

RESEARCH ARTICLE

Strain-specific galactose utilization by commensal *E. coli* mitigates *Salmonella* establishment in the gut

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Abstract

Salmonella enterica serovar Typhimurium (S. Tm) is a major cause of gastrointestinal diseases worldwide. To date, options for prevention or curative therapy remain limited. The gut microbiota plays a protective role against enteric diseases, particularly in preventing establishment and proliferation of S. Tm. While most research has focused on microbiota-mediated pathogen exclusion during the later, inflammation-dominated stages of infection, little is known about how microbiota members mitigate S. Tm early gut colonization. To address this gap, we conducted 24 h *in vivo* competitive experiments using S. Tm and different commensal *E. coli* strains. We observed a significant reduction in pathogen load, which was strain-specific and particularly evident with *E. coli* 8178. To investigate the underlying molecular mechanisms, we performed an *in vivo* screen using a rationally designed S. Tm library—which includes a wide range of carbohydrate utilization mutants—both in the absence and presence of *E. coli* strains. Our findings revealed that *E. coli* 8178-mediated S. Tm competition was driven by the exploitation of galactose during the early stage of infection. Identifying galactose as a key metabolite in pathogen exclusion by gut microbiota members enhances our mechanistic understanding of microbiota-mediated protection and opens new avenues for developing microbiota- and dietary-based strategies to better control intestinal infections.

Author summary

Salmonella infections remain a major global health problem, and options for preventing or treating them are still limited. Our gut microbiota helps protect against such infections. While most research has focused on how the microbiota acts during later, more severe stages of infection, we wanted to understand

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Data availability statement: All data needed to evaluate the conclusions of this study are presented in the Article and [Supporting Information](#). The amplicon sequencing data for the WISH-barcoded *S. Typhimurium* mutant pool experiments has been deposited in the European Nucleotide Archive under the accession number: PRJEB85163.

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how it can stop *Salmonella* early, before it takes hold. In this study, we looked at how different strains of *Escherichia coli*, a common gut bacterium, compete with *Salmonella* Typhimurium. We found that one strain, called 8178, was especially good at restricting *Salmonella* shortly after infection. We discovered that this competition centers around galactose, a simple sugar. Our findings show that some gut bacteria can effectively starve out invading pathogens by beating them to essential nutrients. This work offers new insights into how our gut microbiota protects us and points to strategies - such as using beneficial bacteria or tailored diets - to help prevent or control gut infections.

Introduction

Foodborne diarrhoea is a widespread disease, affecting 1 in 10 people globally and leading to over 420,000 deaths annually [1]. Bacterial enteric pathogens are among the leading causes of foodborne diseases, with *Salmonella* responsible for 180 million cases of gastroenteritis each year [2]. *Salmonella enterica* serovar Typhimurium (*S. Tm*) is among the most common non-typhoidal serovars that affect humans [3,4]. Research using antibiotic-pretreated specific pathogen free (SPF) C57BL/6J and gnotobiotic mouse models have identified two distinct growth phases for *S. Tm* in the gut [5]. In these models, *S. Tm* establishes and grows in the intestinal lumen through monosaccharide-fuelled mixed acid fermentation during the early stage of infection (0–48 hours post-infection) [6–9]. Subsequently, *S. Tm* employs the type III secretion systems encoded within the pathogenicity island 1 (T3SS-1) and 2 (T3SS-2) to trigger inflammation, creating conditions that favour proliferation, evolution and transmission of the pathogen [6,7,9–18].

Under homeostatic conditions, the gut microbiota plays a variety of functions, including restricting the invasion of enteric pathogens [19]. This phenomenon is referred to as colonization resistance and can often be attributed to specific commensal strains exhibiting pathogen-displacing capabilities [20,21]. This is particularly relevant for *S. Tm* infections, where several microbiota members were shown to act as protective strains [22–25]. The prevention of pathogen growth by intestinal commensals can occur through interference mechanisms involving the production of toxic compounds. This is exemplified by commensal *E. coli* or *Bacteroides* spp. which produce toxins and metabolites directly impacting *Salmonella* growth in the gastrointestinal tract [26–28]. Alternatively, commensal bacteria can engage in competitive interactions by exploiting available nutrients, thereby reducing the fitness of their competitors – a mechanism known as nutrient exploitation. This holds true for the human probiotic *E. coli* Nissle strain, which restricts intestinal *S. Tm* growth by scavenging iron [29]. Besides iron, several electron acceptors such as oxygen, fumarate and nitrate were also shown to be actively used by commensal *E. coli* strains, thereby limiting *Salmonella*'s fitness in the gut [7,8,30–32]. While the mechanisms driving *S. Tm* restriction by commensals are increasingly understood, most of this research focuses on the later stages of infection, particularly when inflammation is

already established. In contrast, our understanding of how commensals restrict pathogens during the initial growth phase remains scarce, with galactitol standing as one of the few known carbohydrates exploited by commensal *E. coli* to limit *S. Tm* gut invasion [33].

