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Harnessing engineered symbionts to combat concurrent malaria and arboviruses transmission

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Concurrent malaria and arbovirus infections pose significant public health challenges in tropical and subtropical regions, demanding innovative control strategies. Here, we describe a strategy that employs multifunctional engineered symbiotic bacteria to suppress concurrent transmission of malaria parasites, dengue, and Zika viruses by various vector mosquitoes. The symbiotic bacterium *Serratia* AS1, which efficiently spreads through *Anopheles* and *Aedes* populations, is engineered to simultaneously produce anti-*Plasmodium* and anti-arbovirus effector proteins controlled by a selected blood-induced promoter. Laboratory and outdoor field-cage studies show that the multifunctional engineered symbiotic strains effectively inhibit *Plasmodium* infection in *Anopheles* mosquitoes and arbovirus infection in *Aedes* mosquitoes. Our findings provide the foundation for the use of engineered symbiotic bacteria as a powerful tool to combat the concurrent transmission of malaria and arbovirus diseases.

Mosquitoes serve as vectors for various pathogens and parasites that cause infectious diseases, including malaria and various arbovirus diseases such as dengue and Zika virus disease. More than half of the global population lives in areas at risk of contracting two or more major mosquito-borne diseases, primarily in tropical and subtropical areas, posing a significant threat to human health[1]. *Anopheles* mosquitoes are the primary transmitters of malaria, responsible for ~240 million infection cases and over 600 thousand deaths annually[2]. Concurrently, *Aedes* mosquitoes are the main vectors of various highly pathogenic arboviruses for humans such as dengue virus (DENV), chikungunya virus (CHIKV) and Zika virus (ZIKV), which collectively afflict hundreds of millions worldwide each year[3–6]. Particularly, dengue has emerged as a significant contributor to the escalating disease burden of great concern globally[6–8], with recent epidemics of dengue and Zika virus disease precipitating substantial and rapid multi-regional outbreaks[9–11]. Moreover, prior Zika virus or dengue virus infection increases the risk of severe clinical symptoms during secondary infection due to antibody-dependent enhancement[12].

The geographic distributions of *Anopheles* and *Aedes* mosquitoes are dynamic and largely overlapping. *Ae. aegypti* and *Ae. albopictus* are two primary species involved in the transmission of arboviruses. *Ae. aegypti* is recognized as a primary vector for arboviral diseases such as dengue fever, Zika fever, and chikungunya, primarily inhabiting tropical and subtropical regions. In contrast, *Ae. albopictus* serves as a secondary vector with a broader ecological range, extending into temperate areas. Recent vector surveillance has documented the spread of *Ae. albopictus* from Asia to other continents, presenting an emerging health challenge for DENV transmission in temperate regions, including parts of Eastern European and the Mediterranean basin[13]. Additionally, *Ae. aegypti* has been identified as an invasive vector in regions such as North America

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and China[14]. Moreover, *An. stephensi* has recently emerged as an invasive urban malaria vector in East Africa[15,16]. These expansions have led to pronounced geographic overlap among various *Anopheles* and *Aedes* species, converging the endemic regions of their associated diseases. This convergence results in epidemiological synergy between outbreaks of malaria and various arbovirus diseases transmitted by these mosquitoes. The overlapping distributions highlight the inevitable co-existence and potential co-infection of malaria and arboviruses, or multiple arboviruses, in single host. Such scenarios pose complex and multifaceted public health challenges[11,17–21]. The co-infection and concomitant transmission of malaria and arboviruses have been reported in various regions, including African (e.g., Guiana and Nigeria)[22,23], South Asia (e.g., Kolkata and Timor)[24,25], and South American Brazilian Amazon[26,27]. Moreover, co-circulation and simultaneous co-infection of multiple arbovirus diseases, such as dengue fever, chikungunya, and Zika fever, was prevalent in the Americas and Latin America regions, including Costa Rica, Nicaragua, Colombian, Venezuelan, and Brazil[11,28–34]. This complex pattern of co-infection or sequential infection with different arboviruses[28–31], or in combination with malaria[19–26,35,36], poses significant challenges for disease surveillance, diagnosis, and treatment[37,38].

Due to antimalarial drug resistance and a lack of therapeutics or prophylactics against dengue and Zika diseases, efforts to control and prevent these diseases rely heavily on mosquito management[39]. However, mosquito control is compromised by widespread resistance to commonly used chemical insecticides[40,41]. Moreover, behavioral changes in *Anopheles* mosquito, such as increased outdoor and early biting activities[42], coupled with malaria parasite's growing resistance to drugs[43], have stalled progress in the fight against malaria[44]. The current global outbreaks of malaria and dengue fever are changing disease transmission dynamics and escalating the disease burden, highlighting the inadequacies in existing control tools[39]. This situation underscores the urgent need for innovative intervention strategies to tackle the concurrent transmission of malaria and arbovirus diseases[45].

The transmission of *Plasmodium* and arboviruses is strictly dependent on the completion of their infection cycles in vector mosquitoes. Importantly, the mosquito gut microbiota plays a pivotal role in influencing the outcome of *Plasmodium* and virus infection in mosquitoes[46–52]. Paratransgenesis, which utilizes the genetically manipulated symbionts of the vector insect to inhibit or kill the disease pathogens, is an attractive strategy for blocking pathogen transmission in mosquitoes[39,53,54]. Although this approach has shown promise in targeting malaria transmission in *Anopheles* mosquitoes, its effectiveness for inhibiting arboviruses in *Aedes* mosquitoes, as well as its potential to simultaneously target both malaria parasites and arboviruses—thereby preventing synergistic outbreaks of these major mosquito-borne diseases—requires further exploration.

Here, we explore the potential of parastransgenesis strategy to concurrently inhibit the transmission of *Plasmodium* and arboviruses by *Anopheles* and *Aedes* mosquitoes, respectively. A symbiotic bacterium *Serratia* strain AS1 that efficiently spreads through both *Anopheles* and *Aedes* mosquito populations, was engineered to secrete anti-*Plasmodium* and anti-arbovirus effector proteins. To drive the conditional expression of these effector molecules, we screen and identify a stringent blood-induced promoter, which minimizes potential fitness costs and reduces adverse effects on non-target organisms. The engineered AS1 strains successfully spread through mosquito populations, and strongly inhibit *Plasmodium* infection in *Anopheles* mosquitoes and both dengue and Zika virus infections in *Aedes* mosquitoes. In co-transmission experiments, the engineered strains reduce the proportion of *Aedes* mosquitoes carrying both dengue and Zika viruses, as well as their viral RNA level. These findings show that paratransgenesis strategy has the potential to aid in the elimination of disease transmission in areas endemic with multiple mosquito-borne diseases, particularly in tropical regions with high incidences of malaria and arboviruses.

Results

Serratia AS1 bacteria efficiently spread throughout both *Anopheles* and *Aedes* mosquito populations

The *Serratia* bacteria are the core gut symbionts of *Anopheles* mosquitoes[55–57], and were also found to be associated with *Aedes* mosquitoes[50,56,58,59]. Several species of *Serratia* have been identified in the midgut of both field-caught and laboratory-reared mosquitoes[60]. The genus *Serratia* is highly diverse and shows strong plasticity and niche adaptation[61]. We previously identified a symbiotic *S. marcescens* strain AS1, which can spread horizontally and vertically through the *Anopheles gambiae* mosquito population[62]. To test how well the *Serratia* AS1 colonizes and spreads through *An. stephensi* and *Ae. aegypti* mosquito populations, we conduct laboratory cage experiments in which virgin male mosquitoes, previously fed with *Serratia* AS1-GFP, respectively, were introduced into a cage at a proportion of 5%. All the progeny larvae and newly emerged adults of *An. stephensi* and *Ae. aegypti* mosquitoes carried AS1-GFP (Fig. 1a), indicating that *Serratia* AS1 can efficiently spread throughout these two mosquito populations. After administered to mosquito midguts via sugar meals, AS1 bacteria dramatically proliferated in the midguts of *An. stephensi* and *Ae. aegypti* mosquitoes after a blood meal (Figs. 1b, Supplementary Fig. 1a, b), without causing obvious negative impact on fitness costs in both the mosquito species (Supplementary Figs. 1c–f).

We previously showed that *Serratia* AS1 slightly inhibits *Plasmodium* infection. But, some specific *Serratia* strains were reported to facilitate arboviruses infection[50,58]. We next investigated whether *Serratia* AS1 affects virus infection. AS1 bacteria were fed to *Ae. aegypti* in sugar meals 48 h before mosquitoes fed with blood meals spiked with dengue virus type 2 (DENV2). We found that AS1 did not affect DENV2 infection (Supplementary Fig. 2a). These results support the use of *Serratia* AS1 as a viable bacterial chassis for paratransgenic strategies in both *Anopheles* and *Aedes* mosquitoes.

