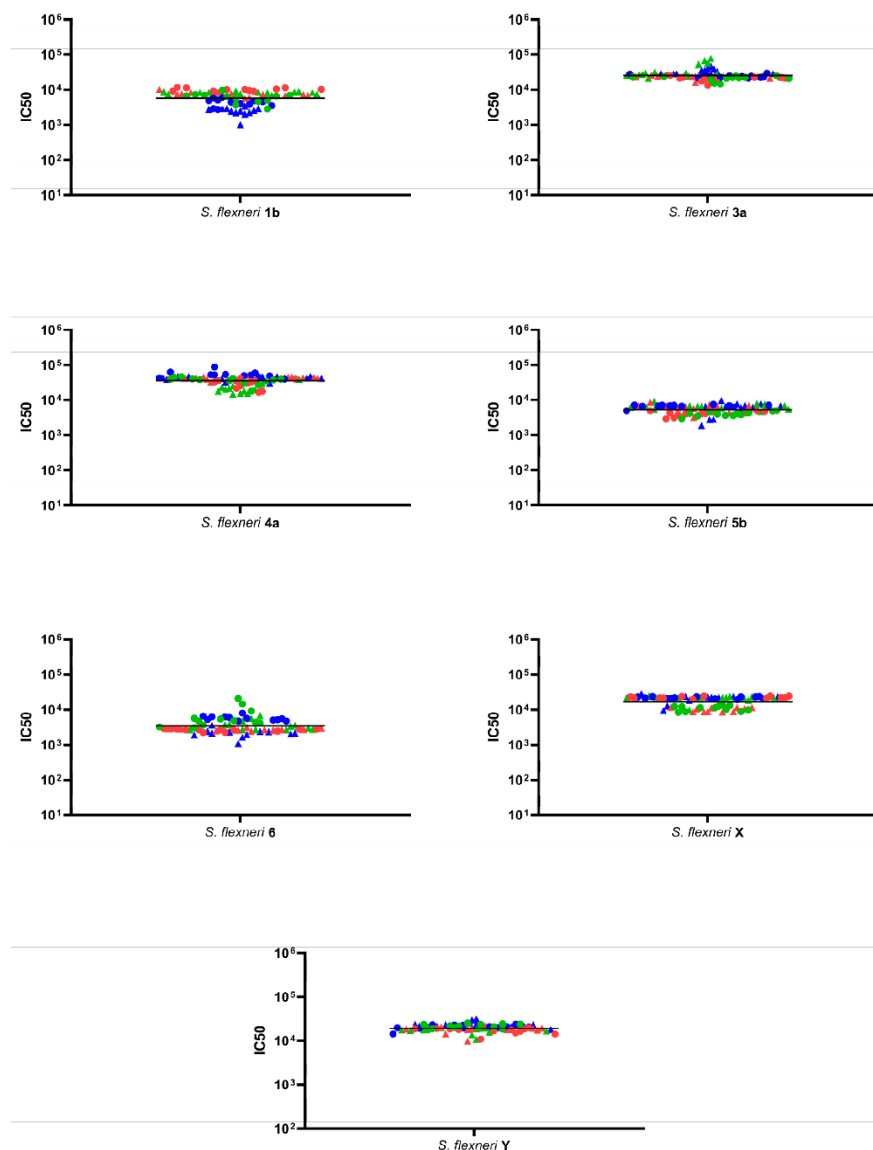


**Figure 4.** SBA titers (IC<sub>50</sub>) of standard sera tested in 12 independent replicates. The assay was conducted against the *S. flexneri* 1b, 3a, 4a, 5b, 6, X, and Y strains. Results from individual repeats by each operator are depicted as circles (for operator 1) and triangles (for operator 2), while results obtained for repeats on different days are color-coded as blue (day 1), green (day 2), and red (day 3). The black line represents the geometric mean of all values.

### 2.3.2. Linearity

In order to assess linearity, the same sample was tested at different serial dilutions. Experimental LogIC<sub>50</sub> was plotted against theoretical LogIC<sub>50</sub>, and a regression analysis was conducted (Figure 5). For each serotype tested, the linear model was significant ( $p$ -value < 0.05), the intercept (named Constant in Table 2) did not significantly differ from zero ( $p$ -value > 0.05), and the slope (named T in Table 2) was not significantly different from 1 (95% CI ranges included 1) other than for *S. flexneri* 4a and X. Indeed, only for SBA against *S. flexneri* 4a and X the intercept was significantly different from zero, and the slope for *S. flexneri* 4a was significantly higher than 1. For these two assays, the %error for the IC<sub>50</sub> values with respect to the theoretical value was calculated for all tested points and was <20%.

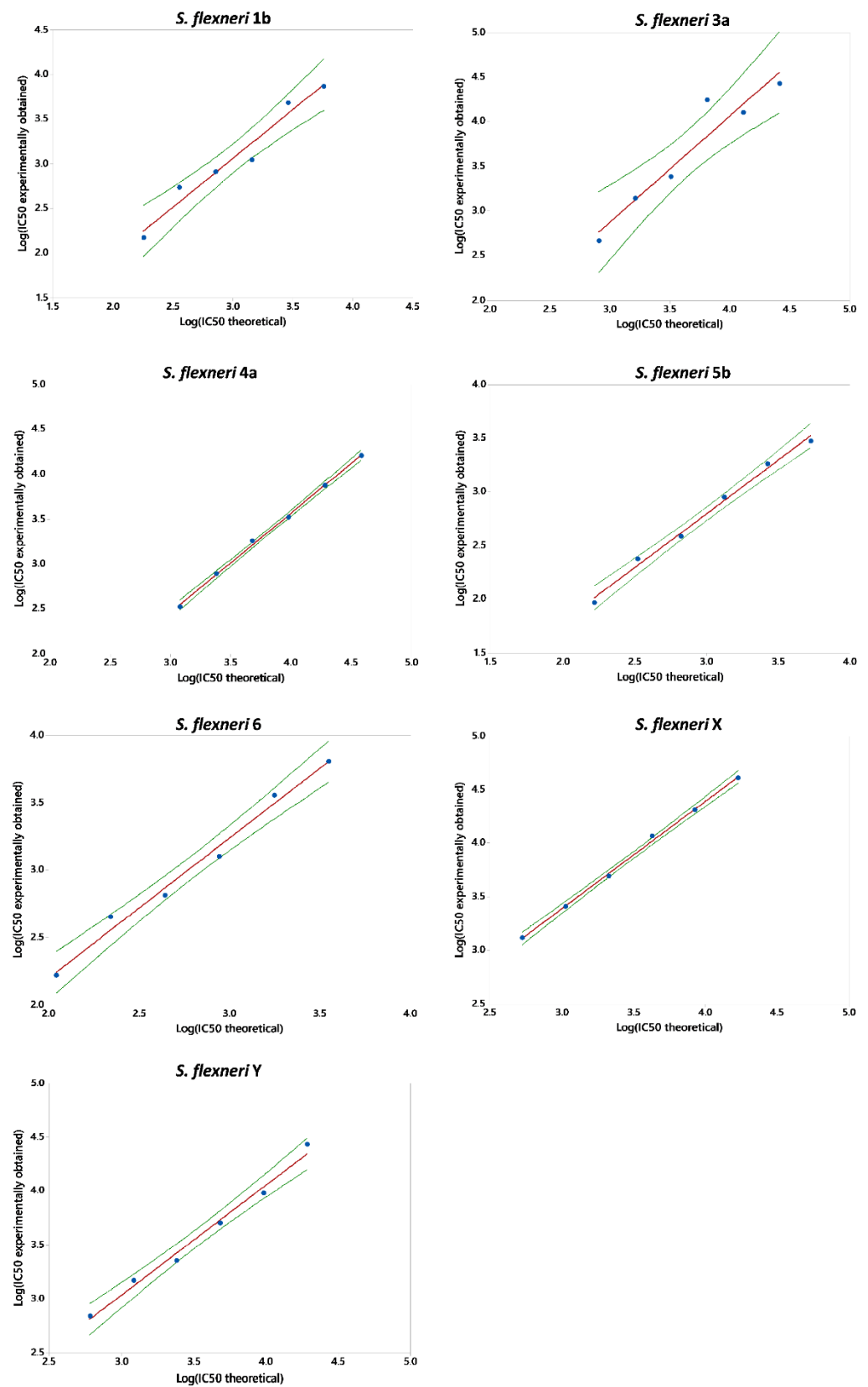


**Figure 5.** Log(IC<sub>50</sub> theoretical) obtained for each sample vs. Log (IC<sub>50</sub> experimentally obtained). Single data points are indicated with blue dots. The red solid line represents the linear regression, and the green dashed line represents the regression 95% confidence interval (CI). Data refer to L-SBA conducted against *S. flexneri* 1b, 3a, 4a, 5b, 6, X, and Y, using human standard sera.

**Table 2.** Coefficients of the regression analysis for assay linearity evaluation of each of the *S. flexneri* serotypes tested. The Constant term represents the intercept of the regression line whereas the T term represents the slope.

|                       | Term     | Coef   | 95% CI             | p-Value |
|-----------------------|----------|--------|--------------------|---------|
| <i>S. flexneri</i> 1b | Constant | −0.205 | (−1.168; 0.759)    | 0.587   |
|                       | T        | 1.088  | (0.772; 1.404)     |         |
| <i>S. flexneri</i> 3a | Constant | −0.681 | (−2.531; 1.170)    | 0.365   |
|                       | T        | 1.185  | (0.684; 1.686)     |         |
| <i>S. flexneri</i> 4a | Constant | −0.857 | (−1.0971; −0.6172) | 0.001   |
|                       | T        | 1.1057 | (1.0436; 1.1678)   |         |
| <i>S. flexneri</i> 5b | Constant | −0.208 | (−0.577; 0.162)    | 0.194   |
|                       | T        | 1.0005 | (0.8780; 1.1229)   |         |
| <i>S. flexneri</i> 6  | Constant | 0.128  | (−0.350; 0.605)    | 0.499   |
|                       | T        | 1.036  | (0.8678; 1.2041)   |         |
| <i>S. flexneri</i> X  | Constant | 0.3808 | (0.1580; 0.6037)   | 0.009   |
|                       | T        | 1.002  | (0.9386; 1.0654)   |         |
| <i>S. flexneri</i> Y  | Constant | −0.018 | (−0.589; 0.552)    | 0.933   |
|                       | T        | 1.0178 | (0.8579; 1.1777)   |         |

### 2.2.3. Specificity

To verify that the L-SBA assay was able to assess the functionality of O-Ag-specific antibodies contained in clinical serum against *S. flexneri* 4a, 5b, 6, X, Y, 1b, and 3a, both the homologous and the heterologous specificity of the assay were assessed.

The lowest homologous *S. flexneri* LPS concentration (among the ones tested) that was able to cause the highest IC<sub>50</sub> reduction in the IC<sub>50</sub> compared to the undepleted control sample was 10 µg/mL for *S. flexneri* 1b, *S. flexneri* 3a, *S. flexneri* 4a, and *S. flexneri* 6, 100 µg/mL for *S. flexneri* 5b and *S. flexneri* X, and 0.5 µg/mL for *S. flexneri* Y. These concentrations were then used to assess the heterologous specificity with the heterologous competitor from the same species (*S. sonnei* LPS) or with the heterologous competitor from a distinct species (*S. Typhimurium* O-Ag).

Depletion with the homologous competitor led to an IC<sub>50</sub> inhibition of at least 60% in all assays, confirming assay specificity. In contrast, depletion with heterologous antigens caused no decrease or a limited ( $\leq 30\%$ ) decrease in the SBA titer, indicating the absence of any non-*S. flexneri* polysaccharide-specific response in the assay (Figure 6).

### 2.3.4. Limit of Detection (LoD) and Limit of Quantification (LoQ)

The limit of detection (LoD) of the assay denotes the lowest SBA titer detectable under the assay conditions and the limit of quantification (LoQ) represents the lowest SBA titer that can be quantified with appropriate precision. To create a worst-case scenario for the assay and assess its performance under challenging conditions, the human standard serum was prediluted in order to obtain an IC<sub>50</sub> of around 100. This prediluted serum was then assayed in L-SBA against *S. flexneri* 4a, 5b, 6, X, Y, 1b, and 3a serotypes. The results obtained under these conditions are detailed in Table 3.

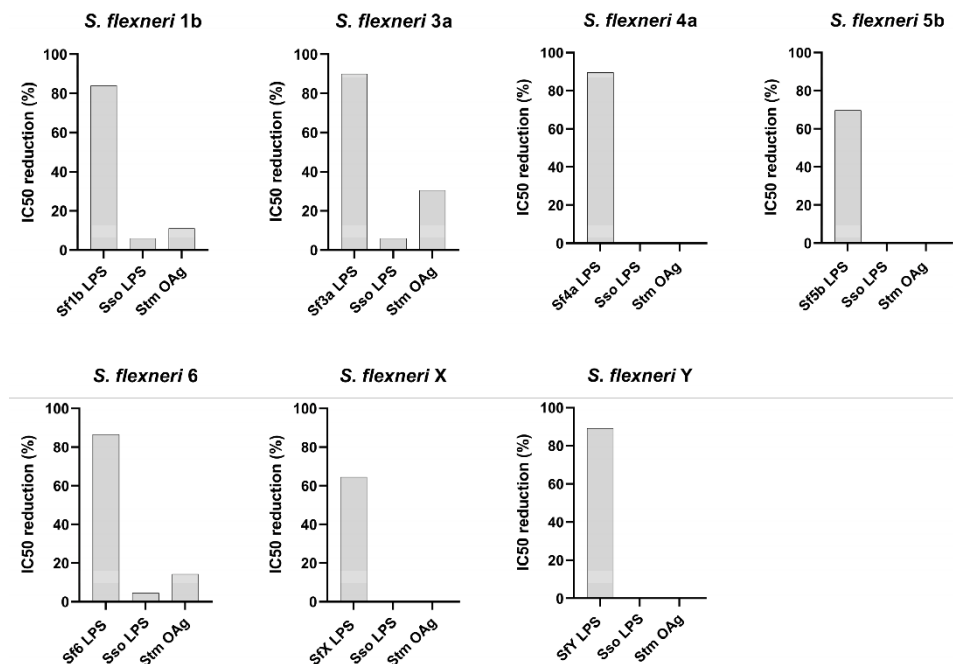


**Figure 6.** IC50 reduction (%) in L-SBA against *S. flexneri* 1b, 3a, 4a, 5b, 6, X, and Y strains compared to undepleted control samples after incubation with homologous or heterologous competitors. Sf = *S. flexneri*; Sso = *S. sonnei*; Stm = *S. Typhimurium*.

**Table 3.** LoD and LoQ (IC50) of the assay assessed by testing prediluted standard serum against each of the tested *S. flexneri* strains. Values have been calculated following the ICH guidelines Q2(R2) as detailed in Section 4.8.

|     | <i>S. flexneri</i> 1b | <i>S. flexneri</i> 3a | <i>S. flexneri</i> 4a | <i>S. flexneri</i> 5b | <i>S. flexneri</i> 6 | <i>S. flexneri</i> X | <i>S. flexneri</i> Y |
|-----|-----------------------|-----------------------|-----------------------|-----------------------|----------------------|----------------------|----------------------|
| LoD | 9                     | 7                     | 5                     | 7                     | 7                    | 10                   | 6                    |
| LoQ | 42                    | 21                    | 8                     | 26                    | 18                   | 68                   | 14                   |

### 3. Discussion

Addressing the significant disease problem of shigellosis in low- and middle-income countries through the introduction of an effective vaccine that can protect against the most widespread and virulent serotypes of *Shigella* is a pressing priority. For *Shigella*, the anti-O-Ag IgG response has been proposed as a correlate of protection [24,25]. Measuring the functionality of the sera also seems important to better characterize the response elicited by a vaccine candidate or after natural exposure to *Shigella*. The SBA is a widely used in vitro method for assessing serum antibodies' ability to kill bacteria through complement activation [40,47–49], and the luminescence-based high-throughput version of the method has been already developed in order to improve time consumption, cost effectiveness, and labor intensity. This advanced method allows us to evaluate 88 individual sera, excluding controls, per day, per operator, and can be easily adapted to a 384-well plate format to further increase the throughput [50]. This assay has been already used to test serum samples from vaccine clinical trials. This methodology was indeed already characterized in preparation for the assessment of antibody functionality in human sera elicited by a mono-component GMMA-based vaccine against *S. sonnei* [44] and a GMMA-based four-component vaccine against *Shigella* including *S. sonnei* and *S. flexneri* 1b, 2a, and 3a serotypes (ClinicalTrials.gov number NCT05073003) [35]. SBA assays against *S. flexneri* 1b and 3a strains requested further optimization in order to reduce bactericidal titers observed at baseline in European adults. Furthermore, human L-SBA characterization

against additional *Shigella* serotypes was necessary in order to evaluate the potential for vaccine cross-protection.

In this study, we described the optimization and intra-laboratory characterization of L-SBA on clinical sera against *S. flexneri* 1b and 3a strains and the intra-laboratory characterization of L-SBA against five additional *Shigella flexneri* serotypes (*S. flexneri* 4a, 5b, 6, X, and Y). Repeatability, intermediate precision, linearity, specificity, LoD, and LoQ were assessed.

SBA against all tested *S. flexneri* serotypes resulted in adequate intermediate precision (CV% IP < 7%) and repeatability (CV% R < 3.9%). The operator and the day and their interaction were confirmed to be non-significant factors in the overall variability. The linearity of the assays was confirmed within the range tested, except for *S. flexneri* 4a and X for which the % error was < 20% at all tested dilutions.

L-SBA also exhibited strong specificity, with  $\geq 60\%$  depletion of SBA titers after depletion with homologous LPS and depletion below 30% when depleted with heterologous LPS both from the same *Shigella* species (*S. sonnei* LPS) and from a different bacterial genus, *Salmonella* (*S. Typhimurium* O-Ag). LoDs and LoQs were also established to understand the lowest SBA titers that can be detected and quantified using the different assays.

#### 4. Materials and Methods

##### 4.1. Bacterial Strains and Reagents

*S. flexneri* strains were purchased from Public Health England, London, UK. Frozen 20% glycerol stocks of *S. flexneri* strains 1b (NCTC9722, NCTC5, NCTC14120, NCTC8517, and NCTC14154), *S. flexneri* 3a (NCTC9724, NCTC9989, NCTC7, NCTC9782, and NCTC9783), *S. flexneri* 4a (H130920147), *S. flexneri* 5b (H130920151), *S. flexneri* 6 (H130920152), *S. flexneri* X (H130920153), and *S. flexneri* Y (H130920154) were prepared from lyophilized cultures and stored at  $-80^{\circ}\text{C}$ .

The LPS from *S. flexneri* 1b, 3a, 4a, 5b, 6, X, Y, and O-Ag from *Salmonella Typhimurium* were extracted and analyzed for sugar content, protein, and nucleic acid impurities, as previously reported [51].

##### 4.2. Serum Samples

The human serum tested to characterize the *S. flexneri* 4a, 5b, 6, X, and Y heterologous strains was obtained by pooling high-responder sera from volunteers originally enrolled in the NCT01229176 clinical study performed in Pakistan and India, which are endemic areas for *Shigella*. High responders were selected based on their anti-*S. flexneri* 1b, 2a, and 3a O-Ag-specific IgG ELISA levels. The frozen 50  $\mu\text{L}$  working aliquots of the serum were stored at  $-80^{\circ}\text{C}$  until use.

A different human serum was used for *S. flexneri* 1b and 3a characterization obtained by pooling high-responder sera from healthy European adults enrolled in the NCT05073003 clinical trial vaccinated with GMMA from *S. sonnei* and *S. flexneri* 1b, 2a, and 3a. High responders were selected based on their anti-*S. sonnei* and *S. flexneri* 2a O-Ag-specific IgG ELISA levels. The frozen 50  $\mu\text{L}$  working aliquots of the serum were stored at  $-80^{\circ}\text{C}$  until use.

All tested samples were heat inactivated (HI) prior to testing in L-SBA at  $56^{\circ}\text{C}$  for 30 min to remove endogenous complement activity. Various aliquots of HI human standard sera were used and treated as described below to determine the different assay parameters.

##### 4.3. Luminescence-Based SBA

The L-SBA was conducted following previously established protocols [46]. Serial dilutions (3-fold dilution steps in 7 dilution points) of the HI sera were made in phosphate-buffered saline (PBS) or Luria Bertani medium (LB) (25  $\mu\text{L}$ /well). After the cultures reached  $\text{OD}_{600} = 0.22 \pm 0.02$  (Log-phase), they were diluted to about  $1 \times 10^6$  Colony Forming Units (CFUs)/mL in PBS. Then, a reaction mixture (75  $\mu\text{L}$ /well) was prepared by combining the previously diluted cultures (10  $\mu\text{L}$ /well), the exogenous BRC (Cederlane or Pel-Freez at

different percentages depending on the strain: 30% for the assay with *S. flexneri* 1b, 20% for the assay with *S. flexneri* 3a, 7% for the assay with *S. flexneri* 4a and 5b, 7.5% for the assay with *S. flexneri* X and Y, and 25% for the assay with *S. flexneri* 6, and the assay buffer (LB for *S. flexneri* 1b, 4a, and 5b and PBS for *S. flexneri* 3a, 6, X, and Y). The mixture was finally added to the volume of HI sera dilutions already contained in the wells of the plate to create a final reaction volume of 100  $\mu$ L. As a control, a row of wells without sera was added to each plate to be sure that the killing was due to the sera and not to the complement (non-specific complement killing). The plates were incubated at 37 °C for 3h. To evaluate bacteria viability at time zero, 5  $\mu$ L of the bacterial culture diluted in PBS to have about  $1 \times 10^6$  CFU/mL were added to 45  $\mu$ L of BacTiter-Glo Reagent (Promega, Southampton, UK) inside a white 96-well plate (Greiner Bio-One, Roma, Italy). After 5 min of incubation on an orbital shaker at 600 rpm in the dark and at RT, the luminescence was detected by a luminometer (Synergy HT, Biotek, Swindon, UK).

After 3 h of incubation, the plate was centrifuged for 10 min at  $4000 \times g$ , and the supernatant was removed. The pellets were resuspended in 100  $\mu$ L of PBS, and then 50  $\mu$ L of the suspension was mixed with 50  $\mu$ L of BacTiter-Glo Reagent (Promega, Southampton, UK) inside a white 96-well plate (Greiner Bio-One, Roma, Italy). After 5 min of incubation on an orbital shaker at 600 rpm in the dark and at RT, the luminescence was detected by a luminometer (Synergy HT, Biotek, Swindon, UK).

#### 4.4. Calculations

The assay results are expressed in IC<sub>50</sub>, with the reciprocal serum dilution causing a 50% reduction in luminescence with respect to the control (indicating 50% growth inhibition). A 4-parameter non-linear regression was applied to the luminescence detected by the luminometer for all tested sera dilutions, and an arbitrary dilution of  $10^{15}$  was assigned to the wells without sera. Since the luminescence levels measured directly correlate with the number of living bacteria in the wells and inversely correlate with the functionality of the antibodies contained in the serum [50], we applied a fitting with a weighting of the data for the inverse of luminescence, constraining the curves to the bottom between 0 and a defined threshold [52]. The threshold was calculated for each serotype tested in the same way: the average luminescence detected in the first 3 dilutions of the tested sera at T180 plus the SD (Standard Deviation) of the luminescence detected. All the values were then rounded to the closest hundred. The plate was validated only if the average luminescence in the wells not containing any sera at T180 was at least 3-fold for *S. flexneri* 1b, 2-fold for *S. flexneri* 3a, 5-fold for *S. flexneri* 4a, 8-fold for *S. flexneri* 5b, 2.5-fold for *S. flexneri* 6, 2-fold for *S. flexneri* X, and 1.5-fold for *S. flexneri* Y regarding the average luminescence at T0.

To be able to calculate an IC<sub>50</sub>, the highest luminescence detected in the 7 dilutions for each serum had to be at least 0.6-fold that of the luminescence measured in the wells without sera. GraphPad Prism 9 software (GraphPad Software, La Jolla, CA, USA) was used for fitting and IC<sub>50</sub> determination.

#### 4.5. Precision

The assay precision signifies its capacity to consistently reproduce measurements. Precision was evaluated in terms of repeatability and intermediate precision. Repeatability gauges precision under identical operating conditions and is also called intra-assay variation. On the other hand, intermediate precision measures other kinds of variations—different operators, days, equipment, and calibrants—and it is also known as inter-assay variation.

To assess the precision of the assay, the IC<sub>50</sub> for the standard sera against *S. flexneri* 1b, 3a, 4a, 5b, 6, X, and Y was determined independently by two operators, 12 replicates per day, and on three different days (72 measurements in total). HI standard serum obtained by pooling high-responder sera from healthy European adults enrolled in the NCT05073003 clinical trial vaccinated with GMMA from *S. sonnei* and *S. flexneri* 1b, 2a, and 3a was used for the assessment of the precision of the assay against *S. flexneri* 1b and 3a, whereas HI standard serum obtained by pooling high-responder sera from adults from the

NCT01229176 clinical study performed in Pakistan and India was used for the assessment of the precision of the assay against *S. flexneri* 4a, 5b, 6, X, and Y. The HI human standard sera were tested starting from 1:100 (final dilution) in L-SBA against *S. flexneri* 5b and 6, from 1:300 (final dilution) in L-SBA against *S. flexneri* 1b and *S. flexneri* X, from 1:500 (final dilution) in L-SBA against *S. flexneri* 4a, and from 1:1000 (final dilution) in L-SBA against *S. flexneri* 3a and *S. flexneri* Y. Log-transformed IC50 results from the three analysis sessions were analyzed with Minitab 18 (Minitab, LLC, State College, PA, USA) applying a mixed-effects model considering the day and operator as random factors to determine the repeatability (CV% R) and intermediate precision (CV% IP) of the assay.

#### 4.6. Linearity

HI standard serum obtained by pooling high-responder sera from healthy European adults enrolled in the NCT05073003 clinical trial vaccinated with GMMA from *S. sonnei* and *S. flexneri* 1b, 2a, and 3a was used for the assessment of the linearity of the assay against *S. flexneri* 1b and 3a, whereas HI standard serum obtained by pooling high-responder sera from adults from the NCT01229176 clinical study performed in Pakistan and India was used for the assessment of the linearity of the assay against *S. flexneri* 4a, 5b, 6, X, and Y. The HI human standard sera were diluted in PBS (undiluted, 2-fold, 4-fold, 8-fold, 16-fold, and 32-fold) before being assayed against the *S. flexneri* strains in L-SBA to evaluate the linearity of the assay. A nominal value, referred to as the average IC50 of the undiluted serum, was obtained through precision analysis (Table 1) and served as the expected value against which the theoretical IC50 values, calculated based on the dilutions performed, were compared in linearity analysis. The %error was calculated for all tested points and expressed as  $\text{LogIC}_{50} \text{ theoretical} - \text{LogIC}_{50} \text{ experimental} / \text{LogIC}_{50} \text{ theoretical} \times 100$ .

#### 4.7. Specificity

Regarding the samples used to assess specificity, to determine the minimum homologous O-Ag concentration able to inhibit  $\text{IC}_{50} \geq 70\%$ , HI standard serum obtained by pooling high-responder sera from healthy European adults enrolled in the NCT05073003 clinical trial vaccinated with GMMA from *S. sonnei* and *S. flexneri* 1b, 2a, and 3a was incubated overnight (16–18h) at 4 °C via shaking in an orbital shaker at 200 rpm with an equal volume (1:1; v:v) of the homologous competitor at the final concentrations of 100, 50, 10, 2, and 0.5 µg/mL or PBS alone prior to being assayed in SBA against each *Shigella* serotype.

The lowest concentration of LPS between the ones tested able to inhibit the  $\text{IC}_{50} > 70\%$  was then used in a second experiment to determine the heterologous specificity. To determine the heterologous specificity, *S. sonnei* LPS (heterologous but from the same species) and *Salmonella* Typhimurium OA-g (heterologous but from a different species) were used as inhibitors. Internal controls for this experiment were represented by a sample preincubated overnight at 4 °C via shaking at 200 rpm with an equal volume of PBS (undepleted) and a sample preincubated with homologous *S. flexneri* O-Ag (homologous). Each spiked sample was assayed on the same day.

Specificity was determined as % IC50 inhibition, calculated using the following formula:

$$\% \text{IC}_{50} \text{ inhibition} = \frac{(\text{IC}_{50} \text{ of the undepleted sample}) - (\text{IC}_{50} \text{ of the depleted sample})}{(\text{IC}_{50} \text{ of the undepleted sample})} \times 100$$

#### 4.8. Limit of Detection and Limit of Quantification Assessment

HI standard serum obtained by pooling high-responder sera from healthy European adults enrolled in the NCT05073003 clinical trial vaccinated with GMMA from *S. sonnei* and *S. flexneri* 1b, 2a, and 3a was used for the assessment of the LoD and LoQ of the assay against *S. flexneri* 1b and 3a, whereas HI standard serum obtained by pooling high-responder sera from adults from the NCT01229176 clinical study performed in Pakistan and India was used for the assessment of the LoD and LoQ of the assay against *S. flexneri* 4a, 5b, 6, X, and Y. HI standard human sera were diluted v:v in PBS 60 times prior to being assayed in SBA

against *S. flexneri* 1b, 70 times prior to being assayed in SBA against *S. flexneri* 3a, 400 times prior to being assayed in SBA against *S. flexneri* 4a, 50 times prior to being assayed in SBA against *S. flexneri* 5b, *S. flexneri* 6, and *S. flexneri* Y, and 90 times prior to being assayed in SBA against *S. flexneri* X to obtain an IC<sub>50</sub> of around 100. Twelve identical diluted samples were assayed on the same day by the same operator starting with a 1:4 dilution.

The LoD and LoQ of the assay were calculated according to the ICH guideline Q2(R2) [53] by applying the following formulas:

$$LoD = 10^{(3.3 \times SD)} \times X$$

$$LoQ = 10^{(10 \times SD)} \times X$$

where X represents the lowest serum dilution tested in the assay (e.g., 4) and SD represents the standard deviation of IC<sub>50</sub> obtained for the samples.

