

Combined oral vaccination with niche competition can generate sterilizing immunity against entero-pathogenic bacteria

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1 **Abstract**

2 Widespread antimicrobial resistance generates an urgent need to develop better disease prophylaxis
3 for intestinal bacterial pathogens. While the first phase of infection with any bacterial pathogen is
4 typically colonization of the mucosal surfaces, current vaccine strategies typically target invasive stages
5 of disease. Here we demonstrate the ability to specifically generate sterilizing immunity against
6 *Salmonella enterica* subspecies *enterica* serovar Typhimurium (S.Tm) at the level of gut lumen
7 colonization using a combination of oral vaccination and a rationally-designed niche competitor strain.
8 This is based on the proven ability of specific secretory IgA to generate a fitness disadvantage for a
9 targeted bacterium, allowing a non-targeted competitor to rapidly overtake its niche. By hugely
10 decreasing the population size of an intestinal pathogen during the early stages of infection, this
11 improves protection of gut tissue compared to standard licensed animal vaccines. We demonstrate
12 that most effective protection is generated when the niche competitor is derived from the pathogen
13 and therefore occupies an identical niche. However, as this is unrealistic in real-world infections, we
14 further demonstrate that robust protection can also be generated with a more distantly related
15 “probiotic” niche competitor from a distinct species. Interestingly, focusing prophylaxis on the gut
16 lumen reveals an uncoupling of protective mechanisms required for protection in the gut and gut
17 tissues and those required for protecting against colonization of the spleen and liver. Therefore, while
18 there is still potential to improve this approach by adding systemic immune activation, we nevertheless
19 believe this is a fundamental step forward in our ability to manipulate colonization of intestinal
20 bacteria with potential application to a wide-range of entero-pathogens, as well as to manipulation of
21 microbiota composition.

22 Introduction

23 World-wide, the frequency of drug-resistant infections with *E. coli* and with non-typhoidal *Salmonella*
24 have been steadily increasing, posing a looming challenge for health systems¹. There is a pressing need
25 to develop control and prevention strategies that are independent of antibiotics. Vaccination of both
26 farm animals and humans is a promising alternative. While high-efficacy vaccine candidates against
27 Typhoid fever are currently well advanced (e.g. intramuscular Vi capsule, typhoid conjugate vaccines
28 Vi-TT, and oral Ty21a live-attenuated vaccines)^{2,3}, there are no licensed human vaccines for
29 non-typhoidal Salmonellosis (NTS)⁴. Moreover, licensed live-attenuated *S.Tm* swine and bovine
30 vaccines show only weak protection from colonization⁵ and can cause disease in highly-susceptible
31 individuals, such that versions currently licensed for animal use are not directly suitable for human
32 translation⁶. An ideal solution would be fully inactivated vaccines that can mimic the immunity and
33 protection generated by live-attenuated vaccines but with a much higher safety profile and robustness.

34 When designing vaccines targeting intestinal bacteria, we have either focused on mimicking natural
35 infection⁷ or on generating immune responses targeting specific pathogen antigens^{8,9}. However, we
36 increasingly realize that infection of the gut involves disruption of a densely populated microbial
37 ecosystem, and the intestinal microbiome is itself part of our defenses¹⁰. Therefore, evidence suggests
38 that naturally arising immunity at our mucosal surfaces does not operate in isolation, but rather
39 interacts with the endogenous microbiota to recover homeostasis and evict colonizing opportunistic
40 pathogens¹¹. For example, fecal microbiota transplantation can be curative in recurrent *Clostridioides*
41 *difficile* infections¹², and introduction of the pneumococcal conjugate vaccines has resulted in
42 displacement of vaccine-targeted *Streptococcus pneumoniae* serovars from the nasopharynx by non-
43 targeted strains in both vaccinated as well as unvaccinated individuals^{13,14}.

44 To prevent tissue invasion in hosts permissive for systemic *S.Tm* growth, inflammation and systemic
45 spread, we have previously estimated that a >1000-fold reduction in invasion rate of *S.Tm* is required
46 during the first day of infection¹⁵. The overall invasion rate is a product of the gut luminal *S.Tm*
47 population size and the ability of each individual bacterium to invade. Antibody-mediated clumping,
48 via enchainment growth and/or agglutination achieves around a 100-fold reduction in the planktonic
49 infectious population size¹⁶, but alone cannot be sufficient to fully prevent invasive disease. Therefore,
50 vaccination strategies are required that more efficiently prevent colonization of the gut lumen.

51 At the simplest level, the population size of *S.Tm* in the gut lumen is determined by its growth rate,
52 clearance rate and the size of the available metabolic niche. The available metabolic niche can be
53 dramatically shrunk by competitors using the same resources. A classic example of this phenomenon
54 occurs when “cheater” mutations spontaneously arise during chronic NTS¹⁷. Mutations in the
55 *Salmonella* Pathogenicity Island 1 (SPI-1) master-regulator of virulence *hilD* can outgrow and displace

56 wildtype Salmonella from the gut lumen. This is attributed to a fast net-growth rate and increased
57 stress resistance of *hild*-mutant strains due to loss of the fitness costs associated with expression of
58 the Hild regulon^{18,19}.

59 Niche competition can also be favoured by increasing the clearance rate of the pathogen. High-affinity
60 intestinal IgA, induced by oral vaccination with whole-cell inactivated vaccines, can increase S.Tm
61 clearance rates by generating bacterial clumps (via enchainned growth or agglutination) that are more
62 efficiently cleared in the flow of the fecal stream¹⁶. We have recently demonstrated that this IgA-
63 mediated selective pressure can be used to manipulate the evolutionary trajectory of *Salmonella*
64 *enterica* subspecies *enterica* serovar Typhimurium (S.Tm) in the gut lumen²⁰. Moreover, part of this
65 work demonstrated the ability of IgA to generate a more than a million-fold ratio of a targeted S.Tm
66 strain over a non-bound strain²⁰. Therefore, IgA protects the intestinal environment not only via
67 immune exclusion (i.e. preventing interaction of pathogenic bacteria with the epithelium) but also by
68 competitively eliminating targeted bacteria from the intestinal ecosystem. This suggested a huge
69 potential for specific high-affinity IgA responses to manipulate the outcome of competition between
70 an invading pathogen and an engineered niche competitor.

71 Critical components of such a prophylactic system are: 1) a vaccine capable of inducing high-affinity
72 specific IgA against the pathogen of interest; 2) a non-pathogenic strain with (ideally) complete
73 metabolic niche overlap with the pathogen of interest, a faster growth-rate than the pathogen of
74 interest and absence of surface antigen cross-reactivity to the pathogen of interest. Additionally,
75 questions arise as to how vaccines and competitors can be combined temporally to give maximum
76 effect, and the extent of protection from invasive disease that can be achieved with such an approach.
77 Here we make use of the well-established and severe, murine model of non-typhoidal Salmonellosis
78 to establish this concept. In this model, oral antibiotics are applied to acutely generate a large open
79 niche for S.Tm in the mouse cecum and upper large intestine²¹. Subsequently, very low numbers of
80 S.Tm can be orally inoculated and will rapidly grow to fill the available niche. Disease depends on the
81 activity of *Salmonella* Pathogenicity Islands 1 and 2 and includes acute typhlocolitis, colonization of the
82 intestinal tissue, mesenteric lymph nodes, spleen and liver²¹⁻²³. In the resistant (Nramp1+/+, also
83 known as Slc11a1) 129SjL mouse strain, the disease is slowly controlled, but full recovery takes more
84 than 1 month²⁴. We also extend our observations to the murine oral Typhoid fever model, in which a
85 high-dose of S.Tm is delivered orally to mice with an intact intestinal microbiota, resulting in a disease
86 that is more reliant on the tissue-invasive stages of disease.

87 Here we demonstrate that combining a benign niche-competitor and a whole-cell inactivated
88 vaccination regimen can profoundly suppress intestinal colonization with virulent *Salmonella* even on
89 high-dose exposure in a very high-susceptibility model. Protection from gut tissue invasion and

90 inflammation was also robust. Interestingly, live-attenuated *Salmonella* vaccines were superior to
91 inactivated vaccines in protecting from colonization of systemic sites, indicating that mechanisms that
92 protect systemic sites are discrete from intestinal protection.

93

94 **Results**

95 **Combining oral inactivated bacterial vaccination with a bacterial niche competitor protects mice** 96 **from intestinal inflammation and leads to rapid *S.Tm*^{WT} clearance in a model of NTS**

97 As a first proof-of-principle we made use of *S.Tm* carrying a mutation in the “Salmonella Pathogenicity
98 Island 1” (SPI-1) master regulator *hilD*, that has previously been demonstrated to outgrow wild-type
99 *S.Tm* in the gut lumen during long-term infections^{18,25}. This was combined with inactivating mutations
100 in *ssaV* to disrupt the function of “Salmonella Pathogenicity Island 2” (SPI-2) and deletion of *oafA* to
101 prevent acetylation of the *S.Tm* O-antigen, i.e. preventing generation of the O:5 epitope. The resulting
102 strain is fully avirulent¹⁸ (**Fig. S1**), fast-growing¹⁸, and less bound by IgA induced by a wild-type O:5,12-0
103 *Salmonella* vaccine than the isogenic wild-type strain²⁰. The resulting mutant *S.Tm*^{*hilD ssaV oafA*} was used
104 as niche competitor hereafter named *S.Tm*^{Comp}.

105 To demonstrate the effect of pathogen-targeting IgA on competition, SPF 129S6/SvEv wildtype mice
106 received an inactivated whole-cell oral *S.Tm* vaccine once weekly for 4 weeks or a mock PBS treatment.
107 Subsequently, mice were antibiotic treated to eliminate a large part of the microbiota and infected
108 with either virulent wildtype *S.Tm* (*S.Tm*^{WT}) alone, or *S.Tm*^{WT} combined 1:1 with *S.Tm*^{Comp}. Intestinal
109 colonization and inflammation were monitored for 10 days post infection and tissue invasion and
110 histopathology were monitored at endpoint (**Fig. 1A**).

111 In line with published data, we could detect high levels of *S.Tm* O:5,12-0-specific IgA in the intestine as
112 well as IgG in serum of vaccinated mice at endpoint, regardless of the subsequent infection (**Fig. 1B**).
113 This *S.Tm* specific antibody response was able to protect the vaccinated mice from weight loss upon
114 infection with WT *S.Tm* (**Fig. 1C**). In contrast, all mice that were not vaccinated experienced significant
115 weight loss until 5 days post infection. One unvaccinated mouse of each of two independent
116 experiments had to be euthanized at 5 days post infection due to excessive weight loss.

117 As antibiotic pre-treatment in this model generates a huge empty niche for *S.Tm* colonization in the
118 gut lumen, *in vivo* binding of IgA to *S.Tm*^{WT} could not prevent initial expansion of *S.Tm*, and total *S.Tm*
119 counts were similar in all groups. However, over time the differences between groups become clear:
120 *S.Tm*^{WT} CFU/g feces remains constant over 10 days in unvaccinated mice without *S.Tm*^{Comp}. The
121 presence of either vaccination alone, or *S.Tm*^{Comp} results in a drop in *S.Tm*^{WT} CFU from day 2-3 post

122 infection, with CFU 10^5 CFU/g feces at day 10. In contrast, the combination of vaccination and *S.Tm*^{Comp}
123 generates an exponential drop in fecal CFU of *S.Tm*^{WT} over the first 5 days of infection, with some mice
124 completely clearing the pathogenic strains by day 5 (**Fig. 1D and E**). The cecum content counts reflected
125 the picture found in feces with a much lower burden of *S.Tm*^{WT} in all treated groups and absence of
126 *S.Tm*^{WT} in 50% of the mice that received vaccination and the niche competitor (**Fig. 1F**). Interestingly,
127 despite a very dramatic clearance of *S.Tm*^{WT} from the gut lumen, colonization of systemic sites was
128 apparently unaltered by either vaccination or by introduction of the niche competitor (**Fig. 1G-I**). This
129 is consistent with quantitative modelling of tissue invasion in murine NTS, which predicted that a more
130 than 1000-fold reduction in *Salmonella* intestinal load is required to completely inhibit systemic
131 spread^{15,16}. As it takes several days for the *S.Tm*^{WT} levels to be reduced below this level in our treatment
132 groups, there remains a considerable time-window for systemic spread to occur. While we cannot
133 exclude that differences may have been more apparent at earlier time-points, this clearly indicates
134 that encounter of a niche competitor simultaneous to infection, even in orally vaccinated mice that
135 display both intestinal IgG and serum IgA responses, is not sufficient to prevent colonization of the
136 spleen and liver of mice.

137 The partial-protective capacity of IgA with and without niche competition was also apparent when
138 looking at intestinal inflammation as measured by lipocalin-2 levels in feces. In both vaccinated groups,
139 inflammation not only started later but also the maximum fecal lipocalin-2 was significantly lower
140 throughout the whole course of infection (**Fig. 1J**). This was underpinned by histological stainings at
141 day 10 post challenge that showed no pathological changes in mice that were both vaccinated and
142 colonized with *S.Tm*^{Comp}, and only mild changes in the vaccine-alone group (**Fig. 1K**). Intestinal
143 inflammation could not be significantly prevented by the presence of the niche-competitor alone when
144 it is introduced simultaneously with the infectious challenge (**Fig. 1J and K**).

145 These experiments indicated the feasibility of combining vaccination and niche competition, while
146 revealing the need to optimize the procedure for protection of both the gut and systemic sites.

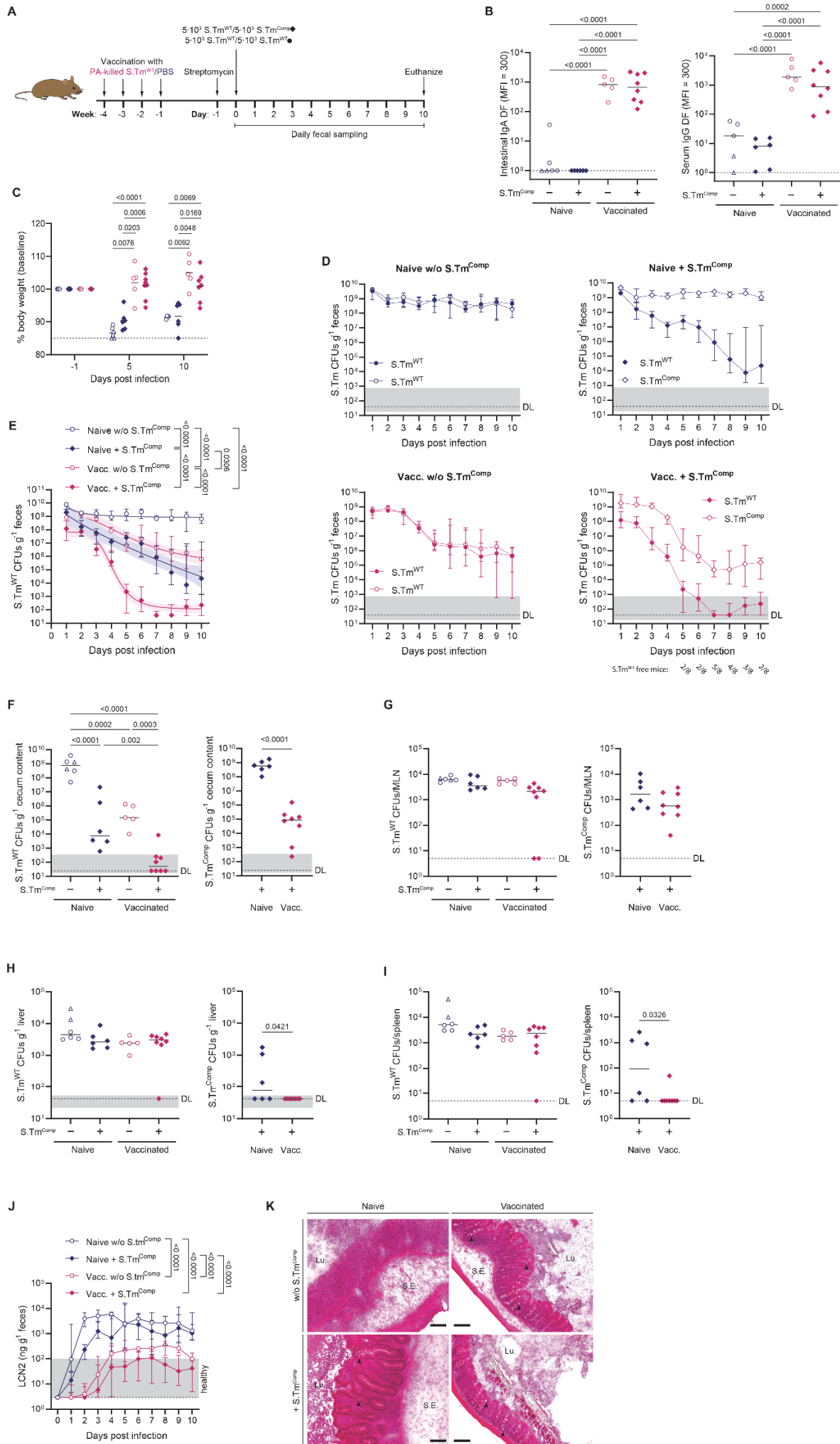


Figure 1. Combining oral vaccination with niche competition protects mice from intestinal inflammation and leads to rapid *S.Tm*^{WT} clearance. PBS (blue symbols) or PA-*S.Tm*-vaccinated (pink symbols) 129S6/SvEv mice were pretreated with streptomycin and infected with a total of 10^4 of a 1:1 mixture of two isogenic *S.Tm*^{WT} strains (open circles) or *S.Tm*^{WT} and *S.Tm*^{Comp} (*S.Tm*^{hilD ssaV oafA}, filled diamonds). **(A)** Experimental procedure. **(B)** *S.Tm*^{WT} (O:4[5]12-0) specific intestinal IgA and serum IgG titres as determined by flow cytometry. **(C)** Change in body weight over the course of infection. Solid lines show the mean. **(D)** Fecal CFUs as determined by selective plating. Open and filled symbols represent either isogenic *S.Tm*^{WT} strains (circles) or *S.Tm*^{WT} and *S.Tm*^{Comp} (diamonds) with different antibiotic resistances. **(E)** A 4-parameter logistic curve fit was fitted to the fecal *S.Tm*^{WT} CFUs and the area under the curve was statistically compared. Shaded areas depict the 95% CI of the fits. **(F-I)** *S.Tm* CFUs in cecum content **(F)**, MLN **(G)**, liver **(H)** and spleen **(I)**. **(J)** Intestinal inflammation was determined by measuring fecal lipocalin-2. **(K)** Histology of cryo-embedded H&E-stained cecal tissue sections. Arrowheads show exemplary goblet cells. Scale bars: 100 μ m. Pooled data from two independent experiments with switched antibiotic resistances (n = 5-8 mice/group). Solid lines depict the median unless stated otherwise, error bars the interquartile range. Dotted lines show the detection limit and the shaded area the range for cases in which the detection limit is dependent on sample weight. Open triangles show mice that had to be euthanized prematurely due to excessive weight loss ($\geq 15\%$) or disease symptoms. Statistics were performed by mixed-effects analysis (C) or one-way analysis of variance (ANOVA) (C) on log-normalized data (B, F-I) or area under the curve (AUC) (E, J). Where only two groups were compared, an unpaired two-tailed t-test on log-normalized data was done (F-I). CFU, colony forming unit; DF, dilution factor; LCN2, lipocalin-2; Lu., Lumen; MFI, median fluorescence intensity; MLN, mesenteric lymph node, S.E., submucosal edema.