In this study, we aimed to address these gaps by investigating how commensal *Enterobacteriaceae* restrict *S. Tm* during the early phase of intestinal growth and identifying the carbohydrates driving this exclusion relationship.

Results

E. coli 8178 limits early *S. Tm* gut establishment independently of interference mechanisms

E. coli 8178 is a commensal strain, originally isolated from the murine gut and capable of outcompeting *S. Tm* at the later stages of infection once inflammation is established [14]. This competitive advantage, visible in the severely inflamed gut between 72- and 96-hours post-infection (p.i) is attributed to an interference mechanism involving the synthesis and secretion of siderophore-bound toxins [28,34]. While investigating this phenomenon, we found out that *E. coli* 8178 was also able to restrict the growth of *S. Tm* at the early stage of infection (24 h p.i) when administered either prior to or concomitantly with *S. Tm* [7,28]. To confirm this observation, we inoculated streptomycin-pretreated 129S6/SvEvTac mice harbouring a specific pathogen-free (SPF) microbiota with a 1:1 mixture of *E. coli* 8178 and *S. Tm* SL1344. Mice co-inoculated with *E. coli* 8178 displayed a 50-fold reduction in faecal pathogen loads at 24 hours post-infection compared to mice infected with *S. Tm* alone (Fig 1A and 1B). This significant reduction during the initial stage of *S. Tm* infection, which remains uncharacterized, was the focus of our investigation.

First, we aimed to determine whether *S. Tm* growth attenuation at 24 h p.i was specific to *E. coli* 8178 or if it was a common effect among other commensal *E. coli* strains. To explore this, we co-inoculated streptomycin-pretreated 129S6/SvEvTac mice with a mixture of *S. Tm* and a different *E. coli* strain. We selected *E. coli* Z1331, a recent and genetically tractable isolate from a healthy human volunteer [35]. This strain has previously been used to study autoinducer-2-mediated chemotaxis in both interspecies and intraspecies signalling [36,37]. Interestingly, in this context, the competitive elimination of *S. Tm* at 24 h p.i was not observed (Figs 1C and S1A) despite *E. coli* Z1331 shedding at similar levels as *E. coli* 8178 at 24 h p.i (Fig 1D). This indicated that *S. Tm* growth attenuation by *E. coli* 8178 is a strain-specific feature. Given the variety of antimicrobial systems encoded by *E. coli* 8178, we next hypothesized that *S. Tm* competition at 24 hours was driven by an interference mechanism, similar to what occurs during the late-stage infection, when inflammation peaks [28,34]. To test this, we systematically mutated each known interference factor in the *E. coli* 8178 genome, including both diffusible and contact-dependent toxin delivery machineries. We then assessed the ability of each *E. coli* 8178 mutant to displace *S. Tm* using streptomycin-pretreated SPF 129S6/SvEvTac mice and found that all strains remain effective in reducing *S. Tm* early gut establishment (Fig 1E). Finally, considering the variety of antagonistic relationships triggered during inflammation [14,27–29,31], we examined whether *E. coli* 8178-mediated exclusion of *S. Tm* was dependent on an inflammatory response. To investigate this, we infected streptomycin-pretreated SPF 129S6/SvEvTac mice with *E. coli* 8178 and an isogenic *S. Tm* strain lacking both type three secretion systems (T3SS-1, $\Delta invG$ and T3SS-2 $\Delta ssaV$), thus unable to trigger a severe gut inflammation [11]. Notably, the faecal loads of the *S. Tm* $\Delta invG \Delta ssaV$ at 24 h p.i were consistently reduced by the presence of *E. coli* 8178 (Fig 1F). Overall, we concluded that *E. coli* 8178-mediated inhibition of *S. Tm* during the initial growth phase was independent of inflammation and did not rely on any known interference mechanisms.