#### 4.9. Statistical Analysis

Statistical analyses were conducted using Minitab 18 (Minitab Inc., Chicago, IL, USA). Variance component analysis using a mixed-effect model with Restricted Maximum Likelihood (REML) was employed to assess the intermediate precision (defined as the variability among different days and different operators), the repeatability (defined as the variability under the same operating conditions over a short interval of time), and the contributions of the day of analysis and operator to the variability. Regression analysis was utilized to evaluate linearity.

## 5. Conclusions

L-SBA has already been characterized as able to obtain good performance against other bacterial pathogens using both animal and human samples [46,52]. Here, the assay was characterized against a broad panel of *Shigella* serotypes, confirming that it can be easily extended to other strains. Such an assay is relatively rapid and cost effective and is being used for the analysis of samples coming from Phase I/II *Shigella* clinical trials. Additional work is planned to further qualify the assay and use it to analyze sera from a Phase III trial, further supporting the development of a vaccine against *Shigella*.

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**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** Data are contained within the article. The raw data supporting the conclusions of this article will be made available by the authors upon reasonable request.

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## CHAPTER 5. Evaluation of cross-functional antibody responses elicited by a four-component GMMA *Shigella* vaccine

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Valentina Caradonna<sup>1</sup>, Eleanna Sarakinou<sup>2</sup>, Roberta Di Benedetto<sup>2</sup>, Agnes Hunyady<sup>2</sup>, Usman Nakakana<sup>2,3</sup>, Renzo Alfini<sup>2</sup>, Marika Pinto<sup>2</sup>, Federica Boretto<sup>2</sup>, Salvatore Gemmellaro<sup>2</sup>, Pietro Ferruzzi<sup>2</sup>, Miren Iturriza<sup>2</sup>, Francesco Berlanda Scorza<sup>2</sup>, Simona Rondini<sup>2</sup>, Valentino Conti<sup>2</sup>, Carlo Giannelli<sup>2</sup>, Ashwani Kumar Arora<sup>2</sup>, Omar Rossi<sup>2</sup>, Francesca Micoli<sup>2</sup>, Francesca Mancini<sup>2</sup>.

<sup>1</sup> University of Siena, Siena, Italy; <sup>2</sup> GSK Global Health Vaccines R&D (GVGH), Siena, Italy;

<sup>3</sup>Present address: Gates Foundation, Seattle WA, USA.

### Abstract

*Shigella* is a major cause of morbidity and mortality in children in low- and middle-income countries. The O-antigen (OAg) component of the lipopolysaccharide is considered a protective antigen, however, their diversity challenges vaccine development, since more than 50 OAg serotypes have been identified. No licensed vaccine against shigellosis is available. A 4-component GMMA-based vaccine, altSonflex1-2-3, delivering *S. sonnei* and *S. flexneri* 1b, 2a and 3a OAg has been developed. Coverage is expected against non-vaccine serotypes, due to cross-reactivity, mediated by structural similarities among *S. flexneri* OAg. The vaccine is currently being tested in phase I and II clinical trials.

In this work, sera from mice, rats, and rabbits injected with altSonflex1-2-3 were analyzed for their ability to bind to and kill *S. flexneri* serotypes not included in the vaccine. Results obtained were compared to corresponding results from vaccinated European adults. While no cross-reactive antibodies were measured in mouse sera, the antibodies elicited by altSonflex1-2-3 in rats, rabbits and humans were able to bind and kill the tested *S. flexneri* X, Y, 6, 4a and 5b strains.

A study in African children and infants will confirm how data from animal models may predict the immune response in different age groups.

Keywords: *Shigella*, GMMA, vaccine, cross-reactivity, functionality

## 1. Introduction

*Shigella* remains a significant public health threat, particularly in low- and middle-income countries (LMICs), where inadequate sanitation and limited access to clean water facilitate its transmission (1-3). As a leading cause of bacillary dysentery (shigellosis), it disproportionately affects young children, immunocompromised individuals, and malnourished populations (2). The increasing prevalence of antimicrobial-resistant strains further exacerbates the disease burden, limiting treatment options. In recognition of this threat, fluoroquinolone-resistant *Shigella* was recently reclassified from a "medium" to a "high" priority pathogen in the 2024 World Health Organization (WHO) Bacterial Priority Pathogens List (4), emphasizing the urgency of developing alternative preventive measures, including vaccines.

While *Shigella* primarily affects LMICs, shigellosis also occurs in high-income countries (HICs), particularly among men who have sex with men in urban settings or young children living in crowded communities with a notable rise in multidrug-resistant outbreaks (5-9). These trends highlight the evolving epidemiology of *Shigella* and reinforce the need for a broadly protective vaccine capable of addressing diverse populations and transmission contexts.

Among the four species of the genus *Shigella*—*S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*—the distribution of *S. flexneri* and *S. sonnei* is of particular epidemiological relevance (10). Specifically, *S. sonnei* is reported to predominate in HICs, whereas *S. flexneri* is identified as the leading causative agent in LMICs (11). In both endemic and non-endemic countries, the predominant serotype of *S. flexneri* reported is consistently *S. flexneri* 2a (6, 12-15). Other serotypes, including *S. flexneri* 1b, 3a, and 6, were observed mainly in endemic countries (12, 15-17).

O-antigen (OAg), a key component of the *Shigella* lipopolysaccharide (LPS), is central to serotype classification and immune recognition (18). *S. flexneri* comprises at least 23 serotypes sharing a conserved OAg backbone composed of repeating tetrasaccharide units containing three rhamnose residues (rhamnose I, II, and III) and N-acetylglucosamine (19). This is true for all *S. flexneri* serotypes except for *S. flexneri* 6, presenting a different OAg backbone composed of two rhamnose residues, one galacturonic acid and one N-acetylgalactosamine (20). The diversity in serotypes arises from the addition of glucosyl and/or O-acetyl groups at various positions within the

tetrasaccharide (18). No cross-functionality of antibodies specific for *S. sonnei* OAg is expected against *S. flexneri* due to the different polysaccharide structure. Therefore, preclinical studies evaluating the cross-reactivity and cross-functionality among 14 *S. flexneri* serotypes guided the design of a four-component *Shigella* vaccine that included *S. flexneri* 1b and 3a OAg for the broad cross-reactivity of induced immune responses, and *S. sonnei* and *S. flexneri* 2a OAg due to the high prevalence of the two bacteria in endemic regions (21). This combination should maximize cross-protection while covering the most relevant disease-causing serotypes.

To address the need for a *Shigella* vaccine, the Generalized Modules for Membrane Antigens (GMMA) platform was selected based on specific characteristics which make it suitable for LMIC vaccine development. GMMA are outer membrane vesicles naturally shed by Gram-negative bacteria, genetically modified to enhance vesicles release and to modulate endotoxin-related potential reactogenicity (22). Their strong immunogenic properties, combined with simple and cost-effective production, make them well-suited for development of vaccines for LMICs.

The quadrivalent GMMA-based vaccine, named altSonflex1-2-3, showed good immunogenicity in animal models (23). A phase 1 trial (NCT05073003 stage 1) has already assessed the safety and immunogenicity of the vaccine in European adults (24), allowing the progression into the target population of 9-month-old infants in Kenya, where shigellosis is endemic (NCT05073003 stage 2 and NCT06663436).

Given that altSonflex1-2-3 is designed to provide broad coverage, it was important to assess the cross-reactivity of the immune response elicited, thereby extending the protective coverage beyond the vaccine's direct components. Different studies have already demonstrated that certain *S. flexneri* serotypes can induce antibodies that are broadly reactive, covering a range of serotypes (21, 25-28).

In this study, we aimed to build a comprehensive profile of the altSonflex1-2-3 vaccine's immunogenic potential by evaluating the contribution of each vaccine component to cross-functional immune responses against *S. flexneri* 4a, 5b, 6, X and Y serotypes that are not included in the vaccine but of important epidemiological importance (15, 12). The cross-functionality of antibodies was assessed in mice, rats and rabbits. In addition, sera from vaccinated European adults were evaluated for cross-functionality and results were compared to pre-clinical data.

## 2. Methods

### 2.1. Strains and reagents

*S. sonnei* 53G was obtained from Walter Reed Army Institute of Research, Washington, D.C., USA. The *S. sonnei*  $\Delta virG::cat$  strain with stabilized OAg used in flow cytometry binding experiments (FACS) and for serum bactericidal assay (SBA) was generated by Caboni *et al.* (29). *S. flexneri* strains were purchased from Public Health England, London, UK, as detailed in Supplementary Table 1. Frozen 20% glycerol stocks were prepared from lyophilized cultures and stored at -80°C.

The LPS from *S. sonnei* and *S. flexneri* strains used in SBA were extracted and analyzed for sugar structural features, protein, and nucleic acid impurities, as previously reported (30).

### 2.2. GMMA formulation and characterization

altSonflex1-2-3 was produced and characterized according to the methods previously described by Rossi *et al.* (23). altSonflex1-2-3 was formulated by adsorbing at 1:1:1:1 ratio GMMA (OAg content based) from *S. sonnei* and *S. flexneri* serotypes 1b, 2a, and 3a in a solution containing 154 mM NaCl and 10 mM NaH<sub>2</sub>PO<sub>4</sub> at pH 6.5 and Alhydrogel at a concentration of 0.7 mg/ml (Al<sup>3+</sup>). The corresponding mono-, bi-, and tri-component formulations were prepared using the same procedure, maintaining the same GMMA content for each component and Alhydrogel amount.

### 2.3. Immunogenicity studies in animals

altSonflex1-2-3 vaccine, and bi-, tri- and mono-component GMMA formulations were used to immunize mice, rats and rabbits.

Eight CD1 mice per group (female, 4 to 6 weeks old), eight Sprague Dawley rats (female, 200–300 g) per group or eight New Zealand white rabbits (female, 2.5 kg) per group were immunized at days 0 and 28. All animals were immunized intramuscularly with either 50 µl (mice), 200 µl (rats) or 500 µl (rabbits) of vaccine formulation. Mice were immunized with 0.15 µg of OAg from a single serotype (*S. sonnei*, *S. flexneri* 1b, 2a, or 3a) when administered with each of the mono-component formulations, or 0.6 µg total OAg (0.15 µg of each serotype, combined in a 1:1:1:1 ratio) when formulated as the altSonflex1-2-3 four-component formulation. Similarly, rats were immunized with 0.6 µg of OAg from a single serotype when administered with mono-component formulations, or 2.4 µg total OAg when formulated as altSonflex1-2-3. Rabbits were immunized

with either 1.5 µg of OAg from a single serotype (6 µg total OAg when formulated as altSonflex1 -2-3) or, for depletion experiments, 60 µg total OAg formulated as altSonflex1 -2-3. Sera were collected at days -1, 27 and 42.

All the animals had unrestricted access to food and water. To minimize stress, anesthesia is administered only during the terminal withdrawal phase. Euthanasia methods vary by species. Carbon dioxide is used for euthanasia of mice and rats, with no wake procedures before bleeding. Once the final bleed is completed, cervical dislocation is performed for mice while for rats rigor mortis is awaited before confirming death.

For rabbits, anesthesia is induced with a pentobarbital overdose, followed by exsanguination via intracardiac puncture for final serum collection. The absence of a heartbeat indicates death, which is confirmed by the onset of rigor mortis or cervical dislocation.

Sera were pooled and used for FACS binding (day -1 and day 42), while individual sera were used to conduct Enzyme-Linked Immunosorbent Assay (ELISA; day -1, day 27 and day 42) and SBA (day -1 and day 42). Pre-immune sera were used as negative controls.

## **2.4. Human sera**

Individual sera from a subset of 25% of healthy European adults enrolled in the NCT05073003 stage 1 clinical trial were tested by SBA (24). These participants were vaccinated with altSonflex1 -2-3 at a dose of 15 µg of each OAg. Vaccine injections were given with either a 3-month or a 6-month interval. Analyzed sera were collected pre-dose 1 (day 1), post-dose 1 (day 29), and post-dose 2 (day 113/197). The study was approved by the ethics committee of the Ghent University Hospital and conducted as per Good Clinical Practice guidelines.

For antigen depletion experiments, a human standard serum was created by pooling sera from the same clinical trial selected based on their anti-*S. sonnei* and *S. flexneri* 2a O-Ag-specific IgG EU/mL elevated levels. Frozen 50 µL working aliquots of the serum were stored at -80°C until use.

## **2.5. Enzyme-linked immunosorbent assay (ELISA)**

To assess OAg-specific IgG concentration by ELISA, 384-well microplates (Greiner Bio-One, Kremsmünster, Austria) were coated with 40 µL/well of coating antigen and incubated overnight (16h) at 4°C. The coating concentrations and buffers used were as follows: *S. sonnei* LPS at 0.5

µg/ml in phosphate-buffered saline (PBS), *S. flexneri* 1b OAg at 2 µg/ml in carbonate buffer, *S. flexneri* 2a OAg at 5 µg/ml in carbonate buffer, and *S. flexneri* 3a OAg at 1 µg/ml in PBS. Coating buffers were selected based on results from preliminary experiments to maximize the ELISA signal given by a standard serum generated by immunizing mice with the respective *Shigella* GMMA. Absence of impact of coating buffer on antigen acetylation status was confirmed by NMR (data not shown). Following coating, plates were aspirated and blocked with 100 µL/well of 5% PBS milk powder (pH 7.4) (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) for 1 hour at 25°C. Plates were then washed 3 times with PBS-Tween 0.05% (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and incubated for 2h at 25°C with 40 µL/well of sera diluted at 1:100, 1:4000, and 1:160000 in PBS-Tween 0.05% with 0.1% BSA (pH 6.5) (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) for mice and rat sera and in 5% PBS milk (pH 7.4) for rabbit sera. Following this, plates were washed 3 times with PBS-Tween 0.05% and IgG detection was carried out using 40 µL/well of an enzyme-labeled secondary antibody (anti-species-specific IgG-alkaline phosphatase) (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) in PBS-Tween 0.05% with 0.1% BSA (pH 6.5) at different concentrations depending on the antigen used and for mice, rabbits and rats, and incubated for 1h at 25°C. After that, plates were washed 3 times with PBS-Tween 0.05%. The presence of anti-*S. sonnei* LPS/*S. flexneri* 1b, 2a, 3a OAg antibodies was identified by adding 40 µL/well of a p-Nitrophenyl phosphate substrate solution (Sigma-fast, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), resulting in a yellow color, which was measured by absorbance at 405 nm minus the absorbance at 490 nm detected via a Synergy HTX reader (Biotek, Winooski, VT, USA). The samples were compared to calibrated species-specific reference standard sera, with results expressed in ELISA units/mL (EU/mL) relative to the reference serum. One ELISA unit is defined as the reciprocal of the dilution of the reference serum that produces an Optical Density (OD) of one in the assay.

## **2.6. Luminescence-Based SBA**

SBA was performed against a panel of homologous (serotypes included in the vaccine) and heterologous (serotypes not included in the vaccine) *Shigella* serotypes by adapting previously published conditions. All tested samples were heat inactivated (HI) prior to testing in SBA at 56°C for 30 min to remove endogenous complement activity. Heat-inactivated sera were diluted in PBS when tested against *S. sonnei* and *S. flexneri* 2a, 3a, 6, X and Y strains and in Luria Bertani broth (LB) when tested against *S. flexneri* 1b, 4a and 5b strains, starting from 1:100 with 6 subsequent

3-fold serial dilutions. Serial dilutions of sera from mice, rats, rabbits or humans were added to 96-well plates, followed by the addition of baby rabbit complement (BRC, Cederlane, Ontario, Canada). For each tested strain we initially determined the optimal lot and percentage of BRC to be used, which lacked intrinsic bactericidal activity. To achieve this, we conducted BRC titration experiments where target bacterial cells were incubated in assay diluents containing various percentages of BRC, ranging from 0% to 50%. At the conclusion of the incubation period ( $T_{180}$ ), we measured the growth of the bacteria by luminescence and compared these values to the initial time point ( $T_0$ ). This approach allowed us to identify the maximum BRC concentration not causing non-specific bacterial killing (data not shown, (31)). The concentration of BRC utilized in each assay was therefore different depending on the strain utilized: 30% for *S. flexneri* 1b, 20% for *S. sonnei* and *S. flexneri* 3a, 7% for *S. flexneri* 4a and 5b, 7.5% for *S. flexneri* 2a, X and Y, and 25% for *S. flexneri* 6.

Working aliquots of all strains stored frozen at  $-80\text{ }^{\circ}\text{C}$  in 20% glycerol stocks were grown overnight (16–18 h) at  $37\text{ }^{\circ}\text{C}$  in a LB medium, stirring at 180 rpm. Bacterial strains were cultured to log-phase ( $\text{OD}_{600} = 0.22 \pm 0.03$ ) as previously described (32) and then diluted to approximately  $1 \times 10^6$  CFU/mL in PBS. An adequate volume of reaction mixture containing the target bacterial cells (10  $\mu\text{L}$ /well) and BRC in assay buffer was prepared; 75  $\mu\text{L}$ /well of the reaction mixture were added to each well of the SBA plate containing HI serum dilutions (final reaction volume 100  $\mu\text{L}$ ), mixed and incubated for 3 h at  $37\text{ }^{\circ}\text{C}$ . The viability of the surviving bacteria was assessed using BacTiter-Glo reagent (Promega Corporation, Madison, WI, USA) to measure ATP production, with luminescence detected via a Synergy HT luminometer (Biotek, Winooski, VT, USA). The bactericidal activity of each serum was quantified by determining the  $\text{IC}_{50}$  value, defined as the reciprocal serum dilution that leads to a 50% reduction in luminescence (indicating 50% inhibition of bacterial growth) with respect to the maximum growth of bacteria (no serum control). For titers below the minimum measurable signal, a value equal to half of the lowest dilution tested was assigned for animal sera (therefore 50), or equal to half of the limit of quantification in case of human sera (32–34). Curve fitting and  $\text{IC}_{50}$  calculations were performed using GraphPad Prism version 9 (GraphPad Software, LLC, San Diego, CA, USA). A 4-parameter non-linear regression was applied to the raw luminescence (no normalization of data was applied) obtained for all the sera dilutions tested for each serum; an arbitrary serum dilution of  $10^{15}$  was

assigned to the well containing no sera. Fitting was performed by weighing the data for the inverse of luminescence.

To assess the impact of antigen-specific antibody depletion on SBA, HI rabbit or human standard sera were subjected to a pre-incubation process with LPS. HI standard sera were mixed with an equal volume (1:1; v) of the competitor (or PBS as control) and incubated overnight (16–18 hours) at 4°C in an orbital shaker set at 200 rpm. Following the pre-incubation, each spiked sample was assayed on the same day by SBA against each *Shigella* serotype. The percentage of IC<sub>50</sub> inhibition was calculated using the following formula:

$$\%IC_{50} \text{ inhibition} = \frac{(IC_{50} \text{ of the undepleted sample}) - (IC_{50} \text{ of the depleted sample})}{(IC_{50} \text{ of the undepleted sample})} \times 100$$

## **2.7. Binding of cross-antibodies measured by flow cytometry**

Bacteria were cultured in LB to the log-phase (OD<sub>600</sub> = 0.22±0.03) as described in previous paragraph. Approximately 5 x 10<sup>6</sup> bacterial cells were then washed with PBS and fixed with BD Cytofix™ (BD Biosciences, CA, USA) by incubating them for 20 minutes at room temperature (RT). After fixation, the bacteria were washed and resuspended in PBS. Pooled serum samples from mice, rats, and rabbits, pre-diluted in PBS with 1% BSA (1:200 for mice and rabbits, 1:500 for rats), were added to the bacterial suspension, which had been previously distributed in 96-well plates, and incubated for 20 minutes at RT. Sera dilutions were established through preliminary titration experiments. The plates were washed, and AlexaFluor488-conjugated IgG anti-mouse, anti-rabbit or anti-rat antibodies (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) (diluted 1:200 for mice and rabbits, and 1:1000 for rats) were added and incubated for an additional 20 minutes at RT. The plates were washed and read in a BD Accuri™ C6 flow cytometer (BD Biosciences, CA, USA) and signal was measured in the fluorescein isothiocyanate (FITC) channel. The mean fluorescence intensity (MFI) was used to generate heatmaps depicting the binding of antisera to *Shigella* bacterial strains.

## **2.8. Statistical Analyses**

For clinical results, the following metrics were calculated for SBA titers against each serotype: geometric mean titers (GMTs) with their associated 2-sided 95% confidence intervals (CIs), within-participant geometric mean ratios (GMRs) with their associated 2-sided 95% confidence intervals (CIs), and the number and percentage of participants showing a ≥4-fold increase in titers

from baseline (seroresponse rate) with their exact (Clopper-Pearson) 96% Cis. For animal studies, GMTs and within-animal GMRs were calculated.

For the comparison of anti-OAg IgG responses from sera at day 27 or day 42 of mice immunized using altSonflex1-2-3 and tri-, bi- or mono-component formulations, the Mann-Whitney test was used.

Pearson's correlation coefficient (r) was calculated to assess the relationship between SBA responses induced after immunization with altSonflex1-2-3 and tri-, bi- or mono-component formulations against homologous versus heterologous strains.

### 3. Results

#### 3.1. Assessing the serological response to altSonflex1-2-3 vaccine components

In this study, we aimed to dissect the serological response induced by each of the four vaccine GMMA components of altSonflex1-2-3 (23) to understand the relative contribution to the OAg-specific IgG response (measured by ELISA) and antibody functionality (measured by SBA). To do this, we compared the immune response elicited by altSonflex1-2-3 GMMA vaccine in mice with that elicited by corresponding mono-, bi- or tri-component formulations. The bi-component formulation was composed of *S. sonnei* GMMA and *S. flexneri* 2a GMMA, which represent the most medically and epidemiologically relevant serotypes (6, 12, 15). The tri-component formulations were composed of *S. sonnei* GMMA, *S. flexneri* 2a GMMA, and either *S. flexneri* 1b GMMA or *S. flexneri* 3a GMMA. The comparison was also conducted between all these multi-component formulations and the four mono-component ones, each consisting of a single GMMA from one of the four strains. Anti-OAg specific IgG levels (Fig. 1) against *S. sonnei*, *S. flexneri* 2a, *S. flexneri* 3a, and *S. flexneri* 1b and related serum bactericidal activity (Fig. 2) were observed when the corresponding GMMA was present in the formulation. The anti-OAg IgG responses measured in ELISA at day 42 was significantly higher when the four-component formulation was compared to the mono-component formulations and this was verified for each of the tested antigens, except for *S. flexneri* 3a (*S. sonnei*, P=0.014; *S. flexneri* 1b, P=0.002; *S. flexneri* 2a, P=0.014; *S. flexneri* 3a, P=0.075). When comparing the groups immunized with bi- or tri-component formulations with groups immunized with the four-component altSonflex1-2-3 formulation, in each ELISA assay the IgG responses were generally comparable, except for *S.*

*flexneri* 1b OAg, where the four-component formulation elicited higher IgG compared to the tri-component formulation containing *S. flexneri* 1b GMMA (P=0.0115).

Regarding functionality against the four vaccine strains, bactericidal activity was significantly higher in mice immunized with altSonflex1-2-3 compared to the mono-component formulations for *S. sonnei* (P=0.0011) and *S. flexneri* 2a (P=0.0007). When comparing the functional response of altSonflex1-2-3 to that elicited by the bi- and tri-component formulations containing the respective antigen, no significant differences were observed, except for a notably lower IC<sub>50</sub> against the *S. sonnei* strain in mice immunized with the bi-component formulations.

Although some limited cross-reactivity between *S. flexneri* 3a and 1b was observed in both ELISA and SBA assays, no significant cross-reactivity was detected between the other vaccine components. The results further demonstrated that the 4-component vaccine elicited higher IgG titers and functional antibody responses against homologous antigens and strains, indicating the necessity of including all four components to achieve an optimal immune response against all the vaccine serotypes.

All tested formulations induced higher anti-OAg IgG specific response on day 42 (14 days after the second immunization) compared to day 27 (27 days after the first immunization), indicating a boost effect following the second immunization, which occurred one month after the first immunization for all the GMMA formulations tested.

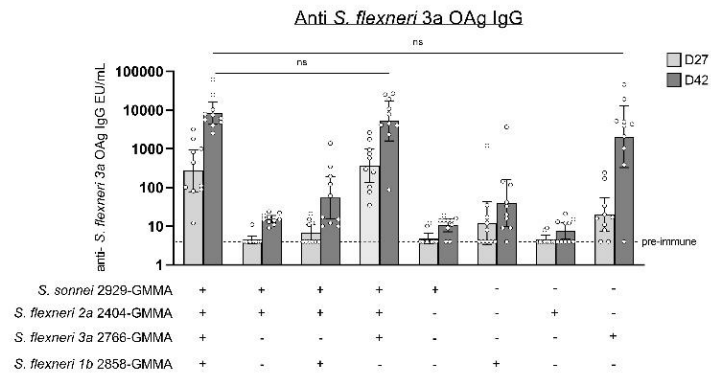
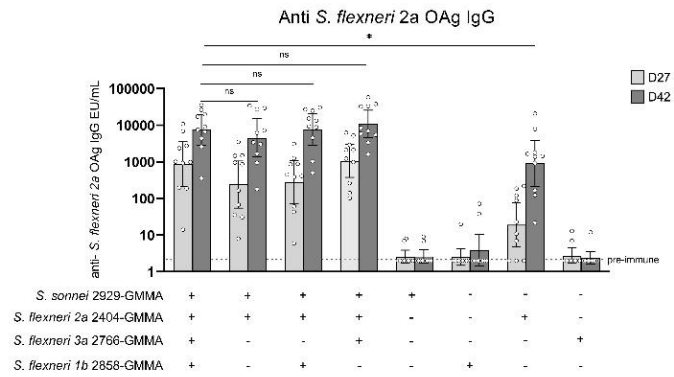
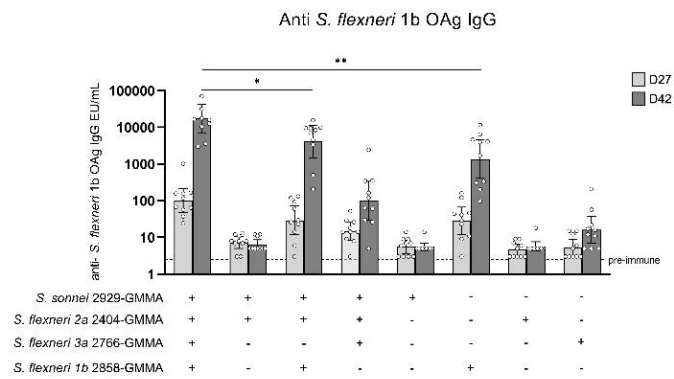
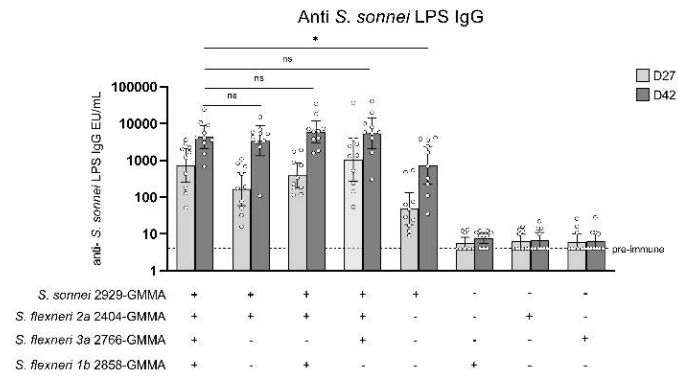


*Figure 1. Anti-OAg IgG responses in sera collected at day 27 (D27) and day 42 (D42) from mice injected with the four-component formulation (altSonflex1-2-3), tri-component formulations (S. sonnei GMMA + S. flexneri 2a GMMA and either S. flexneri 1b GMMA or S. flexneri 3a GMMA), a bi-component formulation (S. sonnei GMMA + S. flexneri 2a GMMA) or mono-component formulations (S. sonnei GMMA, S. flexneri 2a GMMA, S. flexneri 1b GMMA and S. flexneri 3a GMMA). All formulations contained Alhydrogel. Geometric mean (bar) with 95%CI (error bars) and individual IgG values (EU/ml; white dots) of each group are reported. Sample size per group (N) = 8. + = component present in the composition of the formulation; - = component not present in the composition of the formulation; pre-immune = baseline values before immunization. ns =  $P > 0.05$ ; \* =  $P \leq 0.05$ ; \*\* =  $P \leq 0.01$ .*

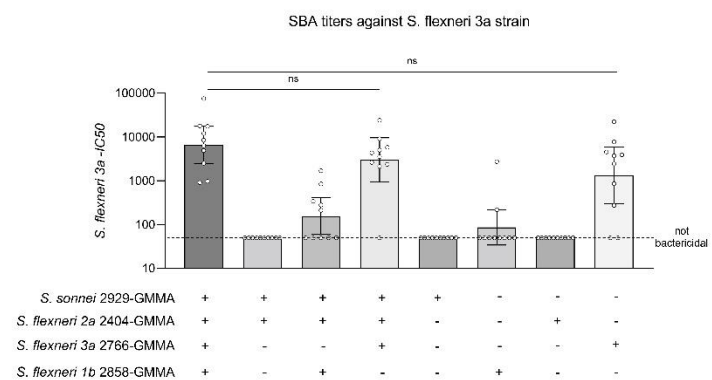
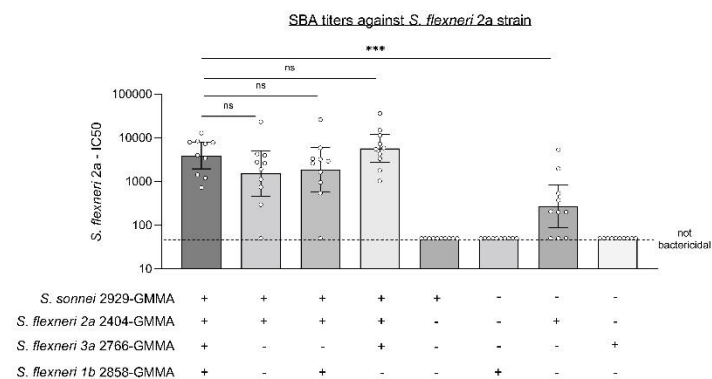
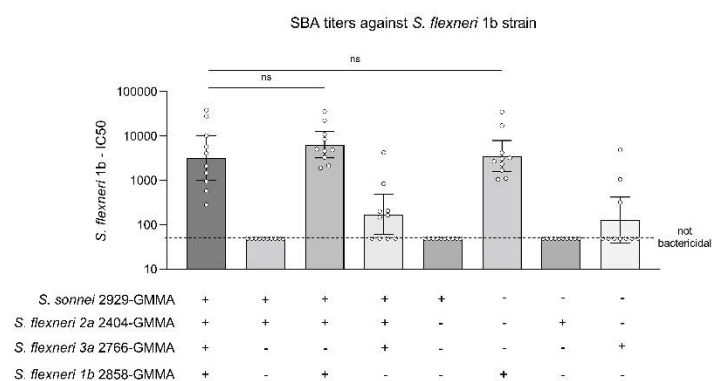
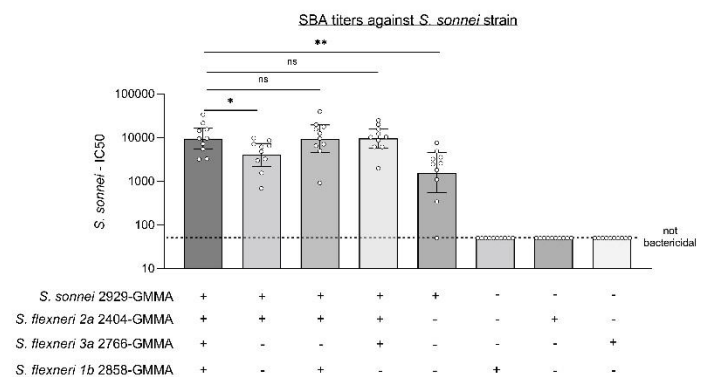


Figure 2 - Bactericidal titers of sera collected at day 42 from mice immunized with the four-component formulation (altSonflex1-2-3), tri-component formulations (*S. sonnei* GMMA + *S. flexneri* 2a GMMA and either *S. flexneri* 1b GMMA or *S. flexneri* 3a GMMA), a bi-component formulation (*S. sonnei* GMMA + *S. flexneri* 2a), or mono-component formulations (*S. sonnei* GMMA, *S. flexneri* 2a GMMA, *S. flexneri* 1b GMMA or *S. flexneri* 3a GMMA). Geometric mean (bar) with 95%CI (error bars) and individual values (EU/ml; white dots) of each group are reported. The dotted line represents the pre-immune IC50. Sample size per group (N) = 8. + = component present in the composition of the formulation; - = component not present in the composition of the formulation. ns =  $P > 0.05$ ; \* =  $P \leq 0.05$ ; \*\* =  $P \leq 0.01$ ; \*\*\* =  $P \leq 0.001$ .