147 **Modelling the interaction of niche competition and vaccination**

148 As intestinal colonization is necessarily a highly dynamic process, we built a simple mathematical
149 model to generate predictions on the requirements for extinction of *S.Tm*^{WT} and the time-to-
150 extinction. We predict that minimizing the time-to-extinction in the gut lumen will best inhibit systemic
151 spread of *S.Tm* and can minimize the risk of immune escape.

152 In this model we assumed that the microbiota (of size $M(t)$), *S.Tm*^{Comp} (of size $C(t)$) and *S.Tm*^{WT} (of size
153 $W(t)$) compete for undefined shared nutrient resources, which leads to a carrying capacity K_1 .
154 Additionally, we assumed a second independent nutrient source for *S.Tm*^{Comp} and *S.Tm*^{WT} on the one
155 hand, and for the microbiota on the other hand leading to carrying capacities K_2 and K_3 . In a
156 deterministic view, the size of the populations evolves as

$$\text{Eq. 1} \quad \left\{ \begin{array}{l} \frac{dW}{dt} = W \cdot \left(r_w \cdot \left(1 - \frac{1}{\frac{K_1}{M+C+W} + \frac{K_2}{C+W}} \right) - c_w \right) \\ \frac{dC}{dt} = C \cdot \left(r_c \cdot \left(1 - \frac{1}{\frac{K_1}{M+C+W} + \frac{K_2}{C+W}} \right) - c_c \right) \\ \frac{dM}{dt} = M \cdot \left(r_m \cdot \left(1 - \frac{1}{\frac{K_1}{M+C+W} + \frac{K_3}{M}} \right) - c_m \right) \end{array} \right.$$

157 where r_w , r_c and r_m correspond to the growth rate of $S.Tm^{WT}$, $S.Tm^{Comp}$ and the microbiota, respectively.

158 The parameters c_w , c_c and c_m correspond to the clearance rates of these populations.

159 In order to predict “extinction probability” and “extinction time”, we required realistic estimates of
 160 the population dynamics of the two $S.Tm$ strains and the “average microbiome”. $S.Tm$ population
 161 dynamics parameters in the presence or absence of IgA were estimated by fitting the competition data
 162 shown in Figure 1 and Figure S2A (see materials and methods) (**Fig. 2A**). This confirmed the predicted
 163 higher net growth rate for $S.Tm^{Comp}$ as compared to $S.Tm^{WT}$ and the elevated clearance rate of $S.Tm^{WT}$
 164 in vaccinated mice (see **Table 1**).

Table 1: Kinetic parameter values inferred from the competition data shown in Fig. 1 (for vaccinated + $S.Tm^{Comp}$ group). r_w , r_c and r_m : growth rate of $S.Tm^{WT}$, $S.Tm^{Comp}$ and the microbiota, respectively. c_w , c_c and c_m : clearance rates of $S.Tm^{WT}$, $S.Tm^{Comp}$ and the microbiota. K_1 , K_2 , K_3 : carrying capacities of $S.Tm$ and the microbiota, $S.Tm^{WT}$ and $S.Tm^{Comp}$ and the microbiota alone. m_0 : size of microbiota after antibiotic clearance at time point of infection with $S.Tm^{WT}$.

r_w	r_c	r_m	
33.7 day ⁻¹	38.7 day ⁻¹	18.7 day ⁻¹	
c_w	c_c	c_m	
6.4 day ⁻¹	5.9 day ⁻¹	1.7 day ⁻¹	
K_1	K_2	K_3	m_0
$5.9 \cdot 10^9$	$2.3 \cdot 10^4$	10^7	10^5

165 Using 2500 iterations of a stochastic version of this model, we could derive extinction-related
166 parameters. Based on continuous growth and clearance of the gut luminal *Salmonella* population,
167 these models indicate clearance of virulent *Salmonella* within eight days of infection in the mice
168 treated with vaccination and *S.Tm^{Comp}*, compared to continuous colonization in mice untreated or
169 receiving vaccine only (**Fig. 2B and C**). Interestingly, the model predicted that the time-point for
170 introduction of the niche competitor was not a major variable, as long as this occurred prior to
171 infection. Major determinants of success are the relative growth rates and loss rates of the competitor
172 versus the virulent bacterium as well as the carrying capacity, which is in turn determined by the
173 regrowth of the microbiota. Therefore, a major conclusion of the model is that the niche competitor
174 should be already present in the gut for effective protection (**Fig. 2B and D**), which is also a more
175 realistic situation for disease prophylaxis.

176 Longer duration of infection and more intense *Salmonella* shedding increase the probability of
177 transmission²⁶. Both can be dramatically reduced by vaccination/competition, and the model reveals
178 a major potential for this intervention to reduce the *Salmonella* burden in farm animals.

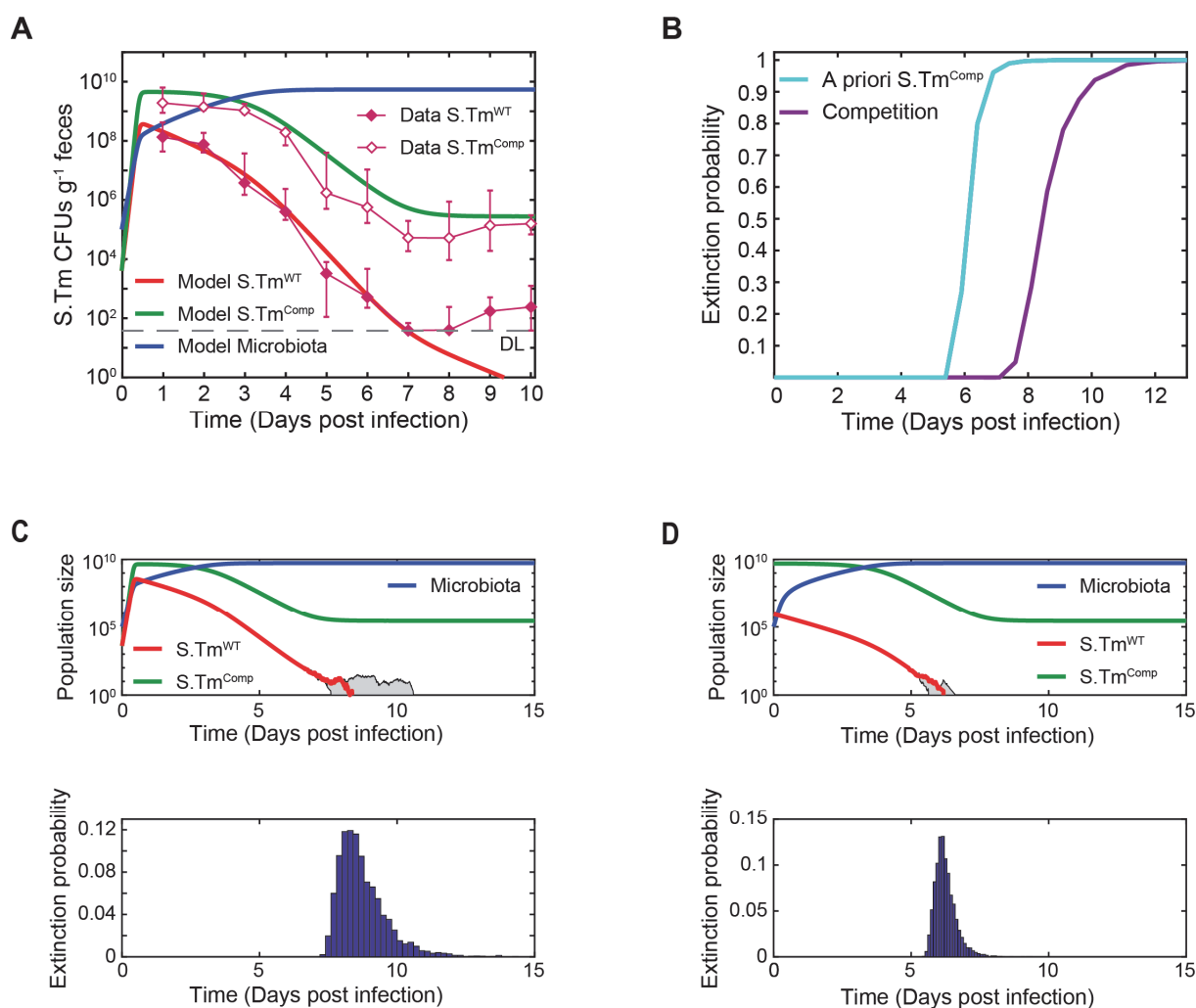


Figure 2: Modelling of *S.Tm*^{WT} extinction for vaccination and/or niche competition. (A) Adjustment of the model to the data shown in Figure 1. The thick lines correspond to the prediction from the model and the thin lines with diamonds to the experimental data (median \pm interquartile range). Initial *S.Tm*^{WT} and *S.Tm*^{Comp} CFUs: $4 \cdot 10^3$. (B) Extinction probability of *S.Tm*^{WT} over time for vaccinated mice + *S.Tm*^{Comp}. The purple line shows the prediction of the model when *S.Tm*^{WT} and *S.Tm*^{Comp} are given at the same time, the turquoise line for the case that *S.Tm*^{Comp} is given 3 days prior to *S.Tm*^{WT}. The simulations were performed with the deterministic model for large population size and with the Gillespie algorithm (1000 realizations) for small size of *S.Tm*^{WT} population (*S.Tm*^{WT} < 100). (C) Extinction time probability distribution for *S.Tm*^{WT} and *S.Tm*^{Comp} given at the same time. Simulations were performed with the deterministic model for large population size. For small size of *S.Tm*^{WT} population (*S.Tm*^{WT} < 100), simulations were performed with the Gillespie algorithm (5000 realizations). Upper panel: the red line corresponds to a trajectory whose extinction time is around the median extinction time. The gray area shows trajectories of the 2.5th percentile extinction time and the 97.5th percentile extinction time. Lower panel: extinction time probability distribution. The y-axis corresponds to (bin height*bin width). Median extinction time: 8.5 days. 2.5th percentile: 7.6 days. 97.5th percentile: 11 days. Initial *S.Tm*^{WT} CFUs: $4 \cdot 10^3$. Initial *S.Tm*^{Comp} CFUs: $4 \cdot 10^3$. (D) Extinction time probability distribution for a priori colonization with *S.Tm*^{Comp}. Simulations were performed with the deterministic model for large population size. For small size of *S.Tm*^{WT} population (*S.Tm*^{WT} < 100), simulations were performed with the Gillespie algorithm (5000 realizations). Upper panel: the red line corresponds to a trajectory whose extinction time is around the median extinction time. The gray area shows trajectories of the 2.5th percentile extinction time and the 97.5th percentile extinction time. Lower panel: extinction time probability distribution. The y-axis corresponds to (bin height*bin width). Median extinction time: 6 days. 2.5th percentile: 5.7 days. 97.5th percentile: 7.2 days. Initial *S.Tm*^{WT} CFUs: $5 \cdot 10^9$. Initial *S.Tm*^{Comp} CFUs: $4 \cdot 10^3$. The dotted line depicts the mean detection limit of the experimental data. (A, C, D) Parameter values: $r_w = 33.7 \text{ day}^{-1}$, $c_w = 6.4 \text{ day}^{-1}$, $r_c = 38.7 \text{ day}^{-1}$, $c_c = 5.9 \text{ day}^{-1}$, $r_m = 18.7 \text{ day}^{-1}$, $c_m = 1.7 \text{ day}^{-1}$, $K_1 = 5.9 \cdot 10^9$, $K_2 = 2.3 \cdot 10^4$, $K_3 = 10^7$ and $m_0 = 10^5$.

r_w , r_c and r_m : growth rate of $S.Tm^{WT}$, $S.Tm^{Comp}$ and the microbiota, respectively. c_w , c_c and c_m : clearance rates of $S.Tm^{WT}$, $S.Tm^{Comp}$ and the microbiota. K_1 , K_2 , K_3 : carrying capacities of $S.Tm$ and the microbiota, $S.Tm^{WT}$ and $S.Tm^{Comp}$ and the microbiota alone. m_0 : size of microbiota after antibiotic clearance.

179 Introduction of the niche competitor a priori allows complete pathogen clearance when combined 180 with oral vaccination

181 Based on the modelling data, we carried out a vaccination and challenge experiment, this time
182 introducing the competitor strain carrying the same Streptomycin resistance as $S.Tm^{WT}$ three days prior
183 to infection (**Fig. 3A**). We additionally increased our $S.Tm^{WT}$ infection dose to $1 \cdot 10^6$ CFU, in order to
184 increase the stringency of the challenge. Again, we could confirm that vaccination leads to a robust
185 induction of $S.Tm$ -specific intestinal IgA as well as serum IgG (**Fig. 3B**), and this protected animals from
186 weight loss upon $S.Tm^{WT}$ infection, regardless of the higher dose. In contrast to simultaneous infection,
187 introducing $S.Tm^{Comp}$ a priori into naïve mice also prevented weight loss (**Fig. 3C**). In animals infected
188 with $S.Tm^{WT}$ only, we observe very similar infection kinetics to those induced by challenging with $5 \cdot 10^3$
189 CFU. By contrast, $S.Tm^{WT}$ struggled to expand at all in mice already colonized with the
190 competitor (**Fig. 3D**) and was eliminated as soon as four days post infection in mice protected by both
191 vaccination and pre-colonization with $S.Tm^{Comp}$. $S.Tm^{WT}$ was undetectable in the cecum content in 5 of
192 8 “vaccinated + $S.Tm^{Comp}$ colonized” mice on the final day of infection (**Fig. 3E**). This fitted well to
193 predictions from the model derived above (**Fig. 3F**). The predictions of the model were less accurate
194 for $S.Tm^{WT}$ at later time points, which might be due to the emergence of escape mutants. Intestinal
195 inflammation could also be completely prevented by the combined prophylaxis, remaining within the
196 range of healthy animals for the full duration of the infection (**Fig. 3G-I**). Moreover, systemic spread of
197 $S.Tm^{WT}$ could be effectively prevented by the combination of vaccination and $S.Tm^{Comp}$ in around 50%
198 of treated animals (**Fig. 3J-L**), and correlated well with early loss of $S.Tm^{WT}$ from the gut, rendering
199 some mice completely pathogen free at 10 days post infection. Therefore, combining an inactivated
200 oral vaccine with a live niche competitor can generate sterilizing immunity against virulent *Salmonella*
201 in a very severe model of non-typhoidal Salmonellosis.

202 To further corroborate our findings of sterilizing immunity, we performed fecal microbial transplants
203 from vaccinated/ $S.Tm^{Comp}$ mice into naïve Streptomycin pre-treated 129S6/SvEv mice. All but one
204 mouse from this group, did not efficiently transmit $S.Tm^{WT}$ by fecal microbial transplantation (FMT)
205 (**Fig. S3**). In contrast, all untreated control mice used for this experiment efficiently transferred $S.Tm^{WT}$
206 and disease to the naïve mice (**Fig. S3**).

207 In summary, we could show that our niche competitor when given a priori is able to establish a partial
208 colonization resistance thereby limiting intestinal $S.Tm^{WT}$ expansion. When combined with vaccination,
209 a major fraction of the animals could completely clear $S.Tm^{WT}$ from all examined sites i.e. generating

210 sterilizing immunity. Importantly, we could not only greatly ameliorate disease in animals treated with
211 this method, but we could also prevent transmission in almost 90% of the cases.