Engineering *Serratia* AS1 for simultaneous expression of anti-*Plasmodium* and anti-arbovirus effector molecules

We next explored the potential of engineered AS1 to simultaneously inhibit *Plasmodium* and arbovirus infections in *Anopheles* and *Aedes* mosquitoes, respectively. Employing the highly efficient hlyA secretion system encoded by the pDB47 plasmid[46], we modified this plasmid to co-express Shiva1, a potent anti-*Plasmodium* lytic peptide[46,63,64], and DN59, a peptide known for its robust inhibition of DENV and West Nile virus (WNV)[65]. DN59 targets the membrane-interacting region of the dengue virus envelope glycoprotein (E protein) and has shown inhibitory effects against all four serotypes of dengue viruses in vitro[66]. We constructed the pDB47-DK (DK denoting double killing) plasmid to harbor dual copies of DN59 and Shiva1, to maximize peptide expression levels. Under the control of the broad constitutive *neomycin phosphotransferase II* gene promoter (*PnptII*)[67], DN59 and Shiva1 were arranged in tandem, interspersed with trypsin recognition sites to facilitate their separation in the mosquito's digestive midgut (Fig. 1c).

The recombinant *Serratia* AS1 strains, transformed either with the empty pDB47 plasmid (AS1-Vector) or the engineered pDB47-DK plasmid (AS1-DK), were administered to axenic *An. stephensi* and *Ae. aegypti* mosquitoes (Supplementary Fig. 3) via cotton pads soaked with bacteria suspended in 5% (wt/vol) sucrose solution (Fig. 1c). Forty-eight hours later, the mosquitoes were fed with an infectious blood meal. We found that AS1-DK strongly inhibited *P. berghei* ANKA (Pb ANKA) development in *An. stephensi* mosquitoes (Fig. 1d) and DENV2 infection in *Ae. aegypti* mosquitoes (Fig. 1e).

The Asian tiger mosquito *Ae. albopictus* is also an important vector for arboviruses such as dengue, chikungunya, and Zika virus, and has emerged as one of the most invasive species worldwide, posing a

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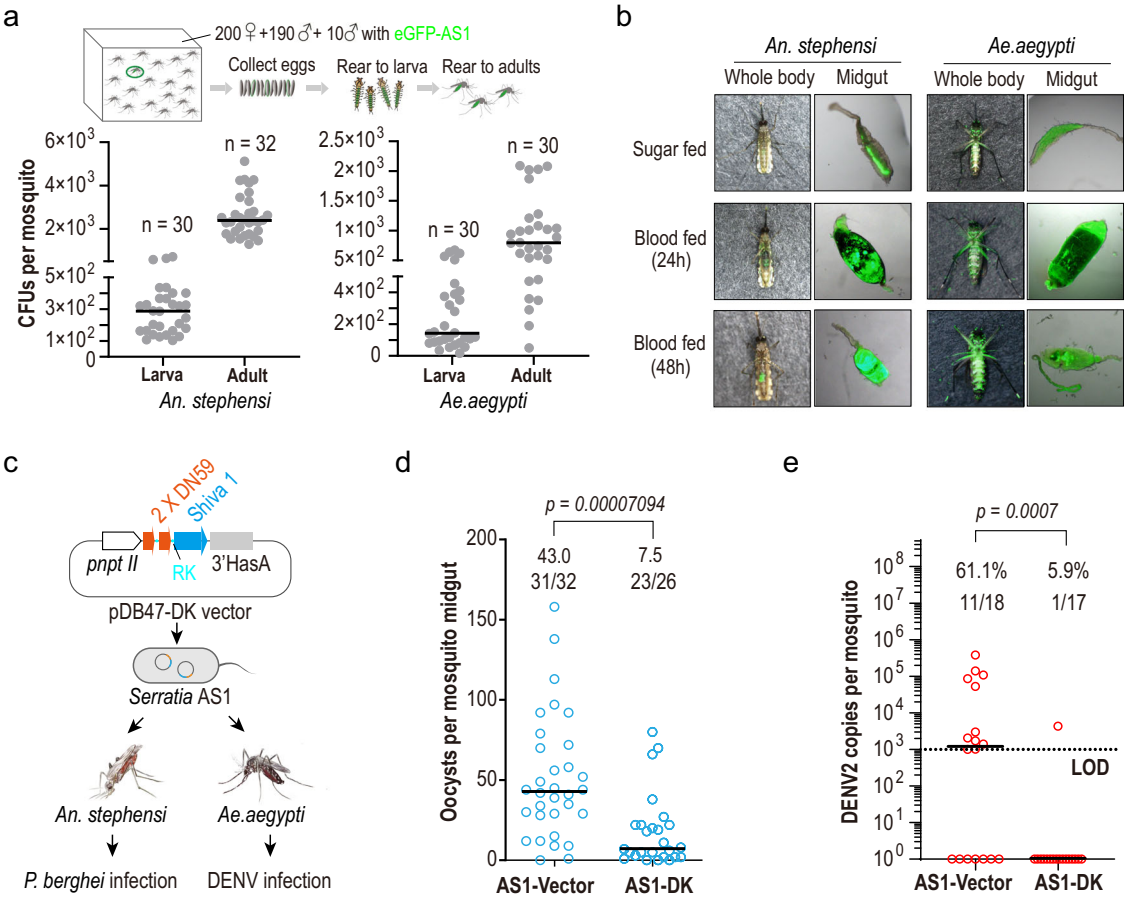

Fig. 1 | *Serratia* AS1 colonization and spread in *Anopheles* and *Aedes* mosquitoes, and the engineered strains inhibit *Plasmodium* parasite and dengue virus infection. a Cage experiment of *Serratia* AS1 dissemination in populations of *An. stephensi* and *Ae. aegypti* mosquitoes. Ten male mosquitoes fed with *Serratia* AS1-GFP bacteria were introduced into a cage containing 190 non-bacterium-fed males and 200 virgin females. Eggs were collected and reared to larval and adult stages. The presence of fluorescent bacteria was examined at both larval and adult (female) stages. Data points represent CFUs in individual mosquitoes and the horizontal lines indicate the median values ($n = 30$-32). **b** Visualization of GFP-fluorescent AS1 bacteria in the midgut of *An. stephensi* and *Ae. aegypti* mosquitoes after ingestion via a sugar meal, at 24 h and 48 h after a blood meal. Bright-field images are merged with corresponding fluorescent images. **c** Schematic diagram of the pDB47-DK plasmid depicting the fusion constructs of effectors with HasA. Trypsin cleavage sites (RK) were introduced as spacers between the peptides, as indicated. AS1 bacteria transformed with pDB47-DK (AS1-DK) were administered via sugar meal to *Anopheles* and *Aedes* mosquitoes, and subsequently challenged with *P. berghei* ANKA and DENV2 infections, respectively. **d** Oocyst loads in *An. stephensi* mosquitoes carrying AS1 bacteria transformed with pDB47 vector (AS1-Vector) and AS1 bacteria transformed with pDB47-DK (AS1-DK). The circles represent the number of oocysts in individual midguts and the horizontal lines indicate the median values ($n = 26$-32), p values were determined by a two-tailed Mann-Whitney test. The infection rate and median values is displayed at the top of each column. **e**, DENV2 copy number in *Ae. aegypti* mosquitoes carrying AS1 bacteria transformed with pDB47 vector (AS1-Vector) and AS1 bacteria transformed with pDB47-DK (AS1-DK). The dots represent copy number in individual mosquitoes and the horizontal lines indicate the median (n = 18-17), p values were determined by two sided Mann-Whitney test. The infection rate is displayed at the top of each column. Similar results were obtained from two biological repeats. LOD (black grid line), limit of detection. Source data are provided as a Source Data file.

significant global threat[68,69]. We confirmed that *Serratia* AS1 also colonized the midgut of *Ae. albopictus* and dramatically proliferated after a blood meal (Supplementary Fig. 2b). Moreover, the recombinant strain AS1-DK strongly inhibited DENV2 infection in *Ae. albopictus* mosquitoes (Supplementary Fig. 2c). These results indicate that engineered *Serratia* AS1 bacteria could efficiently inhibit infections of *Plasmodium* parasites and the dengue virus in *Anopheles* and *Aedes* mosquitoes, respectively.

Identification of promoters for driving blood-induced expression of effector molecules

The *pnptII* promoter used in pDB47-DK is a constitutive expression promoter derived from *Escherichia coli*[70]. However, the constitutive expression of effector molecules may impose a fitness burden on the bacterial host, potentially compromising AS1's competitiveness with other gut microbiota and limiting its persistence and dissemination across the mosquito population. Moreover, constitutive expression of effector molecules may cause adverse effects on mosquitoes or lead to resistance against the effector molecules. To mitigate these challenges, conditionally expressing elements were used in both transgenesis[71–76] and paratransgenesis strategies[77]. The use of blood-inducible promoters proves particularly advantageous for gut bacteria-based symbiotic control strategies, as the expression of effectors remains inactive in non-hematophagous arthropods, and is activated only upon blood meal ingestion, coinciding with pathogen entry into the mosquito midgut and the proliferation of symbiotic bacteria.