### 3.2. Cross-functionality of vaccine-induced antibodies in different animal models

After having dissected the response induced against the vaccine components of altSonflex1-2-3, we then assessed the ability of antibodies elicited by altSonflex1-2-3 and its corresponding mono-component formulations (*S. sonnei* GMMA, *S. flexneri* 2a GMMA, *S. flexneri* 1b GMMA, or *S. flexneri* 3a GMMA) to bind to (by FACS) and kill (by SBA) a panel of epidemiologically relevant heterologous *S. flexneri* serotypes (*S. flexneri* 4a, 5b, 6, X and Y). This evaluation was conducted in three different animal models: mice, rats, and rabbits.

In mice, vaccine-induced antibodies exhibited strong binding and bactericidal activity against homologous strains, while the responses against the heterologous strains tested were very low, indicating limited cross-reactivity (Fig. 3A, B).

In rats, a broader cross-reactivity pattern was observed. Antibodies generated by the altSonflex1-2-3 four-component formulation showed high binding and bactericidal activity across most tested heterologous strains, except for *S. flexneri* 6 (Fig. 3C, D). Interestingly, the mono-component vaccines demonstrated that individual antigens could elicit cross-reactive responses against specific heterologous strains. In particular, strong responses were elicited against *S. flexneri* 4a, 5b, X, and Y when rats were immunized with *S. flexneri* 1b GMMA; against 4a, X, and Y when immunized with *S. flexneri* 2a GMMA; and against 4a, 5b, X, and Y when immunized with *S. flexneri* 3a GMMA, both in terms of antibody binding (FACS) and bactericidal activity (SBA).

In rabbits, the results aligned with those observed in rats, with cross-reactivity patterns largely maintained. Differently from rats, antibody binding and bactericidal activity were also detected against *S. flexneri* 6.

In many cases, across the three tested species, stronger responses were observed in sera of animals immunized with altSonflex1-2-3 compared to the corresponding mono-component formulations.

Overall, these findings highlight species-specific differences in vaccine-induced immune responses and underscore the role of individual antigens in mediating cross-reactivity against heterologous strains.

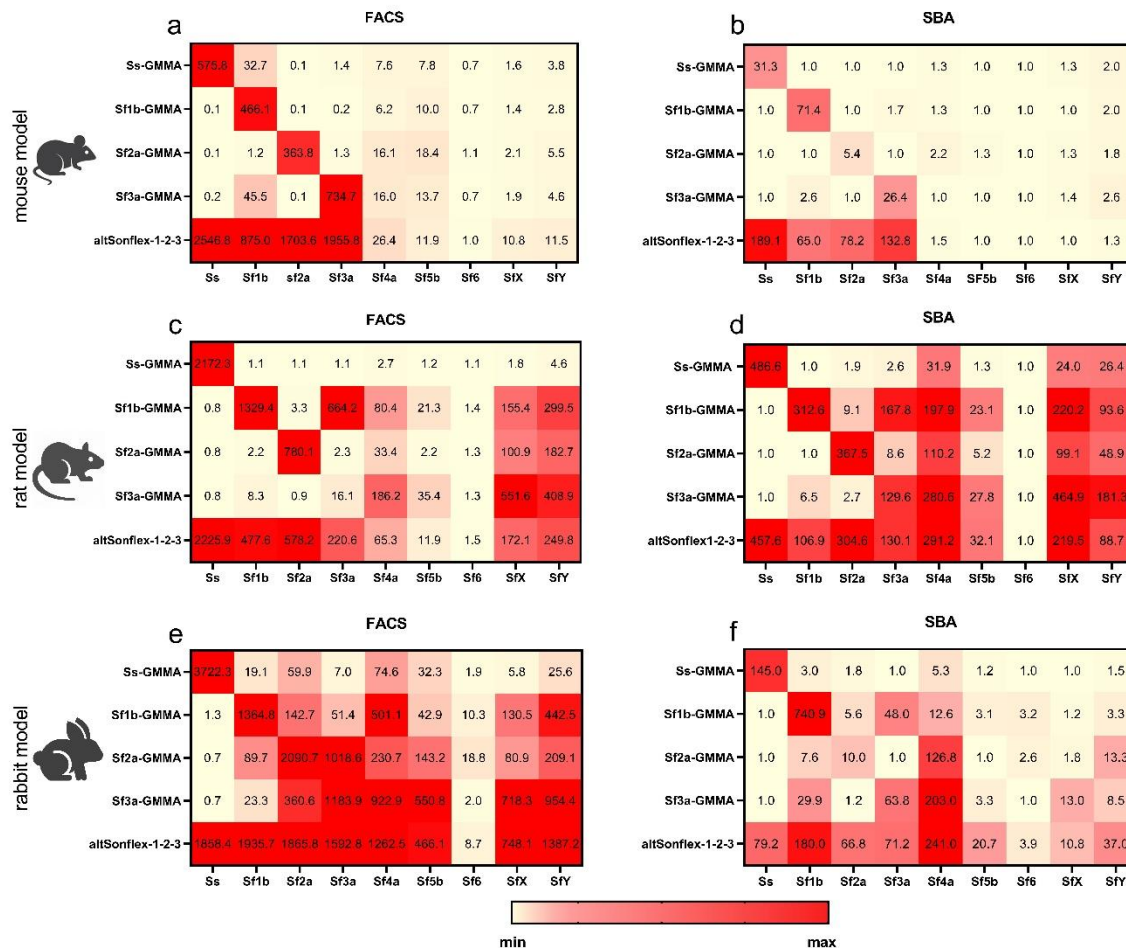


**Figure 3.** Heatmaps of antibody binding measured by FACS and bactericidal activity measured by SBA using sera obtained from mice (a, b), rats (c, d) and rabbits (e, f) immunized with *S. sonnei* GMMA (Ss-GMMA), *S. flexneri* 2a GMMA (Sf2a-GMMA), *S. flexneri* 1b GMMA (Sf1b-GMMA), *S. flexneri* 3a GMMA (Sf3a-GMMA) or altSonflex1-2-3. Values are expressed as geometric mean ratio (fold increase) between day 42 and pre-immune IC50 values for SBA and Median Fluorescence Intensity day 42 values divided pre-immune values for FACS; Ss= *S. sonnei*; Sf = *S. flexneri*; Red = high antibody recognition/functionality; Yellow = low antibody recognition/functionality.

Further, we explored the association between bactericidal responses elicited by altSonflex1-2-3 and its corresponding mono-component formulations against homologous and heterologous strains by using Pearson correlation analysis (Table 1). The results indicate that there is no correlation

between the magnitude of the response to homologous antigen and the extent of cross-reactivity (functionality) to heterologous antigens in the mouse model. In the rat model, a moderate positive correlation was observed between the response against *S. flexneri* 1b and *S. flexneri* 5b (Pearson  $r=0.57$ ), while SBA against *S. flexneri* 3a showed strong associations with SBA against *S. flexneri* 5b (Pearson  $r=0.73$ ) and *S. flexneri* X (Pearson  $r=0.70$ ), as well as moderate correlation with SBA against *S. flexneri* Y (Pearson  $r=0.61$ ). In the rabbit model, moderate correlations were observed between SBA against *S. flexneri* 2a and *S. flexneri* 5b and Y, and between SBA against *S. flexneri* 3a and SBA against *S. flexneri* 4a, 5b, and X (Pearson  $r\sim 0.5$ ).

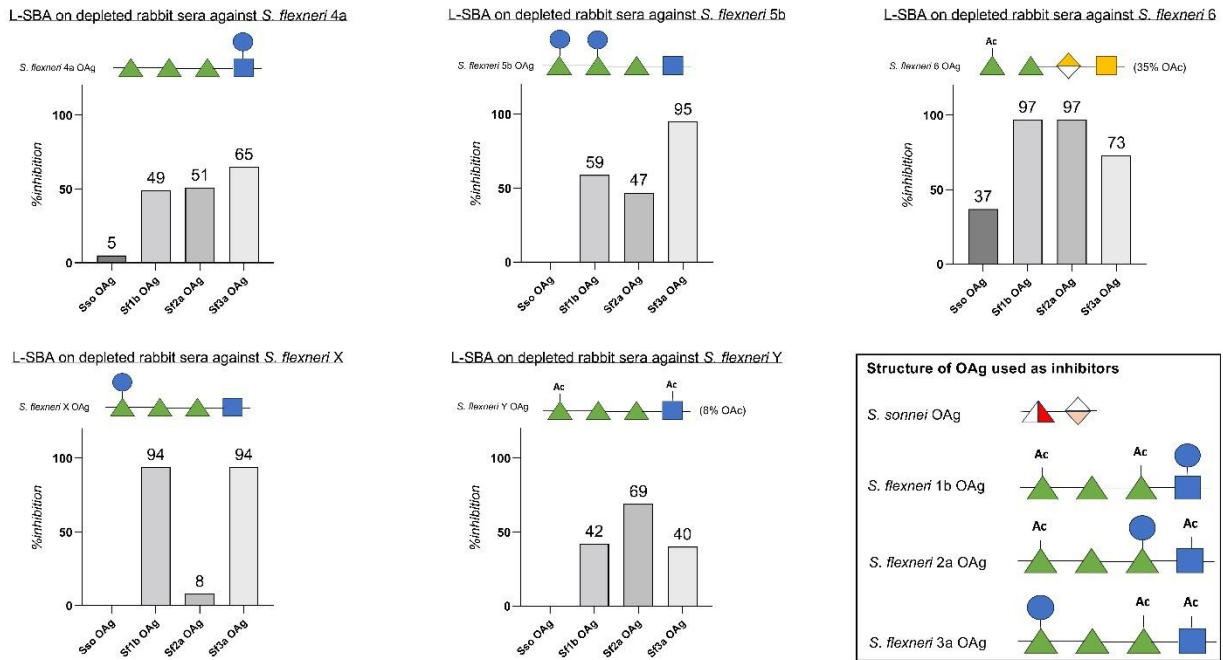
*Table 1. Pearson correlation results are reported as Pearson  $r$  values, derived from the correlation of serum bactericidal activity (SBA) log-transformed data from a combined dataset of five vaccine groups: altSonflex1-2-3, *S. sonnei*-GMMA, *S. flexneri* 1b-GMMA, *S. flexneri* 2a-GMMA, and *S. flexneri* 3a-GMMA. The table presents correlations of SBA results against homologous (SBA against *S. sonnei*, *S. flexneri* 1b, 2a or 3a) versus heterologous (SBA against *S. flexneri* 4a, 5b, 6, X or Y) strains in mice, rats, and rabbits. N/A = values within a group are identical, and therefore the Pearson correlation coefficient could not be calculated. Values close to 1 or -1 indicate a strong correlation while values close to 0 indicate a low correlation.  $P$  values are reported as \* =  $P \leq 0.05$ ; \*\* =  $P \leq 0.01$ ; \*\*\* =  $P \leq 0.001$ .  $n = 40$  for all comparisons.*

| <b>Homologous SBA-heterologous SBA correlation</b> |                       |                       |                      |                      |                      |
|----------------------------------------------------|-----------------------|-----------------------|----------------------|----------------------|----------------------|
| <b>Mouse model</b>                                 | <i>S. flexneri</i> 4a | <i>S. flexneri</i> 5b | <i>S. flexneri</i> 6 | <i>S. flexneri</i> X | <i>S. flexneri</i> Y |
| <i>S. sonnei</i>                                   | -0.06                 | -0.11                 | N/A                  | -0.19                | -0.18                |
| <i>S. flexneri</i> 1b                              | -0.04                 | -0.12                 | N/A                  | -0.21                | -0.10                |
| <i>S. flexneri</i> 2a                              | 0.19                  | 0.06                  | N/A                  | -0.10                | -0.16                |
| <i>S. flexneri</i> 3a                              | -0.09                 | -0.11                 | N/A                  | 0.001                | -0.13                |
| <b>Rat model</b>                                   | <i>S. flexneri</i> 4a | <i>S. flexneri</i> 5b | <i>S. flexneri</i> 6 | <i>S. flexneri</i> X | <i>S. flexneri</i> Y |
| <i>S. sonnei</i>                                   | -0.19                 | -0.23                 | N/A                  | -0.40*               | -0.28                |
| <i>S. flexneri</i> 1b                              | 0.32*                 | 0.57***               | N/A                  | 0.46**               | 0.34*                |
| <i>S. flexneri</i> 2a                              | 0.31*                 | 0.17                  | N/A                  | 0.18                 | 0.08                 |
| <i>S. flexneri</i> 3a                              | 0.42**                | 0.73***               | N/A                  | 0.70***              | 0.61***              |
| <b>Rabbit model</b>                                | <i>S. flexneri</i> 4a | <i>S. flexneri</i> 5b | <i>S. flexneri</i> 6 | <i>S. flexneri</i> X | <i>S. flexneri</i> Y |
| <i>S. sonnei</i>                                   | -0.30                 | 0.21                  | -0.08                | -0.04                | -0.02                |
| <i>S. flexneri</i> 1b                              | 0.34*                 | 0.39*                 | 0.26                 | 0.12                 | 0.16                 |
| <i>S. flexneri</i> 2a                              | 0.34*                 | 0.52***               | 0.45**               | 0.22                 | 0.54***              |
| <i>S. flexneri</i> 3a                              | 0.50***               | 0.53***               | 0.09                 | 0.59***              | 0.38*                |

### 3.3. Impact of antigen depletion on functionality of antibodies

To further investigate the contribution of each antigen to the wide cross-functionality observed in rabbits and rats, sera from rabbits immunized with altSonflex1-2-3 were depleted of *S. sonnei*, *S. flexneri* 1b, 2a and 3a OAg-specific antibodies. The concentration of each OAg was selected to have >70% inhibition against the homologous strain, and same amounts were then tested to look at residual bactericidal activity against heterologous strains. Depletion using the four different competitors led to an IC<sub>50</sub> inhibition >90% in many cases, such as when the altSonflex1-2-3 serum was depleted with *S. flexneri* 3a OAg and tested against *S. flexneri* 5b; when it was depleted with *S. flexneri* 1b OAg or *S. flexneri* 2a OAg and tested against *S. flexneri* 6; when it was depleted with *S. flexneri* 1b or *S. flexneri* 3a OAg and tested against *S. flexneri* X (Fig. 4A). These results confirm that the previously observed cross-reactivity (Fig. 3E, F) is primarily mediated by OAg-specific antibodies, as the depletion experiments revealed consistent patterns that align with those observed in regular SBA testing using non-depleted sera.

a



b

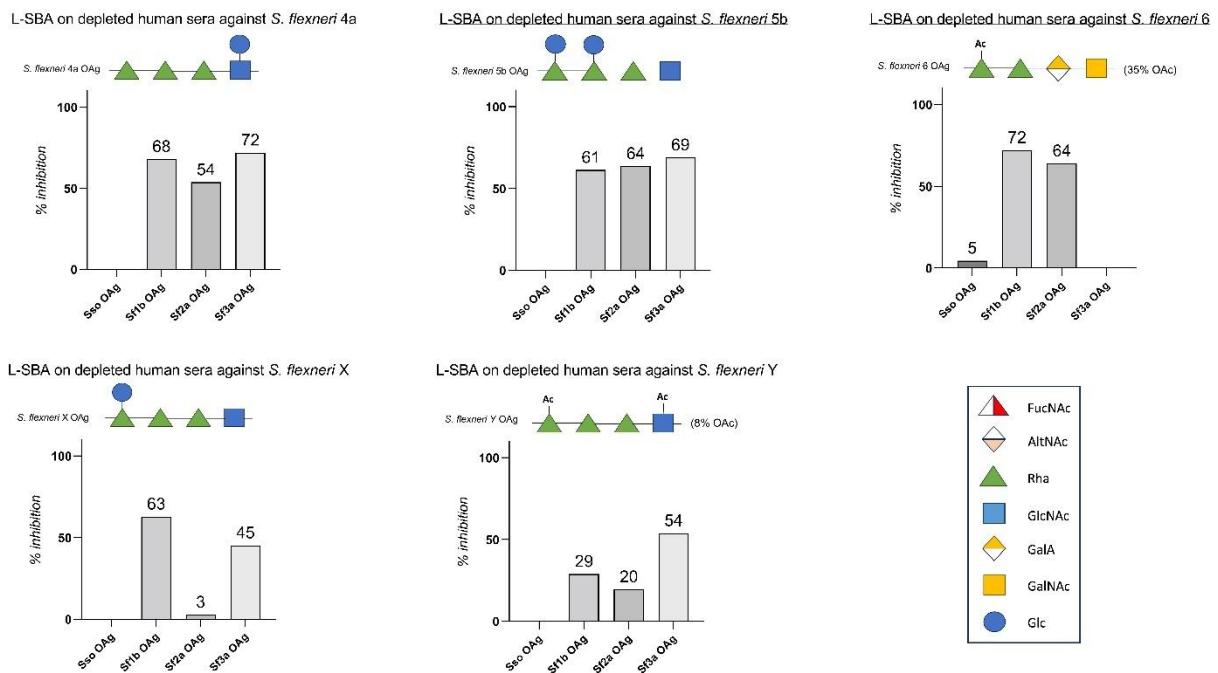


Figure 4. Inhibition of bactericidal activity (%) against *S. flexneri* 4a, 5b, 6, X, and Y strains using pooled sera from rabbits (a) and European adults (b) vaccinated with altSonflex1-2-3 previously incubated with *S. sonnei* LPS, *S. flexneri* 1b LPS, *S. flexneri* 2a LPS and *S. flexneri* 3a LPS. Sso = *S. sonnei*; Sf = *S. flexneri*. Structures of each *Shigella* strain tested is reported above each graph and described in Supplementary Table 1. Structures of *S. sonnei*, *S. flexneri* 1b, 2a and 3a OAg

displayed on GMMA have been already described in <sup>21</sup>. FucNAc = N-Acetyl-L-Fucosamine; AltNAcA= N-acetyl-alturonic acid; Rha = Rhamnose; GlcNAc = N-Acetylglucosamine; GalA = Galacturonic acid ; GalNAc = N-Acetylgalactosamine; Glc = glucose.

To assess whether this trend was consistent between animal models and clinical outcomes, the assay was replicated using a human standard serum obtained by pooling sera of European adults vaccinated with altSonflex1-2-3 (NCT05073003 clinical trial, stage 1)(24). The results revealed very similar patterns in the inhibition of bactericidal activity against heterologous strains of *S. flexneri* (Fig. 4B).

### 3.4. Cross-functionality of vaccine-induced antibodies in European adults

Human sera from European adults were collected before and after each one of the 2 vaccinations with an interval of 3 or 6 months and assessed for their ability to elicit cross-functional antibodies against the same panel of five heterologous strains tested in the animal models (Supplementary Table 2).

For *S. flexneri* 4a and 6, a slight increase from baseline (GMR ~2-fold) was observed in participants vaccinated with an immunization schedule of either 0-3 months or 0-6 months. Specifically, the seroconversion rates (proportion of participants achieving a 4-fold change from baseline), were 42.9% and 33.3% for *S. flexneri* 4a, and 0% and 22.2% for *S. flexneri* 6, respectively, after two doses with a 0-3 or 0-6 month schedule (Table 2).

In the case of *S. flexneri* 5b, X, and Y, there was a more significant increase from baseline (GMR ~4-fold). For *S. flexneri* 5b, the seroconversion rates were 14.3% and 22.2%; for *S. flexneri* X and Y, the seroconversion rates were 28.6% and 55.6%, respectively, after two doses with the 0-3 month and 0-6 month schedules (Table 2).

The data in Table 2 demonstrate a higher immune response elicited by the vaccine compared to the placebo in terms of SBA titers from *S. flexneri* 5a, X and Y GMTs. Post-dose 2, the geometric mean ratios (GMR) of the immunized participants exceed those of the placebo group. Additionally, although seroconversion rates were modest for some serotypes, a higher percentage of participants in the vaccine group exhibit a 4-fold increase in SBA titers compared to the placebo group.

Table 2. Geometric mean ratio (GMR) versus baseline and % of participants with 4-fold increase from baseline measured in a subset of 25% of healthy European adults vaccinated with altSonflex1-2-3 in the NCT05073003 clinical trial. 3M = participants who received 2 altSonflex1-2-3 immunizations at a 3-month interval; 6M = participants who received 2 altSonflex1-2-3 immunizations at a 6-month interval; placebo = participants who received placebo. N = number of tested participants; PD1 = post-dose 1 (day 29); PD2 = post-dose 2 (day 113/197).

| GMR                        |     |     |                       |      |                       |      |                      |      |                      |      |                      |      |
|----------------------------|-----|-----|-----------------------|------|-----------------------|------|----------------------|------|----------------------|------|----------------------|------|
|                            | N   |     | <i>S. flexneri</i> 4a |      | <i>S. flexneri</i> 5b |      | <i>S. flexneri</i> 6 |      | <i>S. flexneri</i> X |      | <i>S. flexneri</i> Y |      |
|                            | PD1 | PD2 | PD1                   | PD2  | PD1                   | PD2  | PD1                  | PD2  | PD1                  | PD2  | PD1                  | PD2  |
| 3M                         | 9   | 7   | 1.9                   | 2.4  | 3.2                   | 2.1  | 1.7                  | 2    | 4.4                  | 3.6  | 4.8                  | 3.1  |
| 6M                         | 8   | 9   | 2.2                   | 2.1  | 4.2                   | 2.9  | 1.3                  | 2.4  | 3.1                  | 4.6  | 3.3                  | 4.5  |
| placebo                    | 10  | 10  | 1.6                   | 1.2  | 1                     | 0.8  | 1                    | 0.9  | 1.7                  | 1.4  | 0.9                  | 1.2  |
| % 4-fold increase (95% CI) |     |     |                       |      |                       |      |                      |      |                      |      |                      |      |
|                            | N   |     | <i>S. flexneri</i> 4a |      | <i>S. flexneri</i> 5b |      | <i>S. flexneri</i> 6 |      | <i>S. flexneri</i> X |      | <i>S. flexneri</i> Y |      |
|                            | PD1 | PD2 | PD1                   | PD2  | PD1                   | PD2  | PD1                  | PD2  | PD1                  | PD2  | PD1                  | PD2  |
| 3M                         | 9   | 7   | 22.2                  | 42.9 | 22.2                  | 14.3 | 0                    | 0    | 55.6                 | 28.6 | 66.7                 | 28.6 |
| 6M                         | 8   | 9   | 12.5                  | 33.3 | 50.0                  | 22.2 | 0                    | 22.2 | 25.0                 | 55.6 | 37.5                 | 55.6 |
| placebo                    | 10  | 10  | 20.0                  | 0    | 0                     | 0    | 10.0                 | 0    | 10.0                 | 0    | 0                    | 0    |

### 3.5. Comparison of preclinical and clinical results

Animal sera tested against *S. flexneri* 6 showed relatively low SBA titers GMRs in all species and this is consistent with the results obtained in humans. However, the bactericidal activity of human sera against *S. flexneri* 4a showed GMRs of ~2, which is less than what would have been expected and not aligned with results in rats and rabbits, where the bactericidal activity against *S. flexneri* 4a was much higher (291 GMR in rats and 241 GMR in rabbits).

In addition, variations in cross-reactivity patterns were observed among different animal models and humans. In mice, no cross-reactivity was detected. In rats, cross-reactivity was observed with the following relative order: bactericidal titers against *S. flexneri* 4a were high and approximately equal to titers against *S. flexneri* X, higher than titers against *S. flexneri* Y, and much higher than titers against *S. flexneri* 5b. No cross-reactivity was detected for *S. flexneri* 6. In rabbits, titers against *S. flexneri* 4a were significantly higher than titers against all other strains. Cross-reactivity to *S. flexneri* Y was approximately equal to cross-reactivity to *S. flexneri* 5b, and both were higher than functional titers against *S. flexneri* X and *S. flexneri* 6. In humans, *S. flexneri* 6 and *S. flexneri*

4a exhibited similar levels of cross-reactivity. The cross-reactivity against *S. flexneri* 5b was greater. Higher SBA titers were observed against *S. flexneri* X and *S. flexneri* Y (Fig. 5).

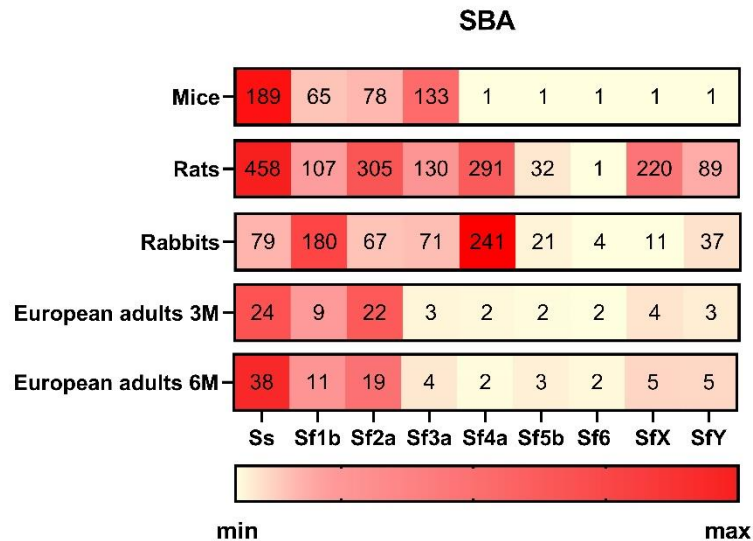


Figure 5. Heatmaps of bactericidal activity (SBA) in clinical sera from European adults who received 2 immunizations with altSonflex1-2-3 with an interval of 3 or 6 months and preclinical sera from mice, rats and rabbits. Values are expressed as GMR from baseline values ( $IC_{50}$ ); Ss = *S. sonnei*; Sf = *S. flexneri*; Red = high GMR; Yellow = low GMR.

## 4. Discussion

This study aimed to evaluate the capability of the 4-component altSonflex1-2-3 vaccine to generate functional antibodies against a range of epidemiologically significant heterologous *S. flexneri* serotypes. Additionally, it sought to analyze the contribution of each antigen in the vaccine to the cross-functionality against these heterologous serotypes. This research utilized three different animal models and compared the pre-clinical findings with clinical outcomes.

Importantly, in all animal species it was observed that the combination of the four GMMA resulted in no negative immune-interference and increased coverage against heterologous *Shigella* serotypes (21, 23). Indeed, by testing the ability of altSonflex1-2-3-elicited antibodies to bind and kill a panel of epidemiologically relevant *Shigella* strains we confirmed that the altSonflex1-2-3 four-component formulation enhanced the breadth of the immune response, suggesting potential cross-protection against additional *S. flexneri* serotypes.

Data from Rossi et al. (23) prefigured some key findings of the current work. In mice, altSonflex1-2-3 elicited a dose-dependent anti-OAg IgG response at very low doses (from 0.58 to 37 ng of each OAg), with a booster effect observed 14 days post-second vaccination. In rabbits, the full human dose (60 µg of total OAg) induced significant responses and mediated bactericidal activity against the homologous strains and heterologous *S. flexneri* serotypes (4a, 5b, 6, X, and Y) (23).

In this study, we observed cross-reactivity against all heterologous *S. flexneri* strains tested in both rat and rabbit models, with the exception of *S. flexneri* 6 in the rat model.