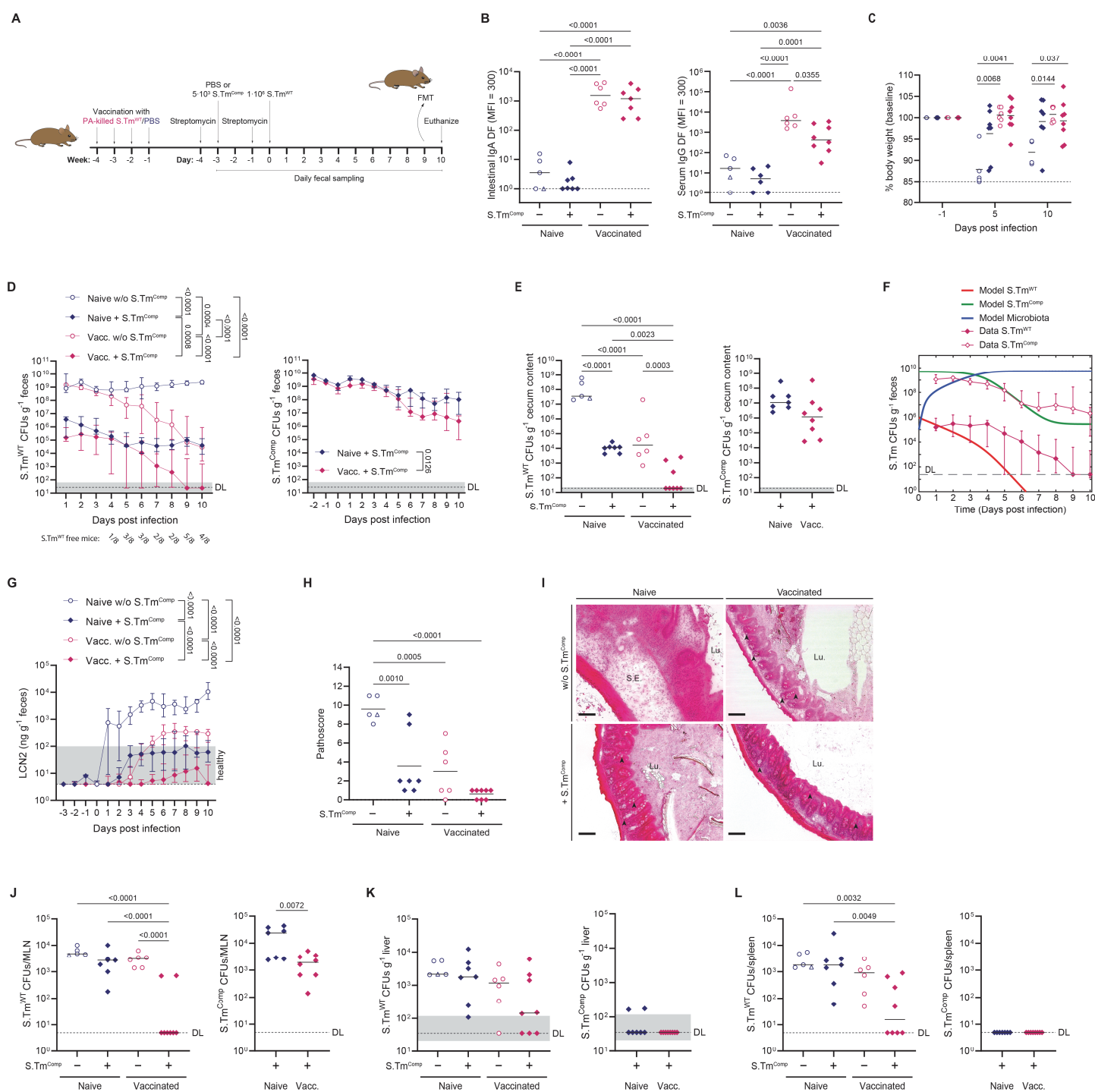


Figure 3. A priori colonization with *S.Tm^{Comp}* can generate sterilizing immunity in the gut when combined with vaccination. PBS (blue symbols) or PA-*S.Tm*-vaccinated (pink symbols) 129S6/SvEv mice were pretreated with streptomycin and infected with $1 \cdot 10^6$ *S.Tm^{WT}*. Two groups were pre-colonized with $5 \cdot 10^3$ *S.Tm^{Comp}* 3 days before infection. **(A)** Experimental procedure. **(B)** *S.Tm^{WT}*-specific intestinal IgA and serum IgG titres as determined by flow cytometry. **(C)** Change in body weight over the course of infection. Solid lines depict the mean. Fecal **(D)** and cecum content **(E)** *S.Tm* CFUs as determined by selective plating. **(F)** Comparison of the predictions of the model to the experimental data. The thick solid lines correspond to the prediction from the model, using the value of the dynamical parameter inferred from the experiment with simultaneous *S.Tm^{WT}* and *S.Tm^{Comp}* infection. The thin lines with diamonds to the experimental data (median \pm interquartile range). Initial *S.Tm^{WT}* CFUs: $5 \cdot 10^9$. Initial *S.Tm^{Comp}* CFUs: $4 \cdot 10^3$. Parameter values: $r_w = 33.7 \text{ day}^{-1}$, $c_w = 6.4 \text{ day}^{-1}$, $r_c = 38.7 \text{ day}^{-1}$, $c_c = 5.9 \text{ day}^{-1}$, $r_m = 18.7 \text{ day}^{-1}$,

$c_m = 1.7 \text{ day}^{-1}$, $K_1 = 5.9 \cdot 10^9$, $K_2 = 2.3 \cdot 10^4$, $K_3 = 10^7$ and $m_0 = 10^5$. r_w , r_c and r_m : growth rate of $S.Tm^{WT}$, $S.Tm^{Comp}$ and the microbiota, respectively. c_w , c_c and c_m : clearance rates of $S.Tm^{WT}$, $S.Tm^{Comp}$ and the microbiota. K_1 , K_2 , K_3 : carrying capacities of $S.Tm$ and the microbiota, $S.Tm^{WT}$ and $S.Tm^{Comp}$ and the microbiota alone. m_0 : size of microbiota after antibiotic clearance. (G-I) Intestinal inflammation as determined by fecal lipocalin-2 (G) and histopathological scoring of cecal tissue sections (H) Solid lines in (H) show the mean. (I) Representative images of H&E-stained cecal tissue sections. Arrowheads show exemplary goblet cells. Scale bars: 100 μm . (J-L) $S.Tm$ CFUs in MLN (J), liver (K) and spleen (L).

Pooled data from two independent experiments with switched antibiotic resistances ($n = 5-8$ mice/group). Solid lines depict the median unless stated otherwise, error bars the interquartile range. Dotted lines show the detection limit and the shaded area the range for cases in which the detection limit is dependent on sample weight. Open triangles show mice that had to be euthanized prematurely due to excessive weight loss ($\geq 15\%$) or disease symptoms. Statistics were performed by mixed-effects analysis (C) or one-way ANOVA (C, G) on log-normalized data (B, E, I-K) or area under the curve (AUC) (D, F). Where only two groups were compared, an unpaired two-tailed t-test on log-normalized data was done (E, I-K). CFU, colony forming unit; DF, dilution factor; FMT, fecal microbial transplant; LCN2, lipocalin-2; Lu., lumen; MFI, median fluorescence intensity; MLN, mesenteric lymph node; S.E., submucosal edema.

212 Commensal competitors and vaccination

213 While the combination of inactivated oral vaccines and an engineered *Salmonella*-derived competitor
214 is highly effective, there remain potential safety/legislative issues associated with the application of
215 live GMOs and a very low, but non-zero, risk of reversion of the competitor strain via horizontal gene
216 transfer²⁷. An attractive alternative would be to develop a non-pathogenic commensal bacterial strain
217 that can nevertheless compete efficiently with $S.Tm^{WT}$. For this purpose, we chose the mouse
218 commensal ECOR B2 strain *E. coli* 8178 (Ec^{8178}). This has been demonstrated to grow well in $S.Tm$ -
219 permissive conditions²⁸ and could limit $S.Tm$ infection after dietary perturbation²⁹. Moreover, this
220 *E. coli* produces an unrelated O-antigen structure, allowing us to use an “evolutionary trap” version²⁰
221 of our $S.Tm$ vaccine covering all possible variations of the $S.Tm$ O-antigen. This adds robustness to the
222 technique as selective pressure exerted by vaccine-induced IgA can provide a selective advantage both
223 to the competitor strain, and to naturally emerging $S.Tm$ variants with a short O-antigen. As mutants
224 with short LPS are susceptible to complement, bile acids and other membrane-targeting stresses, this
225 is associated with decreased virulence of $S.Tm^{WT}$ in vaccinated mice²⁰.

226 We therefore repeated our previous experiment using the evolutionary trap (EvoTrap) vaccine
227 together with Ec^{8178} as a competitor given three days prior to challenge (Fig. 4A). Vaccination with the
228 EvoTrap vaccine led to high levels of $S.Tm$ O:5,12-0 specific antibodies in small intestine and blood
229 (Fig. 4B) as well as to the other $S.Tm$ O-antigen variants (Fig. S4). Similarly, to what we have observed
230 with giving $S.Tm^{Comp}$ prior to $S.Tm^{WT}$ infection, also Ec^{8178} alone could reduce weight loss after challenge
231 and weight loss was prevented in both vaccinated groups (Fig. 4C). Ec^{8178} colonized the mouse gut to
232 high levels and was able to decrease initial $S.Tm^{WT}$ expansion. However, Ec^{8178} was clearly less effective

233 as a competitor in the intestine when compared with *S.Tm*^{Comp}, as *S.Tm*^{WT} levels remained high (>10⁶)
234 in feces and cecum of all groups at day 10 post infection (**Fig. 4D and E**). Despite the high *S.Tm*^{WT}
235 numbers in the gut, we observed a significant reduction in systemic counts (mesenteric lymph nodes,
236 spleen and liver) in the vaccinated group that received *Ec*⁸¹⁷⁸ (**Fig. 4F-H**). When analysing intestinal
237 inflammation, we again found that the intestinal inflammation marker lipocalin-2 was almost
238 completely absent from the “vaccinated + *Ec*⁸¹⁷⁸ pre-colonized” group, indicating robust protection
239 from tissue invasion and disease, despite incomplete clearance of *S.Tm*^{WT} from the gut lumen
240 (**Fig. 4I-K**). As predicted²⁰ with EvoTrap vaccination, a major fraction of luminal *S.Tm*^{WT} re-isolated from
241 vaccinated mice carried a spontaneous deletion resulting in loss of *wzyB* and therefore short O-antigen
242 production, which may explain this discrepancy (**Fig. S5**).

243 In summary, we could show that a more distantly related competitor does not compete as well in the
244 gut but nonetheless is able to abolish systemic invasion and intestinal inflammation, and this may
245 represent a more easily translatable model. Moreover, future analysis of the metabolism of *S.Tm* and
246 commensal *E. coli* strains may allow for the identification of strain combinations with a more complete
247 metabolic niche overlap.

248 Taken together, we here demonstrate that the combination of an inactivated oral vaccine and a
249 competitor strain in the gut lumen can protect from *S.Tm* colonization and disease in murine models.
250 The extent of gut lumen clearance was well-correlated with fecal lipocalin-2 and intestinal
251 inflammation.

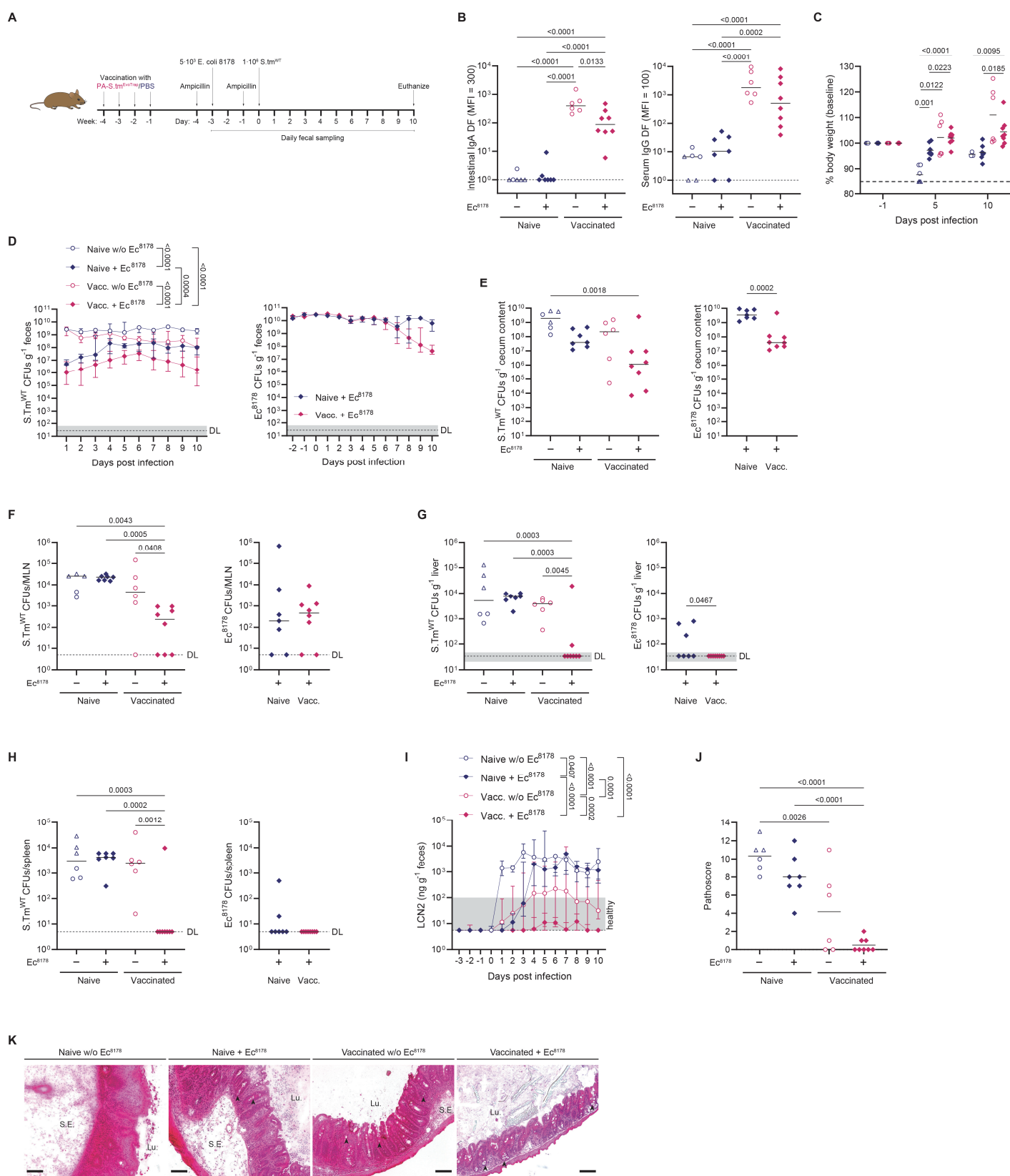


Figure 4. Vaccination together with the commensal competitor *E. coli* 8178 can prevent intestinal pathology albeit *S.Tm*^{WT} cannot be cleared from the gut. PBS (blue symbols) or EvoTrap-vaccinated (pink symbols) 129S6/SvEv mice were pretreated with ampicillin and infected with $1 \cdot 10^6$ *S.Tm*^{WT}. Two groups were pre-colonized with $5 \cdot 10^3$ *E. coli* 8178 3 days before infection. **(A)** Experimental procedure. **(B)** *S.Tm*^{WT}-specific intestinal IgA and serum IgG titres as determined by flow cytometry. **(C)** Change in body weight over the course of infection. Solid lines depict the mean.

(D-H) CFUs in feces (D) cecum content (E), MLN (F), liver (G) and spleen (H). Intestinal inflammation was determined by fecal lipocalin-2 (I) and histopathological scoring of cecal tissue sections (J). Solid lines in (J) show the mean. (K) Representative images of H&E-stained cecal tissue sections. Arrowheads show exemplary goblet cells. Scale bars: 100 μ m.

Pooled data from two independent experiments (n = 6-8 mice/group). Solid lines depict the median unless stated otherwise, error bars the interquartile range. Dotted lines show the detection limit and the shaded area the range for cases in which the detection limit is dependent on sample weight. Open triangles show mice that had to be euthanized prematurely due to excessive weight loss ($\geq 15\%$) or disease symptoms. Statistics were performed by mixed-effects analysis (C) or one-way ANOVA (C, J) on log-normalized data (B, E-H) or area under the curve (AUC) (D, I). Where only two groups were compared, an unpaired two-tailed t-test on log-normalized data was done (E-H). CFU, colony forming unit; DF, dilution factor; LCN2, lipocalin-2; Lu., lumen; MFI, median fluorescence intensity; MLN, mesenteric lymph node; S.E., submucosal edema.

253 Comparing vaccination/niche competition to licensed animal vaccines reveals mechanistic 254 differences in protection of the intestine and systemic sites.