Impact of *E. coli* 8178 on *S. Tm* sugar metabolism during initial growth phase

Since exclusion of *S. Tm* by *E. coli* 8178 at 24 h post-infection is likely not due to an interference mechanism, we hypothesized that this effect may be explained by nutrient exploitation. Recently, hexoses have been identified as the primary nutrient source driving *S. Tm* colonization during the initial growth phase [6,8,9], making them a potential prime target for

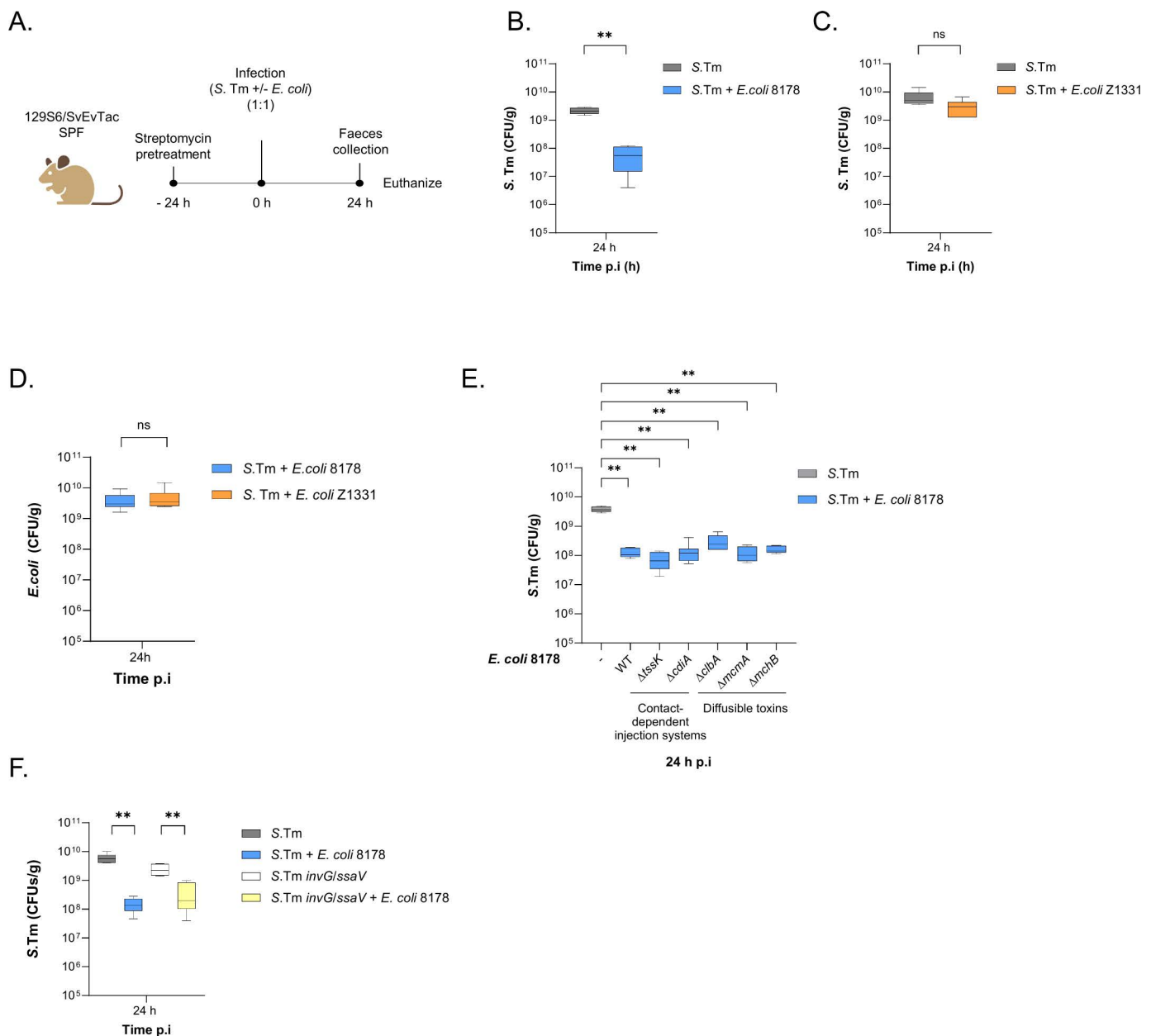


Fig 1. *E. coli* 8178-mediated *S. Tm* early growth restriction is independent of interfering competition interactions. (A) Experimental scheme. Streptomycin-pretreated specific pathogen-free (SPF) 129S6/SvEvTac mice were infected with either *S. Tm* alone or a 1:1 mixture of *S. Tm* and the indicated *E. coli* strain. The *E. coli* and *S. Tm* loads were determined by selective plating from faecal samples collected 24 h post-infection (p.i.) prior to euthanasia. Created in BioRender. Cherrak, Y. (2025) <https://BioRender.com/n43x499>. (B-C) *E. coli* mitigates the early growth of *S. Tm* in a strain-dependent manner. The *S. Tm* loads at 24 h p.i. are plotted and compared between *S. Tm* mono-infected and *S. Tm* + *E. coli* co-infected mice. *E. coli* 8178 (B) and Z1331 (C) were tested as competitors. (D) *E. coli* loads at 24 h p.i. from Fig 1B and 1C are shown. (E) *E. coli* 8178 restricts *S. Tm* establishment independently of interference mechanisms. The faecal load of *S. Tm* collected 24 h p.i. is plotted and compared between *S. Tm* mono-infected and *S. Tm* + *E. coli* 8178 co-infected mice. The *E. coli* 8178 mutant strains tested are listed and categorized based on the nature of the interference system disrupted. (F) Inflammation does not trigger *E. coli* 8178-mediated *S. Tm* competition 24 h p.i. *E. coli* 8178 competitiveness was tested against an avirulent *S. Tm* mutant ($\Delta invG/\Delta ssaV$). (B-F) All competitive experiments are presented in a box-and-whiskers plot, showing the minimum