This finding aligns with the results of a study by Noriega et al., in which guinea pigs were immunized intranasally with attenuated strains of *S. flexneri* 2a and 3a. Following immunization, the guinea pigs were challenged with wild-type *S. flexneri* strains from various serotypes, and cross-protection was observed against all strains tested, except for *S. flexneri* 6 (27). In particular, efficacy percentages of 92% against *S. flexneri* 2b, 80% against *S. flexneri* Y, 64% against *S. flexneri* 5b, and 56% against *S. flexneri* 1b were reported, with lower percentages of 42% against *S. flexneri* 1a, 20% against *S. flexneri* 4b, and 4% against *S. flexneri* 6 (27). Similarly, in our study, consistent cross-reactivity with *S. flexneri* Y and 5b was observed when animals were immunized with either the altSonflex1-2-3 4-component formulation or mono-component formulations of *S. flexneri* 2a and 3a. Furthermore, the cross-reaction we observed between *S. flexneri* 3a and 1b aligns with the findings of Noriega *et al.*

In our study, the rabbit model demonstrated the most comprehensive response, with antibodies binding to and effectively killing all five heterologous strains tested. This behavior does not correlate with magnitude of the response against vaccine antigens, and indicates an enhanced cross-reactivity in the rabbit model, in line with results previously reported (23).

Another study, that explored cross-reactivity in humans and monkeys challenged with *S. flexneri* 2a and in humans and guinea pigs immunized with a recombinant vaccine expressing the *S. flexneri* 2a OAg, found that human sera exhibited cross-reactive IgA, particularly against *S. flexneri* 2b and other serotypes (1a, 5a, Y). Guinea pig sera showed cross-reactive IgA and IgG antibodies against 1a, 5a, and Y, but not 2b, while monkey sera displayed broad cross-reactivity with multiple *S. flexneri* serotypes, with 1a, 2b, and Y being most recognized, and with IgG responses being more prominent than IgA (28). In the present work, IgA responses were not assessed as the study focused on IgG-mediated bactericidal activity measured by SBA. However, given the demonstrated

importance of IgA in protection, future studies will evaluate OAg-specific IgA responses in sera from vaccinees in the target population.

Notably, the results indicate that stronger cross-functionality can be elicited when all four antigens are present together, rather than in mono-component formulations. This could suggest a synergistic effect on cross-reactivity elicited by the four GMMA components, or higher adjuvanticity due to the increased amount of GMMA components in the multi-component formulation (22). Additionally, the altSonflex1-2-3 formulation contains a 4-fold higher content of the common *S. flexneri* OAg tetrasaccharide chain domain than the correspondent mono-component *S. flexneri* formulations, which may contribute to increased OAg-specific IgG titers and enhanced functionality against both the homologous and heterologous *S. flexneri* serotypes tested. A similar pattern was observed in a previous study conducted in rabbits, in which a quadrivalent bioconjugate *Shigella* vaccine (containing *S. sonnei* and *S. flexneri* 2a, 3a, and 6 OAg) and the corresponding monovalent vaccines were compared for their ability to elicit bactericidal activity against non-targeted serotypes. Specifically, cross-reactivity with *S. flexneri* 4a and 4b was observed only when the quadrivalent formulation was tested (25).

Discrepancies between FACS and SBA results were observed in the rabbit model, with strong FACS binding not reflected in high SBA titers. This suggests that while vaccine-induced antibodies were capable of binding to bacterial targets, they were not always functional in mediating killing. This behavior could be attributed to higher anti-GMMA protein and/or anti-LPS core antibody response in this animal model, which we previously demonstrated to be non-functional under the experimental conditions used (35, 36).

In the study by Citiulo *et al.* (21), the functional outcomes measured by SBA using sera from animals immunized with *S. flexneri* GMMA against the same panel of *S. flexneri* strains did not align with cross-reactivity predictions among serotypes based solely on group-specificities (serogroups 3(4), 6, 7(8), 9 and 10). This suggests that factors beyond classical serogrouping may contribute to mediating binding and bactericidal activity across different *S. flexneri* serotypes and underscores the complexity of immune response against *Shigella*. Indeed, several other mechanisms might explain the observed pattern of cross-reactivity. For instance, the functional response against *S. flexneri* 6 seen in rabbits immunized with *S. flexneri* 2a GMMA could be linked to an O-acetylated disaccharide shared by both *S. flexneri* 6 and 2a. Specifically, the shared O-

acetylated disaccharide Rhap-(1→2)-Rhap has been identified as a common epitope. However, in the study from Farzam et al., this cross-reactivity mechanism between *S. flexneri* 2a and *S. flexneri* 6 was investigated using a competitive inhibition assay with antibodies induced by a *S. flexneri* 2a conjugate vaccine in humans. Inhibition was observed with both O-acetylated and non-O-acetylated *S. flexneri* 6 OAg, with the inhibition curve slightly higher when the non-acetylated *S. flexneri* 6 OAg was used as inhibitor (26). This finding evidences the need for further studies to clarify the impact of O-acetylation on cross-reactivity (26).

In rabbits, we were able to observe also the contribution of *S. flexneri* 1b OAg in the bactericidal activity against *S. flexneri* 6 and the two OAg share the same O-acetylated disaccharide. The cross-reactivity between *S. flexneri* 3a and 5b is likely caused by their shared O-factor 7,8 (glucosylation on O-3 of rhamnose III), which influences OAg conformation and antibody recognition (37). Although O-acetylation may be the basis for the observed cross-reactivity, other studies conducted on GMMA indicate that OAg O-acetylation is not critical for generating functional antibodies in mice against either homologous or heterologous strains (38).

To clarify the immune response against the heterologous panel of *S. flexneri* and the role of each antigen in the altSonflex1-2-3 vaccine, we depleted sera of rabbits and humans of LPS-specific antibodies. In both cases, this revealed that most of the response to each heterologous strain is mediated by LPS/OAg rather than protein contained in GMMA. If, in theory, we could speculate that they may still contribute to some of the remaining response observed, recent studies have shown that the presence of OAg chains on the bacterial surface can mask protein antigens and limit anti-protein antibody binding (22, 35, 39). Consequently, anti-OAg antibodies were confirmed as the primary mediators of bactericidal activity against OAg-positive bacteria.

Sera from human participants immunized with altSonflex1-2-3 exhibited stronger bactericidal activity against *S. flexneri* X, and Y compared to *S. flexneri* 5b, *S. flexneri* 4a and 6. The fold rise in bactericidal titers observed after vaccination against heterologous *Shigella* strains was lower compared to the rat and rabbit model. However, this trend can be linked to the numerically lower response specific for *S. flexneri* 1b and 3a OAg in comparison to *S. sonnei* and *S. flexneri* 2a, which we showed to be important components of the cross-reactive response (24). As the clinical study is currently still blinded, we are not able to correlate, at the subject level, bactericidal titers

observed against *Shigella* strains included in the vaccine with bactericidal titers against heterologous serotypes.

A limitation of the study is that the number of human sera samples tested is low. Moreover, a threshold for protection has not yet been established for any of the serotypes, and this threshold might vary among the different serotypes. Finally, using strain-specific complement concentrations in SBA testing may restrict direct head-to-head comparisons among serum bactericidal results against different strains. However, this tailoring is critical to ensure that the bactericidal effect observed is primarily attributable to the antibody response rather than differences in intrinsic complement susceptibility.

The study shows that the altSonflex1-2-3 vaccine can generate cross-functional antibodies against various *Shigella* serotypes in different animal models and in humans. It confirmed the necessity of the four selected GMMA components to produce a strong IgG response with bactericidal activity against the most common *Shigella* serotypes and highlighted the importance of the OAg in facilitating cross-serotype immunity. However, in mice, the immune response against the heterologous strain showed very low titers. Cross-reactivity was observed in the rat and rabbit models, but each of them had distinct patterns and specificities, especially when compared to clinical data. This could indicate that the same epitopes might be recognized differently by the immune system of different species. Therefore, it is essential to explore how these findings apply to the target population, e.g. infants in LMICs. Testing three doses of altSonflex1-2-3 in African infants with different immunization schedules (NCT05073003 stage 2 and NCT06663436) will determine if the results observed in European adults are consistent across different age groups and will help understand which animal model is mostly predictive for this kind of studies.

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## **Author Contributions**

Conceived and designed the experiments: all authors; Performed the experiments: V.Ca., F.B., S.G., F.Ma., O.R., R.D.B., R.A., M.P.; Analyzed the data: V.Ca., F.Ma., O.R., S.G., F.B., V.Co., E.S., C.G., M.P., R.A., R.D.B., F.Mi.; Contributed to the writing of the manuscript: all authors. All authors have read and agreed to the published version of the manuscript and take accountability for all aspects of the work.

## **Data availability**

All data associated with this study are present in the paper or the Supplementary Materials. Requests for resources, data and reagents should be directed to the corresponding author. Material Transfer Agreements would be required for any sample requests. Clinical data are currently not available as the study remains blinded. Once the study will be unblinded, please refer to GSK weblink to access GSK's data sharing policies and as applicable seek anonymized subject level data via the link <https://www.gsk-studyregister.com/en/>.

## **Competing interests**

V. Caradonna is a student at the University of Siena and participates in a post-graduate studentship program at GSK. Usman Nakakana was a Senior Project Physician in GSK at the time of the study. All other authors are currently employed by the GSK group of companies. The authors declare no other financial or non-financial competing interests.

## **Ethics**

GSK is committed to the Replacement, Reduction and Refinement of animal studies (3Rs). Non-animal models and alternative technologies are part of our strategy and employed where possible. When animals are required, the application of robust study design principles and peer review minimizes animal use, reduces harm, and improves benefit in studies. The animal husbandry and experimental procedures were ethically reviewed and carried out in the GSK Animal Research center (mice and rabbits) and in Charles River Laboratories (France, rats) in compliance with the relevant guidelines (European directive 2010/63/UE) and the institutional policies of GSK.

## Supplementary material

*Supplementary Table 1. Name, source and OAg characterization of S. sonnei and S. flexneri strains used for FACS/SBA experiments. WRAIR = Walter Reed Army Institute of Research; PHE = Public Health England; HMW = High Molecular Weight; MMW= Medium Molecular Weight; HPLC-SEC = High-Performance Liquid Chromatography - Size Exclusion Chromatography; Hestrin = Hestrin method. HPAEC-PAD = High-Performance Anion-Exchange Chromatography - Pulsed Amperometric Detection; Dische = Dische reaction. RU = Repeating Unit.*

| Strain name                          |                                                            | <i>S. sonnei</i><br>53G               | <i>S. flexneri</i><br>1b<br>NCTC5 | <i>S. flexneri</i><br>2a<br>2457T | <i>S. flexneri</i><br>3a<br>NCTC998<br>9 | <i>S. flexneri</i><br>4a<br>H1309201<br>47 | <i>S. flexneri</i><br>5b<br>H1309201<br>51 | <i>S. flexneri</i><br>6<br>H1309201<br>52 | <i>S. flexneri</i><br>X<br>H1309201<br>53 | <i>S. flexneri</i><br>Y<br>H13092015<br>4 |
|--------------------------------------|------------------------------------------------------------|---------------------------------------|-----------------------------------|-----------------------------------|------------------------------------------|--------------------------------------------|--------------------------------------------|-------------------------------------------|-------------------------------------------|-------------------------------------------|
| Strain source                        |                                                            | WRAIR,<br>Washington,<br>D.C.,<br>USA | PHE,<br>London,<br>UK             | PHE,<br>London,<br>UK             | PHE,<br>London,<br>UK                    | PHE,<br>London,<br>UK                      | PHE,<br>London,<br>UK                      | PHE,<br>London,<br>UK                     | PHE,<br>London,<br>UK                     | PHE,<br>London,<br>UK                     |
| OAg characterization method and test | HPLC-SEC: OAg molecular weight Distribution (kDa)          | HMW<br>209.2                          | HMW 55.1                          | HMW<br>58.4                       | HMW<br>58.7                              | -                                          | -                                          | -                                         | HMW<br>67.6                               | HMW<br>65.9                               |
|                                      |                                                            | MMW<br>19.2                           | MMW<br>14.6                       | MMW<br>15                         | MMW<br>16.3                              | MMW<br>16.9                                | MMW<br>18.3                                | MMW<br>21.3                               | MMW<br>15.2                               | MMW<br>13.9                               |
|                                      |                                                            | LMW/core<br>only<br>2.7-5.1           | LMW/core<br>only<br>2.5           | LMW/core<br>only<br>2.5           | LMW/core<br>only<br>5.1                  | LMW/core<br>only<br>2.5                    | LMW/core<br>only<br>3.2                    | LMW/core<br>only<br>3.0                   | LMW/core<br>only<br>2.5                   | LMW/core<br>only<br>2.5                   |
|                                      | Hestrin (OAc) and HPAEC-PAD or Dische (RU): % OAc (OAc/RU) | -                                     | 136                               | 84                                | 137                                      | -                                          | -                                          | 35                                        | -                                         | 8                                         |
|                                      | HPAEC-PAD: Glc/RU molar ratio                              | -                                     | 1                                 | 1                                 | 1                                        | 1                                          | 2                                          | 0                                         | 1                                         | 0                                         |

*Supplementary Table 2. Bactericidal activity measured by SBA against heterologous S. flexneri serotypes in adults 18 to 50 years of age in Europe. Dose C GR1 = altSonflex1-2-3 Dose C (15 µg per OAg serotype) scheduled on Day 1 and 85; Dose C GR2 = altSonflex1-2-3 Dose C scheduled on Day 1 and 169; Dose C GR1+GR2 = altSonflex1-2-3; Dose C scheduled on Day 1 and 85 or 169; Placebo = altSonflex placebo administered on Day 1 and 85 or 169; n/% = number/percentage of participants in a category; 95% CI = 95% confidence interval; LL = Lower Limit; UL = Upper Limit; N = number of participants with available results; GMT = geometric mean titres measured in IC50; GMR = within-subject geometric mean ratio.*

| Serotype: S flexneri 4a Ab |                    |            |                  |        |        |            |                  |        |        |                |                  |        |        |         |                  |        |        |       |
|----------------------------|--------------------|------------|------------------|--------|--------|------------|------------------|--------|--------|----------------|------------------|--------|--------|---------|------------------|--------|--------|-------|
|                            |                    | DOSE C GR1 |                  |        |        | DOSE C GR2 |                  |        |        | DOSE C GR1+GR2 |                  |        |        | PLACEBO |                  |        |        |       |
|                            |                    | 95% CI     |                  |        |        | 95% CI     |                  |        |        | 95% CI         |                  |        |        | 95% CI  |                  |        |        |       |
|                            |                    | n          | %<br>or<br>value | LL     | UL     | n          | %<br>or<br>value | LL     | UL     | n              | %<br>or<br>value | LL     | UL     | n       | %<br>or<br>value | LL     | UL     |       |
| Visit 1 (Day 1)            | N                  |            | 9                |        |        |            | 9                |        |        |                | 18               |        |        |         | 10               |        |        |       |
|                            | GMT                |            | 8262.8           | 4020.5 | 16981  |            | 9058.5           | 4847.9 | 16926  |                | 8651.5           | 5662   | 13219  |         | 5769.8           | 2689.6 | 12378  |       |
| Visit 4 (Day 29)           | N                  |            | 9                |        |        |            | 8                |        |        |                | 17               |        |        |         | 10               |        |        |       |
|                            | GMT                |            | 15973            | 7001.7 | 36439  |            | 17689            | 8847.5 | 35365  |                | 16759            | 10349  | 27137  |         | 9355.2           | 3633.5 | 24087  |       |
|                            | Comparison/Visit 1 |            |                  |        |        |            |                  |        |        |                |                  |        |        |         |                  |        |        |       |
|                            | N                  |            | 9                |        |        |            | 8                |        |        |                | 17               |        |        |         | 10               |        |        |       |
|                            | 4-fold increase    | 2          | 22.2             | 2.8    | 60.0   | 1          | 12.5             | 0.3    | 52.7   | 3              | 17.6             | 3.8    | 43.4   | 2       | 20.0             | 2.5    | 55.6   |       |
|                            | GMR                |            | 1.9              | 0.7    | 5.1    |            | 2.2              | 1      | 4.6    |                | 2                | 1.2    | 3.6    |         | 1.6              | 0.8    | 3.3    |       |
| Visit 5 (Day 85/169)       | N                  |            | 8                |        |        |            | 8                |        |        | -              | -                | -      | -      |         | 9                |        |        |       |
|                            | GMT                |            | 19823            | 12948  | 30350  |            | 17004            | 9349.8 | 30923  |                | -                | -      | -      | -       |                  | 8498   | 4116.9 | 17542 |
|                            | Comparison/Visit 1 |            |                  |        |        |            |                  |        |        |                | -                | -      | -      | -       |                  |        |        |       |
|                            | N                  |            | 8                |        |        |            | 8                |        |        |                | -                | -      | -      | -       |                  | 9      |        |       |
|                            | 4-fold increase    | 2          | 25.0             | 3.2    | 65.1   | 1          | 12.5             | 0.3    | 52.7   | -              | -                | -      | -      | 1       | 11.1             | 0.3    | 48.2   |       |
|                            | GMR                |            | 2.2              | 1      | 5      |            | 2.1              | 1.2    | 3.6    |                | -                | -      | -      | -       |                  | 1.5    | 0.9    | 2.4   |
| Visit 7 (Day 113/197)      | N                  |            | 7                |        |        |            | 9                |        |        |                | -                | -      | -      | -       |                  | 10     |        |       |
|                            | GMT                |            | 20412            | 10500  | 39682  |            | 19407            | 13869  | 27156  |                | -                | -      | -      | -       |                  | 7097.6 | 3837.5 | 13127 |
|                            | Comparison/Visit 1 |            |                  |        |        |            |                  |        |        |                | -                | -      | -      | -       |                  |        |        |       |
|                            | N                  |            | 7                |        |        |            | 9                |        |        |                | -                | -      | -      | -       |                  | 10     |        |       |
|                            | 4-fold increase    | 3          | 42.9             | 9.9    | 81.6   | 3          | 33.3             | 7.5    | 70.1   |                | -                | -      | -      | -       | 0                | 0      | 0.0    | 30.8  |
|                            | GMR                |            | 2.4              | 1      | 5.8    |            | 2.1              | 1.1    | 4      |                | -                | -      | -      | -       |                  | 1.2    | 0.9    | 1.6   |
|                            | Comparison/Visit 5 |            |                  |        |        |            |                  |        |        |                | -                | -      | -      | -       |                  |        |        |       |
|                            | N                  |            | 7                |        |        |            | 8                |        |        |                | -                | -      | -      | -       |                  | 9      |        |       |
| 4-fold increase            | 0                  | 0          | 0.0              | 41.0   | 0      | 0          | 0.0              | 36.9   |        | -              | 0                | -      | -      | 0       | 0                | 0.0    | 33.6   |       |
| GMR                        |                    | 0.9        | 0.5              | 1.7    |        | 1.2        | 0.8              | 1.8    |        | -              | -                | -      | -      |         | 0.8              | 0.6    | 1.2    |       |
|                            |                    |            |                  |        |        |            |                  |        |        |                |                  |        |        |         |                  |        |        |       |
| Serotype: S flexneri 5b Ab |                    |            |                  |        |        |            |                  |        |        |                |                  |        |        |         |                  |        |        |       |
|                            |                    | DOSE C GR1 |                  |        |        | DOSE C GR2 |                  |        |        | DOSE C GR1+GR2 |                  |        |        | PLACEBO |                  |        |        |       |
|                            |                    | 95% CI     |                  |        |        | 95% CI     |                  |        |        | 95% CI         |                  |        |        | 95% CI  |                  |        |        |       |
|                            |                    | n          | %<br>or<br>value | LL     | UL     | n          | %<br>or<br>value | LL     | UL     | n              | %<br>or<br>value | LL     | UL     | n       | %<br>or<br>value | LL     | UL     |       |
| Visit 1 (Day 1)            | N                  |            | 9                |        |        |            | 9                |        |        |                | 18               |        |        |         | 10               |        |        |       |
|                            | GMT                |            | 1919.9           | 1096   | 3363.3 |            | 1637.4           | 743.2  | 3607.7 |                | 1773.1           | 1151.3 | 2730.6 |         | 1339.5           | 517.4  | 3468.1 |       |
| Visit 4 (Day 29)           | N                  |            | 9                |        |        |            | 8                |        |        |                | 17               |        |        |         | 10               |        |        |       |
|                            | GMT                |            | 6127.5           | 3100.2 | 12111  |            | 6122.2           | 3075.1 | 12189  |                | 6125             | 3996.5 | 9387.2 |         | 1306             | 510.4  | 3342   |       |
|                            | Comparison/Visit 1 |            |                  |        |        |            |                  |        |        |                |                  |        |        |         |                  |        |        |       |
|                            | N                  |            | 9                |        |        |            | 8                |        |        |                | 17               |        |        |         | 10               |        |        |       |
|                            | 4-fold increase    | 2          | 22.2             | 2.8    | 60.0   | 4          | 50.0             | 15.7   | 84.3   | 6              | 35.3             | 14.2   | 61.7   | 0       | 0                | 0.0    | 30.8   |       |
|                            | GMR                |            | 3.2              | 1.5    | 6.8    |            | 4.2              | 1.9    | 9.4    |                | 3.6              | 2.2    | 6      |         | 1                | 0.8    | 1.2    |       |

|                       |                    |        |        |        |      |        |        |        |      |   |   |   |   |        |       |        |      |
|-----------------------|--------------------|--------|--------|--------|------|--------|--------|--------|------|---|---|---|---|--------|-------|--------|------|
| Visit 5 (Day 85/169)  | N                  | 8      |        |        |      | 8      |        |        |      | - | - | - | - | 9      |       |        |      |
|                       | GMT                | 5131.2 | 3148.1 | 8363.7 |      | 3146   | 1730.8 | 5718.5 |      | - | - | - | - | 1391.9 | 444.6 | 4357.9 |      |
|                       | Comparison/Visit 1 |        |        |        |      |        |        |        |      | - | - | - | - |        |       |        |      |
|                       | N                  | 8      |        |        |      | 8      |        |        |      | - | - | - | - | 9      |       |        |      |
|                       | 4-fold increase    | 1      | 12.5   | 0.3    | 52.7 | 2      | 25.0   | 3.2    | 65.1 | - | - | - | - | 0      | 0     | 0.0    | 33.6 |
|                       | GMR                |        | 2.3    | 1.3    | 3.8  |        | 2.3    | 1.2    | 4.2  | - | - | - | - | 1      | 0.8   | 1.1    |      |
| Visit 7 (Day 113/197) | N                  | 7      |        |        |      | 9      |        |        |      | - | - | - | - | 10     |       |        |      |
|                       | GMT                | 4649.9 | 2669.6 | 8099.4 |      | 4679.8 | 2061.2 | 10625  |      | - | - | - | - | 1039.4 | 370.6 | 2914.7 |      |
|                       | Comparison/Visit 1 |        |        |        |      |        |        |        |      | - | - | - | - |        |       |        |      |
|                       | N                  | 7      |        |        |      | 9      |        |        |      | - | - | - | - | 10     |       |        |      |
|                       | 4-fold increase    | 1      | 14.3   | 0.4    | 57.9 | 2      | 22.2   | 2.8    | 60.0 | - | - | - | - | 0      | 0     | 0.0    | 30.8 |
|                       | GMR                |        | 2.1    | 1.2    | 3.9  |        | 2.9    | 1.4    | 5.8  | - | - | - | - | 0.8    | 0.7   | 0.9    |      |
|                       | Comparison/Visit 5 |        |        |        |      |        |        |        |      | - | - | - | - |        |       |        |      |
|                       | N                  | 7      |        |        |      | 8      |        |        |      | - | - | - | - | 9      |       |        |      |
|                       | 4-fold increase    | 0      | 0      | 0.0    | 41.0 | 0      | 0      | 0.0    | 36.9 | - | 0 | - | - | 0      | 0     | 0.0    | 33.6 |
|                       | GMR                |        | 1      | 0.9    | 1.1  |        | 1.2    | 0.8    | 1.9  | - | - | - | - | 0.8    | 0.7   | 1      |      |

**Serotype: S flexneri 6 Ab**

|                       |                    | DOSE C GR1 |            |       |        | DOSE C GR2 |            |       |        | DOSE C GR1+GR2 |            |       |        | PLACEBO |            |       |        |
|-----------------------|--------------------|------------|------------|-------|--------|------------|------------|-------|--------|----------------|------------|-------|--------|---------|------------|-------|--------|
|                       |                    | 95% CI     |            |       |        | 95% CI     |            |       |        | 95% CI         |            |       |        | 95% CI  |            |       |        |
|                       |                    | n          | % or value | LL    | UL     | n          | % or value | LL    | UL     | n              | % or value | LL    | UL     | n       | % or value | LL    | UL     |
| Visit 1 (Day 1)       | N                  |            | 9          |       |        |            | 9          |       |        |                | 18         |       |        |         | 10         |       |        |
|                       | GMT                |            | 469.4      | 296.6 | 742.9  |            | 515.2      | 284.5 | 932.9  |                | 491.8      | 352.1 | 686.7  |         | 673.3      | 373.3 | 1214.7 |
| Visit 4 (Day 29)      | N                  |            | 9          |       |        |            | 8          |       |        |                | 17         |       |        |         | 10         |       |        |
|                       | GMT                |            | 780.2      | 409.1 | 1488   |            | 726.7      | 414.1 | 1275.2 |                | 754.6      | 515   | 1105.6 |         | 694.3      | 411.5 | 1171.5 |
|                       | Comparison/Visit 1 |            |            |       |        |            |            |       |        |                |            |       |        |         |            |       |        |
|                       | N                  |            | 9          |       |        |            | 8          |       |        |                | 17         |       |        |         | 10         |       |        |
|                       | 4-fold increase    | 0          | 0          | 0.0   | 33.6   | 0          | 0          | 0.0   | 36.9   | 0              | 0          | 0.0   | 19.5   | 1       | 10.0       | 0.3   | 44.5   |
|                       | GMR                |            | 1.7        | 1     | 2.7    |            | 1.3        | 0.7   | 2.4    |                | 1.5        | 1     | 2.1    |         | 1          | 0.7   | 1.5    |
| Visit 5 (Day 85/169)  | N                  |            | 8          |       |        |            | 8          |       |        |                | -          | -     | -      |         | 9          |       |        |
|                       | GMT                |            | 830.2      | 560.6 | 1229.6 |            | 964.2      | 501.8 | 1852.5 |                | -          | -     | -      |         | 673.9      | 349.2 | 1300.5 |
|                       | Comparison/Visit 1 |            |            |       |        |            |            |       |        |                | -          | -     | -      |         |            |       |        |
|                       | N                  |            | 8          |       |        |            | 8          |       |        |                | -          | -     | -      |         | 9          |       |        |
|                       | 4-fold increase    | 0          | 0          | 0.0   | 36.9   | 1          | 12.5       | 0.3   | 52.7   |                | -          | -     | -      | 0       | 0          | 0.0   | 33.6   |
|                       | GMR                |            | 1.6        | 1.3   | 2.1    |            | 1.6        | 0.8   | 3.1    |                | -          | -     | -      |         | 0.9        | 0.8   | 1.1    |
| Visit 7 (Day 113/197) | N                  |            | 7          |       |        |            | 9          |       |        |                | -          | -     | -      |         | 10         |       |        |
|                       | GMT                |            | 1093.9     | 634.4 | 1886   |            | 1230.2     | 561.7 | 2694.3 |                | -          | -     | -      |         | 597.9      | 337.7 | 1058.4 |
|                       | Comparison/Visit 1 |            |            |       |        |            |            |       |        |                | -          | -     | -      |         |            |       |        |
|                       | N                  |            | 7          |       |        |            | 9          |       |        |                | -          | -     | -      |         | 10         |       |        |
|                       | 4-fold increase    | 0          | 0          | 0.0   | 41.0   | 2          | 22.2       | 2.8   | 60.0   |                | -          | -     | -      | 0       | 0          | 0.0   | 30.8   |
|                       | GMR                |            | 2          | 1.4   | 2.7    |            | 2.4        | 1.2   | 4.8    |                | -          | -     | -      |         | 0.9        | 0.7   | 1.1    |
|                       | Comparison/Visit 5 |            |            |       |        |            |            |       |        |                | -          | -     | -      |         |            |       |        |
|                       | N                  |            | 7          |       |        |            | 8          |       |        |                | -          | -     | -      |         | 9          |       |        |
|                       | 4-fold increase    | 0          | 0          | 0.0   | 41.0   | 1          | 12.5       | 0.3   | 52.7   |                | -          | -     | -      | 0       | 0          | 0.0   | 33.6   |
|                       | GMR                |            | 1.3        | 1     | 1.6    |            | 1.5        | 0.9   | 2.4    |                | -          | -     | -      |         | 1          | 0.8   | 1.2    |