255 Live-attenuated non-typhoidal *Salmonella* vaccines, carrying a mutation in *aroA*, which renders the
256 strain auxotrophic for aromatic amino acids, are already used for broilers³⁰ and very similar
257 auxotrophic strains are used in pig-farming³¹. In order to benchmark our approach to a known *S.Tm*
258 vaccine we therefore compared protective efficacy and safety of the combined inactivated oral
259 vaccine+*Ec*⁸¹⁷⁸ treatment to that of *S.Tm*^{aroA} vaccination. To gain mechanistic insight into protection at
260 the tissue level versus the gut lumen, we also compared protection in the NTS model to protection in
261 the murine oral typhoid model, in which no major gut luminal niche is generated³².

262 In the NTS model (Fig. 5A), both the classical *S.Tm*^{aroA} vaccination as well as EvoTrap vaccination+*Ec*⁸¹⁷⁸
263 led to the induction of *S.Tm*^{WT} specific antibodies (Fig. 5B) and prevented weight loss (Fig. 5C). EvoTrap
264 vaccination+*Ec*⁸¹⁷⁸ resulted in significantly lower levels of *S.Tm*^{WT} in feces and cecum (Fig. 5D and E)
265 and completely prevented intestinal inflammation (Fig. 5F-H). *S.Tm*^{aroA} vaccination, in contrast, showed
266 no effect on fecal or cecal *S.Tm* loads (Fig. 5D and E) and only a very small suppression of intestinal
267 inflammation (Fig. 5F-H). Interestingly, although EvoTrap vaccination+*Ec*⁸¹⁷⁸ showed better protection
268 at the level of the gut and mesenteric lymph nodes (Fig. 5D, E and I), the classical *S.Tm*^{aroA} vaccination
269 provided better protection from systemic *S.Tm*^{WT} infection. Liver and spleen CFU of *S.Tm*^{WT} were not
270 dramatically decreased in this experiment by EvoTrap vaccination+*Ec*⁸¹⁷⁸ but were strongly reduced by
271 vaccination with live *S.Tm*^{aroA} (Fig. 5J and K). Interestingly, although *S.Tm*^{aroA} was absent from cecum
272 and feces it could still be found in MLNs and liver at day 46 post-vaccination (Fig. 5I-K), consistent with
273 known safety challenges associated with these live-attenuated vaccines⁶.

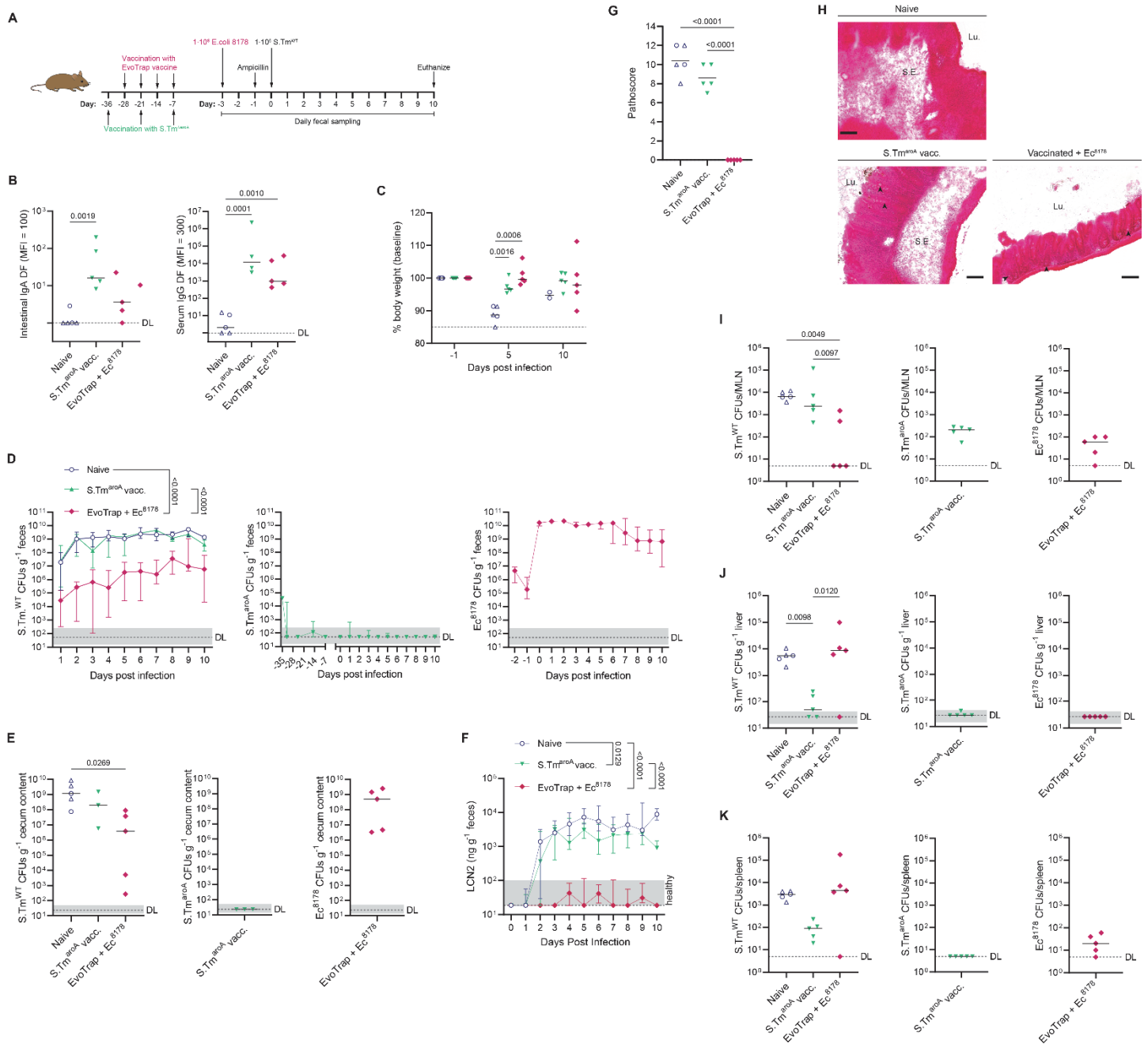


Figure 5. EvoTrap vaccination together with niche competition provides better intestinal protection than a licensed animal vaccine in the murine NTS model. PBS (blue circles), EvoTrap-vaccinated (pink diamonds) or *S.Tm^{aroA}* vaccinated (green triangles) 129S6/SvEv mice were pretreated with ampicillin and infected with $1 \cdot 10^6$ *S.Tm^{WT}*. EvoTrap-vaccinated mice were pre-colonized with $1 \cdot 10^9$ *E. coli* 8178 3 days before infection. **(A)** Experimental procedure. **(B)** *S.Tm^{WT}*-specific intestinal IgA and serum IgG titres as determined by flow cytometry. **(C)** Change in body weight over the course of infection. Solid lines show the mean. Fecal **(D)** and cecum content **(E)** CFUs as determined by selective plating. Intestinal inflammation as determined by fecal lipocalin-2 **(F)** and histopathological scoring of cecal DL tissue sections **(G)**. Solid lines in **(G)** show the mean. **(H)** Representative images of H&E-stained cecal tissue sections. Arrowheads show exemplary goblet cells. Scale bars: 100 μ m. **(I-K)** CFUs in MLN **(I)**, liver **(J)** and spleen **(K)**.

N = 5 mice/group. Solid lines depict the median unless stated otherwise, error bars the interquartile range. Dotted lines show the detection limit and the shaded area the range for cases in which the detection limit is dependent on sample weight. Open triangles show mice that had to be euthanized prematurely due to excessive weight loss (\geq

15%) or disease symptoms. Statistics were performed by mixed-effects analysis (C) or one-way ANOVA (C, G) on log-normalized data (B, E, I-K) or area under the curve (AUC) (D, F). CFU, colony forming unit; DF, dilution factor; LCN2, lipocalin-2; Lu., lumen; MFI, median fluorescence intensity; MLN, mesenteric lymph node; S.E., submucosal edema.

275 In the murine typhoid model (**Fig. 6A**), both vaccine regimens could prevent intestinal colonization
276 (**Fig. 6B and C**) and suppress any detectable increase in intestinal lipocalin-2 levels (**Fig. 6D**). MLN
277 colonization (**Fig. 6E**) correlated well with intestinal colonization levels at day 10 post infection. As in
278 the murine NTS model, vaccination with *S.Tm^{aroA}* resulted in superior protection against infection of
279 liver and spleen (**Fig. 6F and G**). However, persistent *S.Tm^{aroA}* was again found in systemic sites a full
280 17 days after the last vaccination.

281 These data imply that protection of the gut tissue and of systemic sites require different mechanisms
282 and can be uncoupled: vaccination/niche competition provides superior protection of all examined gut
283 sites and gut-draining lymphoid tissues, while live-attenuated vaccines provide better protection of
284 deep systemic sites. This therefore reveals both a promising approach for improved safe prophylaxis
285 of intestinal bacterial infections and a system to investigate missing stimuli from whole-cell inactivated
286 oral vaccines to improve their protection of systemic sites.

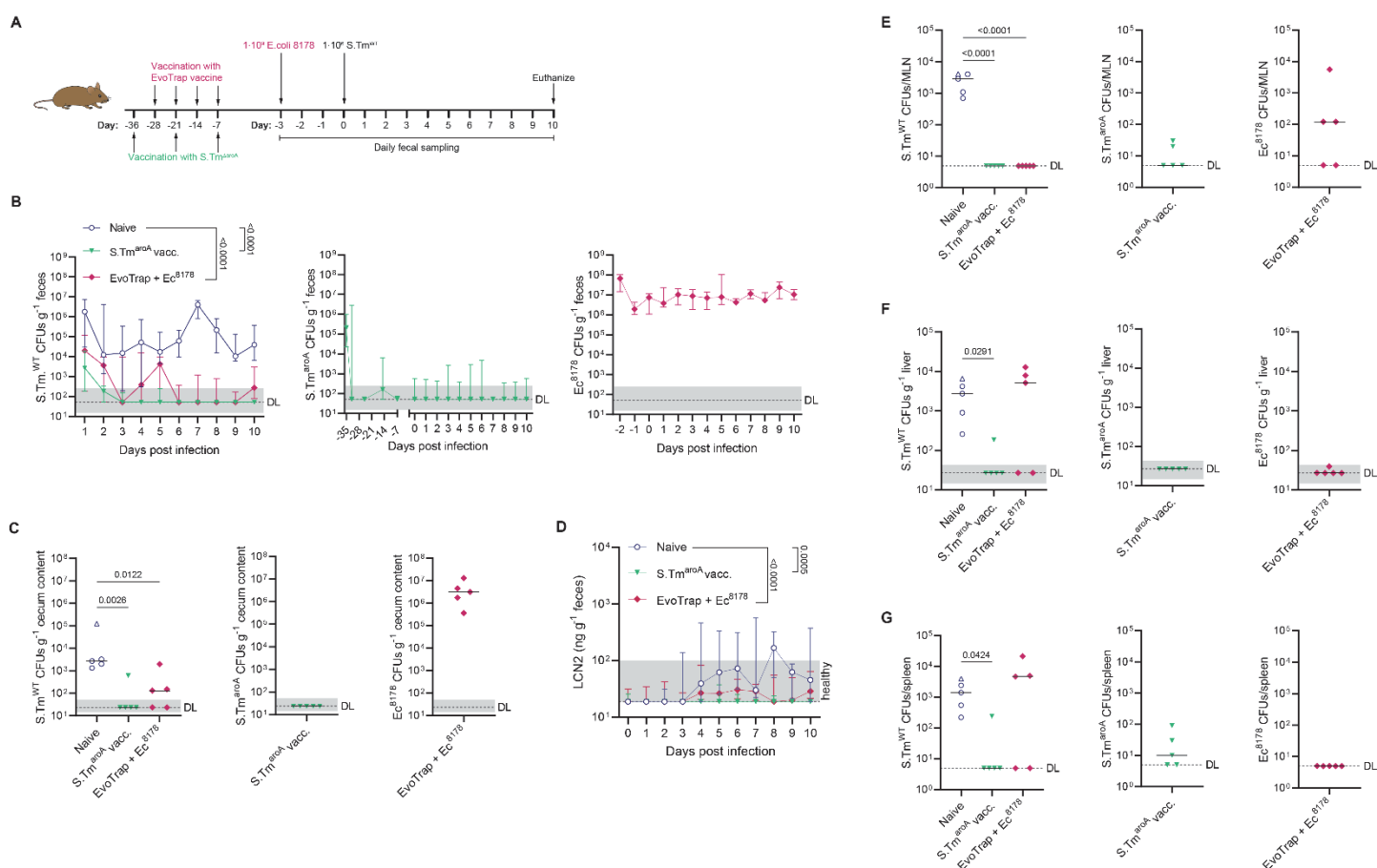


Figure 6. *S. Tm^{aroA}* vaccination protects better against systemic invasion of *S. Tm^{WT}* in the murine Thyphi model. PBS (blue circles), EvoTrap-vaccinated (pink diamonds) or *S. Tm^{aroA}* vaccinated (green triangles) 129S6/SvEv mice were infected with 1·10⁶ *S. Tm^{WT}* without antibiotic pretreatment. EvoTrap-vaccinated mice were pre-colonized with 1·10⁹ *E. coli* 8178 3 days before infection. **(A)** Experimental procedure. Fecal **(B)** and cecum content **(C)** CFUs as determined by selective plating. **(D)** Intestinal inflammation as determined by fecal lipocalin-2. **(E-G)** CFUs in MLN **(E)**, liver **(F)** and spleen **(G)**. N = 5 mice/group. Solid lines depict the median, error bars the interquartile range. Dotted lines show the detection limit and the shaded area the range for cases in which the detection limit is dependent on sample weight. Open triangles show mice that had to be euthanized prematurely due to excessive weight loss ($\geq 15\%$) or disease symptoms. Statistics were performed by ANOVA on log-normalized data (C, E-G) or area under the curve (AUC) (B, D). CFU, colony forming unit; LCN2, lipocalin-2; MLN, mesenteric lymph node.

288 Discussion

289 Previous work from our group showed the importance of IgA in pathogen immune exclusion and
290 provided a mechanistic explanation for this phenotype based on IgA-driven enchained growth¹⁶.
291 However, the extent of tissue protection that can be provided by IgA alone is typically too weak to
292 completely protect intestinal tissues. A further feature of secretory IgA is its ability to increase the
293 clearance rate of bacteria from the gut lumen¹⁶, but in the case of a large open niche this function
294 affects evolutionary dynamics without affecting total population size. In contrast, if a niche competitor
295 is present and either replicates faster, or is cleared more slowly, or ideally both, this competitor has a
296 fitness advantage and can rapidly take over the niche. Exploiting IgA to increase the clearance rate of
297 the pathogen allows fast and efficient niche competition, generating high-efficacy prophylaxis with a
298 very safe inactivated whole-cell oral vaccine.

299 This observation may explain some controversies in the existing *Salmonella* literature. For example,
300 the extent of protection from NTS obtained with live-attenuated vaccines in different laboratories
301 varies extensively³³. Our data indicate that the efficacy of *Salmonella* niche competitors in the
302 microbiota of mice, which likely varies in SPF facilities around the world, is a critical determinant of
303 protective efficacy. Moreover, this is generally in line with the concept of colonization resistance³⁴: the
304 microbiome itself provides extensive protection from *Salmonella* gut colonization, in part due to direct
305 competition for metabolic resources. It makes complete sense that intestinal antibody responses have
306 evolved to work with this protective effect of the microbiota.

307 A major question remaining is how to ideally select niche competitors that are genuinely benign but
308 also highly effective. Niche competition will occur most extensively when the environmental and
309 metabolic requirements of the competitor strain are as close as possible to the pathogen. Using
310 modified versions of the pathogen itself provides the ideal candidate as by definition this must
311 compete for an identical niche. However, genetically modified pathogens may be a very hard sell as
312 “probiotics”. An alternative would be to make use of commensal bacterial isolates that are rigorously
313 checked for potential pathogenicity. *E. coli* strains, such as Nissle 1917 are of course attractive
314 propositions for this both due to proven safety³⁵ and relatively short genetic distances from the
315 pathogen. However, non-typhoidal *Salmonella* are metabolic generalists, and it remains possible that
316 there is no one ideal competitor for all microbiota perturbations. Using a bacterial consortium instead
317 of only one strain as competitor might be able to circumvent this problem by using bacterial strains
318 that complement each other. A mix of three *E. coli* strains has shown increased protection against *S.Tm*
319 infection after dietary perturbation when compared to only one of those *E. coli* strains²⁹. We clearly
320 demonstrate that a murine commensal *E. coli* 8178 is less effective in clearing *Salmonella* from the gut
321 lumen than a *Salmonella*-derived competitor. Nevertheless, this is sufficient to prevent intestinal

322 inflammation over a long course of infection. An adaption in the administration of *E. coli* 8178 to a
323 more realistic setting i.e. giving it without prior antibiotic pre-treatment, led to a dramatic decrease in
324 protection of systemic sites from *S.Tm*^{WT}. We can currently only speculate regarding the underlying
325 mechanism of protection by high-density *E. coli* colonization. As *E. coli* overgrowth is generally not
326 considered to be part of a healthy microbiota, this is not promising for translation. However, we have
327 a few clues: We do not assume that this is due to direct competition in these sites as we could not
328 detect *E. coli* 8178 in spleen or liver of vaccinated mice in case of antibiotic pre-treatment. An
329 alternative would be *E. coli*-induced changes in intestinal innate immune signalling³⁶ with either direct
330 effects on *Salmonella* invasion, or indirect effects via changes in abundance of factors such as bile
331 acids³⁷ or short-chain fatty acids^{38,39} that repress major virulence factor expression. Another concern
332 is potential immunogenicity of the inoculated niche competitors over time, which could decrease their
333 efficacy or lead to clearance before they are needed, as well as waning of the intestinal IgA response
334 over time. Novel techniques to identify robust niche competitor strains will be needed in order to
335 translate the approach into human use, as well as further studies on long-term robustness of
336 protection. Within veterinary applications, we would hope that sterilizing-immunity could be
337 generated acutely across whole farms resulting in permanent de-colonization of the herds, and
338 potentially circumventing any potential problems with response longevity.