to maximum values, median, and interquartile range (25th to 75th percentiles). The bar plots show the median. Two-tailed Mann-Whitney U tests to compare 2 groups in each panel. $P \geq 0.05$ not significant (ns), $p < 0.05$ (*), $p < 0.01$ (**). A minimum of 5 mice ($n \geq 5$) were used for each experimental group. CFU: colony forming units.

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nutrient exploitation. To explore this further, we employed a targeted library of 35 *S. Tm* mutants, each defective in specific carbohydrate-utilizing enzymes [8] and uniquely labelled with a chromosomal, fitness-neutral DNA barcode (WISH tag, [38]) (Fig 2A and 2B). We screened for metabolic-deficient *S. Tm* strains and tested whether mutant fitness was impacted by the presence of *E. coli* 8178. We also included *E. coli* Z1331 for comparison to highlight specific *E. coli* 8178-driven changes (Fig 2B). To control the quality of the generated pool data, seven wildtype (WT) strains carrying distinct WISH tags were included. This allowed to assess the bottleneck severity by calculating the Shannon evenness score, with mice scoring below 0.9 being excluded from the competitive index analysis [8,39]. Furthermore, two control *S. Tm* mutants ($\Delta dcuABC$, Δfrd), which are deficient in fumarate respiration and exhibit an initial growth defect, were included as internal standards to validate our findings [7,8]. Streptomycin-pretreated 129S6/SvEvTac mice were infected with the targeted *S. Tm* carbohydrate-mutant pool, both in the absence and presence of *E. coli* 8178 or *E. coli* Z1331. The raw fitness data for each *S. Tm* metabolic mutants across these 3 conditions are presented in the S1 Data file. The Shannon evenness score and the fitness of the *S. Tm* control mutants for each dataset are presented in S2A and S2B Fig. As previously reported, mutants deficient in fumarate respiration are already attenuated by day 1 post-infection [8], further validating the reliability of our downstream analysis.