**Serotype: S flexneri X Ab**

|                 |     | DOSE C GR1 |            |        |       | DOSE C GR2 |            |        |       | DOSE C GR1+GR2 |            |        |       | PLACEBO |            |        |       |
|-----------------|-----|------------|------------|--------|-------|------------|------------|--------|-------|----------------|------------|--------|-------|---------|------------|--------|-------|
|                 |     | 95% CI     |            |        |       | 95% CI     |            |        |       | 95% CI         |            |        |       | 95% CI  |            |        |       |
|                 |     | n          | % or value | LL     | UL    | n          | % or value | LL     | UL    | n              | % or value | LL     | UL    | n       | % or value | LL     | UL    |
| Visit 1 (Day 1) | N   |            | 9          |        |       |            | 9          |        |       |                | 18         |        |       |         | 10         |        |       |
|                 | GMT |            | 13235      | 7838.5 | 22347 |            | 13674      | 8683.2 | 21533 |                | 13453      | 9888.8 | 18301 |         | 10036      | 3972.3 | 25354 |

|                       |                    |   |       |       |       |   |       |       |       |   |       |       |       |   |       |        |       |
|-----------------------|--------------------|---|-------|-------|-------|---|-------|-------|-------|---|-------|-------|-------|---|-------|--------|-------|
| Visit 4 (Day 29)      | N                  |   | 9     |       |       |   | 8     |       |       |   | 17    |       |       |   | 10    |        |       |
|                       | GMT                |   | 58562 | 38386 | 89342 |   | 39875 | 19285 | 82448 |   | 48873 | 33728 | 70818 |   | 16980 | 5192.9 | 55524 |
|                       | Comparison/Visit 1 |   |       |       |       |   |       |       |       |   |       |       |       |   |       |        |       |
|                       | N                  |   | 9     |       |       |   | 8     |       |       |   | 17    |       |       |   | 10    |        |       |
|                       | 4-fold increase    | 5 | 55.6  | 21.2  | 86.3  | 2 | 25.0  | 3.2   | 65.1  | 7 | 41.2  | 18.4  | 67.1  | 1 | 10.0  | 0.3    | 44.5  |
|                       | GMR                |   | 4.4   | 2.3   | 8.6   |   | 3.1   | 1.5   | 6.2   |   | 3.7   | 2.4   | 5.7   |   | 1.7   | 0.8    | 3.4   |
| Visit 5 (Day 85/169)  | N                  |   | 8     |       |       |   | 8     |       |       | - | -     | -     | -     |   | 9     |        |       |
|                       | GMT                |   | 50875 | 36096 | 71705 |   | 33562 | 21014 | 53601 | - | -     | -     | -     |   | 14555 | 4931.4 | 42960 |
|                       | Comparison/Visit 1 |   |       |       |       |   |       |       |       | - | -     | -     | -     |   |       |        |       |
|                       | N                  |   | 8     |       |       |   | 8     |       |       | - | -     | -     | -     |   | 9     |        |       |
|                       | 4-fold increase    | 3 | 37.5  | 8.5   | 75.5  | 2 | 25.0  | 3.2   | 65.1  | - | -     | -     | -     | 0 | 0     | 0.0    | 33.6  |
|                       | GMR                |   | 3.6   | 2.4   | 5.3   |   | 2.8   | 1.9   | 4.2   | - | -     | -     | -     |   | 1.4   | 1      | 2     |
| Visit 7 (Day 113/197) | N                  |   | 7     |       |       |   | 9     |       |       | - | -     | -     | -     |   | 10    |        |       |
|                       | GMT                |   | 51906 | 32643 | 82535 |   | 62856 | 31421 | 126E3 | - | -     | -     | -     |   | 14169 | 5925.3 | 33883 |
|                       | Comparison/Visit 1 |   |       |       |       |   |       |       |       | - | -     | -     | -     |   |       |        |       |
|                       | N                  |   | 7     |       |       |   | 9     |       |       | - | -     | -     | -     |   | 10    |        |       |
|                       | 4-fold increase    | 2 | 28.6  | 3.7   | 71.0  | 5 | 55.6  | 21.2  | 86.3  | - | -     | -     | -     | 0 | 0     | 0.0    | 30.8  |
|                       | GMR                |   | 3.6   | 1.9   | 7     |   | 4.6   | 2.1   | 9.9   | - | -     | -     | -     |   | 1.4   | 1      | 1.9   |
|                       | Comparison/Visit 5 |   |       |       |       |   |       |       |       | - | -     | -     | -     |   |       |        |       |
|                       | N                  |   | 7     |       |       |   | 8     |       |       | - | -     | -     | -     |   | 9     |        |       |
|                       | 4-fold increase    | 0 | 0     | 0.0   | 41.0  | 1 | 12.5  | 0.3   | 52.7  | - | -     | -     | -     | 0 | 0     | 0.0    | 33.6  |
|                       | GMR                |   | 1     | 0.7   | 1.6   |   | 1.8   | 1     | 3.3   | - | -     | -     | -     |   | 1     | 0.8    | 1.4   |

**Serotype: S flexneri Y Ab**

|                       |                    | DOSE C GR1 |                  |        |       | DOSE C GR2 |                  |        |       | DOSE C GR1+GR2 |                  |        |       | PLACEBO |                  |        |       |
|-----------------------|--------------------|------------|------------------|--------|-------|------------|------------------|--------|-------|----------------|------------------|--------|-------|---------|------------------|--------|-------|
|                       |                    | 95% CI     |                  |        |       | 95% CI     |                  |        |       | 95% CI         |                  |        |       | 95% CI  |                  |        |       |
| Time point            |                    | n          | %<br>or<br>value | LL     | UL    | n          | %<br>or<br>value | LL     | UL    | n              | %<br>or<br>value | LL     | UL    | n       | %<br>or<br>value | LL     | UL    |
| Visit 1 (Day 1)       | N                  |            | 9                |        |       |            | 9                |        |       |                | 18               |        |       |         | 10               |        |       |
|                       | GMT                |            | 11176            | 6486.3 | 19258 |            | 12605            | 7546.2 | 21056 |                | 11869            | 8504.8 | 16565 |         | 12331            | 5827.3 | 26093 |
| Visit 4 (Day 29)      | N                  |            | 9                |        |       |            | 8                |        |       |                | 17               |        |       |         | 10               |        |       |
|                       | GMT                |            | 53149            | 28494  | 99137 |            | 36182            | 16084  | 81394 |                | 44351            | 28169  | 69831 |         | 11114            | 4564.2 | 27061 |
|                       | Comparison/Visit 1 |            |                  |        |       |            |                  |        |       |                |                  |        |       |         |                  |        |       |
|                       | N                  |            | 9                |        |       |            | 8                |        |       |                | 17               |        |       |         | 10               |        |       |
|                       | 4-fold increase    | 6          | 66.7             | 29.9   | 92.5  | 3          | 37.5             | 8.5    | 75.5  | 9              | 52.9             | 27.8   | 77.0  | 0       | 0                | 0.0    | 30.8  |
|                       | GMR                |            | 4.8              | 2.5    | 9.2   |            | 3.3              | 1.4    | 7.7   |                | 4                | 2.5    | 6.4   |         | 0.9              | 0.7    | 1.2   |
| Visit 5 (Day 85/169)  | N                  |            | 8                |        |       |            | 8                |        |       | -              | -                | -      | -     |         | 9                |        |       |
|                       | GMT                |            | 50893            | 31669  | 81787 |            | 35303            | 16058  | 77612 | -              | -                | -      | -     |         | 15155            | 5843.5 | 39305 |
|                       | Comparison/Visit 1 |            |                  |        |       |            |                  |        |       | -              | -                | -      | -     |         |                  |        |       |
|                       | N                  |            | 8                |        |       |            | 8                |        |       | -              | -                | -      | -     |         | 9                |        |       |
|                       | 4-fold increase    | 5          | 62.5             | 24.5   | 91.5  | 2          | 25.0             | 3.2    | 65.1  | -              | -                | -      | -     | 0       | 0                | 0.0    | 33.6  |
|                       | GMR                |            | 3.9              | 2.1    | 7.3   |            | 3                | 1.7    | 5.3   | -              | -                | -      | -     |         | 1.2              | 0.9    | 1.6   |
| Visit 7 (Day 113/197) | N                  |            | 7                |        |       |            | 9                |        |       | -              | -                | -      | -     |         | 10               |        |       |
|                       | GMT                |            | 39012            | 25171  | 60463 |            | 56908            | 29096  | 111E3 | -              | -                | -      | -     |         | 14398            | 6142.8 | 33747 |
|                       | Comparison/Visit 1 |            |                  |        |       |            |                  |        |       | -              | -                | -      | -     |         |                  |        |       |
|                       | N                  |            | 7                |        |       |            | 9                |        |       | -              | -                | -      | -     |         | 10               |        |       |
|                       | 4-fold increase    | 2          | 28.6             | 3.7    | 71.0  | 5          | 55.6             | 21.2   | 86.3  | -              | -                | -      | -     | 0       | 0                | 0.0    | 30.8  |
|                       | GMR                |            | 3.1              | 1.7    | 5.6   |            | 4.5              | 2.5    | 8.3   | -              | -                | -      | -     |         | 1.2              | 1      | 1.4   |
|                       | Comparison/Visit 5 |            |                  |        |       |            |                  |        |       | -              | -                | -      | -     |         |                  |        |       |
|                       | N                  |            | 7                |        |       |            | 8                |        |       | -              | -                | -      | -     |         | 9                |        |       |
|                       | 4-fold increase    | 0          | 0                | 0.0    | 41.0  | 0          | 0                | 0.0    | 36.9  | -              | 0                | -      | -     | 0       | 0                | 0.0    | 33.6  |
|                       | GMR                |            | 0.8              | 0.5    | 1.4   |            | 1.6              | 1      | 2.6   | -              | -                | -      | -     |         | 1                | 0.8    | 1.3   |

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## **CHAPTER 6. Phylogenomic analysis of cross-reactivity and antigenic cartography of the functional response induced by altSonflex1-2-3**

### **6.1. Aims and rationale**

Assessing serologically the cross-reactivity induced by altSonflex1-2-3 is critical for inferring the potential of antibodies induced by the vaccine to bind and kill strains from serogroups not included in the vaccine. To perform this analysis, a discrete panel of heterologous strains representing unique and prevalent serogroups was experimentally used. With the aim to evaluate whether the heterologous strains used in cross-reactivity studies were representative of circulating *Shigella* isolates in endemic regions, we constructed a phylogenetic tree including both heterologous *S. flexneri* strains used for the assessment of altSonflex1-2-3 cross-reactivity described in Chapter 5 and publicly available genomes from the GEMS epidemiological study. Moreover, we applied antigenic cartography to SBA data obtained from rat, rabbit, and mouse studies to visualize antigenic relationships among *Shigella* strains and to better understand the degree of cross-reactivity elicited by altSonflex1-2-3.

### **6.2. Datasets and methods**

The heterologous panel of strains used in SBA included *S. flexneri* 4a (H130920147), *S. flexneri* 5b (H130920151), *S. flexneri* 6 (H130920152), *S. flexneri* X (H130920153), and *S. flexneri* Y (H130920154), that were originally collected and characterized by Public Health England (PHE). These isolates were serotyped using both agglutination and flow cytometry-based surface staining with commercial antisera and genotyped by PCR for group-specific markers as previously described (1). Whole-genome sequencing was performed at the Wellcome Trust Sanger Institute. To place the heterologous strains into a broader epidemiological context, we phylogenetically mapped them with *Shigella* isolates collected in endemic settings by the GEMS study, which enrolled children with moderate-to-severe diarrhoea and matched controls across seven sites in Africa and Asia between 2007 and 2011 (2, 3).

Raw sequencing reads of *S. flexneri* heterologous isolates were processed through a standard quality-control and mapping pipeline. Read quality was inspected using FastQC and summarized with MultiQC. Low-quality bases and adapters were trimmed with fastp (4) or Trimmomatic (5),

and assemblies available only as long-read contigs were shredded into pseudo-Illumina reads using ART-Illumina (6) to allow uniform downstream analysis. High-quality reads were assembled de novo using Shovill and annotated with Bakta (7). Reads were also mapped to the *S. flexneri* 2a 301 reference chromosome (NC\_004337.2) using Burrows-Wheeler Alignment (BWA) tool (8) and were processed with Samtools(9) (Li et al., 2009). Variant calling was performed with bcftools (10), applying quality filters (minimum depth  $\geq 4$ , QUAL  $\geq 20$ , strand balance) to exclude low-confidence SNPs. Pseudogenomes were generated with bcftools consensus, masking plasmid, phage, and insertion-sequence regions. Core-genome alignments were produced by concatenating pseudogenomes, and recombination was identified and masked using Gubbins (11). Invariant sites were recovered with snp-sites (12), and maximum likelihood phylogenetic trees were reconstructed with IQ-TREE(13) under a GTR+I+G substitution model with 1000 ultrafast bootstrap replicates.

For antigenic mapping, SBA data were the same as those described in Chapter 5. Sera were obtained from groups of 10 mice, 8 rats, and 6 rabbits immunized with GMMA derived from *S. sonnei*, *S. flexneri* 1b, *S. flexneri* 2a, or *S. flexneri* 3a. The sera were tested against a panel of nine strains, including the four vaccine strains and the five heterologous strains (*S. sonnei*, *S. flexneri* 1b, 2a, 3a, 4a, 5b, 6, X, and Y). Individual SBA titers expressed as IC<sub>50</sub> were log<sub>2</sub>-transformed ( $\log_2[\text{titer}/10]$ ) and organized into antigen  $\times$  serum matrices. For each serum, geometric mean titers were computed at the serotype level, and sera with maximum titers  $\leq 50$  were excluded from the column basis (a titer of 50 represents half of the first SBA dilution and is considered not bactericidal). Antigenic maps were generated in Racmacs (14, 15) using simulated annealing with 1000 independent optimizations and 100 random restarts, retaining the solution with the lowest stress. In these maps, the distance between points is inversely related to the log<sub>2</sub>-transformed SBA titer: higher titers correspond to shorter distances, while lower titers correspond to longer distances. Antigens that are positioned close to each other are those recognized in a similar way by multiple sera, whereas antigens placed far apart elicit more distinct recognition profiles. Likewise, sera cluster if they exhibit similar reactivity patterns across the antigen panel, and separate if their profiles differ. Thus, the two-dimensional layout provides an interpretable visualization of cross-reactivity patterns.

### 6.3. Results and discussion

Maximum likelihood phylogenies constructed from core SNP alignments including the heterologous *S. flexneri* isolates (4a, 5b, X, Y) together with genomes from the GEMS study revealed clustering by serotype (Figure 1). The *S. flexneri* 4a strain consistently grouped within endemic lineages classified as serotype 4a. *S. flexneri* 5b showed close relatedness to *S. flexneri* 3a strains, consistent with the cross-reactivity observed between these serotypes in SBA data (Chapter 5). *S. flexneri* X and Y clustered with field *S. flexneri* X and Y isolates within the GEMS dataset, confirming their representativeness. These results indicate that the heterologous panel is genomically representative of *S. flexneri* diversity circulating in endemic regions, while also highlighting that certain sublineages are not represented and could be targeted in future panel expansions. Additionally, deeper insights can be obtained by focusing only on genes encoding the O-antigen (OAg) or group 4 capsule (which shares the same composition as OAg), as well as on GMMA proteins. These targeted analyses could be beneficial for better understanding the immunogenic profiles associated with OAg (the serotype determinant) or GMMA proteins present in altSonflex1-2-3, which elicit a sustained immune response (16, 17). Ultimately, this approach could enhance our understanding of the potential to generate cross-reactive antibodies.

Tree scale: 0.01

### Geographic Location

- Bangladesh
- India
- Kenya
- Mali
- Mozambique
- Pakistan
- The Gambia

### Case/Control

- Case
- Control

### Cross-reactivity isolates

- Cross-reactivity isolates

### Serotype

- 1a
- 1b
- 2a
- 2b
- 3a
- 3b
- 4a
- 5b
- 6
- 7a
- X
- Y
- 4b
- 4c
- 1d

### ShigaPass

- 2a
- Xv
- SF6
- 1b
- 3a
- 3b
- 2b
- Yv
- 1a
- Unknown
- 4av
- 1c(7a)
- 5b
- Not Shigella/EIEC
- 5a
- EIEC

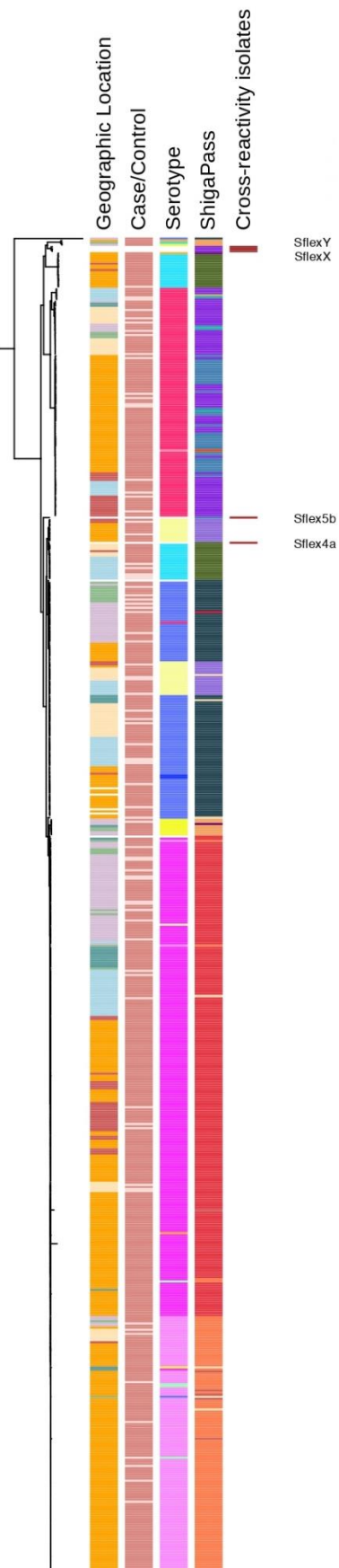


*Figure 1. Maximum-likelihood phylogeny of heterologous strains with GEMS isolates. Branches are colored by geographic location and annotated by case/control status and serotype. Isolates used for cross-reactivity assessment in Chapter 5 (labeled SflexX, SflexY, Sflex4a, and Sflex5b) are shown in brown colons alongside field isolates.*

Antigenic maps constructed from SBA titers revealed species-specific patterns of cross-reactivity (Figure 2). In mice, homologous sera were positioned far apart, indicating poor cross-reactivity in this model, as shown in Chapter 5. Heterologous antigens clustered together and were positioned close to the *S. flexneri* 2a homologous antigen, suggesting that *S. flexneri* 2a is the most cross-reactive homologous strain within this animal model. In contrast, rat sera positioned *S. flexneri* 1b and 3a close together, while *S. flexneri* 4a, X, and Y clustered near both 3a and 2a, consistent with strong cross-reactivity observed in functional assays. *S. flexneri* 5b mapped near 1b and 3a, reflecting its cross-reactive profile, while *S. sonnei* and *S. flexneri* 6 were distant from all other antigens, suggesting limited cross-reactivity of these two strains with all the other represented strains. Rabbit sera produced a similar topology but with greater antigenic separation: *S. flexneri* 4a mapped closer to 1b and 3a, while *S. flexneri* Y and X were positioned nearer to 2a; *S. flexneri* 6 was closer to 2a and 1b than in the rat model. Indeed the rabbit model is the only model where *S. flexneri* 6 showed cross-reactivity, especially with *S. flexneri* 2a. Together, these results help dissect the ability of altSonflex1-2-3 to elicit cross-reactive responses against heterologous *S. flexneri* strains and clearly show how the breadth and topology of antigenic relationships vary across animal models. This underscores the importance of using multiple animal models in preclinical evaluation and supports the inclusion of diverse heterologous strains to better capture altSonflex1-2-3 ability to protect against widespread *S. flexneri* strains that are not included in the vaccine formulation. Looking forward, a critical future prospect is conducting similar analyses using human sera to verify these findings in humans. This would enable confirmation of cross-reactivity patterns and antigenic relationships observed in animal models, offering a closer approximation to how the vaccine formulation performs in a clinical context.

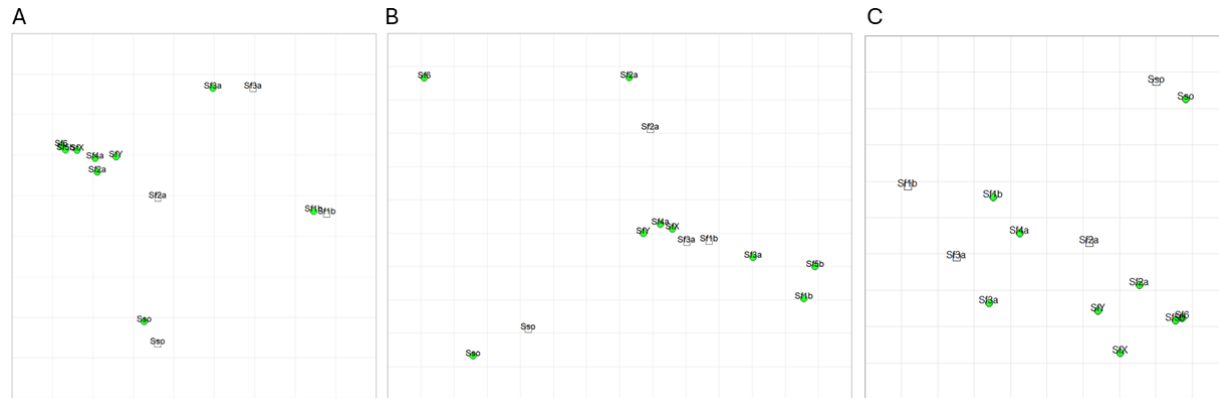


Figure 2. Antigenic maps from SBA data in mice (A), rats (B), and rabbits (C). Antigens are shown as green circles and antisera as open squares. The distance between points is calculated from  $\log_2$ -transformed SBA titers.

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## CHAPTER 7. Novel insights into O-antigen-specific cellular and humoral immunity induced by the altSonflex1-2-3 *Shigella* vaccine

*Manuscript under preparation*

Valentina Caradonna<sup>1</sup>, Valentina Mocci<sup>1</sup>, Roberta Di Benedetto<sup>2</sup>, Omar Rossi<sup>2</sup>, Francesca Micoli<sup>2</sup>, Francesca Mancini<sup>2</sup>, Donata Medaglini<sup>1</sup>, Elena Pettini<sup>1</sup>

<sup>1</sup>. University of Siena, Siena, Italy; <sup>2</sup>. GSK Global Health Vaccines R&D (GVGH), Siena, Italy;

### Abstract

Shigellosis continues to pose a major public health burden, especially among young children in low- and middle-income countries, where effective vaccines are urgently needed. Generalized Modules for Membrane Antigens (GMMA), derived from genetically engineered *Shigella*, represent a promising vaccine platform due to their ability to present native O-antigens (OAg) and bacterial proteins. In this study, we conducted a comprehensive immunological assessment of altSonflex1-2-3, a 4-component GMMA-based *Shigella* vaccine, focusing on both short and long-term immune responses in a murine model. Vaccination rapidly induced robust OAg-specific IgG responses, with high levels achieved after the prime immunization and further boosted following the second dose, along with strong bactericidal activity. These responses were supported by immunological findings observed 10 days after the booster immunization. At that timepoint, analyses revealed the formation of germinal center (GC), the engagement of T follicular helper (Tfh) cells, and the early activation of plasma cells (PCs). Moreover, antigen-positive GCs and PCs were found in the vaccinated group with altSonflex1-2-3. The CD4<sup>+</sup> T cell response was predominantly Th1-polarized, with significant IFN- $\gamma$  and TNF- $\alpha$  production, with a modest Th17 component. Over time the IgG response and bactericidal activity were sustained, demonstrating the persistence of the response up to 22 weeks after the boost. At this long-term timepoint, the cellular immune response transitioned from active GC activity to the establishment of durable memory, characterized by the presence of memory B cells (MBCs) subsets and the persistence of OAg<sup>+</sup> long-lived plasma cells (LLPCs). These findings demonstrate that altSonflex1-2-3 elicits a coordinated and sustained adaptive immune response, engaging both B and T cell arms of immunity and provide key insights into the durability and quality of GMMA-induced immune

response. Ongoing clinical trials in the target infant population will be critical to translating these preclinical findings into real-world impact.

## 1. Introduction

Shigellosis, caused by *Shigella* species, remains a major public health threat worldwide, particularly in low- and middle-income countries where limited access to sanitation and healthcare contributes to its high transmission and disease burden (1). Characterized by diarrhoea, fever, and intestinal inflammation, *Shigella* infections result in significant morbidity and mortality, especially among children under five years of age (2, 3). Despite this substantial burden, there is still no licensed vaccine available for shigellosis, underscoring an urgent need for effective and accessible immunization strategies.

The O-antigen (OAg), a polysaccharide component of *Shigella* lipopolysaccharides (LPS), is a key target of protective immune responses (4-6). Building on this insight, recent innovations have focused on Generalized Modules for Membrane Antigens (GMMA), outer membrane vesicles derived from genetically modified Gram-negative bacteria, as a platform for OAg delivery. GMMA offer several advantages, including presentation of OAg in its native membrane context, intrinsic adjuvanticity via Toll-like receptor (TLR) activation (7), and simplicity of production (8, 9).

To address the unmet need for a broad and effective *Shigella* vaccine, the GSK Vaccines Institute for Global Health (GVGH) has developed altSonflex1-2-3, a four-component GMMA-based vaccine incorporating the OAg of *S. sonnei* and *S. flexneri* serotypes 1b, 2a, and 3a (10). The vaccine has been extensively tested in preclinical studies using various animal models (10, 11) and has advanced to Phase II clinical trials in infants in Kenya. This advancement follows positive safety and immunogenicity results, with the evaluation of the humoral immune response in European adults during a Phase I trial (12). These studies have shown that altSonflex1-2-3 induces robust IgG response and strong bactericidal activity, not only against the targeted strains but also against heterologous *Shigella* isolates (10, 12). However, the OAg-specific cellular immune response elicited by the altSonflex1-2-3 vaccine has not yet been characterized. Moreover, questions remain about the kinetics and persistence of the vaccine-induced humoral and cellular responses over time.

There is growing evidence that GMMA-based vaccines can stimulate both innate and adaptive immune responses (13). Due to their complex membrane composition, including LPS, lipoproteins, and outer membrane proteins, GMMA activate innate immune cells via TLR4 signaling (14). *In vitro*, *S. sonnei* GMMA have been shown to activate a range of human immune cells, including monocytes, dendritic cells, natural killer cells, B cells, and  $\gamma\delta$  T cells, accompanied by robust cytokine secretion (15). In murine models, GMMA have also been shown to be captured by follicular dendritic cells (FDCs), which facilitate antigen presentation to B cells and promote the development of high-affinity antibody responses (16). Furthermore, a GMMA-based vaccine has previously demonstrated the ability to elicit strong cellular responses against other Gram-negative pathogens, such as *Salmonella* (17), highlighting the broader immunostimulatory potential of this platform. The primary aim of this study was to characterize, for the first time, the OAg-specific cellular immune response elicited by the altSonflex1-2-3 vaccine. A secondary objective was to evaluate the kinetics and persistence of humoral and cellular immune responses, including antibody titers and functionality, as well as short- and long-term cellular immunity. A secondary objective was to evaluate the kinetics and persistence of humoral and cellular immune responses, including antibody titers and functionality, as well as short- and long-term cellular immunity.

## **2. Materials and methods**

### **2.1. GMMA formulation**

To determine the optimal dose of the GMMA-based *Shigella* vaccine for animal administration to investigate cellular immune response, a preliminary dose selection study was conducted. For this purpose, groups of animals were immunized with different doses of two formulations, designated as altSonflex1-2-3 and 4-C GMMA (with and without Alhydrogel, respectively), along with their respective control formulations (altSonflex1-2-3 placebo and 4-C GMMA placebo), made of same matrix without antigens (Supplementary Figure 1). Following evaluation of the humoral and cellular immune responses, formulation altSonflex1-2-3 at the dose of 5  $\mu$ g of total OAg was selected for all further studies.

The altSonflex1-2-3 and 4-C GMMA vaccine formulations, as well as the control formulations (altSonflex1-2-3 placebo and 4-C GMMA placebo), were supplied by GVGH (Siena, Italy). The

GMMA utilized were derived from genetically engineered strains of *S. sonnei*, *S. flexneri* 1b, 2a, and 3a (*S. sonnei* 53G  $\Delta tolR:kan$   $\Delta virG:nadAB$   $\Delta msbB2:cat$   $\Delta msbB:erm$ , *S. flexneri* 1b Stansfield  $\Delta tolR:frt$   $\Delta msbB1a:frt$   $\Delta msbB1b:frt$ , *S. flexneri* 2a 2457T  $\Delta tolR:kan$ ,  $\Delta msbB:cat$ , and *S. flexneri* 3a 6885  $\Delta tolR:kan$ ,  $\Delta msbB:cat$ ), prepared as detailed in previous work (10). The vaccine formulations were designed to contain a 1:1:1:1 ratio (based on OAg content) GMMA from the four strains.

For the dose selection study and the subsequent studies, altSonflex1-2-3 formulation was prepared by adsorbing GMMA in NaCl 154 mM NaH<sub>2</sub>PO<sub>4</sub> 10 mM pH 6.5 on 0.7 mg/ml (Al<sup>3+</sup>) Alhydrogel, at the final concentration of 0.5 or 5  $\mu$ g/ml total OAg (0.125 or 1.25  $\mu$ g/ml each OAg). The correspondent placebo formulation (altSonflex1-2-3 placebo) was composed by NaCl 154 mM NaH<sub>2</sub>PO<sub>4</sub> 10 mM pH 6.5 and 0.7 mg/ml (Al<sup>3+</sup>) Alhydrogel.

The 4-C GMMA vaccine formulation, used exclusively in the dose selection study, was prepared without the addition of Alhydrogel. The formulation was obtained by diluting *S. sonnei* and *S. flexneri* 1b, 2a and 3a GMMA in NaCl 154 mM NaH<sub>2</sub>PO<sub>4</sub> 10 mM pH 6.5 at the final concentration of 10  $\mu$ g/ml total OAg (2.4  $\mu$ g/ml each OAg). The correspondent placebo formulation (4-C GMMA placebo) was composed by NaCl 154 mM NaH<sub>2</sub>PO<sub>4</sub> 10 mM pH 6.5.

## **2.2. Animals and studies design**

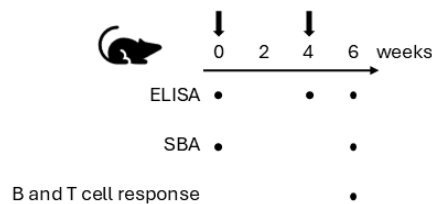
Six-week-old female C57BL/6 mice were purchased from Charles River Laboratories (Lecco, Italy) and handled in compliance with national regulations (Decreto Legislativo 26/2014). Animals were housed in specific pathogen-free conditions at the animal facility of the Laboratory of Molecular Microbiology and Biotechnology (LA.M.M.B.), Department of Medical Biotechnologies, University of Siena, in cages with food and water ad libitum. The study design and procedures adhered to the principles of the three R's principles (reduce, replace and refine), incorporating measures such as environmental enrichment, nesting materials, veterinary supervision, statistically powered sample sizes, and humane practices, including the use of anesthesia prior to cervical dislocation for euthanasia. All animal experiments received approval from the Italian Ministry of Health with authorization number 731/2024-PR.