339 Due to improved efficacy, the field of non-typhoidal *Salmonella* vaccine research has been strongly
340 focused on live-attenuated vaccines. *S.Tm* vaccines are licensed only for the veterinary sector and both
341 the poultry and swine vaccine are live, auxotrophic *S.Tm* strains^{30,31}. These vaccine strains were
342 originally tested in mice and showed protection against subsequent lethal infection with wildtype
343 *S.Tm*⁷. Although *S.Tm*^{aroA} strains were stated to be replication incompetent and avirulent, later studies
344 with these mutants showed repeatedly higher bacterial counts than the applied infection doses, long-
345 lasting persistence in liver and spleen and severe splenomegaly^{40,41}. Continuous shedding of a *S.Tm*
346 *aroA ssaV* double mutant strain was also observed in a small study with healthy human volunteers for
347 up to 23 days⁶ and a *S.Tm phoP/phoQ* deletion strain although in this study they treated volunteers
348 still shedding the vaccine strain with antibiotics from day nine onwards⁴². Moreover, while
349 immunocompetent mice do survive infection with *S.Tm*^{aroA}, mice lacking CD4+ T cells or IFN γ succumb
350 to this type of vaccination^{43,44}. However, individuals with immunodeficiencies or a not yet fully
351 developed immune system are the demographic group most requiring effective *Salmonella*
352 prophylaxis, and it is therefore vital to develop a vaccine that is also safe to use in high-risk individuals.

353 Because of this we have directly compared protective efficacy of the *aroA* live-attenuated vaccine and
354 the combination of an “Evolutionary Trap” version of the inactivated whole-cell oral vaccine with an
355 *E. coli* niche competitor. Our data clearly demonstrate a much stronger protection of the intestinal
356 environment by the inactivated vaccine/niche competitor approach, indicating that combined

357 secretory IgA and competition robustly prevent invasion of intestinal tissues and intestinal
358 inflammation. In contrast, the live-attenuated vaccines provided superior protection of systemic sites.
359 As both vaccines induce broad and robust IgG responses, we could exclude this mechanism as the
360 grounds for protection. Instead, there are two potential explanations: 1) the live-attenuated vaccine
361 permanently colonizes systemic sites, generating low-grade inflammation and niche competition in
362 these environment (i.e. protection independent of adaptive immunity) or 2) live-attenuated vaccines
363 more efficiently induce cell-mediated immunity which can control the intracellular stages of
364 *Salmonella* infection. We cannot currently distinguish between these phenomena based on our data,
365 but note that CFU of live *S.Tm^{aroA}* could be detected in the spleen and liver of vaccinated mice more
366 than two weeks after the final vaccination which would be consistent with explanation (1).
367 Nevertheless, live-attenuated vaccines are also expected to induce stronger systemic T cell responses
368 than inactivated bacterial cells⁴⁵. Overall, our conclusion is that vaccination/niche competition is a
369 promising approach to prevent intestinal disease and its transmission using a very safe vaccine. There
370 is still room for improvement in preventing *Salmonella* invasion and replication at systemic sites.
371 However, it possible that this strategy can achieve full protection in more realistic infection scenarios
372 with less microbiota disruption, reduced niche sizes for pathogen blooms and lower inoculum sizes.

373 Overall, this work reveals the ability of rationally designed oral vaccines combined with carefully
374 selected niche competitors to prevent or suppress intestinal colonization by a pathogen. Even in the
375 very stringent non-typhoidal Salmonellosis model, this strategy is capable of generating a type of
376 “sterilizing immunity”, i.e. complete clearance of the pathogen, in the gut lumen within one week of
377 challenge. This is also highly suggestive of a role of secretory IgA in controlling competition between
378 intestinal microbiota members, which is consistent with observations of instability of *E. coli* at the
379 strain level, but not the species level, over time in healthy volunteers⁴⁶. Therefore, this approach also
380 has potential applications in microbiota engineering where strain replacement could either eliminate
381 a detrimental strain or could introduce a strain with beneficial properties into an otherwise complete
382 microbiota.

383 **Materials and Methods**

384 All materials are available upon request to the corresponding authors.

385

386 **Strains and plasmids**

387 All strains and plasmids used in this study are listed **Table S1**.

388 Bacteria were cultivated in lysogeny broth (LB) containing appropriate antibiotics (100 µg/ml
389 streptomycin (AppliChem); 15 µg/ml chloramphenicol (AppliChem); 50 µg/ml kanamycin (AppliChem);
390 50 µg/ml ampicillin (AppliChem)). Dilutions were prepared in Phosphate Buffered Saline (PBS, Difco).

391 Gene-deletion mutants were created by generalized transduction with bacteriophage P22 HT105/1
392 *int-201* as described in⁴⁷. When needed, antibiotic resistance cassettes were removed using the
393 temperature-inducible FLP recombinase encoded on pCP20⁴⁸. Deletions originated from in-frame
394 deletions made in S.Tm 14028S, kind gifts from Prof. Michael McClelland (University of California,
395 Irvine). Primers used for verifications of gene deletions or genetic background are listed **Table S2**.

396 Plasmids were transferred by electro-transformation into competent cells^{29,49}.

397

398 **Mice**

399 All animal experiments were performed in accordance with Swiss Federal regulations approved by the
400 Commission for Animal Experimentation of the Kanton Zurich (licenses 193/2016, 158/2019 and
401 120/2019; Kantonales Veterinäräm t Zürich, Switzerland). Specific opportunistic pathogen-free (SPF,
402 containing a complete microbiota free of an extended list of opportunistic pathogens) 129S6/SvEvTac
403 WT mice were used in all experiments except for Fig. S1 A-F, where C57BL/6J WT mice were used. Mice
404 were bred and housed in individually ventilated cages with a 12 h light/dark cycle in the ETH Phenomics
405 Center (EPIC, RCHCI), ETH Zürich and were fed a standard chow diet. All mice included in experiments
406 were 4 weeks or older and objectively healthy as determined by routine health checks. Wherever
407 possible an equal number of males and females was used in each group. Mice were allocated cage-
408 wise to groups with a minimum of two cages per group. As strong phenotypes were expected, we
409 adhered to standard practice of analysing at least five mice per group. Researchers were not blinded
410 to group allocation to decrease the risk of contamination and because the majority of readouts were
411 quantitative and not subjective (CFU determination, ELISA). The one exception to this was
412 histopathology scoring, for which a blinded researcher (SAF) not otherwise involved in the experiment
413 carried out all scoring.

414

415 **Vaccinations**

416 Mice were either vaccinated with peracetic acid (PA) killed vaccines or live-attenuated *S.Tm*^{aroA}.

417 Peracetic acid killed vaccines were produced as previously described⁵⁰. Briefly, bacteria were grown
418 overnight to late stationary phase, harvested by centrifugation and re-suspended to a density of
419 10^9 - 10^{10} per ml in sterile PBS. Peracetic acid (Sigma-Aldrich) was added to a final concentration of
420 0.4% v/v. The suspension was mixed thoroughly and incubated for 60 min at room temperature.
421 Bacteria were washed three times in 50-100 ml sterile PBS. The final pellet was resuspended to yield a
422 density of 10^{11} - 10^{12} particles per ml in sterile PBS. The exact number was determined by flow cytometry
423 with counting beads (Fluoresbrite® Multifluorescent Microspheres). Vaccines were stored at 4 °C for
424 up to three weeks. Each batch of vaccine was tested for sterility before use. Vaccine lots were released
425 for use only when a negative enrichment culture had been confirmed. Mice were vaccinated with
426 10^{10} - 10^{11} PA-killed bacteria by oral gavage, once weekly for 4 weeks. Where multiple strains were used,
427 equal numbers of each strain were given.

428 Live-attenuated *S.Tm*^{aroA} was grown overnight in LB containing chloramphenicol. The cells were
429 washed in PBD and resuspended at a density of 10^{10} bacteria per ml. Mice were orally vaccinated with
430 10^9 *S.Tm*^{aroA} in 100 μ l three times in bi-weekly intervals without antibiotic treatment.

431 Vaccinations were started in mice at an age of 4-6 weeks.

432

433 **Colonization with the bacterial niche competitor and *Salmonella* challenge infections**

434 The competitor strain was grown overnight in LB containing the appropriate antibiotics. In the
435 morning, the bacteria were washed with sterile PBS and diluted. The competitor was introduced by
436 oral gavage into the respective groups either at $5 \cdot 10^3$ CFUs after antibiotic pre-treatment or at
437 $1 \cdot 10^9$ CFUs without antibiotic pre-treatment of the animals.

438 Non-typhoidal *Salmonella* infections were carried out as previously described²¹. In brief, mice were
439 orally pretreated 24 h before infection with 25 mg streptomycin or 20 mg ampicillin. Strains were
440 cultivated overnight separately in LB containing the appropriate antibiotics. Subcultures were
441 prepared before infections by diluting overnight cultures 1:20 in fresh LB without antibiotics and
442 incubation for 3 h at 37 °C. The cells were washed in PBS, diluted, and 100 μ l of bacteria were used to
443 infect mice by orogastric gavage with either $5 \cdot 10^3$ or $1 \cdot 10^6$ *S.Tm* CFUs, as indicated in the respective
444 figure legends/text. Competitions were performed by inoculating 1:1 mixtures of each competitor

445 strain. For mouse typhoid-like infection, the animals were infected with $1 \cdot 10^6$ S.Tm CFUs without prior
446 antibiotic treatment. A detailed layout of the vaccination and infection schedule is shown in the figures.

447 Feces were sampled daily, homogenized in 500 μ l PBS by bead beating (3 mm steel ball, 25 Hz for
448 2.5 min in a TissueLyser (Qiagen)), and large particles were sedimented by centrifugation at 500x *g* for
449 1 minute. Bacteria were enumerated by selective plating on MacConkey agar supplemented with the
450 appropriate antibiotics. Fecal samples for lipocalin-2 measurements were kept homogenized in PBS at
451 -20 °C. At endpoint, blood was collected from the heart into 1.1 ml serum gel tubes (Sarstedt).
452 Intestinal lavages were harvested by flushing the small intestinal content with 2 ml of PBS using a
453 cannula. The middle part of the cecum was placed into OCT Compound (Tissue-Tek), snap-frozen and
454 stored at -80 °C until analysis. Spleen, liver, mesenteric lymph nodes were collected and homogenized
455 in 1 ml PBS at 30 Hz for 3 min. Cecum content was collected and homogenized in 500 μ l PBS at 25 Hz
456 for 2.5 min. After centrifugation at 500x *g* for 1 minute, bacteria were plated on selective MacConkey
457 agar.

458

459 **Quantification of fecal lipocalin-2**

460 Fecal pellets were processed as described above. Homogenized feces was centrifuged at 16000x *g* for
461 5 min and the resulting supernatant was analysed in duplicates using the mouse lipocalin-2 ELISA
462 duoset (R&D, DY1857) according to the manufacturer's instructions.

463

464 **Analysis of specific antibody titres by bacterial flow cytometry**

465 Specific antibody titres in mouse intestinal washes and serum were measured by flow cytometry as
466 described⁵¹. Briefly, intestinal washes and blood were collected as described above. Blood was
467 centrifuged at 10000x *g* for 5 min to obtain serum, heat-inactivated at 56 °C for 30 min and stored
468 at -20 °C until further analysis. Intestinal lavage was centrifuged at 16000x *g* for 5 min to clear all
469 bacterial-sized particles and stored at -20 °C until analysis. Bacterial targets (antigen against which
470 antibodies are to be titred) were grown overnight in LB, then gently pelleted for 2 min at 7000x *g*. The
471 pellet was washed with 0.2 μ m-filtered PBS before resuspending at a density of approximately 10^7
472 bacteria per ml. After thawing, intestinal washes were centrifuged again at 16000x *g* for 5 min.
473 Supernatants were used to perform serial dilutions. 50 μ l of the dilutions were incubated with 50 μ l
474 bacterial suspension for 15 min at room temperature. Bacteria were washed twice with 150 μ l PBS by
475 centrifugation at 7000x *g* for 5 min, before resuspending in 25 μ l of 0.2 μ m-filtered PBS containing
476 polyclonal Alexa Fluor 647 Rabbit Anti-Mouse IgG (Jackson ImmunoResearch, 15 μ g/ml, 315-605-003,
477 AB_2340239) or monoclonal Brilliant Violet 421 Rat Anti-Mouse IgA (BD Bioscience, 2 μ g/ml, 743293,

478 AB_2741405). After 5 min of incubation at RT, bacteria were washed twice with PBS as above and
479 resuspended in 100 μ l PBS for acquisition on a Beckman Coulter Cytoflex S using FSC and SSC
480 parameters to threshold acquisition in logarithmic mode. Data were analysed using FlowJo (Treestar).
481 After gating on bacterial particles, log-median fluorescence intensities (MFI) were plotted against
482 lavage dilution factor for each sample and 4-parameter logistic curves were fitted using Prism
483 (Graphpad, USA). Titers were calculated from these curves as the dilution factor giving an above-
484 background signal (typically MFI=300).

485

486 **Histological procedures**

487 Tissue embedded in OCT Compound was cut into 5 μ m cryosections and mounted on glass slides.
488 Cryosections were air dried overnight at room temperature and stained with hematoxylin and eosin
489 (H&E). Scoring of cecal inflammation was done in a blinded manner assessing the following four criteria
490 as previously described²¹.

491 (i) Submucosal edema. Submucosal edema was scored as follows: 0 = no pathological changes;
492 1 = mild edema (the submucosa accounts for <50% of the diameter of the entire intestinal
493 wall - tunica muscularis to epithelium); 2 = moderate edema (the submucosa accounts for 50
494 to 80% of the diameter of the entire intestinal wall); and 3 = profound edema (the submucosa
495 accounts for >80% of the diameter of the entire intestinal wall).

496 (ii) PMN infiltration into the lamina propria. Polymorphonuclear granulocytes (PMN) in the lamina
497 propria were enumerated in 10 high-power fields (x400 magnification; field diameter of
498 420 μ m), and the average number of PMN/high-power field was calculated. The scores were
499 defined as follows: 0 = <5 PMN/high-power field; 1 = 5 to 20 PMN/high-power field; 2 = 21
500 to 60/high-power field; 3 = 61 to 100/high-power field; and 4 = >100/high-power field.
501 Transmigration of PMN into the intestinal lumen was consistently observed when the number
502 of PMN was >60 PMN/high-power field.

503 (iii) Goblet cells. The average number of goblet cells per high-power field (magnification, x400)
504 was determined from 10 different regions of the cecal epithelium. Scoring was as follows:
505 0 = >28 goblet cells/high-power field; 1 = 11 to 28 goblet cells/high-power field; 2 = 1 to 10
506 goblet cells/high-power field; and 3 = <1 goblet cell/high-power field.

507 (iv) Epithelial integrity. Epithelial integrity was scored as follows: 0 = no pathological changes
508 detectable in 10 high-power fields (x400 magnification); 1 = epithelial desquamation;
509 2 = erosion of the epithelial surface (gaps of 1 to 10 epithelial cells/lesion); and 3 = epithelial
510 ulceration (gaps of >10 epithelial cells/lesion; at this stage, there is generally granulation tissue
511 below the epithelium). The combined pathological score for each tissue sample was

512 determined as the sum of these scores. It ranges between 0 and 13 arbitrary units and covers
513 the following levels of inflammation: 0 = intestine intact without any signs of inflammation; 1
514 to 2 = minimal signs of inflammation (this was frequently found in the ceca of SPF mice; this
515 level of inflammation is generally not considered as a sign of disease); 3 to 4 = slight
516 inflammation; 5 to 8 = moderate inflammation; and 9 to 13 = profound inflammation.

517

518 **Statistical analysis**

519 Sample size was determined based on previous experiments²⁰. Where large effect sizes were
520 expected a minimum of five mice/group were used. Researchers were not blinded for the assignment
521 of the experiments and the data analysis except for histopathological scoring. Where errors are
522 expected to be log-normal distributed (e.g., CFU determination or ELISA data based on serial dilutions),
523 all statistical tests were carried out on log normalized data. Where two groups of data were compared,
524 analysis was carried out using unpaired two-tailed t-test. One-way ANOVA followed by Tukey's Test
525 was used for comparison of three or more groups. Statistical analysis on time course data was either
526 done by mixed-effects analysis or by calculating the area under the curve (AUC) of the log normalized
527 data and then assessing differences in AUC with one-way ANOVA and Tukey's test. Statistical analysis
528 was performed with Graphpad Prism Version 9.2.0 for Windows (GraphPad Software, La Jolla,
529 California USA). P values of less than 0.05 were reported.