To achieve potent and stringent blood-induced expression of effector proteins in AS1, we conducted a comprehensive analysis using transcriptional and secretory proteomic data (Accession codes: PRJNA1031615 and PXD042831, respectively) from *Serratia* to identify suitable gene promoters. We targeted genes that showed strong induction when exposed to blood serum. This search identified nine candidates, including *AprE*, *LipA*, *Hha*, *BssS*, *Ecn*, *YbaY*, *UspA*, *CspC*, and *Lpp* (Supplementary Figs. 3a–c). For comparative

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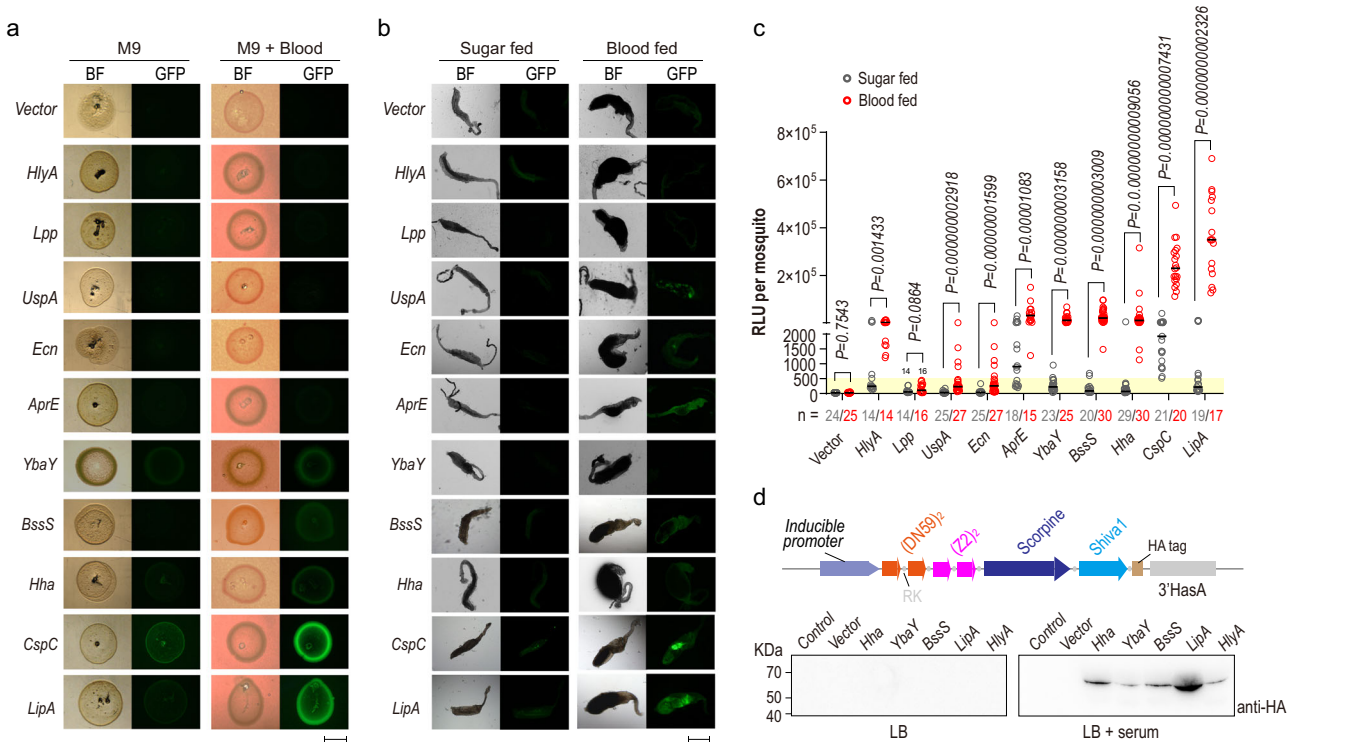

Fig. 2 | Screening of a promoter with strict blood-induced stringency for driving expression of anti-pathogen peptides. a Assessment of reporter activity of candidate promoters on agar plate. AS1 bacteria transfected with either empty vector (Vector) or reporter plasmids were spotted onto M9 minimal medium agar plate or M9 minimal medium agar plate supplemented with 10% sheep blood. The fluorescence signal indicating GFP expression in the bacterial colonies was observed using a fluorescence microscopy. Bright-field images (left) and the corresponding fluorescent images (right) of the bacteria colonies are displayed. Scale bar, 1 mm. **b** Evaluation of reporter activity of candidate promoters in the mosquito midgut. AS1 bacteria transfected with either empty vector (Vector) or reporter plasmids were introduced into *An. stephensi* mosquitoes, which were subsequently fed with either sugar meal or a blood meal. After 24 hours, mosquito midguts were dissected and visualized using fluorescence microscopy. Bright-field images (left) and the corresponding fluorescent images (right) of the midguts are displayed. Scale bar, 1 mm. **c** Blood-induced stringency assay of candidate promoters in *Anopheles* mosquitoes. AS1 bacteria transfected with either empty vector (Vector) or reporter plasmids were introduced into *An. stephensi* mosquitoes, which were then fed with either sugar meal or a blood meal. After 24 hours, mosquito midguts were dissected, homogenized in tubes containing D-luciferin substrates, and relative light units (RLUs) were measured. Each data point represents RLU in individual midguts and the horizontal lines indicate the mean ($n = 14–30$), p values were determined by two-tailed Mann–Whitney test. Similar results were obtained from two biological repeats. **d** Western blot analysis of protein secretion. The upper panel shows the arrangement and design of the anti-pathogen effector proteins. The lower panel shows western blot analysis of the secretion of the anti-pathogen effector proteins. Equal amounts of 20 µl bacterial culture supernatant were taken and subjected to blotting using anti-HA antibody. Similar results were obtained from two independent biological replicates. Source data are provided as a Source Data file.

purposes, the well-established blood-induced gene *HlyA* was also analyzed[77]. We constructed reporter plasmids carrying GFP-Luciferase, driven by the promoters of these genes (Supplementary Fig. 4a). Recombinant *Serratia* strains transformed with these plasmids were cultured on minimal M9 agar plates supplemented with or without sheep blood, and the GFP expression levels were assessed. Notably, promoters from *CspC* and *LipA* genes exhibited robust inducible activity (Fig. 2a).

To validate the functionality of these promoters in the mosquito midgut microenvironment, we introduced these bacterial strains into *An. stephensi* mosquitoes and examined GFP expression after feeding on either sugar or blood. Consistently, the promoters for *CspC* and *LipA* genes showed the most pronounced inducible activities (Fig. 2b). We also conducted a quantitative analysis of blood-induced activity and expression stringency by measuring the relative light units (RLU) in the mosquito midgut under sugar and blood feeding conditions. While the *HlyA* promoter displayed high stringency upon blood induction, its overall inducible activity was modest (Fig. 2c). In contrast, the promoters for *YbaY*, *BssS*, *Hha*, *CspC*, and *LipA* exhibited strong inducible activity. Notably, the *LipA* promoter showed the strongest activity, coupled with the highest stringency for blood-induced expression (Fig. 2c).

Engineered *Serratia* AS1 strains with the *LipA* promotor strongly inhibit the infection of *Plasmodium* parasites, dengue and Zika viruses in vector mosquitoes

To validate the efficacy of these promoters for driving anti-pathogen effector expression, we introduced several modifications. Specifically, we included scorpine, another potent anti-*Plasmodium* protein[46,62,78], to enhance robust anti-*Plasmodium* activity. Additionally, we incorporated peptide Z2[79,80] (Fig. 2 d upper panel, Supplementary Fig. 5) to target Zika virus. The inclusion of both DN59 and Z2 aims to address the growing concern of co-transmission and co-infection of DENV, ZIKV, and other aboviruses in *Aedes* mosquitoes[11,28,29]. Notably, DN59 and Z2 target the viral E protein, disrupting virion membrane integrity, which may enhance their combined inhibitory effects on dengue and Zika viruses.