For the dose selection study, groups of C57BL/6 mice were immunized at weeks 0 and 4 with altSonflex1-2-3 or 4-C GMMA formulations. Immunizations were performed by intramuscular

(IM) route in a volume of 50  $\mu$ L. Control groups were injected with placebo formulations in a volume of 50  $\mu$ L. After the dose selection, two separate studies were designed to evaluate the short- (Figure 1A) and long-term (Figure 1B) OAg-specific humoral and cellular immune responses following vaccination (Figure 1). The dose selection study enabled identification of the altSonflex1-2-3 5  $\mu$ g total OAg dose as the optimal vaccine dose capable of inducing a specific humoral and cellular immune response against the OAg (Supplementary Figure 1).

#### A) study 1: short-term immune response

6 mice/group  
Vaccines:  
• altSonflex1-2-3 (5  $\mu$ g tot. OAg)  
• altSonflex1-2-3 placebo



#### B) study 2: long-term immune response

8 mice/group  
Vaccines:  
• altSonflex1-2-3 (5  $\mu$ g tot. OAg)  
• altSonflex1-2-3 placebo

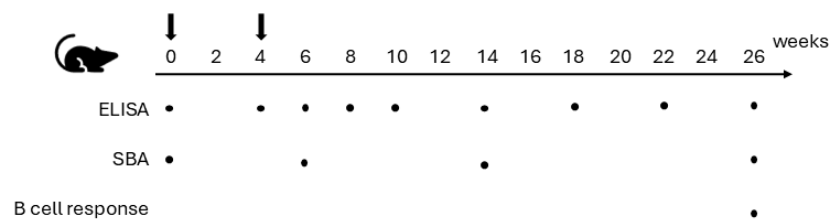


**Figure 1 - Experimental design for immunogenicity studies in mice.** C57BL/6 mice were immunized intramuscularly (IM) with altSonflex1-2-3 at the dose of 5  $\mu$ g of total OAg or altSonflex1-2-3 placebo, administered at weeks 0 and 4 (solid vertical arrows). (A) Study 1: OAg-specific IgG concentration was measured by enzyme-linked immunosorbent assay (ELISA) in sera at week 0, 4 and 6 (10 days post-boost) and bactericidal activity of antibodies was assessed by serum bactericidal assay (SBA) in sera at week 0 and at sacrifice. B cell responses in the draining lymph nodes (dLN) and T cell responses in spleens were evaluated at sacrifice; (B) Study 2: For long-term immune response analysis, OAg-specific IgG levels (ELISA) were monitored in sera at week 0, 4, every two weeks up to week 10, and subsequently until sacrifice at week 26 (22 weeks post-boost). Bactericidal antibody activity (SBA) was measured at week 0, 6, 14 and at the time of sacrifice. B cell responses in the dLN, spleen, and bone marrow (BM) were evaluated at sacrifice.

### 2.3. Samples collection

For the study of humoral response, blood was collected on weeks 0, 4 and 6 (dose selection study and study 1) or 0, 4, 6, 8, 10, 14, 18, 22 and 26 (study 2). Blood was taken from the temporal plexus in the case of *in itinere* blood sampling or by cardiac puncture at the time of sacrifice and centrifuged at 1200× *g* at 4 °C for 12 min and the sera collected. All sera were stored at –80 °C until Enzyme-linked immunosorbent assay (ELISA) or Serum Bactericidal assay (SBA) analysis.

For the study of cellular immune response, draining lymph nodes (dLN), spleen and bone marrow (BM) were collected in tubes with complete medium [cRPMI, RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (both from Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany)] at the sacrifice of animals. Cells from BM were collected by removing the muscles and residue tissues surrounding the femur, eliminating epiphyses, and flushing out the BM with cRPMI by using a syringe with a 25 G needle (BD Plastipak™, Becton Dickinson, USA). All organs were mashed through 70 µm nylon screens (Sefar Italia, Torino, Italy), washed two times in cRPMI and red blood cells (RBC) were removed from samples using RBC lysis buffer, according with the manufacturer's protocol (eBioscience, San Diego, CA, USA). The number of cells was defined using a Countess 3<sup>®</sup> automated cell counter (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA).

## 2.4. ELISA

OAg-specific IgG concentration in mice sera was measured using ELISA as previously described and the protocol was adapted for high-throughput analysis of preclinical sera (18). Briefly, 384-well microplates (Greiner Bio-One, Kremsmünster, Austria) were coated and incubated overnight (16 h) at 4 °C. The coating concentrations and buffers used were as follows: *S. sonnei* LPS (0.5 µg/mL in PBS), *S. flexneri* 1b OAg (2 µg/mL in carbonate buffer), *S. flexneri* 2a OAg (5 µg/mL in carbonate buffer), and *S. flexneri* 3a OAg (1 µg/mL in PBS). Following coating, wells were aspirated and blocked for 1 hour at room temperature (25°C) using fat-free 5% milk powder (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) dissolved in PBS to reduce non-specific binding. After blocking, plates were washed 3 times using PBS containing 0.05% Tween-20 (PBST). Mouse serum samples were diluted at three concentrations (1:100, 1:4000, and 1:160000) in PBS containing 0.05% Tween-20 and 0.1% bovine serum albumin (BSA) (both from Sigma-Aldrich). Diluted sera were added to the wells and incubated for 2 h at 25 °C. Plates were then

washed 3 times with PBS-T. For IgG detection, an alkaline phosphatase-conjugated secondary antibody specific to mice was prepared in PBS-Tween-20 (0.05%) with 0.1% BSA and added to the plates at different concentrations depending on the coating antigen (1:5400 for *S. sonnei* LPS, 1:10000 for *S. flexneri* 1b OAg, 1:6000 for *S. flexneri* 2a OAg and 1:4500 for *S. flexneri* 3a OAg). Plates were incubated for 1 h at 25 °C with secondary antibodies, followed by 3 washed in PBS-T. To reveal the presence of antibodies, a substrate solution of p-Nitrophenyl phosphate substrate was added to each well (Sigma-fast, Sigma-Aldrich, Massachusetts, United States) and incubated for 1h at 25 °C. The enzymatic reaction produced a yellow color, which was quantified by measuring absorbance at 405 nm using a Synergy microplate reader (BioTek Instruments, Winooski, VT, USA). Background absorbance at 490 nm, corresponding to the intrinsic absorbance of the plastic plate, was subtracted from the 405 nm reading to yield the corrected optical density (OD). Antibody concentrations were calculated by comparison to a reference standard serum composed of pooled mouse sera with known OAg-specific IgG titers. A five-parameter logistic (5PL) curve was generated from ten serial dilutions of the standard serum, along with two blank wells (no serum), run in duplicate on each plate. Results were expressed in ELISA Units per milliliter (EU/mL), where one EU was defined as the reciprocal of the dilution of the standard serum that produced a corrected OD (405 nm – 490 nm) equal to 1.

## 2.5. SBA

The bactericidal activity of serum against *S. sonnei*, *S. flexneri* 1b, 2a and 3a was evaluated using a luminescent serum bactericidal assay (L-SBA), as previously described (19). Serum samples were heat-inactivated at 56°C for 30 minutes to eliminate endogenous complement activity prior to testing. Bacterial strains were prepared by culturing to log-phase growth ( $OD_{600} = 0.25 \pm 0.002$ ), followed by dilution at 1:1000 in PBS. Heat-inactivated sera were diluted in PBS for assays involving *S. sonnei*, *S. flexneri* 2a, and *S. flexneri* 3a, and in LB medium for *S. flexneri* 1b assays. Serial dilutions of serum samples were added to 96-well plates. Complement activity was restored by adding Baby Rabbit Complement (BRC, Cederlane, Burlington, Ontario, Canada), with concentrations adjusted based on the bacterial strain being tested: 30% BRC for *S. flexneri* 1b, 20% BRC for *S. sonnei* and *S. flexneri* 3a, 7.5% BRC for *S. flexneri* 2a. The plates were incubated at 37°C for 3 hours to allow complement-mediated killing. Bacterial viability was assessed using BacTiter-Glo reagent (Promega Corporation, Madison, WI, USA), which detects ATP production

as an indicator of surviving bacteria. Luminescence was measured using a Synergy HT luminometer (Biotek, Winooski, VT, USA). Bactericidal activity was quantified by calculating the IC<sup>50</sup> value, defined as the reciprocal serum dilution that results in a 50% reduction in luminescence after 180 minutes of incubation (T<sub>180</sub>) relative to the luminescence measured at the start of the assay (T<sub>0</sub>), indicating 50% bacterial inhibition. For samples with titers below the assay's detectable range, a value of 50 was assigned, corresponding to half of the first dilution tested (1:100). Data analysis, including curve fitting and IC<sup>50</sup> calculations, was performed using GraphPad Prism version 9 (GraphPad Software, LLC, San Diego, CA, USA).

## **2.6. OAg production and tetramerization**

OAg was extracted and purified as previously described (20). Purified OAg were biotinylated using CDAP chemistry (21) as previously described (22), and successful biotin binding was confirmed by biotin assay.

Biotinylated OAg were tetramerized on streptavidin-PE (BD Biosciences), adapting conditions from a previously described protocol (23). Briefly, biotinylated OAg were mixed with streptavidin-PE, added in five steps at 20-minute intervals, incubating in the dark at 4°C on an orbital shaker, to achieve a final OAg:streptavidin molar ratio of 4:1. This procedure yielded four distinct tetramers: [*S. sonnei* OAg]<sub>4</sub>-PE, [*S. flexneri* 1b OAg]<sub>4</sub>-PE, [*S. flexneri* 2a OAg]<sub>4</sub>-PE, and [*S. flexneri* 3a OAg]<sub>4</sub>-PE.

To assess the performance and specificity of tetramer binding, tetramers were validated by adapting a previously described protocol (24). Specifically, 50 µL of UltraComp eBeads Plus Compensation beads (Thermo Fisher Scientific, Waltham, MA, USA) were incubated for 15 minutes on ice with 3 µL of pooled sera from mice vaccinated with altSonflex1-2-3 (study 2, week 8 timepoint). An anti-streptavidin antibody was used as a positive control. After washing, beads were incubated for 25 minutes on ice with tetramers, followed by a second wash. Samples were acquired on a BD Symphony flow cytometer (Supplementary Figure 2).

## **2.7. Multiparametric flow cytometric analysis**

Within study 1, to assess B- and T-cell response induced by altSonflex1-2-3 vaccination at 10 days after the booster vaccine dose, about 3 x 10<sup>6</sup> cells from dLN were incubated for 30 min at 4 °C in Fc-blocking solution (complete medium with 5 µg/mL of CD16/CD32 mAb [clone 2.4G2; BD

Biosciences, San Jose, CA, USA]). Cells were then stained with PE-conjugated OAg tetramers, BUV395-conjugated anti-B220 (clone RA3-6B2; BD Biosciences), FITC-conjugated anti-GL-7 (clone GL-7; BD Biosciences), RB613-conjugated anti-CD95 (clone Jo2; BD Biosciences), BV605-conjugated anti-IgD (clone 11-26c.2a; BD Biosciences), BV711-conjugated anti-IgM (R6-60.2; BD Biosciences), BV650-conjugated anti-IgG1 (clone A85-1, BD Biosciences, USA), BV786-conjugated anti-CD138 (clone 281-2; BD Biosciences), BV421-conjugated anti-CD267 (TACI, clone 8F10 ; BD Biosciences), RB705-conjugated anti-CD3 (clone 145-2C11; BD Biosciences), BUV737-conjugated anti-CD38 (clone 90/CD38; BD Biosciences), APC-conjugated anti-CXCR5 (clone 2G8, BD Biosciences), BV510-conjugated anti-CD4 (clone RM4-5; BD Biosciences), APC700-conjugated anti-CD44 (clone IM-7; Biolegend), RB780-conjugated anti-CD273 (PD-1, clone RMP1-30; BD Biosciences). Samples were labeled with a Live/Dead Fixable Near IR Dead Cell Stain Kit, according to the manufacturer's instruction (Invitrogen, Eugene, OR, USA).

Intracellular cytokine production was assessed in spleen of vaccinated mice by flow cytometry after OAg restimulation. In detail, about  $3 \times 10^6$  splenocytes were collected in sterile conditions and cultured O/N in the presence of anti-CD28, anti-CD49d (both 2  $\mu$ g/mL, eBioscience, Thermo Fisher Scientific, San Diego, CA, USA), and a mix of *S. sonnei*, *S. flexneri* 1b, 2a and 3a GMMA (10  $\mu$ g/mL of total GMMA). Unstimulated or phorbol 12-myristate 13-acetate (PMA) and ionomycin calcium salt (50 ng/mL and 1  $\mu$ M, respectively, Sigma-Aldrich, St Louis, MO, USA) treated cells were used as negative and positive controls, respectively. Brefeldin A (5  $\mu$ g/mL, Sigma-Aldrich) and monensin solution (2 $\mu$ M, eBioscience) were added to all samples for the last 4 h of incubation. Cells were washed twice in PBS and labeled with a Live/Dead Fixable Yellow Stain Kit, according to the manufacturer's instruction (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Fixation and permeabilization were performed using a BD Cytofix/Cytoperm kit, according to the manufacturer's instruction (BD Biosciences) before Fc-blocking and stained with RB705-conjugated anti-CD3 (clone 145-2C11; BD Biosciences), BV510-conjugated anti-CD8a (clone 53-6.7; BD Biosciences), RB613-conjugated anti-CD4 (clone GK1.5; BD Biosciences), RB545-conjugated anti-CD44 (clone IM-7; BD Biosciences), BV786-conjugated anti-IFN- $\gamma$  (clone XMG1.2; BD Biosciences), BV650-conjugated anti-TNF- $\alpha$  (clone MP6-XT22; BD Biosciences), PE-conjugated anti-IL-17A (clone TC11-18H10; BD

Biosciences), BV421-conjugated anti-IL-4 (clone 11B11; eBioscience), BV421-conjugated anti-IL-13 (clone W19-895; BD Biosciences).

Within study 2, to profile the long-term OAg-specific B-cell response, about  $3 \times 10^6$  cells from dLN, BM and splenocytes were stained with PE-conjugated OAg tetramers, BUV737-conjugated anti-CD19 (clone 1D3; BD Biosciences), FITC-conjugated anti-GL-7 (clone GL-7; BD Biosciences), RB613-conjugated anti-CD95 (clone Jo2; BD Biosciences), BV605-conjugated anti-IgD (clone 11-26c.2a; BD Biosciences), BV650-conjugated anti-IgG1 (clone A85-1, BD Biosciences, USA), BV786-conjugated anti-CD138 (clone 281-2; BD Biosciences), BV421-conjugated anti-CD267 (TACI, clone 8F10 ; BD Biosciences), RB705-conjugated anti-CD184 (clone 2B11/CXCR4; BD Biosciences), BUV395-conjugated anti-CD38 (clone 90/CD38; BD Biosciences), BV711-conjugated CD3e (clone 145-2C11, BD Biosciences), APC-conjugated anti-CD73 (clone TY/11.8, BioLegend, San Diego, CA, USA), BV480-conjugated anti-CD80 (clone 16-10A1, BD Biosciences), RB705-conjugated anti-CD184 (clone 2B11/CXCR4, BD Biosciences), RB780-conjugated anti-CD273 (PD-L2, clone MIH37, BD Biosciences). Samples were labeled with a Live/Dead Fixable Near IR Dead Cell Stain Kit, according to the manufacturer's instruction (Invitrogen, Eugene, OR, USA).

All antibodies were titrated for optimal dilution. Samples were acquired on BD LSRFortessa X20 flow cytometer (BD Biosciences). Data analysis was performed using FlowJo v10 (Tree Star, Inc., Ashland, OR, USA).

## **2.8. Statistical analysis**

All samples were tested individually. The Mann–Whitney test was used to assess the statistical difference between vaccinated and placebo groups and the Wilcoxon test was used to assess the statistical difference between timepoints inside the same group. A  $p$ -value  $\leq 0.05$  was considered significant. Statistical analysis was performed using GraphPad Prism version 9 (GraphPad Software, LLC, San Diego, CA, USA).

## **3. Results**

### **3.1. Short-term response**

#### ***Immunization Elicits OAg-Specific Humoral and Cellular Immune Responses***

Group of mice were vaccinated with altSonflex1-2-3 or altSonflex1-2-3 placebo formulation at week 0 and 4. To characterize the humoral immune response induced by vaccination, serum IgG specific for the vaccine antigens were measured at week 0, 4 and 6. As shown in Figure 2A, IgG specific to *S. sonnei* and *S. flexneri* 1b, 2a and 3a OAg significantly increased at week 4 following the primary immunization ( $P \leq 0.001$ , \*\*\*). The rise of IgG before and after the booster immunization (week 4 and week 6) was statistically significant for all the vaccine antigens. In particular, OAg-specific IgG increased from geometric mean titers (GMT) of 82.6 at week 4 to 849.1 at week 6 against *S. flexneri* 1b OAg ( $P \leq 0.001$ , \*\*\*); from GMT of 261 at week 4 to 2328 against *S. flexneri* 2a OAg ( $P \leq 0.001$ , \*\*\*); from GMT of 510.8 at week 4 to 3272 at week 6 against *S. flexneri* 3a OAg ( $P \leq 0.001$ , \*\*\*); from GMT of 247.2 at week 4 to 420.5 at week 6 ( $P \leq 0.001$ , \*\*\*) (Figure 2A). Vaccinated mice also exhibited significantly higher bactericidal activity against all the four vaccine strains compared to those that received placebo ( $P \leq 0.0001$ , \*\*\*\*, Figure 2B), reaching GMTs of 2090 against *S. sonnei*, 4081 against *S. flexneri* 1b, 3202 against *S. flexneri* 2a and 4404 against *S. flexneri* 3a in the vaccinated group compared to GMTs of 50 in the placebo group, confirming that the antibodies were not only abundant but also functionally effective in mediating bacterial killing.

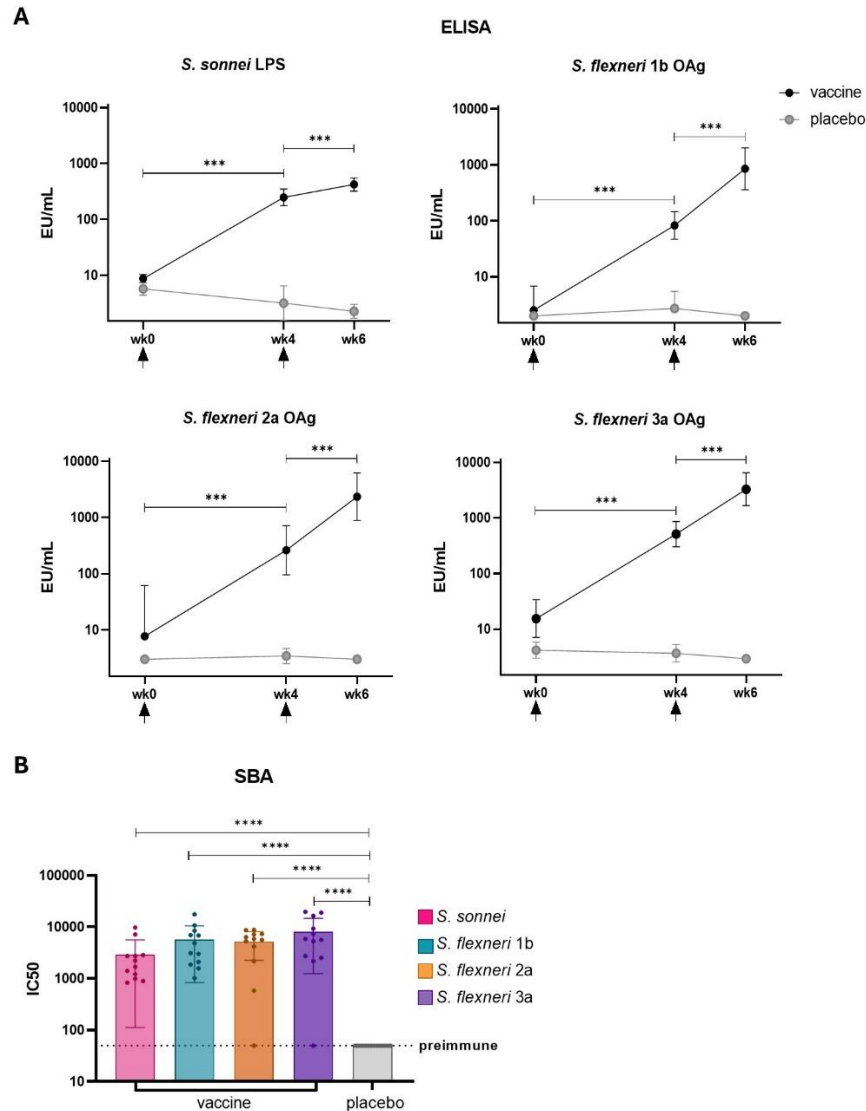


**Figure 2. Short-term antigen-specific IgG immune response and bactericidal activity of antibodies.** C57BL/6 mice were immunized intramuscularly with altSonflex1-2-3 (vaccine) or altSonflex1-2-3 placebo (placebo) at week 0 and week 4 and humoral response was analysed in serum. (A) Serum IgG specific to *S. sonnei* and *S. flexneri* 1b, 2a and 3a OAg were measured by ELISA at week 0 (pre-immune), week 4 (pre-boost), and week 6 (10 days post-boost). Dots indicate the geometric mean titers (GMT) and error bars the 95% CI of 12 mice per group from 2 different experiments. Black dots indicate the vaccinated group and grey dots the control group. Arrows indicates the vaccination timepoints. Wilcoxon test was used to assess statistical difference between timepoints in the vaccine group (\*\* $p \leq 0.001$ ); (B) Bactericidal activity of antibodies was assessed at week 6 using SBA. Serum bactericidal activity was determined as the dilution necessary to obtain 50% CFU reduction at  $T_{180}$  compared with  $T_0$  ( $IC^{50}$ ). Bars indicate the GMT 95% CI of 12 mice per group (dots) from 2 different experiments. Colored bars indicate the vaccinated groups and grey bar the control group. Unpaired Mann-Whitney test was used to assess statistical difference between vaccine and placebo groups (\*\*\*\*  $p \leq 0.0001$ ).

In addition to the assessment of OAg-specific antibody responses against *S. sonnei* and *S. flexneri* 1b, 2a, and 3a and evaluating their effector functionality, for the first time the OAg-specific cellular immune response induced by the altSonflex1-2-3 vaccine was characterized. This was performed through multiparameter flow cytometric analysis of distinct immune cell subsets such as germinal center B cells (GCs), plasma cells (PCs), and follicular helper T cells ( $T_{fh}$ ), as well as the identification of vaccine antigen-specific B cells within both GCs and PCs (Figure 3).

The primary immune response to vaccination is initiated locally in the dLN, where naïve lymphocytes encounter antigen and GC formation occurs (25). The induction of GCs is a critical marker of the quality of the humoral response, as GC is the site of B cell affinity maturation and differentiation to memory B cells (MBCs) and PCs (26-29). GC B cells were identified in dLNs 10 days post-boost as  $B220^{+}IgD^{-}$  cells co-expressing GL7 and CD95 molecules, as shown in representative contour plots in Figure 3A. Significantly higher frequencies of GC/ $B220^{+}$  cells were found in the vaccinated group (35,20%) compared to the placebo group (7%) ( $p < 0.001$ , \*\*\*) (Figure 3A).

$T_{fh}$  cells play an essential role in supporting GC formation and B cell differentiation and for the generation of robust B cell responses (30). Indeed, the GC cycle is initiated when antigen-primed B cells engage with cognate  $T_{fh}$  cells, leading to their extensive proliferation (27).  $T_{fh}$  cells were identified in the dLNs as  $CD4^{+}CD44^{+}PD1^{+}CXCR5^{+}$  cells (Figure 3B). A statistically significant expansion of  $T_{fh}$  cells was observed in the vaccine group (3.9%) compared to placebo controls (0.5%) ( $P < 0.001$ , \*\*\*) (Figure 3B), further supporting the presence of an active and sustained GC response.

After cognate interactions with  $T_{fh}$  cells, some activated B cells follow an extrafollicular pathway and rapidly differentiate into PCs within the dLN (31, 32). To assess whether altSonflex1-2-3 vaccination induced the generation of PCs,  $B220^{-}IgD^{-}TACI^{+}CD138^{+}$  phenotype cells, a well-established profile of terminally differentiated antibody-secreting cells (33), were analysed in dLNs as showed in Figure 3C. Vaccinated mice with altSonflex1-2-3 exhibited a significantly higher frequency of PCs (3.2%) in the dLN respect to the placebo-treated group (0.01%) ( $P < 0.001$ , \*\*\*) (Figure 3C).

It is noteworthy that within the vaccine-induced cellular subpopulations described above, for the first time OAg-specific B cell subsets were identified by flow cytometric analysis using

biotinylated OAgs tetramerized with PE-conjugated streptavidin. As shown in Figure 3D and 3E, this approach enabled the detection of B cells specifically recognizing the vaccine antigens within GCs ( $\text{OAg}^+\text{CD95}^+\text{GL7}^+\text{B220}^+\text{IgD}^-$ ) and among PCs ( $\text{OAg}^+\text{TACI}^+\text{CD138}^+\text{B220}^-\text{IgD}^-$ ). OAg-specific GC B cells were significantly more abundant in the vaccine group (0.99%) respect to the placebo group (0.30%) ( $p < 0.05$ , \*), as showed in histograms in Figure 3D. In addition,  $\text{OAg}^+$ PCs were found in the vaccinated group (6.7%) and undetectable in the placebo-treated controls (0%) ( $P < 0.001$ , \*\*\*) (Figure 3E).

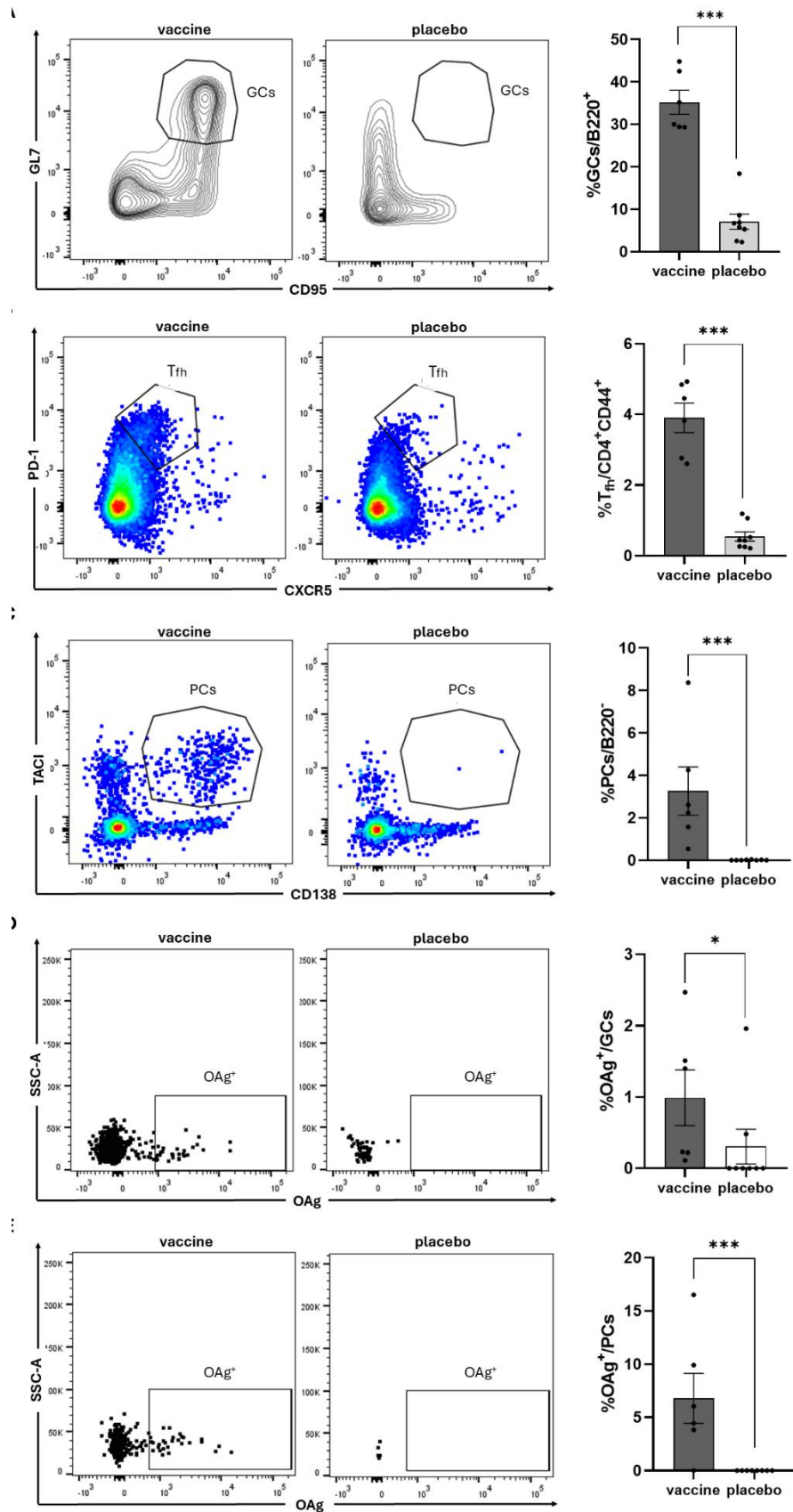


**Figure 3. Short-term cellular immune response.** C57BL/6 mice were immunized intramuscularly with altSonflex1-2-3 (vaccine) or altSonflex1-2-3 placebo (placebo) at week 0 and week 4, and the cellular immune response was analyzed in draining lymph nodes (dLNs) 10 days post-boost. (A) Germinal center B cells (GCs) identified by flow cytometry as B220<sup>+</sup>GL7<sup>+</sup>CD95<sup>+</sup> are shown in contour plots with corresponding histograms reporting frequencies; (B) T follicular helper (T<sub>fh</sub>) cells identified in dLNs by flow cytometry as CD4<sup>+</sup>CD44<sup>+</sup>PD-1<sup>+</sup>CXCR5<sup>+</sup> and (C) Plasma cells (PCs) identified in dLNs by flow cytometry as IgD<sup>+</sup>B220<sup>+</sup>CD138<sup>+</sup>TACI<sup>+</sup>, are shown in pseudocolor plots with corresponding bar graphs reporting frequencies; (D) Antigen-specific GC B cells identified in dLNs by flow cytometry as Ag<sup>+</sup>B220<sup>+</sup>GL7<sup>+</sup>CD95<sup>+</sup> and (E) Antigen-specific PCs identified in dLNs by flow cytometry as Ag<sup>+</sup>IgD<sup>+</sup>B220<sup>+</sup>CD138<sup>+</sup>TACI<sup>+</sup> are shown in pseudocolor plots with corresponding histograms reporting frequencies. For all panels (A–E), flow cytometry plots display one representative animal from the vaccine and placebo groups, selected from two different experiments. Bar graphs report frequencies of the indicated populations, reported as mean  $\pm$  SEM from 6 vaccinated and 8 placebo mice per group (dots) representative of two different experiments. Statistical differences between vaccine and placebo groups were assessed using the Mann–Whitney test for multiple pairwise comparisons (\*  $p \leq 0.05$ , \*\*\*  $p \leq 0.001$ ).