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545

546 **Author contributions**

547 Verena Lentsch, Conceptualization, Investigation, Methodology, Validation, Data Curation, Project
548 administration, Formal Analysis, Visualization, Supervision, Writing – original draft, Writing – review
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555 Writing – review and editing; Emma Slack, Conceptualization, Funding acquisition, Project
556 administration, Resources, Supervision, Writing – original draft, Writing – review and editing.

557

558 **Conflict of interest**

559 The authors declare no conflict of interest.

560 **Supplementary Materials and Methods**

561 **Estimation of *in vivo* growth rates in the gut by plasmid dilution**

562 Absolute *S.Tm* growth rates in the gut were assessed using replication-incompetent plasmid pAM34,
563 which has been described previously^{29,49}. Briefly, pAM34 is a ColE1-like vector in which the replication
564 of the plasmid is under the control of the LacI repressor, whereby plasmid replication only occurs in
565 the presence of isopropyl β -D-thiogalactopyranoside (IPTG). *S.Tm* carrying the pAM34 plasmid was
566 therefore cultured overnight in the presence of 1 mM IPTG in LB containing streptomycin. Cultures
567 were diluted 1:20 into fresh LB broth without IPTG or antibiotics and sub-cultured for 3 h at 37 °C.
568 Inocula for infection were prepared as described above. Concurrently, the inoculum was serially
569 diluted into fresh LB broth without IPTG and cultured for 20 h at 37 °C to generate a standard curve
570 relating plasmid loss to generations undergone for each experiment. pAM34-carrying bacteria within
571 the overnight cultures and the feces were determined by selective plating on agar plates containing
572 50 μ g/ml ampicillin and 1 mM IPTG. To quantify the total population size, samples were further plated
573 on agar plates containing 100 μ g/ml streptomycin. The fraction of pAM34-carrying bacteria was
574 calculated using the ratio of pAM34-carrying CFU to the total population CFU and generations
575 estimated by interpolation from the matched standard curve.

576

577 **Non-typhoidal *Salmonella* transmission**

578 Donor mice were vaccinated with PA-*S.Tm* as described above, orally pretreated with 25 mg
579 streptomycin, and colonized 24 h later with $5 \cdot 10^3$ *S. Tm*^{Comp}. 2 days later, mice were treated again with
580 25 mg Streptomycin by orogastric gavage, and 24 h later infected with $1 \cdot 10^6$ *S.Tm*^{WT}. On day 9 post
581 infection, one fecal pellet was collected from each mouse, weighed, and homogenized in 200 μ l PBS.
582 Large debris was removed by centrifugation at 500x *g* for 1 minute and 50 μ l of the supernatant were
583 immediately given by oral gavage to streptomycin pretreated naïve recipient mice. As a control, the
584 same procedure was done using naïve mice without competitor colonization as donor mice. Recipient
585 mice were euthanized, and organs were collected on day 3 post transmission. In both donor and
586 recipient mice, fecal pellets were collected daily and selective plating was used to enumerate
587 *Salmonella* and determine the relative proportions of both competing bacterial strains.

588

589 **Flow cytometry for analysis of O:5 and O:12-0 intensity on *Salmonella* clonal cultures**

590 Overnight cultures (1 μ l) made in 0.2 μ m-filtered lysogeny broth was stained with 0.2 μ m-filtered
591 solutions of STA5 (human recombinant monoclonal IgG2 anti-O:12-0, 3.2 μ g/ml)¹⁶ or rabbit anti-

592 Salmonella O:5 (Difco, 1:200, 226601). After incubation at 4 °C for 30 min, the bacteria were washed
593 twice by centrifugation at 7000x *g* and resuspension in PBS/2% BSA. The bacteria were then
594 resuspended in 0.2 µm-filtered solutions of appropriate secondary reagents (Alexa 647-anti-human
595 IgG (Jackson ImmunoResearch, 1:100, 109-605-098, AB_2337889) and Brilliant Violet 421-anti-rabbit
596 IgG (BioLegend, 1:100, 406410, AB_10897810)). This was incubated for 30 min at 4 °C before the cells
597 were washed as above and resuspended for acquisition on a Beckman Coulter Cytoflex S.

598

599 LPS purification and silver staining

600 LPS was isolated by applying the hot phenol–water method⁵², followed by buffer exchange against
601 15 ml PBS and concentration in 500 µl PBS. LPS samples were separated on a 13% Tricin gel by gel
602 electrophoresis and silver staining was performed as described previously⁵³.

603

604 Mathematical modeling of *S.Tm*^{WT} extinction in the gut

605 All mathematical modelling is based on the arithmetic mean of the experimental data.

606 For Eq. 1 we consider a case where $K_2 \ll K_1$ because the height of final *S.Tm*^{Comp} “plateau” observed in
607 competition data (which is proportional to K_2 , see calculations below) is much lower than the size of
608 *S.Tm*^{Comp} population at day 1 (which is proportional to K_1 , see calculations below). We successively
609 consider the cases $K_1 \ll K_3$ and $K_3 \ll K_1$ and show that the latter is more compatible with the
610 competition data. In the competition data (vaccinated group with *S.Tm*^{Comp}), several distinct regimes
611 can be observed (**Fig. S6**), which is also highlighted below with the model.

612

613 Case where $K_3 \gg K_1$

614

615 Regime R_1 with $W, M, C \ll K_1$

616 In this case, the equations become

$$\left\{ \begin{array}{l} \frac{dW}{dt} = W \cdot (r_w - c_w) \\ \frac{dC}{dt} = C \cdot (r_c - c_c) \\ \frac{dM}{dt} = M \cdot (r_m - c_m) \end{array} \right.$$

617 Thus, we have

$$\left\{ \begin{array}{l} W(t) \sim \exp(t \cdot (r_w - c_w)) \\ C(t) \sim \exp(t \cdot (r_c - c_c)) \\ M(t) \sim \exp(t \cdot (r_m - c_m)) \end{array} \right.$$

618 Regime R_2 with $W, M \ll C \sim \Theta(K_1)$

619 In this case, the equations become

$$\left\{ \begin{array}{l} \frac{dW}{dt} = W \cdot \left(r_w \cdot \left(1 - \frac{C}{K_1} \right) - c_w \right) \\ \frac{dC}{dt} = C \cdot \left(r_c \cdot \left(1 - \frac{C}{K_1} \right) - c_c \right) \\ \frac{dM}{dt} = M \cdot \left(r_m \cdot \left(1 - \frac{1}{\frac{K_1}{C} + \frac{K_3}{M}} \right) - c_m \right) \end{array} \right.$$

620 Note: in this regime, $\frac{K_3}{M} \gg \frac{K_1}{C}$. Thus, we have

$$\left\{ \begin{array}{l} W(t) \sim \exp\left(t \cdot \left(r_w \cdot \frac{c_c}{r_c} - c_w \right)\right) \\ C(t) \sim K_1 \cdot \left(1 - \frac{c_c}{r_c} \right) \\ M(t) \sim \exp(t \cdot (r_m - c_m)) \end{array} \right.$$

621 Note that we assume C is the first to be close to the carrying capacity K_1 . Next, at $t = \tau$, we have

622 $M \sim C$, that is

$$K_1 \cdot \left(1 - \frac{c_c}{r_c} \right) \sim m_0 \cdot \exp(t \cdot (r_m - c_m))$$

623 Regime R_3 with $C, W \ll M$ and $C \gg K_2$

624 In this regime, we have $\frac{K_1}{M+W+C} + \frac{K_2}{C} \sim \frac{K_1}{M} + \frac{K_2}{C} \ll 1$. This implies that there are no nutrients

625 available to C and W. In this case, the equations become

$$\left\{ \begin{array}{l} \frac{dW}{dt} = W \cdot (-c_w) \\ \frac{dC}{dt} = C \cdot (-c_c) \\ \frac{dM}{dt} = M \cdot \left(r_m \cdot \left(1 - \frac{M}{K_3} \right) - c_m \right) \end{array} \right.$$

626 Thus, we have

$$\left\{ \begin{array}{l} W(t) \sim \exp(-t \cdot (c_w)) \\ C(t) \sim \exp(-t \cdot (c_c)) \\ M(t) \sim K_3 \cdot \left(1 - \frac{c_m}{r_m} \right) \cdot \left(1 + \left(\frac{K_3}{m_0} \cdot \left(1 - \frac{c_m}{r_m} \right) - 1 \right) \cdot (\exp(t \cdot (r_m - c_m))) \right)^{-1} \end{array} \right.$$

627 Thus, in this regime, the slopes expected for $\log(C)$ and $\log(W)$ are $-c_c$ and $-c_w$, respectively. Thus, if
 628 we estimate the slopes of $\log(C)$ and $\log(W)$ from *in vivo* data (vaccinated group) for R_3 , we should get
 629 an estimation of the clearance rate values. Table S3 shows the slopes obtained from experiments: they
 630 range from -1.8 to -3.9, which would correspond to extremely slow clearance rates. This suggests that
 631 the case $K_1 \gg K_3$ is probably not well suited to describe the data.

Table S3: Estimation of the slopes of log (C) and log (W) from *in vivo* data (vaccinated group + S.Tm^{Comp}). The estimation was performed for different possible durations of this regime.

Width of the regime	Slope for log (C)	Slope for log (W)
day 3 to day 7	-3.4 day ⁻¹	-2.2 day ⁻¹
day 3 to day 6	-3.7 day ⁻¹	-1.8 day ⁻¹
day 4 to day 7	-3.3 day ⁻¹	-2.4 day ⁻¹
day 4 to day 6	-3.9 day ⁻¹	-1.9 day ⁻¹

632 Regime R₄ with $W, M \ll C \sim \Theta(K_2)$

633 In this case, the equations become

$$\left\{ \begin{array}{l} \frac{dW}{dt} = W \cdot \left(r_w \cdot \left(1 - \frac{C}{K_2} \right) - c_w \right) \\ \frac{dC}{dt} = C \cdot \left(r_c \cdot \left(1 - \frac{C}{K_2} \right) - c_c \right) \\ \frac{dM}{dt} = M \cdot \left(r_m \cdot \left(1 - \frac{M}{K_3} \right) - c_m \right) \end{array} \right.$$

634 Thus, at long times we have

$$\left\{ \begin{array}{l} W(t) \sim \exp \left(t \cdot \left(r_w \cdot \frac{c_c}{r_c} - c_w \right) \right) \\ C(t) \sim K_2 \cdot \left(1 - \frac{c_c}{r_c} \right) \\ M(t) \sim K_3 \cdot \left(1 - \frac{c_m}{r_m} \right) \end{array} \right.$$

635 Case where $K_1 \gg K_3$

636

637 Regime R_1 with $W, M, C \ll K_1$

638 If we also have $M \ll K_3$, the equations become

$$\left\{ \begin{array}{l} \frac{dW}{dt} = W \cdot (r_w - c_w) \\ \frac{dC}{dt} = C \cdot (r_c - c_c) \\ \frac{dM}{dt} = M \cdot (r_m - c_m) \end{array} \right.$$

639 Thus, we have

$$\left\{ \begin{array}{l} W(t) \sim \exp(t \cdot (r_w - c_w)) \\ C(t) \sim \exp(t \cdot (r_c - c_c)) \\ M(t) \sim \exp(t \cdot (r_m - c_m)) \end{array} \right.$$

640 Regime R_2 with $W, M \ll C \sim \Theta(K_1)$

641 In this case, the equations become

$$\left\{ \begin{array}{l} \frac{dW}{dt} = W \cdot \left(r_w \cdot \left(1 - \frac{1}{\frac{K_1}{C}} \right) - c_w \right) \\ \frac{dC}{dt} = C \cdot \left(r_c \cdot \left(1 - \frac{1}{\frac{K_1}{C}} \right) - c_c \right) \\ \frac{dM}{dt} = M \cdot \left(r_m \cdot \left(1 - \frac{1}{\frac{K_1}{C} + \frac{K_3}{M}} \right) - c_m \right) \end{array} \right.$$

642 Thus, we have

$$\left\{ \begin{array}{l} W(t) \sim \exp\left(t \cdot \left(r_w \cdot \frac{c_c}{r_c} - c_w\right)\right) \\ C(t) \sim K_1 \cdot \left(1 - \frac{c_c}{r_c}\right) \end{array} \right.$$

643 When M is in a regime such that $K_3 \ll M \ll K_1$, then $M(t) \sim \exp\left(t \cdot \left(r_m \cdot \frac{c_c}{r_c} - c_m\right)\right)$.

644

645 Regime R_3 with $C, W \ll M$ and $C \gg K_2$

646 In this case, the equations become

$$\left\{ \begin{array}{l} \frac{dW}{dt} = W \cdot \left(r_w \cdot \left(1 - \frac{M}{K_1}\right) - c_w\right) \\ \frac{dC}{dt} = C \cdot \left(r_c \cdot \left(1 - \frac{M}{K_1}\right) - c_c\right) \\ \frac{dM}{dt} = M \cdot \left(r_m \cdot \left(1 - \frac{M}{K_1}\right) - c_m\right) \end{array} \right.$$

647 Thus, we have

$$\left\{ \begin{array}{l} W(t) \sim \exp\left(t \cdot \left(r_w \cdot \frac{c_m}{r_m} - c_w\right)\right) \\ C(t) \sim \exp\left(t \cdot \left(r_c \cdot \frac{c_m}{r_m} - c_c\right)\right) \\ M(t) \sim (K_1) \cdot \left(1 - \frac{c_m}{r_m}\right) \end{array} \right.$$

648 Regime R_4 with $W, M \ll C \sim \Theta(K_2)$

649 In this case, the equations become

$$\left\{ \begin{array}{l} \frac{dW}{dt} = W \cdot \left(r_w \cdot \left(1 - \frac{1}{\frac{K_1}{M} + \frac{K_2}{C}} \right) - c_w \right) \\ \frac{dC}{dt} = C \cdot \left(r_c \cdot \left(1 - \frac{1}{\frac{K_1}{M} + \frac{K_2}{C}} \right) - c_c \right) \\ \frac{dM}{dt} = M \cdot \left(r_m \cdot \left(1 - \frac{M}{K_1} \right) - c_m \right) \end{array} \right.$$

650 Thus, we have

$$\left\{ \begin{array}{l} W(t) \sim \exp\left(t \cdot \left(r_w \cdot \frac{c_c}{r_c} - c_w \right)\right) \\ C(t) \sim \frac{K_2}{\left(\frac{1}{1 - \frac{c_c}{r_c}}\right) - \left(\frac{1}{1 - \frac{c_m}{r_m}}\right)} \\ M(t) \sim (K_1) \cdot \left(1 - \frac{c_m}{r_m}\right) \end{array} \right.$$

651 Kinetic parameter estimation

652 Below we describe the different methods use to estimate the value of the different kinetic parameters
 653 of the model from the competition data (see Figure 1). Table 1 recapitulates these results. These results
 654 suggest that $S.Tm^{Comp}$ has a higher replication rate than the $S.Tm^{WT}$ and that vaccination induces a
 655 higher clearance rate for $S.Tm^{WT}$ strain.

656 *Replication rates*

657 The replication rates can be estimated from pAM34 experiments (see Fig. S2). pAM34 has been
658 engineered to replicate only in the presence of IPTG and pAM34 carrying bacteria can be tracked by
659 ampicillin resistance encoded on the plasmid. Therefore, the final proportions of plasmid-carriers y_0
660 can be linked to the number of generations, g_0 :

$$\log 2(y_0) = d - (1 - \varepsilon) \cdot g_0$$

661 where 2^d and ε are related to the initial number of plasmid copies and to the residual replication rate,
662 respectively. The measurement of the *in vitro* proportion of plasmid-carriers y_0 for successive dilutions
663 enables the estimation of d and ε . Table S4 shows the estimation of d and ε values from *in vitro* data.

Table S4: Estimation of d and ε from *in vitro* data. The value in brackets for $S.Tm^{Comp}$ gives the day when $S.Tm^{Comp}$ was given as compared to $S.Tm^{WT}$.

Strain	d	ε
$S.Tm^{WT} Kan^R$	2.15	0.11
$S.Tm^{WT} Cm^R$	1.9	0.1
$S.Tm^{Comp} Cm^R$ (D0)	3.43	0.22
$S.Tm^{Comp} Cm^R$ (D-3)	3.38	0.16

664 The replication rates can then be obtained from *in vivo* competition data. Indeed, in these experiments,
665 each strain followed an exponential growth regime at short times. In this regime, we can estimate the
666 number of generations g at time t given access to the maximum replication rate r as

$$2^g = \exp(r \cdot t)$$

667 Here g was estimated from the *in vivo* proportion of plasmid-carriers at 12 h post infection and from
668 the *in vitro* estimation of d and ε . Table S5 shows the growth rate calculated for each strain for the
669 different competition conditions.

Table S5: Estimation of the growth and clearance rates from *in vivo* competition data at $t = 12$ h. For each experiment k , we have expressed the value of the growth rate r_k as $r_k = \bar{r}_k \pm \delta r_k$ with $\bar{r}_k = \frac{1}{n} \cdot \sum_{i=1}^n r_{i,k}$ and $\delta r_k = \frac{1}{n-1} \cdot \sum_{i=1}^n (r_{i,k} - \bar{r}_k)^2$. The index n (column #) corresponds to the number of mice having not out-diluted plasmids at $t = 12$ h. Initial $S.Tm^{WT}$ Kan^R/ $S.Tm^{WT}$ Cm^R CFU: 6000-7500. Initial $S.Tm^{WT}$ Kan^R/ $S.Tm^{Comp}$ Cm^R CFU: 3600.