We selected the promoters of the genes *HlyA*, *YbaY*, *BssS*, *Hha*, and *LipA* to test their ability to drive the secretory expression of effector molecules in response to blood serum. Western blotting analysis of bacterial culture supernatants further confirmed that the *LipA* promoter exhibited the highest inducible activity. Moreover, the secretion of effector proteins, driven by these serum-induced promoters, was nearly undetectable in the absence of serum (Fig. 2d lower panel). Based on these results, we selected the *LipA* promoter for subsequent investigations. To facilitate blood-induced expression of effector

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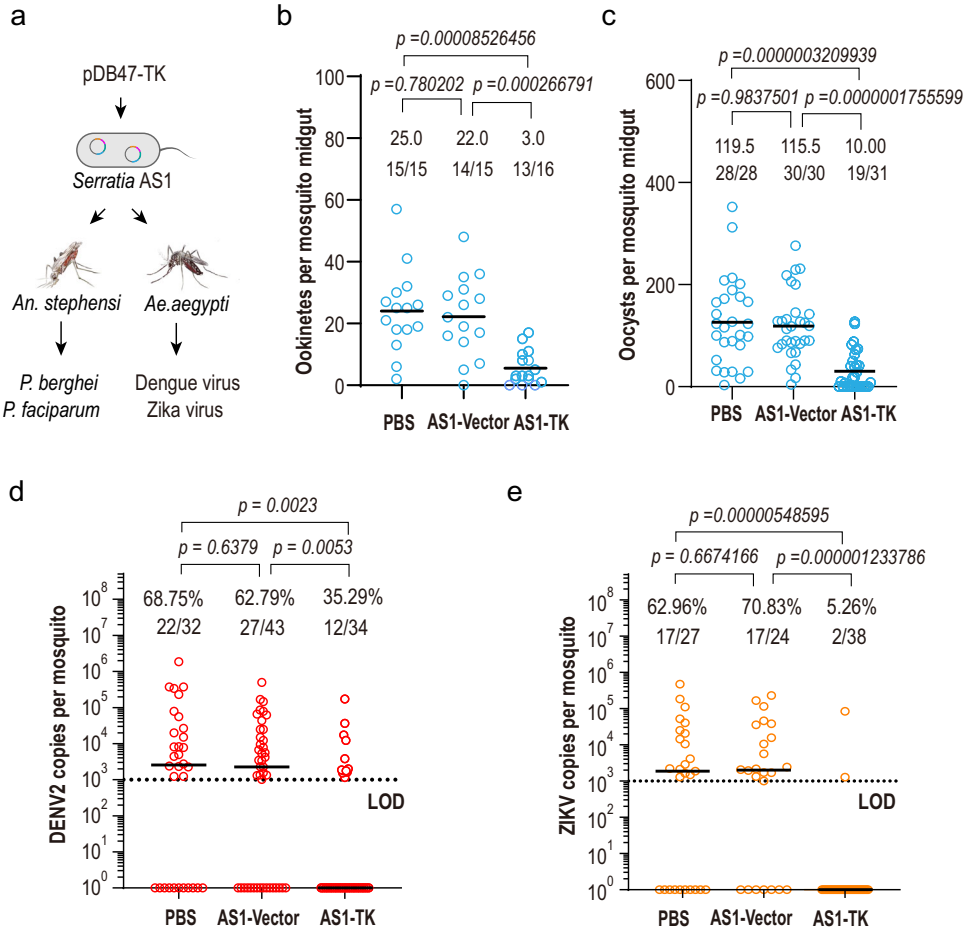

Fig. 3 | The engineered AS1-TK strain efficiently suppresses *Plasmodium* parasite infection in *Anopheles* mosquitoes and viral infection in *Aedes* mosquitoes. **a** Schematic diagram showing experimental procedure of introducing engineered AS1 bacteria transformed with pDB47-TK (AS1-TK) into *An. stephensi* and *Ae. aegypti* mosquitoes, to test its efficacy in inhibiting *P. berghei* and *P. falciparum* infection in *An. stephensi* mosquitoes, and DENV-2 and Zika virus infection in *Ae. aegypti* mosquitoes. **b** Ookinete load of *P. falciparum* NF54 in *An. stephensi* mosquitoes fed with PBS (PBS), pDB47 empty vector-transformed AS1(Vector), or pDB47-TK transformed AS1 (AS1-TK). In the graph, the circles represent the number of ookinetes in individual midguts, and horizontal lines indicate the median values (n = 15-16), p values were determined by two-tailed Mann-Whitney test. The infection rate and median values are displayed at the top of each column. **c** Oocyst load of *Pb.ANKA* in *An. stephensi* mosquitoes fed with PBS, pDB47 empty vector-transformed AS1(Vector), or pDB47-TK transformed AS1 (AS1-TK). The circles represent oocyst numbers in individual midguts and the horizontal lines indicate the median (n = 28-31), p values were determined by two-tailed Mann−Whitney test. The infection rate and median values are displayed at the top of each column. **d** DENV2 copy number in *Ae. aegypti* mosquitoes fed with PBS, pDB47 empty vector-transformed AS1(Vector), or pDB47-TK transformed AS1 (AS1-TK). The infection rate is displayed at the top of each column. The circles represent copy number in individual mosquitoes and the horizontal lines indicate the median values(n = 32-43), p values were determined by two-sided Mann-Whitney test. **e** Zika virus copy number in *Ae. aegypti* mosquitoes fed with PBS, pDB47 empty vector-transformed AS1(Vector), or pDB47-TK transformed AS1 (AS1-TK). The infection rate is displayed at the top of each column. The circles represent copy number in individual mosquitoes and the horizontal lines indicate the median values(n = 24-38), p values were determined by two-sided Mann-Whitney test.Similar results were obtained from two biological repeats, n.s., not significant; LOD (black grid line), limit of detection. The primers used for viral titers qPCR are shown in Supplementary Table 1. Source data are provided as a Source Data file.

molecules targeting *Plasmodium*, dengue and Zika viruses, we cloned genes encoding these molecules into the expression vector under control of the *LipA* promoter. The resulting vector was designated as pDB47-TK (TK denoting triple killing) (Fig. 3a).

Next, pDB47-TK was transformed into *Serratia* AS1. The resulting strain was designated as AS1-TK. To validate whether AS1-TK could indeed simultaneously inhibit *Plasmodium*, DENV and Zika virus infections, we introduced the AS1-TK bacteria into *An. stephensi* and *Ae. aegypti* mosquitoes via sugar meals. The mosquitoes were then provided different pathogen-infected blood meal (Fig. 3a). Notably, the recombinant AS1-TK strain did not impose obvious fitness cost in either *Anopheles* or *Aedes* mosquitoes (Supplementary Figs. 1c–f, Supplementary Fig. 6), suggesting that AS1-TK would spread as well as the AS1 strain in these mosquito species. The recombinant strain AS1-TK strongly inhibit development of the human malaria parasite *P. falciparum* NF54 infection (determined by ookinetes in midguts 24 h post infection) (Fig. 3b) and rodent malaria parasite *P. berghei* ANKA (determined by oocysts in midgut at day 7 post infection) (Fig. 3c) in *An. stephensi* mosquitoes. Moreover, AS1-TK effectively inhibit both DENV2 (Fig. 3d) and Zika virus infections (Fig. 3e) in *Ae. aegypti* mosquitoes (determined by virus titers at day 10 post infection). We further monitor DENV2 titers for a various time points post infection and find AS1-TK persistently reduce DENV2 titers when virus RNA can be readily detected (Supplementary Fig. 7).

Engineered *Serratia* AS1-TK strain efficiently spreads throughout *Anopheles* and *Aedes* mosquito populations and strongly inhibits the transmission of *Plasmodium* and arboviruses

Inspired by the robust and extensive transmission-blocking activity observed with AS1-TK in laboratory settings, we further tested whether the recombinant strains could impede the transmission of *Plasmodium* and arboviruses under semi-natural environment. To this end, we set

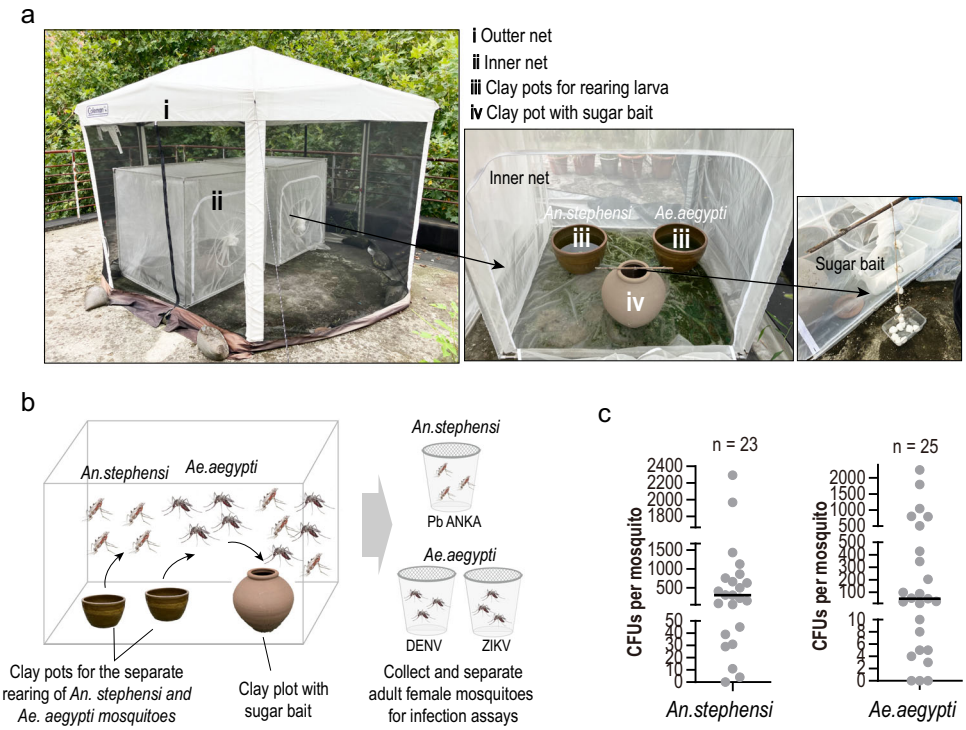

Fig. 4 | The engineered AS1-TK bacteria efficiently spread through *Anopheles* and *Aedes* mosquito populations in outdoor field-cage conditions. **a** Photograph showing the setup of enclosed large field-cages. (i) represents exterior net; (ii) represents inner mosquito cages, (iii) represents two wide-mouth crocks within the mosquito cages used for rearing *An. stephensi* larvae (left) and *Ae. aegypti* larvae (right); (iv) shows a narrow-mouth crock used as a mosquito trap containing sugar bait. **b** Schematic diagram showing the rearing of the *An. stephensi* and *Ae. aegypti* mosquitoes in the large field-cage, the introduction of bacteria into the mosquito population, and collection of adult female mosquitoes for pathogen infection assays. **c** AS1-TK bacterial load in the midguts of *An. stephensi* (left) and *Ae. aegypti* (right), as determined from mosquito samples collected in (**a**). The circles represent bacteria Colony-Forming Units (CFU) in individual mosquito midguts and the horizontal lines indicate mean values ($n = 23–25$). Similar results were obtained from two biological repeats. Source data are provided as a Source Data file.