### **altSonflex1-2-3 vaccine Drives a Predominant Th1 and Th17 Skewing of the immune Response**

To further characterize the cellular immune response and define the profile of vaccine-induced T cells, cytokine production by activated CD44<sup>+</sup>CD4<sup>+</sup> T cells, isolated from spleens 10 days after the booster immunization with altSonflex1-2-3 and restimulated with a total of 10  $\mu$ g/mL of a mix of GMMA vaccine antigens, was evaluated. By intracellular cytokine staining, the frequencies of interferon (IFN)- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-17, IL-4, and IL-13 secreting cells were measured (Figure 4A). Significantly higher production of IFN- $\gamma$ <sup>+</sup> (1.47% in the vaccine group vs. 0.26% in the placebo group) ( $P \leq 0.0001$ , \*\*\*\*), TNF- $\alpha$ <sup>+</sup> (0.43% in the vaccine group v. 0.14% in the placebo group) ( $P \leq 0.001$ , \*\*\*) and IL-17<sup>+</sup> (0.42% in the vaccine group vs. 0.26% in the placebo group) ( $P \leq 0.05$ , \*) was observed (Figure 4B). In contrast, IL-4<sup>+</sup> and IL-13<sup>+</sup> CD4<sup>+</sup> T cell frequencies remained at background levels in the vaccine and in the placebo groups (%0.01 for both groups). To evaluate the functional complexity of the response, Boolean gating was applied to identify T cells producing one or more cytokines simultaneously. This analysis revealed a significantly increased frequency of CD4<sup>+</sup> CD44<sup>+</sup> T cells co-expressing IFN- $\gamma$  and TNF- $\alpha$  in animals vaccinated with altSonflex1-2-3 (0.35%) than in placebo group (0.030%) ( $p < 0.0001$ , \*\*\*\*; Figure 4B). No other multifunctional subsets—cells producing two or three different cytokines—showed significant differences between groups (Figure 3B). Overall, the cytokine-producing CD4<sup>+</sup> T cell population was dominated by IFN- $\gamma$  and TNF- $\alpha$  expression, with a smaller

subset producing IL-17, as depicted in Figure 4C. These findings support a Th1-skewed immune profile with a modest Th17 contribution after the administration of the altSonflex1-2-3 vaccine.

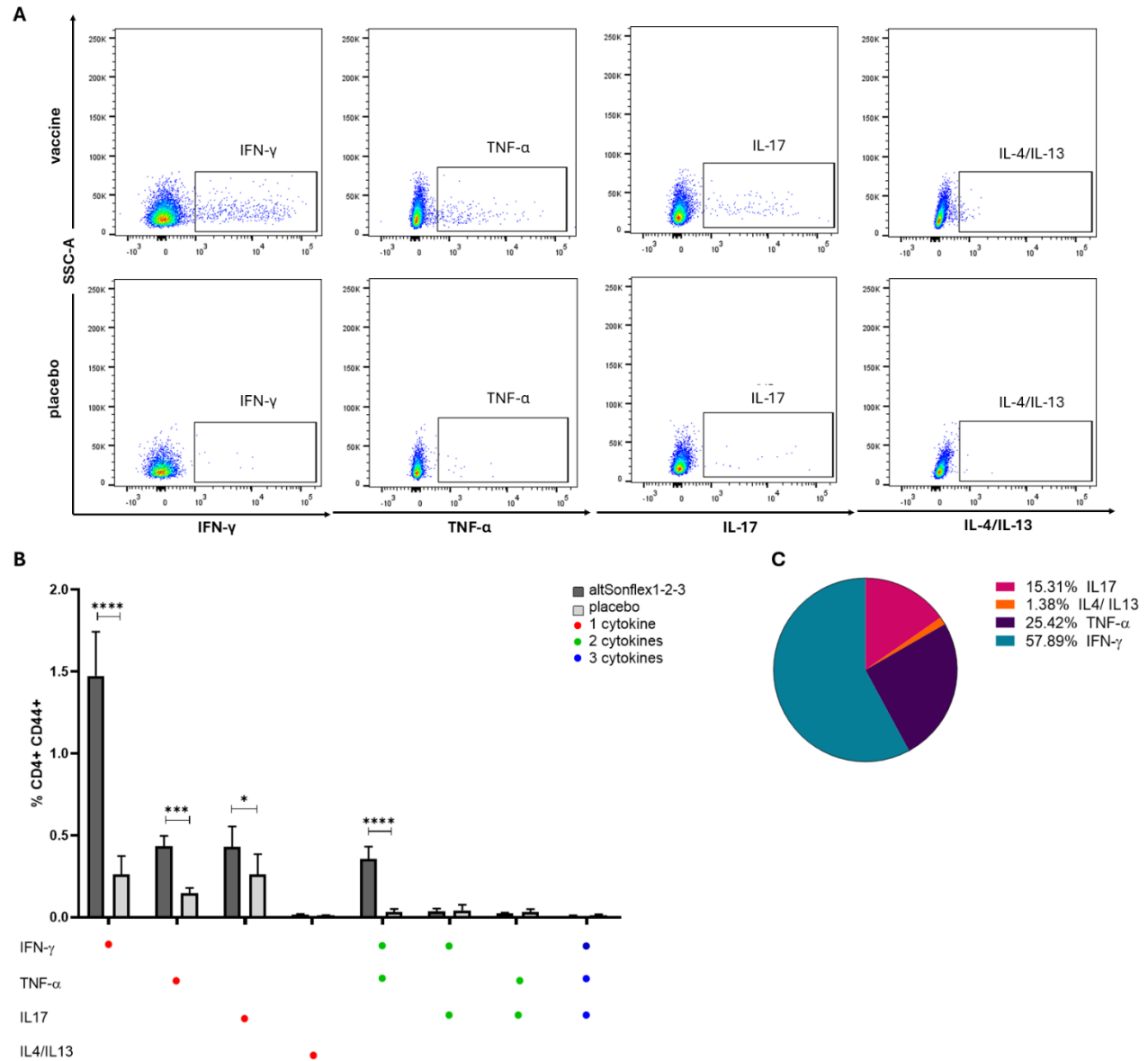


**Figure 4. Characterization of CD44<sup>+</sup>CD4<sup>+</sup> T cell cytokine production.** Cells were isolated from spleens 10 days after booster immunization with altSonflex1-2-3 (vaccine) or altSonflex1-2-3 placebo (placebo) and analyzed by intracellular cytokine staining. (A) Expression of IFN- $\gamma$ , TNF- $\alpha$ , IL-17, and IL-4/IL-13 was evaluated in CD44<sup>+</sup>CD4<sup>+</sup> T cells by flow cytometry to define the cytokine profile of vaccine-induced T cells. Representative pseudocolor plots are shown for one animal from the vaccine and placebo group, selected from two independent experiments. (B) Frequencies of CD4<sup>+</sup> T cells producing a single cytokine or combinations of two or three cytokines,

determined by Boolean gating analysis are reported. Responses are color coded according to functionality (red, single cytokine; green, two cytokines; blue, three cytokines). Data are reported as mean  $\pm$  SEM of 12 mice from two independent experiments. Statistical significance between vaccine and placebo groups was assessed using the Mann–Whitney test for multiple pairwise comparisons (\*  $p \leq 0.05$ , \*\*\*  $p \leq 0.001$ , \*\*\*\*  $p \leq 0.0001$ ). (C) Pie chart illustrating the proportional representation of CD44<sup>+</sup>CD4<sup>+</sup> T cells producing one cytokine. Frequencies of cells were normalized to the total frequency of CD44<sup>+</sup>CD4<sup>+</sup> T cells producing any of the cytokines shown (IL-17, IL-4/IL-13, TNF- $\alpha$ , IFN- $\gamma$ ) and expressed as percentages.

### 3.2. Long-term response

#### ***altSonflex1-2-induces long-lasting functional OAg-specific IgG and OAg-specific LLPCs and MBCs***

To evaluate the persistence of the immune response following vaccination, both OAg-specific humoral and cellular immune responses were analyzed up to 22 weeks after the booster dose. Figure 5A illustrates the kinetics of OAg-specific serum IgG responses induced by altSonflex1-2-3 prime-boost vaccination, measured by ELISA at nine time points: week 0, 4, 6, 8, 10, 14, 18, 22, and 26 after the first vaccine administration. Following the first immunization (from week 0 to week 4), OAg-specific IgG levels increased for all tested antigens, although the changes did not reach statistical significance. In contrast, the second immunization induced significant increases in IgG titers from week 4 to week 6 ( $P \leq 0.01$ , \*\*), thereby demonstrating a boost effect. In particular, geometric mean titers (GMTs) increased from 49.01 to 175 for *S. sonnei* OAg, from 80.58 to 1321 for *S. flexneri* 1b OAg, from 77.05 to 749.7 for *S. flexneri* 2a OAg, and from 193.8 to 2695 for *S. flexneri* 3a OAg. Peak of OAg-specific IgG were reached at week 6 for *S. sonnei* and remained stable through week 26. By week 26 (22 weeks post-boost), antibody levels had decreased to roughly 50% of their peak values (GMT = 72.3) ( $P \leq 0.001$ , \*\*, week 6 vs 26) but remained numerically higher respect to those measured at week 4 (GMT = 49) ( $P \leq 0.01$ , \*, week 4 vs 26). Antigen specific IgG peaked at week 8 for all the *S. flexneri* OAg (GMT = 1488.1 for *S. flexneri* 1b, GMT = 905.1 for *S. flexneri* 2a and GMT = 3299 for *S. flexneri* 3a) and remained stable thereafter, with a decline observed only at week 26 for *S. flexneri* 1b ( $P \leq 0.001$ , \*\*, week 8 vs 26) and at week 22 for *S. flexneri* 3a ( $P \leq 0.001$ , \*\*, week 8 vs 22). Significantly higher IgG levels were observed at week 26 compared to week 4 for *S. flexneri* 3a (GMT = 193.8)( $P \leq 0.05$ , \*, week 4 vs week 26) and *S. flexneri* 1b (GMT = 230.5)( $P \leq 0.05$ , \*, week 4 vs week 26), suggesting a durable OAg-specific humoral response (Figure 5A). IgG at week 26 for *S. flexneri*

2a (GMT = 241.8) resulted numerically higher than IgG at week 4, but the difference was not statistically significant (Figure 5A).

To determine the functionality of the circulating antibodies, serum bactericidal activity was measured at weeks 6, 14, and 26 (Figure 5B). Although IC<sup>50</sup> titers in vaccinated animals declined over time, they remained significantly higher than placebo group at week 26 against *S. sonnei* and *S. flexneri* 1b ( $P \leq 0.01$ , \*\*) and against *S. flexneri* 2a and 3a ( $P \leq 0.001$ , \*\*\*). Significant reductions in IC<sup>50</sup> of bactericidal activity were observed across all four tested antigens between week 6 and week 26 ( $P \leq 0.01$ , \*\*): from GMT of 8231 at week 6 to 1462 at week 26 against *S. sonnei*; from GMT of 11999 at week 6 to 767.1 at week 26 against *S. flexneri* 1b; from GMT of 22671 at week 6 to 5599 at week 26 against *S. flexneri* 2a; from GMT of 33141 at week 4 to 6419 at week 6 against *S. flexneri* 3a, reflecting a natural waning of functional antibodies over time while still maintaining functionality (Figure 5B).

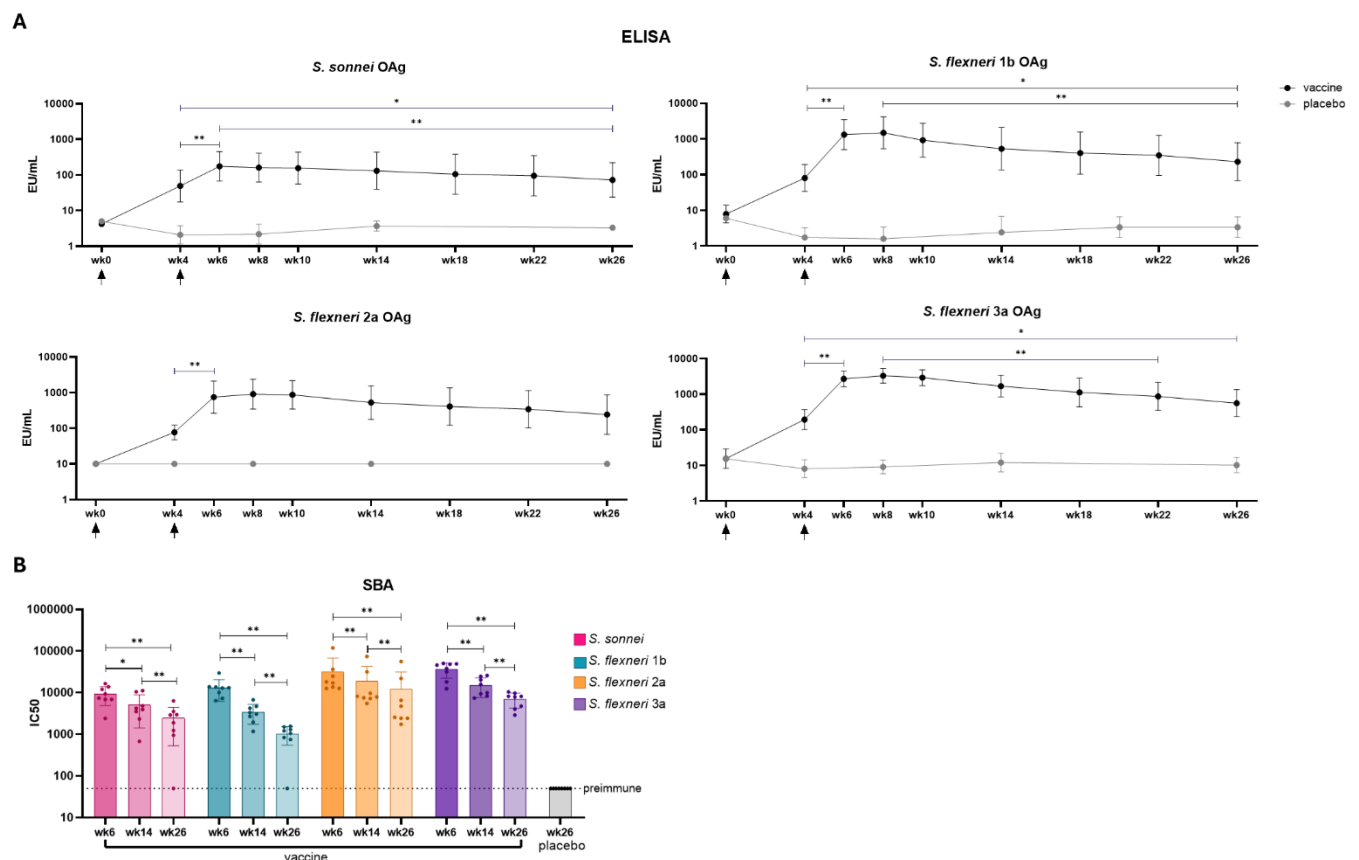


**Figure 5. Long-term OAg-specific IgG immune response and bactericidal activity of antibodies.** C57BL/6 mice were immunized intramuscularly with altSonflex1-2-3 (vaccine) or altSonflex1-2-3 placebo (placebo) at week 0 and week 4 and humoral response was analysed in serum. (A) Serum

*IgG titers specific to S. sonnei and S. flexneri 1b, 2a and 3a OAg were measured by ELISA at weeks 0, 4, 6, 8, 10, 14, 18, 22, and 26. Dots indicate the geometric mean titers (GMT) and error bars the 95% CI of 8 vaccinated mice. Black dots indicate the vaccinated group and grey dots the control group. Arrows indicate the vaccinations timepoints. Wilcoxon test was used to assess statistical difference between timepoints in the vaccinated group (\*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ ); (B) Serum bactericidal activity of antibodies was assessed at week 6, 14 and 26. Bactericidal activity was determined as the dilution necessary to obtain 50% CFU reduction at  $T_{180}$  compared with  $T_0$  ( $IC^{50}$ ). Bars indicate the GMT 95% CI of 8 mice per group, individual values are indicated by dots. Colored bars indicate the vaccinated group and grey bar the control group. Data shown in the placebo group are from SBA against S. sonnei and representative of the other placebo groups. Wilcoxon test was used to assess statistical difference between timepoints in the vaccinated group (\*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ ).*

With the aim of gaining insight into the cellular processes sustaining long-term humoral immunity, GC reaction and memory B cell differentiation in the dLN was investigated. Figure 6A compares short- (10 days post-boost) and long-term (22 weeks post-boost) responses by analyzing GL7 and CD38 molecules expression on total B cells, markers associated with GC B cells and MBCs, respectively. At 10 days post-boost (as shown in section 3.1), in the group vaccinated with altSonflex1-2-3, frequencies of GL7<sup>+</sup> GC B cells on total B cells were abundant (47%), respect to CD38<sup>+</sup> cells (28.8%), consistent with active GC reactions. However, by week 22 post-boost, GL7<sup>+</sup> cell frequencies had diminished (5.8%), while CD38<sup>+</sup> cells became the predominant population (53.6%) (Figure 6A).

MBCs populations were assessed within CD38<sup>+</sup> cells based on co-expression of CD73 and CD80 surface markers, in combination with antigen specificity (Figure 6B-C). CD80 and CD73, ligands for CD28/CTLA-4 and markers of GC activity respectively, are co-expressed on differentiated MBCs, reflecting their roles in T-dependent responses, somatic hypermutation, and class switch recombination (34). Both in BM and in spleen it is possible to observe an higher frequency of OAg<sup>+</sup>CD73<sup>+</sup>CD80<sup>+</sup> MBCs in vaccinated mice respect to placebo-treated mice (0.23% vs. 0.02% in BM and 0.26% vs. 0.11% in spleens; Figure 6B-C), suggesting the establishment of durable MBCs populations. Figure 6D shows the abundance in BM of vaccinated mice respect to placebo treated ones of MBCs co-expressing CD73, CD80, and CD273, a triple-positive phenotype associated with advanced maturation and a heightened ability to rapidly differentiate into antibody-secreting cells upon antigen re-encounter (35, 36). After defining this triple-positive population, the analysis was extended to identify the antigen-specific fraction within it. The OAg<sup>+</sup> subset of

triple-positive MBCs (Figure 6E) was more abundant in mice vaccinated with altSonflex1-2-3 compared to controls (0.45% vs. 0.12% in spleen,  $P \leq 0.05$ , \*; 0.22% vs. 0.03% in BM).

The GC reaction generates plasmablasts that undergo affinity maturation and class switching, with a fraction differentiating into long-lived plasma cells (LLPCs) (28, 37). Sustained and persistent antibody responses rely on the presence of LLPCs, which are characterized by expression of CXCR4 molecule, a chemokine receptor that directs their homing to BM survival niches (38). Plasma cells were initially defined by the co-expression of CD138 and TACI molecules, and within this population, the fraction expressing CXCR4 was gated to delineate LLPCs (Figure 6F). OAg<sup>+</sup> cells were then identified within this CXCR4<sup>+</sup> subset. Antigen-specific LLPCs were present in the vaccine group (2.32%) and absent in the placebo group (0%) ( $P \leq 0.01$ , \*\*), suggesting that vaccination with altSonflex1-2-3 induced long-lived antibody-secreting cells that likely reside within bone marrow survival niches.

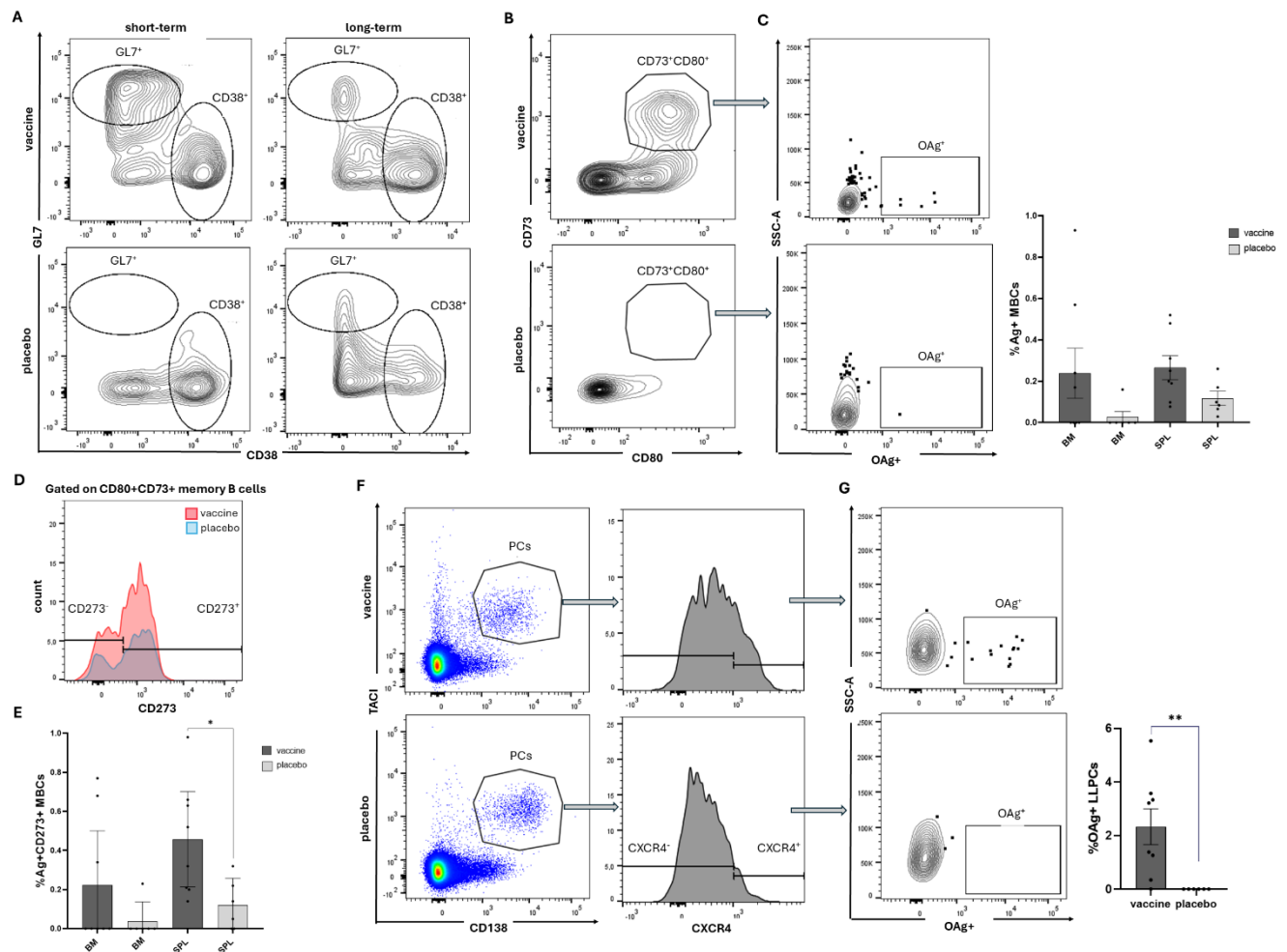


**Figure 6. Long-term OAg-specific cellular immune response.** C57BL/6 mice were immunized intramuscularly with altSonflex1-2-3 (vaccine) or altSonflex1-2-3 placebo (placebo) at week 0 and week 4, and the cellular immune response was analyzed 22 weeks post-boost. (A) Germinal centre B cells (GCs, GL7<sup>+</sup>) and memory B cells (MBCs, CD38<sup>+</sup>) were identified in draining lymph nodes (dLNs) by flow cytometry on B220<sup>+</sup>/CD19<sup>+</sup> total B cells. Contour plots show one representative sample of vaccine or placebo groups at 10 days (short-term) or 22 weeks (long-term) post-boost. (B) CD73<sup>+</sup>CD80<sup>+</sup> expression on CD38<sup>+</sup>IgD<sup>-</sup>CD19<sup>+</sup> MBCs was assessed in bone marrow (BM) 22 weeks post-boost by flow cytometry. Contour plots show one sample representative of vaccine or placebo groups. (C) Antigen-specific MBCs (OAg<sup>+</sup>) were identified within CD73<sup>+</sup>CD80<sup>+</sup> MBCs, as gated in B. Contour plots show one representative sample of the vaccine or the placebo group. The correspondent bar graph summarizes the frequency of Ag<sup>+</sup> MBCs in BM and spleen (SPL), reported as mean  $\pm$  SEM from 8 vaccinated and 6 placebo mice per group (dots). (D) Histogram overlays show CD273 expression on CD80<sup>+</sup>CD73<sup>+</sup> MBCs. One representative vaccinated mouse (red) and placebo mouse (blue) are shown. The x-axis represents CD273 fluorescence intensity and the y-axis the number of events. Vertical markers indicate gating for CD273<sup>-</sup> and CD273<sup>+</sup> subsets. (E) Bar graph showing the frequencies of Ag<sup>+</sup>CD273<sup>+</sup>CD73<sup>+</sup>CD80<sup>+</sup> MBCs in BM and SPL, reported as mean  $\pm$  SEM from 8 vaccinated and 6 placebo mice per group (dots). (F) Long-lived plasma cells (LLPCs) were first identified as TACI<sup>+</sup>CD138<sup>+</sup> cells in BM. Pseudocolor plots show one representative sample of the vaccine and the placebo group. Within this population, CXCR4 expression was assessed, and flow cytometry histograms illustrate the distribution of CXCR4<sup>-</sup> and CXCR4<sup>+</sup> cells in one representative sample in the vaccine and in the placebo group. The x-axis represents CXCR4 fluorescence intensity and the y-axis the number of events. Vertical markers indicate gating for CXCR4<sup>-</sup> and CXCR4<sup>+</sup> subsets. (G) Antigen-specific LLPCs (OAg<sup>+</sup>TACI<sup>+</sup>CD138<sup>+</sup>CXCR4<sup>+</sup>), as gated in F, were identified by flow cytometry. Contour plots show one representative sample of the vaccine or the placebo group. The correspondent bar graph summarizes the frequency of OAg<sup>+</sup> LLPCs expressed as mean (bars)  $\pm$  SEM from 8 vaccinated and 6 placebo mice per group (dots). Statistical differences between vaccine and placebo groups were assessed using the Mann–Whitney test for multiple pairwise comparisons (\*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ , \*\*\*\*  $p \leq 0.0001$ ).

## Discussion

Shigellosis remains a critical public health concern, particularly in low- and middle-income countries, where children under five are disproportionately affected. Despite decades of research, the development of an effective vaccine has been challenging due to the antigenic diversity of *Shigella* serotypes and the ability of the vaccine of inducing a durable and strong immunity. GMMA represent a promising vaccine platform in this context, offering a native presentation of OAg and outer membrane proteins in a self-adjuvanting, low-cost formulation particularly suited for LMICs.

In this study, the immune responses elicited in mice by altSonflex1-2-3, a quadrivalent GMMA-based *Shigella* vaccine, currently undergoing Phase II clinical evaluation (12), was comprehensively characterized. The vaccine was administered twice, four weeks apart, and both

the OAg-specific humoral and cellular immune responses were evaluated at short-term (10 days) and long-term (22 weeks) intervals following the booster dose. In this study robust OAg-specific IgG response was observed against all four vaccine serotypes after a single immunization, with further increases noted following the booster dose (Figure 2A and 5A). Moreover, the functionality of the vaccine induced OAg-specific IgG was confirmed by the sustained bactericidal activity of the antibodies observed at week 6 after the booster dose (Figure 2B and 5B). This immunogenic profile is in line with earlier preclinical findings for this vaccine, underscoring the booster dose's role in enhancing antigen-specific IgG levels and achieving strong bactericidal activity (10).

The strength of a vaccine lies not only in its ability to generate rapid immune responses, but also in its capacity to sustain long-term protection. Here, IgG responses and antibody functionality remained detectable and significantly higher in vaccinated mice than controls for up to 22 weeks post-boost, despite the expected waning over time (Figure 5A). This kinetic pattern mirrors that observed in a Phase 1 study of a monocomponent *S. sonnei* GMMA vaccine (1790GAHB), where functional antibody responses peaked at day 29 and persisted at elevated levels for at least six months in individuals receiving higher OAg/protein doses (39). In this study, a relatively high total OAg dose of 5 µg was used, higher than the amounts typically employed in previous murine studies (10). This dose was selected based on results from a prior dose selection study (Supplementary Figure 1) with the goal of enhancing the antigen-specific cellular responses, evaluated for the first time in this context. However, this higher OAg dose appeared to dampen the overall IgG response and the functional activity of antibodies, particularly in the case of *S. sonnei*, when compared to responses observed using lower antigen doses (10). This reduction is consistent with the hook effect observed with *S. sonnei* antigens at higher concentrations, including the 5 µg dose used here, where excessively high antigen levels may interfere with optimal immune activation and antibody production (Supplementary Figure 1).