Group	Strain	Growth rate (day ⁻¹)	# mice
Naive w/o $S.Tm^{Comp}$	$S.Tm^{WT}$ Kan ^R	34.4±2.4	3
	$S.Tm^{WT}$ Cm ^R	32±3.3	2
Naive + $S.Tm^{Comp}$	$S.Tm^{WT}$ Kan ^R	31.8±4.8	3
	$S.Tm^{Comp}$ Cm ^R	36±4.3	3
Vacc. w/o $S.Tm^{Comp}$	$S.Tm^{WT}$ Kan ^R	30.4	1
	$S.Tm^{WT}$ Cm ^R	25.9±3.6	2
Vacc. + $S.Tm^{Comp}$	$S.Tm^{WT}$ Kan ^R	33.7±3.7	3
	$S.Tm^{Comp}$ Cm ^R	38.7±0.4	3

670 Clearance rates

671 To estimate the clearance rates, we have used the fact that the size of $S.Tm^{WT}$ starts to decrease
672 exponentially after a few days. For the naive group, we can thus write

$$W_{naive}(t) \sim \exp\left(\left(r_w \cdot \frac{c_c}{r_c} - c_{w,naive}\right) \cdot t\right)$$

673 that is

$$\log(W_{naive}(t)) = \Delta r_n \cdot t + \gamma = \left(r_w \cdot \frac{c_c}{r_c} - c_{w,naive}\right) \cdot t + \gamma$$

674 This holds approximatively from day 1 to day 8 post infection, after which the bacterial number
675 plateaus. Here we suppose that the clearance rates of $S.Tm^{WT}$ and $S.Tm^{Comp}$ are similar in the naive
676 case, that is $c_{w,naive} = c_c$. The underlying hypothesis is that the clearance rates of $S.Tm^{WT}$ and $S.Tm^{Comp}$
677 are only different in the vaccinated group due to enchainned growth of $S.Tm^{WT}$. Thus, the slope

678 $\Delta r_n \left(r_w \cdot \frac{c_c}{r_c} - c_c \right)$ can be estimated from the *in vivo* CFUs. If we use the replication rates estimated
679 above and make the assumption that the clearance rate of $S.Tm^{Comp}$ and $S.Tm^{WT}$ are the same in the
680 naive case, we get the naïve clearance rate as

$$c_c = \Delta r_n \cdot \left(\frac{r_w}{r_c} - 1 \right)^{-1}$$

681 Similarly for the vaccinated group from day 1 to day 3 post infection, we can write

$$W_{vacc}(t) \sim \exp \left(\left(r_w \cdot \frac{c_c}{r_c} - c_{w,vacc} \right) \cdot t \right)$$

682 that is

$$\log(W_{vacc}(t)) = \Delta r_v \cdot t + \delta = \left(r_w \cdot \frac{c_c}{r_c} - c_c \right) \cdot t + \delta$$

683 Again Δr_v can be estimated from the data and using the value of c_c obtained from the naive case, we
684 get an estimation for the clearance rate of $S.Tm^{WT}$ in the vaccinated case:

$$c_w = r_w \cdot \frac{c_c}{r_c} - \Delta r_v$$

685 Table S6 shows the values of c_w and c_c estimated with this method.

Table S6: Clearance rate values.

r_w (day ⁻¹)	r_c (day ⁻¹)	c_w (day ⁻¹)	c_c (day ⁻¹)
33.7	38.7	6.4	5.9

686 *Carrying capacities*

687 Three carrying capacities need to be estimated. K_1 can be obtained from the following expression:

$$K_1 \sim C_{max} \cdot \left(1 - \frac{c_c}{r_c} \right)^{-1}$$

688 where C_{max} is the size of the $S.Tm^{Comp}$ population at its maximum (typically at $t = 24$ h) in the vaccinated
 689 group.

690

691 For K_2 , we have

$$K_2 \sim C_{plateau} \cdot \left(\left(\frac{1}{1 - \frac{c_c}{r_c}} \right) - \left(\frac{1}{1 - \frac{c_m}{r_m}} \right) \right)$$

692 where $C_{plateau}$ is the mean size of the $S.Tm^{Comp}$ population when it reaches a plateau around 7-10 days
 693 post infection. This estimation leads to

694 $K_1 = 5.9 \cdot 10^9$ and $K_2 = 2.3 \cdot 10^4$. The value of K_3 needs to be determined by curve fitting (see
 695 below).

696

697 *Microbiota kinetic parameters: ratio between clearance and replication rate*

698 The ratio between clearance and replication rate can be estimated as follows:

699 In the case $K_3 \ll K_1$ when the microbiota has recovered (that is $M \gg C, W$) and where $C \gg K_2$, we have

$$\left\{ \begin{array}{l} W(t) \sim \exp \left(t \cdot \left(r_w \cdot \frac{c_m}{r_m} - c_w \right) \right) \\ C(t) \sim \exp \left(t \cdot \left(r_c \cdot \frac{c_m}{r_m} - c_c \right) \right) \\ M(t) \sim K_1 \cdot \left(1 - \frac{c_m}{r_m} \right) \end{array} \right.$$

700 where r_m and c_m are the replication rate and the clearance rate of the microbiota, respectively.

701

702 We thus have

$$\log(C) = \alpha_c \cdot t + \gamma = \left(r_c \cdot \frac{c_m}{r_m} - c_c \right) \cdot t + \gamma$$

$$\log(W) = \alpha_w \cdot t + \gamma = \left(r_w \cdot \frac{c_m}{r_m} - c_w \right) \cdot t + \gamma$$

703 where the value of the slopes α_c and α_w can be estimated experimentally and gives access to the ratio
704 between clearance and replication rate of the microbiota, $\frac{c_m}{r_m}$. The ratio was calculated as

$$\frac{c_m}{r_m} = 0.5 \cdot \left((\alpha_c + c_c) \cdot \left(\frac{1}{r_c} \right) + (\alpha_w + c_w) \cdot \left(\frac{1}{r_w} \right) \right)$$

705 *Data fitting*

706 Three kinetic parameter values still need to be estimated: the size of the microbiota at S.Tm infection,
707 m_0 , the microbiota replication, r_m (its clearance rate is then directly obtained from the ratio estimated
708 above) and the value of the carrying capacity K_3 .

709 These parameters were estimated by fitting the model to the S.Tm^{WT} and S.Tm^{Comp} data (vaccinated
710 group) with a least squares method (see Fig. 2A for the best fit and Table S7 for the corresponding
711 values).

Table S7: Parameter values obtained from data fitting. The fit was performed with the nonlinear least-squares solver lsqcurvefit of MATLAB R2015a. corresponds to the value of the squared 2-norm of the residual $\sum(f(\rho, xdata) - \log_{10}(ydata))^2$ with $p = r_m$ and where f is the \log_{10} of the solution of the ODE system. The algorithm was run for m_0 values ranging between 10^5 and 10^7 . The algorithm was run for K_3 values ranging between 10^7 and $5 \cdot 10^8$.

m_0 (CFUs g ⁻¹ feces)	r_m (day ⁻¹)	K_3	Res. norm
10^5	18.7	10^7	4

712 **Extinction probability and extinction time**

713 It is also important to evaluate the time needed for the extinction of the S.Tm^{WT} population. For the
714 sake of simplicity, we only consider cases where the initial number of newly introduced bacteria is
715 large, that is $W_0 > 100$. In this case, the dynamics of the invasion process can be divided into two time
716 windows (Fig. 2). The first time window starts with the inoculation and ends when the number of
717 S.Tm^{WT} bacteria becomes < 100 : all populations are thus large so that time lapse spent in this time
718 window is evaluated by integrating the deterministic equations

$$\left\{ \begin{array}{l} \frac{dW}{dt} = W \cdot \left(r_w \cdot \left(1 - \frac{1}{\frac{K_1}{M+C+W} + \frac{K_2}{C+W}} \right) - c_w \right) \\ \frac{dC}{dt} = C \cdot \left(r_c \cdot \left(1 - \frac{1}{\frac{K_1}{M+C+W} + \frac{K_2}{C+W}} \right) - c_c \right) \\ \frac{dM}{dt} = M \cdot \left(r_m \cdot \left(1 - \frac{1}{\frac{K_1}{M+C+W} + \frac{K_3}{M}} \right) - c_m \right) \end{array} \right.$$

719 until the time τ when $W(\tau) = 100$. The second time window starts when the resident population
720 becomes < 100 and ends with its extinction: a stochastic description is used here because of the
721 small size of the resident population. The distribution of the times needed to go from $W = 100$ to
722 extinction can be obtained numerically with the Gillespie algorithm.

723 Supplementary Figures

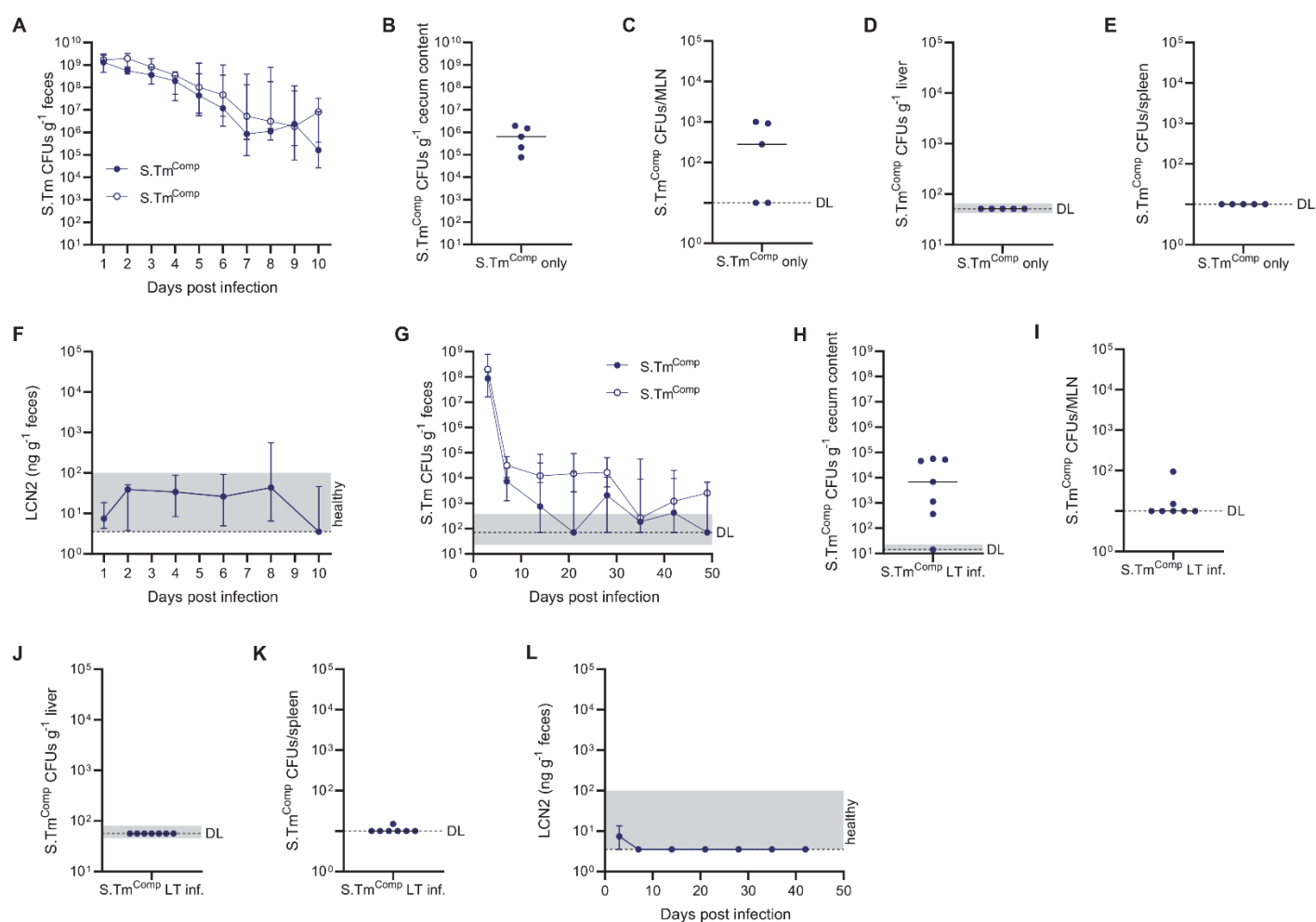


Figure S1. *S.Tm^{Comp}* is not pathogenic in 129S6/SvEv and C57BL/6J mice. (A-F) 129S6/SvEv mice were pretreated with streptomycin and infected with a total of 10^5 of a 1:1 mixture of two isogenic *S.Tm^{Comp}* strains and colonization was followed for 10 days. *S.Tm^{Comp}* CFUs were determined by selective plating in feces (A) cecum content (B), MLN (C), liver (D) and spleen (E). (F) Intestinal inflammation was determined by measuring fecal lipocalin-2. (G-L) C57BL/6 mice were pretreated with streptomycin and infected with a total of 10^4 of a 1:1 mixture of two isogenic *S.Tm^{Comp}* strains and colonization was followed for 7 weeks. *S.Tm^{Comp}* CFUs were determined by selective plating in feces (G) cecum content (H), MLN (I), liver (J) and spleen (K). (L) Intestinal inflammation was determined by measuring fecal lipocalin-2.

N = 5-7 mice. Solid lines depict the median, error bars the interquartile range. Dotted lines show the detection limit and the shaded area the range for cases in which the detection limit is dependent on sample weight. Statistics were performed by an unpaired two-tailed t-test on log-normalized area under the curve (AUC) (A, G). CFU, colony forming unit; LCN2, lipocalin-2; MLN, mesenteric lymph node.

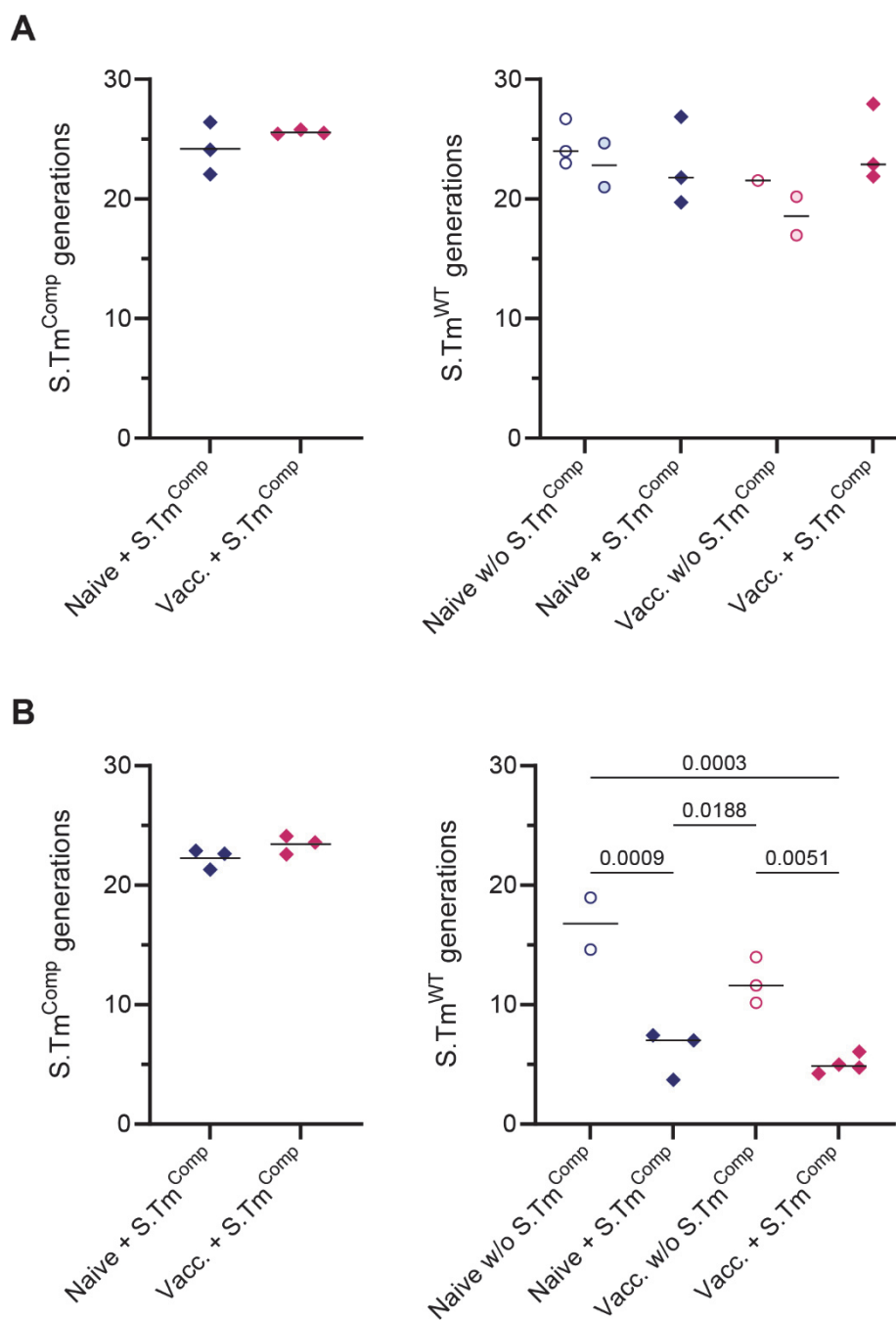


Figure S2. Bacterial generations *in vivo* at 12 hours post infection. (A) PBS (blue symbols) or PA-S.Tm-vaccinated (pink symbols) 129S6/SvEv mice were pretreated with streptomycin and infected with a total of 10^4 of a 1:1 mixture of two isogenic $S.Tm^{WT}$ strains (circles) or $S.Tm^{WT}$ and $S.Tm^{Comp}$ ($S.Tm^{hilD\ ssaV\ oafA}$, filled diamonds). Number of generations 12 hours post infection was estimated based on the loss of pAM34. Open and filled circles show the two isogenic $S.Tm^{WT}$ strains. **(B)** PBS (blue symbols) or PA-S.Tm-vaccinated (pink symbols) 129S6/SvEv mice were pretreated with streptomycin and infected with $1 \cdot 10^6$ $S.Tm^{WT}$. Two groups were pre-colonized with $5 \cdot 10^3$ $S.Tm^{Comp}$ 3 days before infection. Number of generations 12 hours post infection was calculated based on the loss of pAM34. Solid lines depict the mean. Statistics were performed by one-way ANOVA.