up a unique containment facility (netted field-cages) in open field conditions, equipped with dual layers of nets (Fig. 4a). Each of the two inner cages (Size: 1.2 m × 1.2 m × 1.2 m) contains two clay pot refuges filled with rainwater, into which one thousand eggs of *An. stephensi* and *Ae. aegypti* mosquitoes were released, respectively (Fig. 4b). Another clay pot in the inner cages was outfitted deployed with cotton balls laden with either sugar bait alone or sugar bait supplemented with AS1-TK (Fig. 4a–c). After eclosion, adult mosquitoes were captured from the netted chamber, and female *An. stephensi* and female *Ae. aegypti* mosquitoes were subsequently transferred to laboratory settings and placed in paper cups for further analysis (Fig. 4b). These outdoor field-cage experiments were conducted twice in Shanghai at two independent times, during June to October, coinciding with optimal temperature conditions (Supplementary Fig. 8).

The outdoor field-cage experiments showed that the recombinant AS1-TK strain efficiently colonized both *An. stephensi* and *Ae. aegypti* mosquitoes (Fig. 4c), indicating that these recombinant AS1-TK strain may efficiently colonize the guts of wild mosquitoes. Next, these colonized female mosquitoes were provided different pathogen-infected blood meals. Recombinant AS1-TK strain strongly inhibited *P. berghei* ANKA infection in *An. stephensi* mosquitoes (Fig. 5a). Notably, the recombinant AS1-TK strain effectively inhibited the infection of all four serotypes of dengue viruses in *Ae. aegypti* mosquitoes (Figs.5b, Supplementary Fig. 9a–c). Moreover, AS1-TK dramatically inhibited Zika virus infection in *Ae. aegypti* mosquitoes (Fig. 5c).

Engineered *Serratia* AS1-TK strain efficiently blocks the concurrent transmission of dengue and Zika viruses

The concurrent transmission and co-infection of major mosquito-borne diseases in tropical and subtropical regions pose significant public health challenges. Notably, the co-infection and co-circulation of multiple arboviruses, particularly those transmitted by *Aedes* mosquitoes, are frequently reported in epidemiological surveillance[9,10,81,82]. Co-infections of dengue and Zika viruses in *Aedes* mosquitoes have been shown to enhance viral replication[83,84]. In light of this, we sought to investigate whether the recombinant *Serratia* strain could effectively inhibit the co-infection of dengue and Zika viruses in *Aedes* mosquitoes.

Female *Ae. aegypti* mosquitoes were reared in outdoor field-cages as depicted in Fig. 4A. The *Serratia* bacteria were introduced to these colonized female mosquitoes via cotton balls soaked in a *Serratia* suspension mixed with sugar, which were placed in clay pots refuges inside the chambers. These colonized female mosquitoes were then fed on a blood meal containing both DENV2 and Zika viruses (Fig. 5d). The recombinant *Serratia* strain AS1-TK markedly inhibited the viral RNA levels of DENV2 and ZIKV in *Aedes* mosquitoes (Fig. 5e left panel). Moreover, the proportion of mosquitoes carrying both viruses (co-infected) was significantly reduced (Fig. 5e right panel), and the viral RNA levels of DENV2 and Zika virus were markedly suppressed in individual mosquitoes (Supplementary Figs. 10a, b). These results indicate that the engineered AS1-TK strain can effectively suppress the simultaneous co-infection and co-transmission of dengue and Zika viruses.

Discussion

In recent years, there has been a notable increase in the frequency and widespread occurrence of epidemiological synergy among multiple mosquito-borne diseases. This trend poses increasing challenges to public health and exposes populations under multiple threats. New strategies are urgently needed to tackle these complex scenarios.

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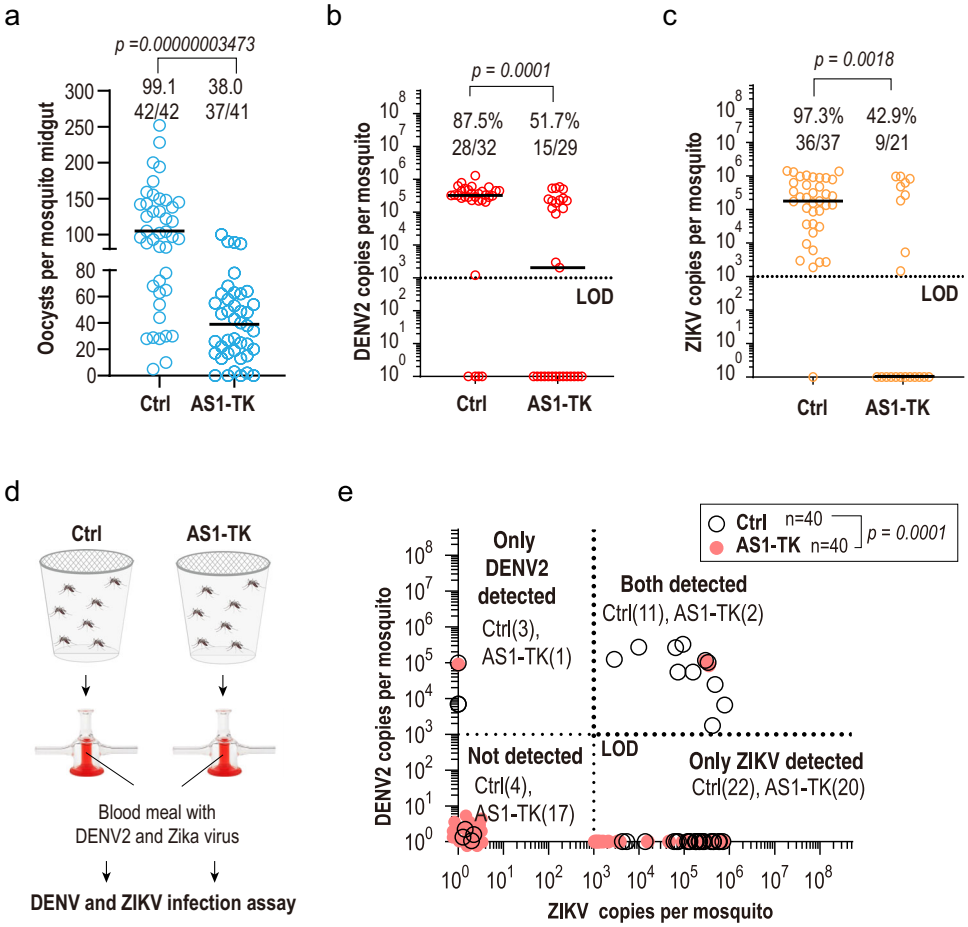

Fig. 5 | Engineered AS1-TK bacteria effectively inhibit *Plasmodium* and arbovirus infections in *Anopheles* and *Aedes* mosquitoes, respectively, as well as co-infections of dengue and Zika viruses. *An. stephensi* and *Ae. aegypti* mosquitoes collected as in Fig. 4 were challenged with Pb.ANKA, DENV and Zika virus infection, as co-infection of DENV2 and Zika viruses. **a** Oocyst loads in *An. stephensi* mosquitoes collected from the control cage (sugar baits with PBS, $n = 42$) and the test cage (sugar baits with AS1-TK, $n = 41$). The circles represent the oocyst number of individual midguts and horizontal lines indicate median values, p values were determined by two-tailed Mann-Whitney test. The infection rate and median values is displayed at the top of each column. **b** DENV2 copy number in *Ae. aegypti* mosquitoes at day 10 post infection collected from the control cage (sugar baits with PBS, n = 32) and the test cage (sugar baits with *Serratia* AS1-TK, n = 29). **c** ZIKV copy number at day 10 post infection in *Ae. aegypti* mosquitoes collected from the control cage (sugar baits with PBS, $n = 37$) and the test cage (sugar baits with AS1-TK, n = 21). **b** and **c**, the infection rate is displayed at the top of each column. The circles represent copy nuber in individual mosquitoes and the horizontal lines indicate the median values, p values were determined by two-sided Mann-Whitney test. LOD (black grid line), limit of detection. **d** Diagram showing the DENV2 and Zika virus co-infection assay in *Ae. aegypti* mosquitoes. Blood meals containing both DENV2 and Zika virus were fed to *Ae. aegypti* mosquitoes collected from the control cage (sugar baits with PBS) and the test cage (sugar baits with AS1-TK). **e** Copy numbers of DENV2 and Zika virus in *Ae. aegypti* mosquitoes from the co-infection assay as described in (**d**). Hollow circles represent individual mosquitoes from the control group, while solid circles represent those from the AS1-TK group. The chart is divided by the limit of detection (LOD) into four areas: both detected, only DENV2 detected, only ZIKV detected, and not detected. The two-sided unadjusted p values for comparison between the two groups were determined using a Generalized Linear Model (GLM) with a 'biomial' link function[96]. Similar results were obtained from two biological repeats (**a**, **b**, **c**, **e**). LOD is indicated by the black grid line. Source data are provided as a Source Data file.