The persistence of humoral responses was accompanied by features that indicate the induction of the cellular immune response by the altSonflex1-2-3 vaccine such as the activation of GC reaction and memory formation, demonstrated for the first time in the present work. At 10 days post-boost timepoint, elevated frequencies of GL7<sup>+</sup>CD95<sup>+</sup> GC B cells in dLN (Figure 3A), a population essential for effective affinity maturation and memory imprinting (40), were detected. Within these GCs, OAg<sup>+</sup> subset was identified, indicating that the vaccine antigen is actively retained within the

GC microenvironment. By 22 weeks post-boost, the GC B cell population had largely transitioned into CD38<sup>+</sup> MBCs, consistent with the contraction of the GC response and development of long-lived humoral memory (Figure 6A). Early studies established CD38 re-expression as a marker of memory commitment following GC exit (41, 42). These CD38<sup>+</sup> MBCs also expressed canonical memory markers including CD73, CD80, and CD273 (Figure 6B-6E), a profile consistent with functionally competent GC-derived memory subsets (36, 43, 44). The combined expression of these markers is widely used to distinguish MBCs with high recall potential, either capable of seeding secondary GC reactions or rapidly differentiating into antibody-secreting cells upon antigen re-encounter (35).

The identification of T<sub>fh</sub> cells in dLN at 10 days after the second vaccination, coinciding with GC formation, supports the T-dependent nature of this vaccine's immunogenicity (Figure 5B). This mechanism was previously confirmed in studies showing that GMMA-induced anti-OAg IgG responses were significantly reduced in T-cell-deficient mice, underscoring the importance of GMMA-associated proteins in promoting T–B cooperation (11). Also, the importance of the evaluation of T<sub>fh</sub> cells after a vaccination against *Shigella* has been previously highlighted for their critical role in promoting humoral responses (45).

At the T cell level, the altSonflex1-2-3 GMMA-based vaccine induced a Th1-skewed immune profile characterized by IFN- $\gamma$  and TNF- $\alpha$  production, accompanied by a detectable IL-17<sup>+</sup> Th17 subset (Figure 4A-C). Boolean analysis revealed polyfunctional CD4<sup>+</sup> T cells co-expressing IFN- $\gamma$  and TNF- $\alpha$ , a functional profile previously associated with infection by intracellular pathogens such as *Salmonella* (46). These findings are also supported by murine pulmonary infection models in which Th17 cells dominated the CD4<sup>+</sup> T cell response to *S. flexneri*, playing a key role in mucosal immunity (47). Human studies also confirm the importance of these cytokines: following oral immunization with inactivated *S. flexneri* vaccines, transient spikes in IL-17, IFN- $\gamma$ , and TNF- $\alpha$  were observed, particularly after the first dose (48).

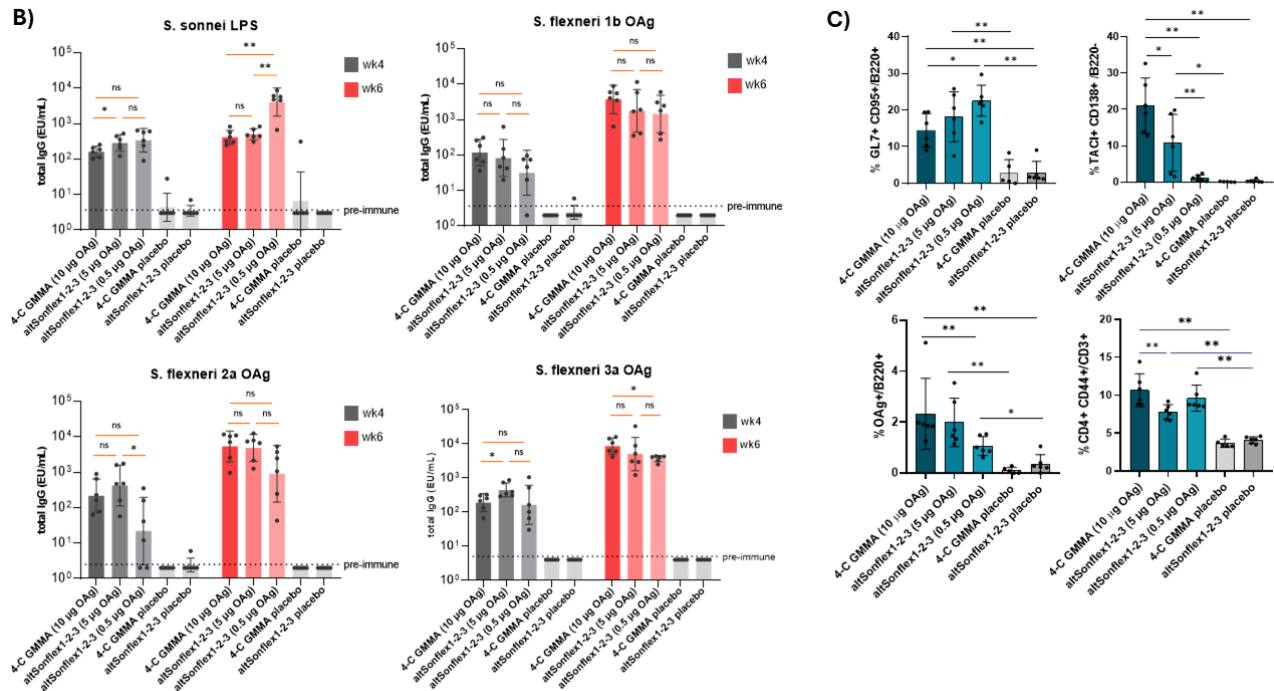
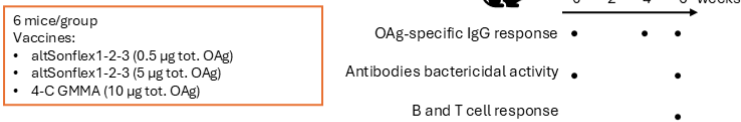
Simultaneously, 10 days after the booster dose, OAg<sup>+</sup> TACI<sup>+</sup>CD138<sup>+</sup> plasmacells in the dLNs (Figure 5C-E) were detected. Typically, such cells appear transiently in the dLNs, about 10–12 days after priming, where they sustain a brief wave of antibody secretion before undergoing apoptosis (27). This process is characteristic of a short-lived plasma cell response often elicited by bacterial antigens like lipopolysaccharides (49). At this early timepoint, it is likely that the detected

PCs include both those generated via the extrafollicular response and GC-derived plasmablasts, some of which may serve as precursors to LLPCs, that eventually migrate to BM survival niches. Indeed, by 22 weeks after the booster immunization, OAg<sup>+</sup> TACI<sup>+</sup>CD138<sup>+</sup>CXCR4<sup>+</sup> LLPCs have been detected in BM (Figure 6F-G). Given the robust GC reaction observed earlier, it is plausible that PCs generated within the GC subsequently disseminated to the bone marrow as LLPCs, thereby contributing to the prolonged high levels of serum antibodies. Similar findings have been reported in GMMA-based vaccine studies for other pathogens such as *Salmonella Typhimurium*, where Alhydrogel-formulated GMMA induced LLPCs and antigen-specific MBCs that persisted beyond six months and conferred protection in systemic challenge models (17).

These findings highlight the capacity of the GMMA platform not only to elicit potent antibody responses but also to induce durable OAg-specific cellular immunity. The quadrivalent GMMA-based *Shigella* vaccine altSonflex1-2-3 is currently being evaluated in Phase II clinical trials in Kenya, where it is administered to 9-month-old infants in an endemic setting (Clinical Trial NCT05073003). This ongoing study includes different schedules of immunization comparisons and longitudinal follow-up of antibody responses up to three years post-vaccination. The next preclinical step involves a follow-up to the current study, evaluating the same vaccine formulation at 22 weeks post-boost but with a booster administered at week 10 instead of week 4. This adjustment is based on growing evidence that delayed booster strategies can enhance the cellular immune response durability and improve the quality of both humoral and cellular memory (50). These insights, along with the accumulating data from ongoing preclinical studies and clinical trials, will be essential for optimizing immunization schedules to maximize both efficacy and durability of protection.

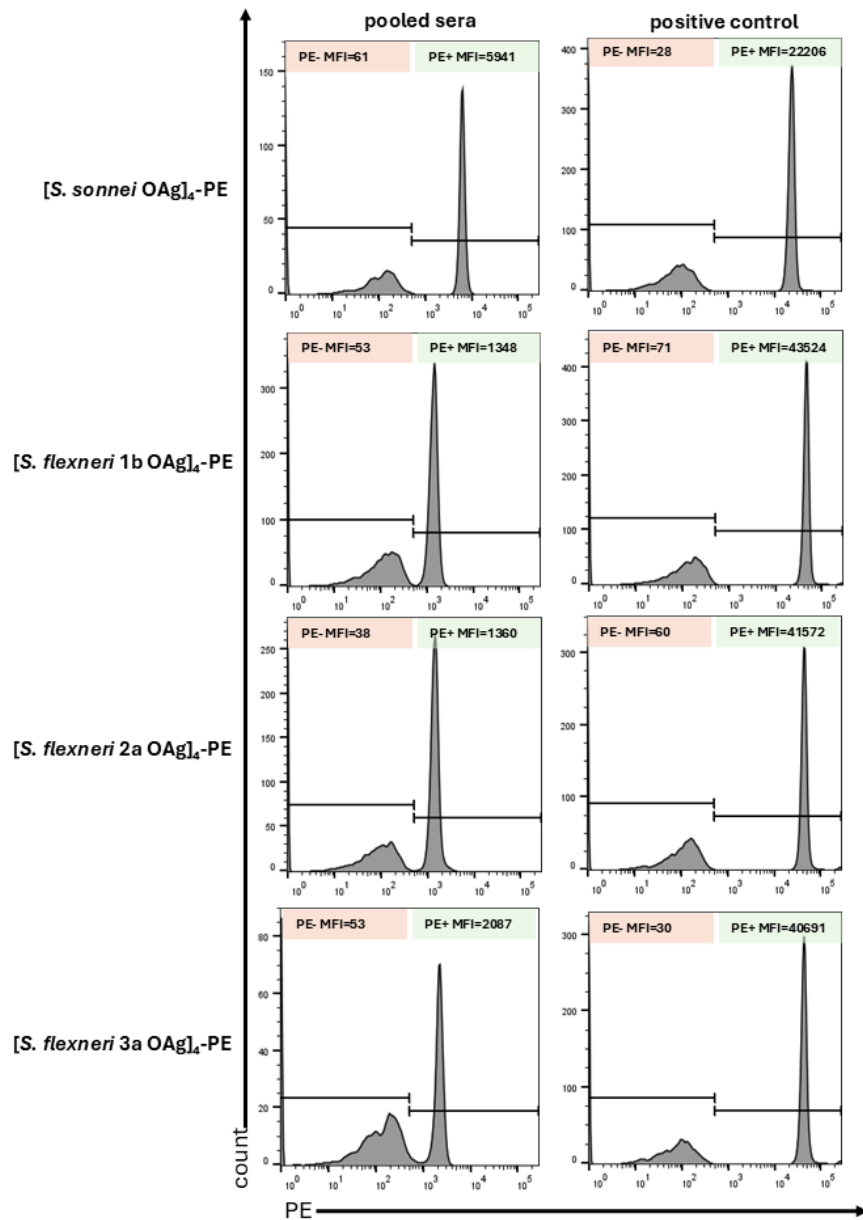
## Supplementary material

### A) setup study



**Supplementary Figure 1. Study for the selection of the optimal dose for subsequent immunological studies.** A) Experimental design of the dose selection study. C57BL/6 mice were immunized intramuscularly (IM) at weeks 0 and 4 (solid vertical arrows) with either altSonflex1-2-3, 4-C GMMA formulations at different doses. Control groups received the corresponding placebo formulations (altSonflex1-2-3 placebo and 4-C GMMA placebo). Serum samples were collected at weeks 0, 4, and 6 to evaluate OAg-specific IgG levels (ELISA), and at weeks 0 and 6 to assess bactericidal antibody activity (SBA). At week 6, draining lymph nodes (dLNs) and spleens were collected for analysis of B and T cell responses. B) Serum IgG titers specific to *S. sonnei* and *S. flexneri* 1b, 2a, and 3a OAg measured by ELISA following immunization with different doses of altSonflex1-2-3 or 4-C GMMA, or their corresponding placebo formulations. Bars show data reported as geometric mean titers (GMT) and the 95% CI of 6 mice/group (dots). Statistical significance between different doses was assessed using the Mann-Whitney test for multiple pairwise comparisons (\*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ , \*\*\*\*  $p \leq 0.0001$ ; ns = not significant). C) Frequencies of total germinal center (GC) B cells (GL7<sup>+</sup>CD95<sup>+</sup>B220<sup>+</sup>), plasma cells (PCs)(IgD<sup>+</sup>B220<sup>+</sup>CD138<sup>+</sup>TACI<sup>+</sup>), antigen-specific B cells (OAg<sup>+</sup>B220<sup>+</sup>), and activated CD4<sup>+</sup> T cells (CD4<sup>+</sup>CD44<sup>+</sup>CD3<sup>+</sup>) in the dLNs, assessed by flow cytometry at week 6 in the same experimental groups. Bars show frequencies reported as mean  $\pm$  SEM of 6 mice /group (dots). Statistical significance between vaccine and placebo groups and between different doses was

assessed using the Mann–Whitney test for multiple pairwise comparisons (\*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ , \*\*\*\*  $p \leq 0.0001$ ).



**Supplementary Figure 2. Validation of tetramer binding performance by flow cytometry.** UltraComp eBeads were incubated with pooled sera from mice vaccinated with altSonflex1-2-3 or with an anti-streptavidin antibody (positive control), followed by staining with PE-labeled tetramers for each of the four GMMA-derived OAGs. Representative histograms for each antigen are shown, comparing PE signal intensity (x-axis), with distinct negative and positive populations (vertical markers), from pooled sera (left) and positive control (right). MFI = median fluorescence intensity.

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## Chapter 8. General conclusions

The research presented in this thesis provides an integrated perspective on how the quadrivalent GMMA-based vaccine, altSonflex1-2-3, shapes both humoral and cellular immune responses against *Shigella* and how these responses may translate into real-world scenarios. The findings collectively contribute to addressing the scientific and public health challenges associated with developing a broadly protective *Shigella* vaccine—a pressing global health priority, particularly in LMICs, where the morbidity and mortality caused by *Shigella* in children is the highest (1-3).

A broad overview of the global distribution of *S. sonnei* and *S. flexneri* serotypes is provided in this thesis, with a focus on *S. flexneri* diversity across LMICs and HICs. The synthesis of epidemiological studies demonstrated that *Shigella* is indeed widespread globally. The review conducted in this thesis further highlighted that *S. flexneri* is often the primary species causing illness in LMICs, with *S. flexneri* 2a frequently reported across multiple countries as the leading cause of shigellosis. Other commonly identified serotypes include *S. flexneri* 3a, 6, and 1b. In HICs, *S. sonnei* typically emerges as the predominant species; however, the prevalence of *S. flexneri* is often comparable, and the distribution of its serotypes mirrors trends seen in LMICs, with *S. flexneri* 2a most common, followed by 3a and 1b, while *S. flexneri* 6 is less frequent. Given the widespread diffusion of this dangerous pathogen and the absence of a widely available vaccine, studying the evolution and distribution of *Shigella* is critical for informing both vaccine design and public health interventions (4-6). This importance is underscored by the establishment of surveillance programs worldwide (7-9) and by multiple epidemiological studies (1, 6, 10) that clarify where *Shigella* circulates and which serotypes require prioritization for vaccine development.

This serotype diversity is driven by structural modifications of the OAg—including glucosylation and O-acetylation substitutions—which not only define *S. flexneri* serotypes but also represent one of the fundamental elements of *Shigella* immunogenicity (11-14). Indeed, many of the most advanced vaccine candidates currently in development are OAg-based (15). However, the OAg itself remains one of the greatest challenges, as it underlies *S. flexneri* serotype diversity and complicates the design of a broadly protective vaccine. The altSonflex1-2-3 vaccine employs GMMA technology as a delivery system for the OAg (16, 17).

The serological studies described in this work had the aim to evaluate the humoral response using ELISA (to measure IgG titers) and SBA (to assess antibody functionality) against *S. sonnei* and *S. flexneri* 1b, 2a, and 3a, which are the altSonflex1-2-3 vaccine components. The vaccine was tested at three different doses and in three different animal models, consistently demonstrating its ability to elicit robust IgG responses and functional activity against all homologous strains, in line with previous findings (17). One of the key contributions of this work is the evaluation of outcomes across multiple animal models, emphasizing the importance of preclinical studies in vaccine development (18). Many countries are actively seeking to reduce or refine animal use in line with the 3Rs principles (19). In this case, testing in different species provided critical insights by identifying a model that is highly predictive of clinical outcomes, ultimately helping minimizing the number of animals needed in future research. For *Shigella*, this is particularly crucial, as it is notoriously difficult to identify a reliable animal model for immune responses against this pathogen and its pathogenesis (20). This work has expanded the understanding of the humoral response against *Shigella*, generating knowledge that is relevant for translational research.

In line with this translational perspective, one of the main objectives of this thesis was to evaluate the ability of altSonflex1-2-3 to induce functional antibodies against *S. flexneri* strains not included in the vaccine, thereby assessing its cross-reactivity both in preclinical models and clinical settings. To bridge preclinical and clinical evaluation, this work included the characterization of a high-throughput SBA assay designed to test vaccine cross-reactivity using clinical human samples against a panel of heterologous *S. flexneri* strains. The ability of the vaccine to stimulate the production of cross-reactive and functional antibodies was therefore evaluated. A central outcome of this work is the demonstration that altSonflex1-2-3 elicits not only strong homologous responses but also functional activity against heterologous *S. flexneri* strains—a critical property given that *Shigella* vaccine development has long been hindered by serotype diversity (3). This was observed especially in rat and rabbit models at the preclinical level, with similarities in cross-reactivity patterns observed but also some differences between species. Results from European adults further confirmed this property: vaccinees generated functional antibodies against non-vaccine serotypes. While preliminary, these findings provide important translational evidence that cross-protection is achievable in humans. Extending such analyses to the target infant population will be essential to determine the full breadth of coverage and to inform the design of broadly protective OAg-based vaccines.

These results gain additional weight when interpreted in light of phylogenetic analyses. By situating the heterologous strains tested in serum bactericidal assays within genomic datasets from large-scale surveillance studies such as GEMS (21), this work confirmed that the strains were representative of circulating *S. flexneri* diversity in endemic countries and reflect responses relevant to real-world epidemiology. Antigenic maps further illustrated how cross-reactivity patterns differ between animal species, offering a visual framework to interpret immune breadth.

Beyond humoral responses, this thesis reports the first detailed characterization of the cellular immune response elicited by altSonflex1-2-3. The vaccine induced germinal center B cells, memory B cells, and long-lived plasma cells, together with polyfunctional Th1 and Th17 responses. These features demonstrate the capacity of GMMA vaccines to establish durable and qualitatively diverse immune memory. Studying cellular immunity is particularly relevant in the case of *Shigella*, an intracellular pathogen where both systemic and mucosal T-cell responses contribute to immunogenicity and probably protection (22-24). Integrating cellular and serological data will therefore be essential to define correlates of protection (25) and to obtain a comprehensive understanding of vaccine-mediated immunity. The results presented here represent a first step in this direction.

Taken together, these findings provide a coherent picture of altSonflex1-2-3 as a rationally designed multicomponent vaccine with the capacity to overcome key challenges in *Shigella* vaccinology. It broadens protection beyond homologous coverage, targets globally relevant strains, shows a good performance across species and in humans (despite inherent differences between species), and induces both humoral and cellular memory. Looking ahead, several priorities emerge. Continuous monitoring of serotype distribution and genomic diversity is needed to ensure vaccine relevance over time (26). Clinical evaluation in infants-the main target population-will be key to confirm immune response and optimize immunization schedules. Finally, the definition of correlates of protection, likely requiring integration of antibody and cellular data, will simplify regulatory approval and future vaccine iterations. If these priorities are successfully met, the development of a *Shigella* vaccine could be accelerated, representing a decisive step in reducing the global burden of shigellosis and addressing one of the most urgent health threats of our time: the spread of antimicrobial resistance.

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Finally, I am profoundly grateful for the collaboration, support, and friendship of all my colleagues at GVGH and LAMMB. Their collective efforts transformed challenges into tangible achievements. This work would not have been possible without the remarkable contributions of every individual and institution mentioned.

## APPENDIX. Curriculum Vitae

# Valentina Caradonna

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### Personal information

**Address:** Via Sandro Pertini, 2, Volte Basse (Sovicille), 53018, Italy

**Phone number:** +39 3316250486

**Email:** [v.caradonna@student.unisi.it](mailto:v.caradonna@student.unisi.it); [valentina.x.caradonna@gsk.com](mailto:valentina.x.caradonna@gsk.com)

[LinkedIn](#)

[ResearchGate](#)

[ORCID](#)

**Date of birth:** 03/11/1996

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### Professional experience

Nov 2022 –

Current

#### **Doctoral program**

**Università degli studi di Siena - GSK Vaccines Institute for Global Health, Siena, Italy**

- Characterization of the immune response elicited by a vaccine developed by GVGH with a focus on cross-functionality and cellular immunity and on the matching of preclinical and clinical data.
- Utilization of preclinical and clinical sera for the characterization and conduction of immunological assays (ELISA, SBA, FACS binding assay, Luminex Multiplex binding assay)
- Bacterial culture growth, characterization and serotyping
- Multiparametric flow cytometry and analysis with the use of FlowJo
- Study of antimicrobial resistant bacteria
- Preparation of sterile formulations for immunological studies in animal models
- Data interpretation and presentation skills
- Team work and collaboration with interdisciplinary groups
- Familiarity with Microsoft Office package and Graphpad for data analysis

Jan 2025 –

Apr 2025

#### **Visiting PhD student**

**University of Cambridge, Cambridge, United Kingdom**

- Construction of phylogenetic trees basing on Shigella circulating genomes in endemic regions with the use of Unix operating system
- Use of R to build antigenic maps based on serological data

Dec 2021 –

Nov 2022

#### **Graduate internship**

**GSK Vaccines Institute for Global Health, Siena, Italy**

- Assessment of the immune response induced by vaccine candidates both at the preclinical level than clinical (phases I and II), using and developing new immunological assays in high throughput.

- Conduction of immunological assays (ELISA, SBA)
- Interpretation of scientific data to support a research project

Aug 2021 –  
Oct 2021

### **Graduate internship**

#### **Faculty of Sciences of the University of Lisbon (FCUL), Lisbon, Portugal**

- Study of symbiotic systems: identification and analysis of lichenic species within the natural parks
- quantification of colonization by mycorrhizal shrubs in *Oryza sativa* and extraction and quantification of the vegetable starch content by spectrophotometry
- study of interactions between thermophilic bacteria and plants used in agriculture
- DNA extraction, gel electrophoresis, PCR

Sep 2019 –  
July 2021

### **Sperimental thesis**

#### **Microbiology and Plant Biotechnology Laboratory, UNITUS, Viterbo, Italy**

- Cytotoxic activity of plant derived compounds on tumoral cells and induction of apoptosis (Cell culture, MTT assay, SDS-page and immunoblotting, fluorescence microscopy)
- Bactericidal and bacteriostatic activity of plant derived compounds (MIC, MBC, agar disk diffusion test, vapour-phase-mediated susceptibility assay)
- Antioxidant activity of plant derived compounds (DPPH, ABTS)

Nov 2020 –  
Feb 2021

### **Undergraduate internship**

#### **CGA – Electron Microscopy section, UNITUS, Viterbo, Italy**

- Fixation and resin inclusion of kidney biopsies and *Drosophila melanogaster* samples for observation and analysis through Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM).

Aug 2018 –  
Nov 2018

### **Undergraduate internship**

#### **Istituto Zooprofilattico del Lazio e della Toscana, Viterbo, Italy**

- Serological assays (ELISA, RBT) and molecular diagnosis for prevention of infectious animal diseases, bacteriological tests on meat and milk in the laboratories of Diagnostics, Serology and Food Microbiology.

Feb 2018 –  
July 2018

### **Undergraduate internship**

#### **Plant Protection Laboratory, UNITUS, Viterbo, Italy**

- Microbiological and molecular diagnosis for the detection of fungi present in non-native plants coming from the Botanical Garden of Rome (DNA extraction and PCR).

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## Education

|           |                                                                                                                                                        |
|-----------|--------------------------------------------------------------------------------------------------------------------------------------------------------|
| July 2021 | Master's degree in Industrial Biotechnology<br>Università degli studi della Toscana                                                                    |
|           | Thesis: "Essential oils of conifers: chemical analysis, cytotoxic activity on human cell models and antimicrobial activity on environmental bacteria". |
| Dec 2018  | Bachelor's degree in Biotechnology<br>Università degli studi della Toscana                                                                             |
|           | Thesis: "Reduction of the incidence of <i>Gnomoniopsis castanea</i> by treatment with gaseous ozone"                                                   |

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## Courses

|             |                                                                                                                                                                                                       |
|-------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| In progress | Certification in Animal Experimentation Techniques, accredited by the Italian Ministry of Health, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna and Università di Siena |
| Feb 2025    | Introduction to R- School of Biological Sciences Bioinformatic Training, University of Cambridge                                                                                                      |
| Feb 2025    | Introduction to the Unix command line – School of Biological Sciences Bioinformatic Training, University of Cambridge                                                                                 |
| Feb 2025    | Working with bacterial genomes – School of Biological Sciences Bioinformatic Training, University of Cambridge                                                                                        |

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## Conferences

|           |                                                                                                                |
|-----------|----------------------------------------------------------------------------------------------------------------|
| Nov 2023  | XV edition of the GSK Phd/Postdoc Global workshop (Siena, Italy)                                               |
| June 2024 | XV edition of GSK Vaccines and Infectious Disease R&D Days 2024 (Siena, Italy)                                 |
| Nov 2024  | XVI edition of the GSK Phd/Postdoc Global workshop (Siena, Italy)                                              |
| Sep 2024  | 7th European Congress of Immunology (Dublin, Ireland)                                                          |
| Mar 2025  | Cambridge Infectious Diseases Annual Symposium 2025 (Cambridge, United Kingdom)                                |
| June 2025 | Palio Meeting "Next-Generation Strategies Against Antimicrobial Resistance". Co-Rapporteur role (Siena, Italy) |

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## Publications

- 2025 Caradonna, V., Hunyady, A., Nakakana, U., Iturriza, M., Micoli, F., Rossi, O., Mancini, F. *Shigella sonnei* and *Shigella flexneri*: epidemiology supporting development of cross-protective vaccines. *Human Vaccines & Immunotherapeutics* (submitted).
- 2025 Caradonna, V., Sarakinou, E., Di Benedetto, R., Hunyady, A., Nakakana, U., Alfini, R., Pinto, M., Boretto, F., Gemmellaro, S., Ferruzzi, P., Iturriza, M., Berlanda Scorza, F., Rondini, S., Conti, V., Kumar Arora, A., Rossi, O., Micoli, F., Mancini, F. Evaluation of cross-functional antibody responses elicited by a four-component GMMA *Shigella* vaccine. *npj Vaccines* (submitted).
- 2024 Caradonna, V., Pinto, M., Alfini, R., Giannelli, C., Iturriza, M., Micoli, F., Rossi, O., & Mancini, F. (2024). High-Throughput Luminescence-Based Serum Bactericidal Assay Optimization and Characterization to Assess Human Sera Functionality Against Multiple *Shigella flexneri* Serotypes. *International journal of molecular sciences*, 25(20), 11123. doi: [10.3390/ijms252011123](https://doi.org/10.3390/ijms252011123)
- 2024 Mancini, F., Caradonna, V., Alfini, R., Aruta, M.G., Vitali C. G., Gasperini G., Piccioli, D., Berlanda Scorza, F., Rossi, O., Micoli, F. (2024). Testing *S. sonnei* GMMA with and without Aluminium Salt-Based Adjuvants in Animal Models. *Pharmaceutics*, 16(4):568. doi: <http://dx.doi.org/10.3390/pharmaceutics16040568>
- 2023 Di benedetto, R., Mancini, F., Caradonna, V., Aruta, M.G., Giannelli, C., Rossi, O., Micoli, F., “Comparison of *Shigella* GMMA and glycoconjugate four-component formulations in animals”, *Front Mol Biosci.*, 2023 Nov 16;10:1284515. doi: [10.3389/fmolb.2023.1284515](https://doi.org/10.3389/fmolb.2023.1284515)
- 2023 Mancini, F., Alfini, R., Caradonna, V., Monaci, V., Carducci, M., Gasperini, G., Piccioli, D., Biagini, M., Giannelli, C., Rossi, O., Pizza, M., Micoli, F., “Exploring the role of GMMA components in the immunogenicity of a 4-valent vaccine against *Shigella*”, *Int. J. Mol. Sci.*, 2023 Feb 1;24(3):2742. doi: [10.3390/ijms24032742](https://doi.org/10.3390/ijms24032742)
- 2021 Garzoli, S., Masci, V.L., Caradonna, V., Tiezzi, A., Giacomello, P., Ovidi, E., “Liquid and Vapor Phase of Four Conifer-Derived Essential Oils: Comparison of Chemical Compositions and Antimicrobial and Antioxidant Properties”, *Pharmaceutics*, 2021, 14, 134. doi: [10.3390/ph14020134](https://doi.org/10.3390/ph14020134)
- 2019 Vettrai, A. M., Bianchini, L., Caradonna, V., Forniti, R., Goffi, V., Zambelli, M., Testa, A., Vinciguerra, V., Botondi, R., “Ozone gas as a storage treatment to control *Gnomoniopsis castanea*, preserving chestnut quality”, *J. Sci. Food Agric*, 2019, 99: 6060–6065. doi: [10.1002/jsfa.9883](https://doi.org/10.1002/jsfa.9883)

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## Languages

Italian Native language

English Advanced listener and speaker. Advanced writing and reading.

Signature: 