Consistent with earlier findings, we found that in absence of competing strains, *S. Tm* relies on multiple carbohydrates to efficiently grow in the murine gut [8] (Fig 2C, left column). Specifically, the Δpgm , Δpgi , and $\Delta pfkA$ mutants, responsible for catalysing the sequential conversion of D-glucose 1-phosphate to D-glucose 6-phosphate (*pgm*), then to D-fructose 6-phosphate (*pgi*), and finally to D-fructose 1,6-bisphosphate (*pfkA*)—exhibited a fitness disadvantage compared to the WT control. This was reflected in competitive index (C.I) values below 1, underscoring the essential role of glycolysis under these conditions. Additionally, mutants deficient in metabolizing arabinose ($\Delta araB$), galactose ($\Delta galK$), fructose ($\Delta fruK$), and mannose ($\Delta manA$) also displayed reduced fitness (Fig 2C). This suggests that *S. Tm* primarily depends on these metabolites and targeting one of these carbohydrates could potentially impede early gut establishment of the pathogen. Interestingly, the presence of additional *E. coli* competitor strains significantly alters the fitness of *S. Tm* carbohydrate mutants *in vivo* (Fig 2C). This was particularly true for the fitness of the *S. Tm* glycerol ($\Delta glpD$) and mannose ($\Delta manA$) metabolic mutants which were affected by both *E. coli* 8178 and *E. coli* Z1331. While these effects were consistent across both *E. coli* strains, we also observed strain-specific changes. Particularly, the fitness of the *S. Tm* $\Delta fruK$ mutant, while relatively unchanged by *E. coli* 8178 (C.I from 0.632 to 0.588), decreased significantly in the presence of *E. coli* Z1331 (C.I=0.117). More interestingly, the addition of *E. coli* 8178 significantly impaired the fitness of the *S. Tm* $\Delta galK$ mutant, as evidenced by an almost 50% reduction in the competitive index, from 0.501 to 0.261—an effect not observed in the presence of *E. coli* Z1331 (Fig 2C). Given the competitive interaction between *S. Tm* and *E. coli* 8178 (but not *E. coli* Z1331, Fig 1B and 1C), we decided to focus on *S. Tm* metabolic genes that were exclusively affected by *E. coli* 8178 while remaining unaffected by *E. coli* Z1331. This highlighted *galK*, and broadly, galactose metabolism as a strong candidate governing *E. coli* 8178-mediated competition against *S. Tm*, which we selected for further investigation.

***E. coli* 8178's restrictions of *S. Tm* early establishment is centred on galactose**

Galactose metabolism is widely conserved across *Enterobacteriaceae* and is considered a core component of their metabolic repertoire [8,40]. This is particularly evident in *E. coli* 8178 and *E. coli* Z1331 strains which we tested and confirmed were capable of using galactose as a carbon source *in vitro* (S3A Fig). However, it remains unclear whether galactose consumption enhances intestinal colonization of these strains. To evaluate the role of galactose metabolism in *E. coli* within an *in vivo* context, we generated $\Delta galK$ mutants in both *E. coli* 8178 and *E. coli* Z1331 backgrounds and assessed their fitness in streptomycin-pretreated 129S6/SvEvTac mice. When inoculated with a 1:1 mixture of WT and *galK*-deficient *E. coli* 8178, the WT strain demonstrated a growth advantage over the $\Delta galK$ mutant (C.I < 1), indicating that galactose metabolism is important for *E. coli* 8178 colonization of the gut (Fig 3A). In contrast, *E. coli* Z1331 did not show a similar dependency on galactose for intestinal colonization, despite both strains metabolizing galactose *in vitro*. We next explored how the galactose utilization of these *E. coli* strains changes in the presence of *S. Tm*. Under these conditions,