Several approaches, including genetically modified mosquitoes, endosymbiotic *Wolbachia* (which has shown success in field trials)[85], and naturally occurring anti-*Plasmodium* *Serratia* Su_YN1[55] or anti-arbovirus *Rosenbergella* bacterium[86], have shown promise in blocking the transmission of mosquito-borne diseases. Paratransgenic approach is a simple and effective strategy that has shown promise in blocking malaria transmission in *Anopheles* mosquitoes[62]. By harnessing the symbiotic bacterium *Serratia* AS1, which efficiently colonizes various *Anopheles* and *Aedes* mosquitoes, this study further demonstrates that engineered multifunctional *Serratia* AS1 strains can effectively inhibit *Plasmodium* parasites in *Anopheles* mosquitoes as well as dengue and Zika virus infections in *Aedes* mosquitoes, both in laboratory and contained field settings (Supplementary Fig. 11). The substantial reduction of *Plasmodium* parasite load (oocyst number) and arbovirus RNA titers in mosquitoes reflects a significant decrease in vector competence. This reduction in pathogen load indicates considerable barriers to pathogen replication within the vector, which would impair the ability of the pathogen to disseminate to the salivary glands- a critical step in transmission[47,87]. The robust inhibitory efficiency and broad-spectrum pathogen inhibition demonstrated by the recombinant strains highlight the potential of the paratransgenesis approach as a powerful tool for simultaneously targeting malaria parasites and arboviruses in various mosquito vectors.

The paratransgenesis strategy rely on the efficient expression of potent effector molecules by the symbiotic bacteria. The scorpine and Shiva-1 are robust anti-*Plasmodium* effectors[88], while the antiviral peptides DN59 and Z2 inhibit various arboviruses, including dengue and Zika viruses. Typically, the bacteria were engineered to express these effector molecules using constitutive expression systems. However, the constitutive expression of effector molecules may impose bacterial fitness cost and potentially elicits adverse effects on host mosquitoes. To circumvent these limitations, our study utilizes a

blood-inducible strategy. Mosquitoes and other hematophagous insects acquire pathogens through the ingestion of host blood. Consequently, ingested pathogens and gut bacteria coexist in the same compartment, the midgut. Following a blood meal, gut symbiotic bacteria undergo dramatic proliferation. Driven by a blood-inducible promoter, anti-pathogen effectors are specifically and robustly produced, precisely targeting the crucial phase of pathogen infection in mosquitoes. This orchestrated response ensures the precise and potent production of anti-pathogen effectors during the critical period of pathogen infection. Through rigorous screening both in vitro and in vivo, we have identified a promoter with minimal leakage expression and pronounced blood-inducibility. This ensures that effector expression remains dormant under non-hematophagous conditions and is selectively activated only upon ingestion of a host blood meal, coinciding with the entry of pathogens into the mosquito midgut.

The concurrent transmission of multiple mosquito-borne diseases, including malaria and arboviral diseases such as dengue fever, Zika fever, West Nile fever, and chikungunya, has led to a plethora of clinical reports of co-infections[17–19,21]. To effectively block the simultaneous transmission of multiple mosquito-borne pathogens, it is crucial that the chassis bacteria efficiently colonize various mosquito species without inducing adverse effects. While *Serratia* bacteria are commonly found in different mosquito species, some strains isolated in one mosquito species may fail to colonize other mosquito species[89]. Moreover, a few *Serratia* strains were reported to facilitate the transmission of some arboviruses[58]. In contrast, the *S. marcescens* strain AS1, used as the chassis in this study, effectively colonizes various arthropod vectors[90]. Here, we further demonstrate that AS1 colonizes the midguts of both *Anopheles* and *Aedes* mosquitoes without imposing any fitness load to the mosquitoes or increasing their susceptibility to pathogen infections. Importantly, AS1 is transmitted vertically from female mosquitoes to the progeny and horizontally from male to female mosquitoes, signifying that it can naturally spread through mosquito populations. Furthermore, engineered AS1 bacteria strongly proliferate in the mosquito midgut following a blood meal, spread rapidly throughout populations of both *Anopheles* and *Aedes* mosquitoes, and drive these mosquitoes resistant to *Plasmodium* and arboviruses, respectively.

Our study demonstrates that *Serratia* AS1 can provide an effective delivery system for effector molecules that target both *Plasmodium* and arboviral infections in the midgut of *Anopheles* and *Aedes* mosquitoes. *Serratia* spp. are commonly found in various insect species and are among the core commensals of mosquitoes[90]. Although AS1 may have the potential to colonize other insects, we employed a blood-inducible system to ensure the strictly-controlled expression of anti-pathogen peptides in vector mosquitoes. This approach minimizes the potential adverse effect of transmitting secreted effectors to non-target insects and hosts. By combining the adaptable mosquito symbiotic *Serratia* AS1 with various anti-pathogen effectors, our findings provide a highly efficient strategy to weaponized the mosquito gut symbiotic bacteria to combat the concurrent transmission of malaria and arboviruses. This multifaceted approach, targeting multiple pathogens, is not only cost-effective but also compatible with existing vector control tools and holds promise for mitigating synergistic outbreaks of multiple significant mosquito-borne diseases. However, more work remains before this approach can be implemented in the field. A key challenge is overcoming the regulatory, ethical, and social issues related to the release of genetically modified organisms.

Methods

Ethics statement

This study was conducted in accordance with the guidelines of the CAS Center for Excellence in Molecular Plant Sciences (Shanghai Institute of Plant Physiology and Ecology) Animal Care and Use Committee (A01MP2001). Six-week-old male SPF (specific pathogen free) grade ICR (Institute of Cancer Research) mice were used for *Plasmodium berghei* infection and mosquito blood feeding. Mice were maintained under a 12-hour light/12-hour dark cycle at 20-24 °C and 40-70% humidity. The O-type human blood used in this study was provided by Shanghai Red Cross Blood Center (Approval number: shblood2019-28).

Mosquito rearing in laboratory and generating axenic female mosquitoes

Anopheles stephensi mosquitoes (Dutch strain), *Aedes aegypti* mosquitoes (Rockfeller strain), and *Ae. albopictus* mosquitoes (Foshan strain) were maintained on 10% sucrose at 27 °C and 70 ± 5% relative humidity (RH) under a 12 h/12 h day-night cycle. Larvae were fed on cat food pellets and ground fish food supplement. Generating axenic female mosquitoes were conducted as described previously[91]. Newly emergenced female mosquitoes were reared with 10% sucrose solution containing penicillin (10 unit/mL), streptomycin (10 μg/mL), and gentamicin (15 μg/mL) from the first day to day 5 post emergence. Then the antibiotic solution was replaced by sterile water, and mosquitoes were starved overnight before providing with oral bacterial solutions for 2 days. Subsequently, the mosquitoes were used for experiments and fed with sugar solution that was replaced twice a day.

Bacterial culture and introduction into *Anopheles* and *Aedes* mosquitoes via sugar meal

Escherichia coli DH5α (Invitrogen) was used for DNA cloning and plasmid amplification. *E. coli* and *Serratia* AS1 bacterial strains were cultured in Luria-Bertani (LB) broth or on agar plates at 37 °C and 30 °C, respectively. Bacteria were precipitated at 4000 rpm for 5 min at 4 °C, washed twice with phosphate buffered saline (PBS), and resuspended in 5% (wt/vol) sterile sucrose solution to obtain a concentration of 10^7 cells/mL. The bacteria sucrose solution was then added to sterile cotton balls and provided to 2-day-old female *Anopheles* and *Aedes* mosquitoes for 48 h.