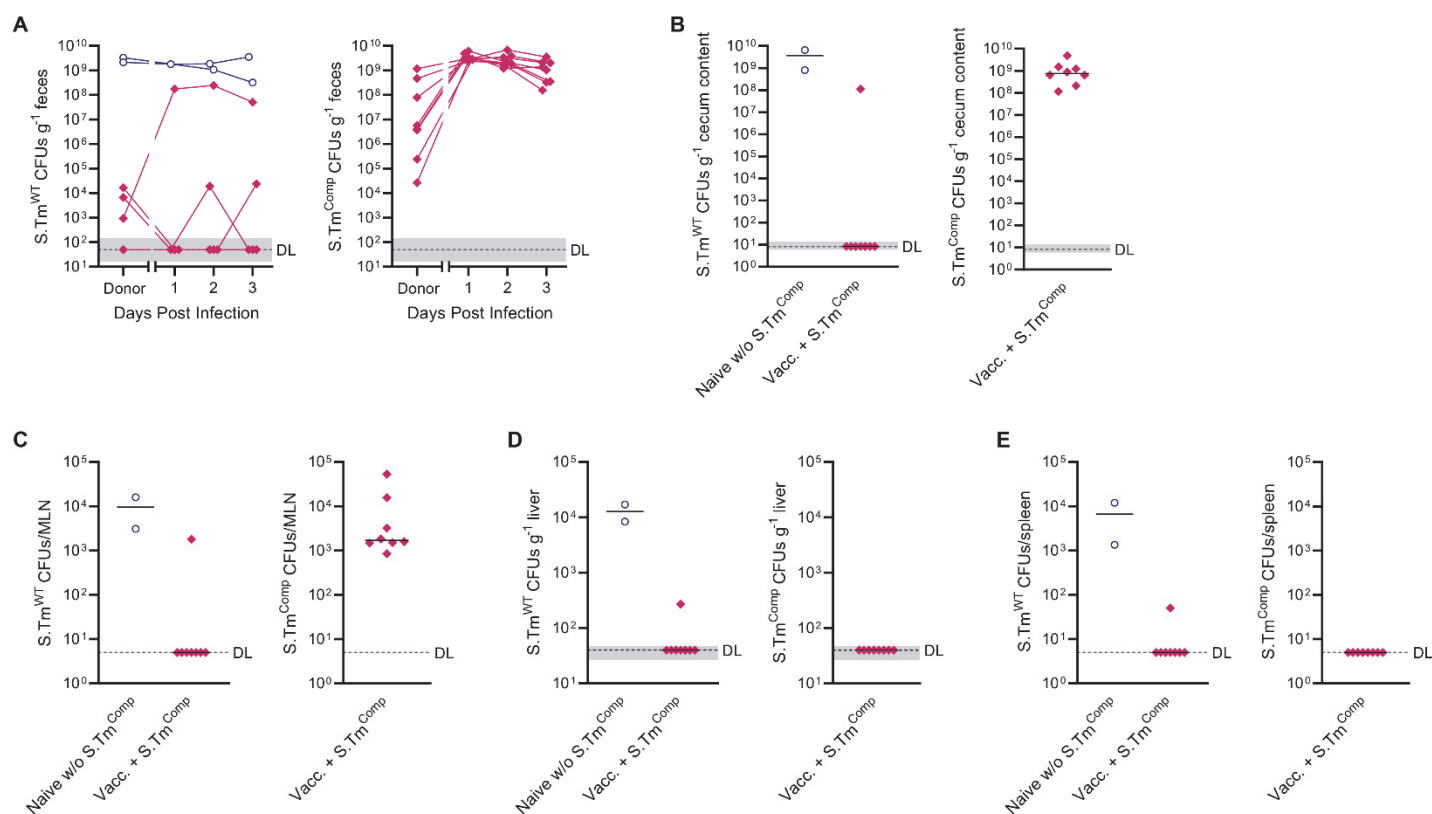


Figure S3. Vaccination together with $S.Tm^{Comp}$ a priori colonization prevents $S.Tm^{WT}$ transmission. Naive 129S6/SvEv mice were pretreated with streptomycin and a FMT was performed with feces collected from 9 days post infection of untreated (blue circles) or vaccinated and $S.Tm^{Comp}$ pre-colonized mice (pink diamonds; see Fig. 3). $S.Tm^{WT}$ and $S.Tm^{Comp}$ CFUs were determined by selective plating in feces (A) cecum content (B), MLN (C), liver (D) and spleen (E).

Pooled data from two independent experiments with switched antibiotic resistances ($n = 2$ or 8 mice/group). Solid black lines depict the median. Dotted lines show the detection limit and the shaded area the range for cases in which the detection limit is dependent on sample weight. CFU, colony forming unit; FMT, fecal microbial transplant; MLN, mesenteric lymph node.

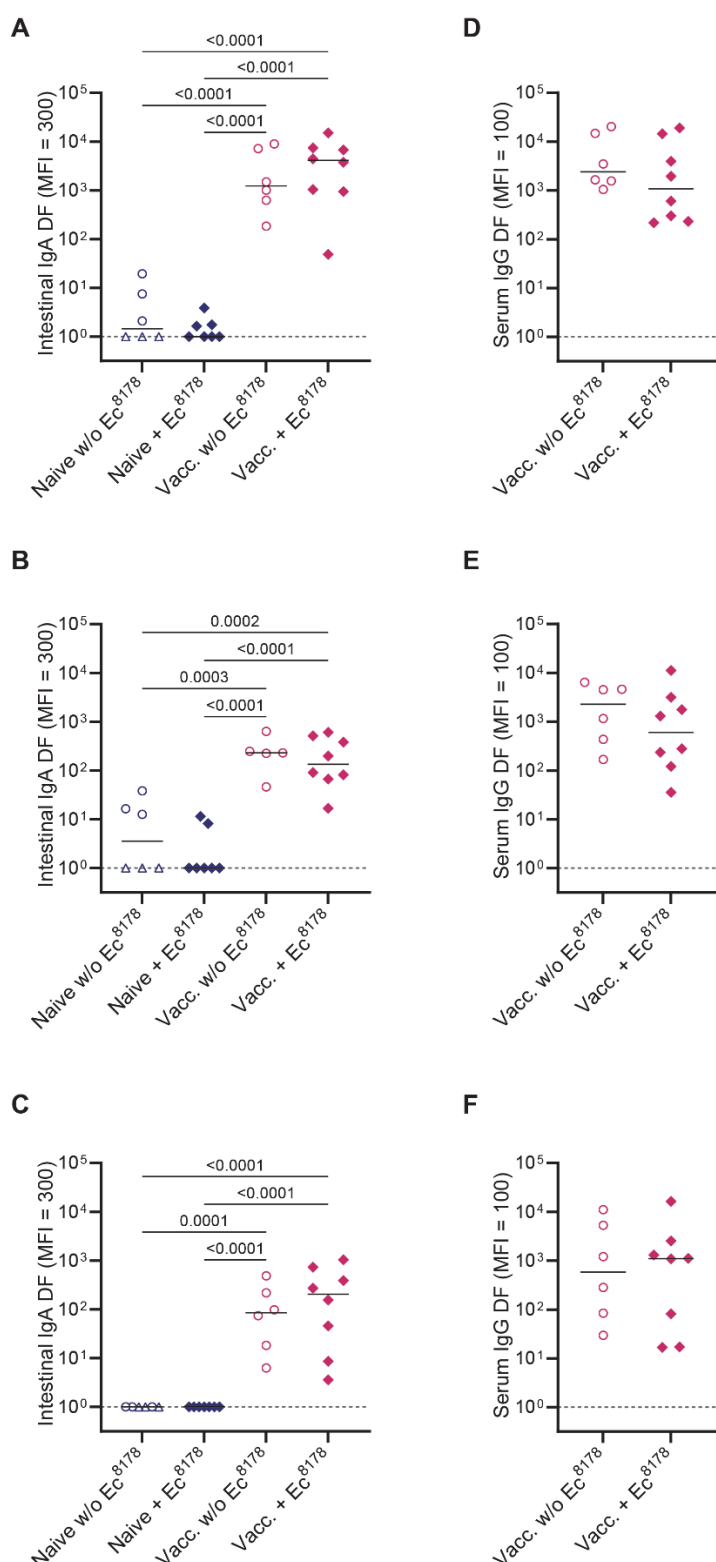


Figure S4. Vaccination with the EvoTrap vaccine generates an intestinal IgA and serum IgG response against all *S.Tm* O-antigen variants. 129S6/SvEv mice were mock-vaccinated with PBS (blue symbols) or EvoTrap-vaccinated (pink symbols) and later infected with $1 \cdot 10^6$ *S.Tm*^{WT} with or without pre-colonization with $5 \cdot 10^3$ *E. coli* 8178. 10 days after *S.Tm*^{WT} infection *S.Tm* specific antibody responses were determined in intestinal lavage (A-C) and serum (D-E) by flow cytometry. (A+D) *S.Tm* O:4,12-0. (B+E) *S.Tm* O:4[5],12-2. (C+F) *S.Tm* O:4,12-2.

Pooled data from two independent experiments ($n = 6-8$ mice/group). Solid lines depict the. Dotted lines show the detection limit. Open triangles show mice that had to be euthanized prematurely due to excessive weight loss ($\geq 15\%$) or disease symptoms. Statistics were performed one-way ANOVA on log-normalized data (A-C). Where only two groups were compared, an unpaired two-tailed t-test on log-normalized data was done (D-F). DF, dilution factor; MFI, median fluorescence intensity.

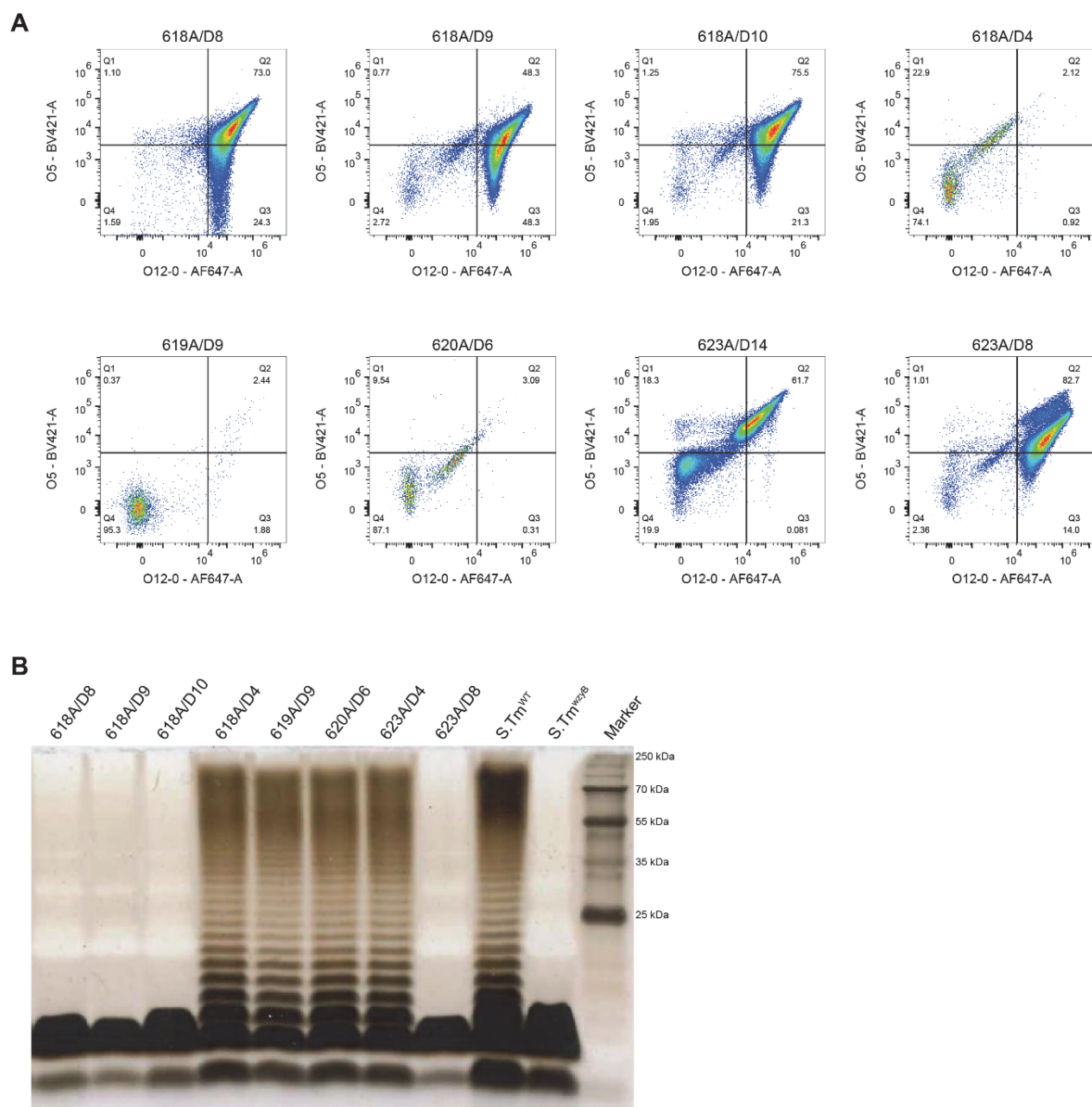


Figure S5. Vaccination with the EvoTrap vaccine leads to emergence of *S.Tm*^{WT} clones with short O-antigen. 129S6/SvEv mice were EvoTrap-vaccinated and later infected with $1 \cdot 10^6$ *S.Tm*^{WT} with or without pre-colonization with $5 \cdot 10^3$ *E. coli* 8178. *S.Tm*^{WT} clones were isolated from feces from 4 days post infection onwards. **(A)** *S.Tm*^{WT} clones were monitored for decreased O:5 and O:12-0 staining intensity by flow cytometry. **(B)** Silver-stained gel of LPS from selected *S.Tm*^{WT} clones from 4 different EvoTrap vaccinated mice.

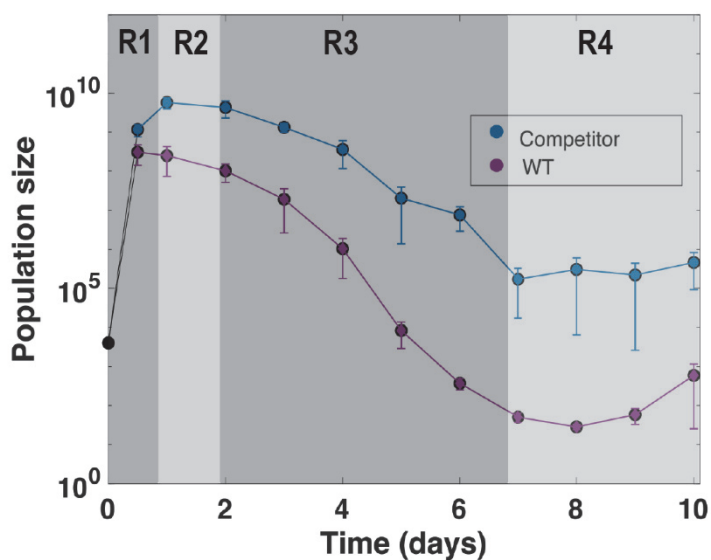


Figure S6. Different regimes used for mathematical modelling. PA-S.Tm-vaccinated 129S6/SvEv mice were pretreated with streptomycin and infected with a total of 10^4 of a 1:1 mixture of $S.Tm^{WT}$ (blue symbols) and $S.Tm^{Comp}$ (purple symbols). R_1 defines the time window where $S.Tm^{WT}$, $S.Tm^{Comp}$ and the microbiota are all below their carrying capacity. R_2 is defined by $S.Tm^{Comp}$ being more abundant than $S.Tm^{WT}$ and the microbiota, and being in the order of magnitude of K_1 . R_3 is defined by the microbiota being more abundant than $S.Tm^{WT}$ and $S.Tm^{Comp}$ but $S.Tm^{Comp}$ is still present in higher numbers than its carrying capacity in equilibrium K_2 . R_4 is defined by the microbiota being much more abundant than $S.Tm^{WT}$ and $S.Tm^{Comp}$ and $S.Tm^{Comp}$ being in the order of magnitude of K_2 .

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