Colonization assay of bacteria in the mosquito midgut

After feeding GFP-labeled AS1 bacteria to *Anopheles* and *Aedes* mosquitoes as described above, the mosquitoes were then fed on a cotton pad moistened with 10% sterile sucrose solution. Two days later, a portion of mosquitoes were sampled for quantifying AS1 bacteria in the mosquito midgut (0 hr), and the remaining mosquitoes were fed on a blood meal. After blood meal, the mosquitoes were sampled at various time points (from 1 day to 24 days) for quantifying AS1 bacteria in the mosquito midgut. The bacterial load in each mosquito was determined as Colony-Forming Units (CFU) as following: the individual mosquitoes were surface-sterilized by washing them in 75% cold ethanol for 3 min and then rinsed in sterile PBS three times. The midguts were dissected and homogenized in sterile PBS under sterile conditions. The bacterial numbers were determined by plating ten-fold serial dilutions of the homogenates on LB agar plates supplemented with 100 μg/mL carbenicillin and 100 μg/mL kanamycin, followed by incubation at 30 °C for 24 h. The fluorescent colonies of AS1-GFP were counted by fluorescent microscopy.

Dissemination of AS1 into *Anopheles* and *Aedes* mosquito populations in cage experiments

We conducted laboratory cage experiments to test whether *Serratia* AS1 bacteria can spread through *Anopheles* and *Aedes* mosquito populations. GFP-labeled AS1 bacteria were introduced into newly emerged virgin male *An. stephensi* or *Ae. aegypti* adult mosquitoes as described above. Then 10 AS1 fed virgin males were mixed with 190 unfed virgin males and 200 uninfected virgin females in a laboratory cage, resulting AS1-fed mosquitoes constitute 5% of the total population. Two days later, the female mosquitoes were allowed to feed a

blood meal. Three days later, an oviposition cup was placed in the cage to collect eggs, and then rear the larvae to adults followed standard protocol. The fourth-instar larvae, and female adults were sampled and examined for the presence of GFP-tagged AS1 bacteria by plating larvae and adult midgut homogenates on LB agar plates containing 100 μg /mL carbenicillin and 100 μg /mL kanamycin.

Construction of pDB47-DK and pDB47-TK plasmids

The HasA (heme-binding protein) secretion expression plasmid pDB47[92,93] was used as backbone for construction of pDB47-DK and pDB47-TK plasmids. The DNA sequences coding effector peptides were synthesized by Sangon Biotech (Shanghai) Co., Ltd., and provided in Figure S4. The DNA sequences coding effector peptides were fused in frame with the HasA secretion elements of the pDB47 plasmid by homologous recombination cloning. For construction pDB47-DK plasmid, the original pDB47 plasmid was digested with restriction enzymes of *Nco*I and *Bam*HI, and DNA sequence coding for two copies of DN59 and one copy of Shiva1 was cloned to fuse with the plasmid by homologous recombination cloning. The constitutive *neomycin phosphotransferase II* (*PnptII*) gene promoter[67] of the original pDB47 plasmid was retained to drive the effectors expression. For construction pDB47-DK plasmid, the original pDB47 plasmid was digested with restriction enzymes of *Xba*I and *Bam*HI, and DNA sequence coding for two copies of DN59, two copies of Z2, one copy of scorpine and one copy of Shiva1 was cloned to fuse with the plasmid by homologous recombination cloning. Then the *LipA* promoter DNA sequence was cloned to the *Nco*I site, to conditionally drive the expression of the effectors.

Effect of *Serratia* AS1 bacteria strains on mosquito fitness

The effect of AS1 bacteria strains on mosquito fitness was monitored, including lifespan, blood-feeding and fecundity. Two-day-old adult *An. stephensi* and *Ae. aegypti* mosquitoes were administered with AS1 bacteria or no bacteria (control) as described above. Two days later, the mosquitoes were fed on a healthy mouse. Mosquito survival was monitored daily. Numbers of engorged and non-engorged mosquitoes were counted. Individual female mosquitoes were allowed to lay eggs on a damp filter paper in an oviposition tube 2 day post a blood meal. After three days, the number of eggs were counted.

Anti-*Plasmodium* and anti-arbovirus peptides selection and design

We selected the anti-*Plasmodium* peptides Shiva1 (sequence: 5′-MPRWRLFRRIDRVGKQIKQGILRAGPAIALVGDARAVG-3′) and scorpine (sequence: 5′-GWINEEKIQKKIDERMGNTVLGGMAKAIVHKMAKNEFQC MANMDMLGNCEKHCQTSGEKGYCHGTKCKCGTPLSY-3′). We selected the anti-dengue virus peptide DN59 (sequence: 5′- MAILGDTAWDFG SLGGVFTSIGKALHQVFGAIY-3′)[65] and anti-Zika virus peptide Z2 (sequence: 5′-MAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAF-3′)[79,80]. We organized the peptides in tandem and interspersed them with trypsin digestion sequences of Lysine- Arginine to facilitate their separation in the mosquito midgut. For anti-arbovirus peptides DN59 and Z2, we incorporated two copies of each peptide. Please also see Fig. S4.

Effect of bacteria on *P. berghei* infection in *Anopheles* mosquitoes

Engineered AS1 bacteria were administered for 48 h to axenic female *An. stephensi* mosquitoes with a cotton pad soaked with a 5% (wt/vol) sucrose solution containing bacteria or no bacteria (PBS control). Mice were infected with 5×10^5 parasites through intraperitoneal injection, and on day 4 post infection, mice were used for mosquito blood feeding. Different groups of mosquitoes were allowed to feed on anesthetic ICR mice infected with *P. berghei*-mCherry for 10 min, and were subsequently maintained at 19 °C and 70% relative humidity. Fully engorged mosquitoes were separated within 24 h and provided with a cotton pad soaked with 5% (wt/vol) sterile sucrose solution. Midguts were dissected on day 7 after the blood meal, and examined for the presence of oocysts, as indicated by mCherry fluorescence signal.

Effect of engineered bacteria on ookinete formation of *P. falciparum* in *Anopheles* mosquitoes

P. falciparum NF54 gametocyte culture was initiated at 0.5% asexual parasitemia and 4% hematocrit as previously described[94]. Mature gametocyte culture was used for infection. Engineered AS1 bacteria were administered for 48 h to axenic female *An. stephensi* mosquitoes with a cotton pad soaked with a 5% sucrose solution containing bacteria or no bacteria (PBS control), and then allowed to feed on *P. falciparum* NF54 gametocyte–containing blood as described previously[46]. The mosquito midguts were dissected 20 hours post the blood feeding. The blood bolus was spread onto glass slides and stained with Giemsa. Ookinetes of individual mosquito midgut were counted under microscopy.

Mosquito cell maintenance and preparation of DENV or Zika viruses

C6/36 cells were cultured in RPMI 1640 medium (HyClone) containing 25 mM HEPES supplemented with 2% (vol/vol) heat-inactivated fetal bovine serum (Gibco) at 28 °C. The following strains of DENV were utilized: DENV1 (16007 strain), DENV2 (New Guinea C strain, AF038403.1), DENV3 (16562 strain), DENV4 (1036 strain), and ZIKV (ZJ03 strain). These viruses were propagated in C6/36 cells. Subsequently, virus particles are collected from the cell culture supernatant, filtered through 0.22 μm filter (Millex-GP), aliquoted into 0.5 mL portions of virus supernatant, and stored at −80 °C until further use.

Feeding of DENV and Zika viruses to infect *Aedes* mosquitoes

The virus supernatant (with starting titers adjusted to 1×10^6 Pfu/mL) was mixed with heat-inactivated sheep blood (Yuanye Biotech) in a 1:1 ratio. The viral-blood mixture was used for the infection of female *Ae. aegypti* or *Ae. albopictus* mosquitoes that had been starved for 12–24 hours. Blood feeding was carried out using a glass feeder covered with parafilm film, which is maintained at 37.5 °C using a circulating water bath. The feeding process sustained for 20 minutes. After feeding, fully engorged mosquitoes were separated and transferred to new paper cups, then maintained in a culture incubator at 27 °C and 70% humidity. The copy number was measured by qPCR 10 days after infection.

Detection of viral titers by qPCR and LOD determination

Viral titers in *Aedes* mosquitoes were determined by standard curve method[86]. The viral titers were determined by qPCR and calculated with standard curves set for each of the viruses of known titers. Viral RNA extracted using TGuide S32 Magnetic Viral DNA/RNA Kit (Tiangen, Cat. No# DP604). Individual whole *Aedes* mosquitoes from each experimental group were anaesthetized and transferred into a 1.5 ml EP tubes containing 200 μl of PBS and 20 μl of protease K. Subsequently, the mosquitoes were homogenize using a tissue grinder (ShanMi, Cat. No# 12109009). Viral RNA was then extracted according to the manufacture's instruction of the TGuide S32 Magnetic Viral DNA/RNA Kit. After viral RNA extraction, cDNA was synthesized using Hifair ® III 1st Strand cDNA Synthesis SuperMix (Yeasen, Cat. No# 11141ES60). The cDNA was then used for qPCR to quantitatively determine the copy number. The limitation of detection (LOD) indicates limit copies numbers that can be detected by this method. LOD was determined by using uninfected mosquitoes as reference, which defined 10^3 DENV1-4/ZIKV genomic copies per mosquito as the LOD threshold. In the virus titers assays, data below LOD were replaced with 1 genome copy[86]. The primers used for detecting DENV1-4 and ZIKV are listed in Supplementary table 1.

Reporter plasmid construction for promoter activity test

For the construction of GFP-luciferase-based reporter plasmids to assess promoter activity, we employed the pDB47 plasmid as the backbone. The plasmid backbone was PCR amplified from pDB47 plasmid with the following primer sets: Forward: 5′-TGTGGTCT CCCTATAGTGAGTCG-3′, and Reverse: 5′-TTGCGGTAATGTGGTATTAC AG-3′. The GFP-Luciferase sequence was PCR amplified from GOMO-G FP-LUC plasmid[95] with the primer sets: Forward: 5′-ATGAGTAAAGGAG AAGAACTTTTCAC-3′, and Reverse: 5′-TTACACGGCGATCTTTCCGC-3′. The promoter sequences to be tested were PCR amplified from *Serratia* genomic DNA using primer sets listed in the supplementary Table S1. The plasmid backbone, GFP-Luciferase sequence and promoters of interest were assembled by homologous recombination cloning.

In vitro screening for blood-induced expression promoters

Reporter plasmids designed for assessing promoter activity were constructed as describe above. Each plasmid was individually transfected into AS1 bacterium. The transfected bacterial strains were cultured in LB medium at 30 °C for 12 h, and adjusted to 0.5 OD. Equal volume of 3 µl of bacterial culture was spotted onto either M9 minimal medium (Sangon Biotech, A507024) agar plates, or M9 minimal medium agar plate supplemented with 10% sheep blood. These plates were incubated in a 30 °C incubator for 12 hours. The expression of GFP in the bacterial spots, indicative of promoter activity, was visualized using a fluorescence microscopy (Olympus, MVX10), and images were captured accordingly.

In vivo screening of blood-induced expression promoters in *Anopheles* mosquitoes

Two-day-old adult female *An. stephensi* mosquitoes were fed with 5% sucrose solution containing 0.1 OD (1×10^7 cells/ml) of *Serratia* AS1 engineered strains transfected with each respective reporter plasmid, while a control group comprised AS1 bacteria transfected with the empty pDB47 vector. Mosquitoes were either maintained on a sugar meal (Sugar-fed), or subjected to a blood meal and sampled 24 h post a blood meal (Blood-fed). Midguts of the mosquitoes were dissected, and fluorescence signals indicative of GFP expression by the bacteria in the mosquito midgut were visualized. Photomicrographs were taken using a fluorescence microscopy (Olympus, BX53).

Blood-induced stringency assay of expression promoters in *Anopheles* mosquitoes

AS1 bacterial strains (along with a vector control strain) transfected with reporter plasmids were administered to two-day-old adult female *An. stephensi* mosquitoes as described above. The mosquitoes were divided into two groups: one group was provided with a sugar meal, while the other group fed on a healthy ICR mouse. Twenty-four hours post blood meal, the whole mosquitoes from both groups were individually transferred to 1.5 mL tubes, each containing a single mosquito, and subsequently homogenized in 100 µl of PBS solution supplemented with 150 µg/mL D-luciferin (MedChemExpress, HY-12591A). Relative fluorescence units (RLUs) were monitored using Modulus™ single tube reader.

Western blot analysis

Western blot analysis was used to detect the expression levels of the anti-*Plasmodium* and antiviral effector molecules. To compare the expression of effector proteins induced by various inducible promoters, the wild-type *Serratia* AS1 (used as a control) and AS1 bacterial transformants were cultured in Luria Broth (LB) liquid medium or LB liquid medium containing 10% serum (wt/vol) at 28 °C for 24 hours. An equal amount of culture supernatant was taken and added with Dual Color SDS-PAGE Protein Sample Loading Buffer (Beyond time, Cat. No # P0295-15ml). The protein samples were separated by 4–20% gradient SDS-PAGE (bio-rad, Cat. No #1658004) and transferred to a PVDF membrane (Millipore, Cat. No #ISEQ00010). Membranes were blocked in blocking buffer (5% BSA in 1 × TBST) and probed with the primary rabbit anti-HA mAb (CST, Cat. No #3724S), at a dilution of 1:1000. After incubation, the membranes were washed three times with TBST and then incubated with HRP-conjugated secondary antibody (Proteintech, Cat. No # SA00001-2) at 1:5000 dilution. The membrane was then washed four times with TBST before visualizing the protein bands through enhanced chemiluminescence detection.

Setup of outdoor large field-cage facility for mosquito rearing

To test the potential spread of AS1 bacteria into *Anopheles* and *Aedes* mosquito populations and its capacity to inhibit pathogen transmission under outdoor conditions, we established a temporary outdoor field-cage testing facility. Rainwater was collected in a tank to rear mosquitoes in the facility. A tend measuring 3 m × 3 m × 2.5 m (length × width × height) was erected, equipped with exterior nets procured from Costco. Inside the tent, two large mosquito cages, each measuring 1.2 m × 1.2 m × 1.2 m (length × width × height) was positioned–one designated for control purposes and the other for conducting bacterial spreading tests. Two wide-mouth crocks and two narrow-mouth crocks were placed. The wide-mouth crocks were filed with rainwater to approximately two-thirds of the volume. One wide-mouth crock in each cage received 1000 *An. stephensi* eggs, and another received 1000 *Ae. aegypti* eggs. After the eggs hatched, the mosquito larvae in the wide-mouth crocks were regularly fed cat food until they reached adulthood. The experiments were conducted between July and September of the year 2023, as depicted in Figure S5.

Effect of engineered bacterial strains on blocking pathogen infection in mosquitoes under large cage conditions

After the emerging of adult *An. stephensi* and *Ae. aegypti* mosquitoes in the designated mosquito cages as described above, cotton pads soaked with a 5% (wt/vol) sucrose solution containing either bacteria or no bacteria (Control) were provided in narrow-mouth clay pot refuges in each mosquito cage. The cotton pads were tethered on a rope attached to a twig and placed at the mouth of the crock. The mosquitoes exhibited a natural inclination to fly into the narrow-mouth clay pot refuges, where they would rest, hide, and consume sugar meals. Five days after deploying the cotton pads, mosquitoes in the cages were collected using electric insect suckers and transferred to the laboratory. Female *An. stephensi* and *Ae. aegypti* mosquitoes were separated. *Serratia* AS1 bacterial load (CFU) in the mosquito was determined. *An. stephensi* mosquitoes were then subjected to infections with *P. berghei* and *P. falciparum*, whereas the *Ae. aegypti* mosquitoes were challenged with infections of dengue virus or Zika virus, as describe previously.

Statistical analysis

The statistical significance of differences in bacterial load, virus copies number, oocyst or ookinete intensities between two treatments was analyzed using a nonparametric Mann-Whitney test. The statistical significance of mosquito survival curves was analyzed with the logrank test, by using R packages “survival” (version 3.7.0) and “survminer” (version 0.5.0) to compare the survival rates of the mosquitoes. Other statistical significance was calculated using a two-tailed Student’s *t*-test for unpaired comparisons between two groups, or one-way analysis of variance (or one-way ANOVA) between more than two groups. In the dengue and Zika virus titers assay, samples with undetectable viral loads were included in the statistical analysis. In the DENV and ZIKV dual infection assay, the *p*-value for comparisons between the two groups was determined by the Generalized Linear Model (GLM) with a ‘biomial’ link function[96]. A value of $P < 0.05$ was considered to be statistically significant. Statistical analyzes were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software).

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The data supporting the findings of this study are available in the article and the Supplementary Materials. The transcriptome data used in this study have been deposited in the National Center for Biotechnology Information Sequence Read Archive under accession code PRJNA1031615. The secretion proteomics data used in this study have been deposited to the ProteomeXchange Consortium via the PRIDE66 partner repository under accession code PXD042831. Source data are provided with this paper.

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Author contributions

S.W. conceived the project. S.W., H.G., and W.Q.H. designed the study. W.Q.H. and H.G. performed the majority of the experiments. H.G. and W.Q.H. designed the multifunctional plasmids. W.Q.H. and H.G. constructed the plasmids and the transformed bacterial strains. W.Q.H., H.G., and F.L. performed the bacterial spreading assay in *Anopheles* and *Ades* mosquito populations. W.Q.H., H.G., and L.H.W. conducted the *Plasmodium* infection tests in *Anopheles* mosquitoes. W.Q.H., C.L.C., Y.F.L., Y.T.Z., and H.G. performed the dengue virus and Zika virus infection tests. W.Q.H., H.G. C.L.C., Y.F.L, and T.Y.X. conducted field-cage experiments. W.Q.H., H.G., Y.G.W, and S.W. analyzed data. H.G., W.Q.H., and S.W. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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