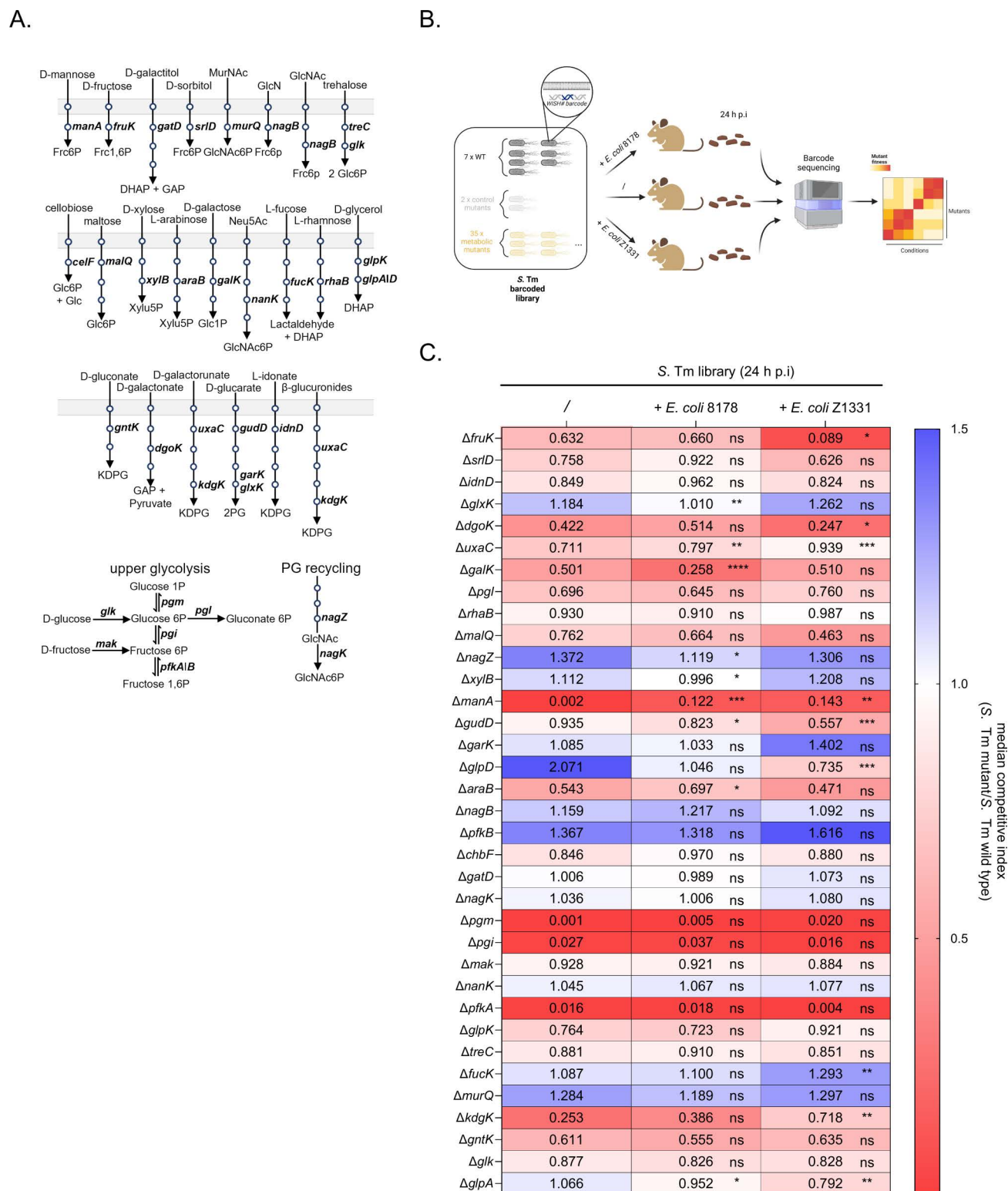


Fig 2. Evaluation of *S. Tm* metabolic requirement in presence of *E. coli* strains. (A) Schematic representation of the *S. Tm* metabolic mutant pool, illustrating the targeted carbohydrate utilization pathways, adapted from [8] (<https://www.nature.com/articles/s41467-025-56890-y#Fig1>). The mutants affect key enzymatic steps, including dehydratases, kinases, and isomerases, positioned between transport reactions and central metabolic

intermediates in glycolysis, the Entner-Doudoroff pathway, and the pentose phosphate pathway. Each circle represents an enzymatic step. Abbreviations are listed in [S4 Table](#) PG, peptidoglycan. **(B)** Experimental scheme. A WISH-barcode *S. Tm* mutant pool library comprising 7 SL1344 wild type (WT), 2 control mutants (Δ frd and Δ dcuABC) and 35 deficient strains in carbohydrate utilization was pooled and used in this study. Streptomycin-pretreated SPF 129S6/SvEvTac mice were infected with the *S. Tm* pool alone or in combination with an *E. coli* strain (8178 or Z1331). The fitness of each *S. Tm* mutant was assessed by WISH-barcode sequencing from faecal samples. Created in BioRender. Cherrak, Y. (2025) <https://BioRender.com/c18e944>. **(C)** Effect of *E. coli* strains on *S. Tm* carbohydrate mutant fitness *in vivo*. The competitive index for each *S. Tm* mutant (listed on the left) is calculated relative to the WT and depicted as a heat map, across 3 different conditions: without *E. coli* (I), in presence of *E. coli* 8178 (+ *E. coli* 8178) in presence of *E. coli* Z1331 (+ *E. coli* Z1331). Median values from a minimum of 6 mice ($n \geq 6$) are shown. Two-tailed Mann–Whitney U tests to compare 2 groups (*S. Tm* library alone vs *S. Tm* library + *E. coli* 8178 or *S. Tm* library alone vs *S. Tm* library + *E. coli* Z1331). $P \geq 0.05$ not significant (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.005$ (***), $p < 0.001$ (****).

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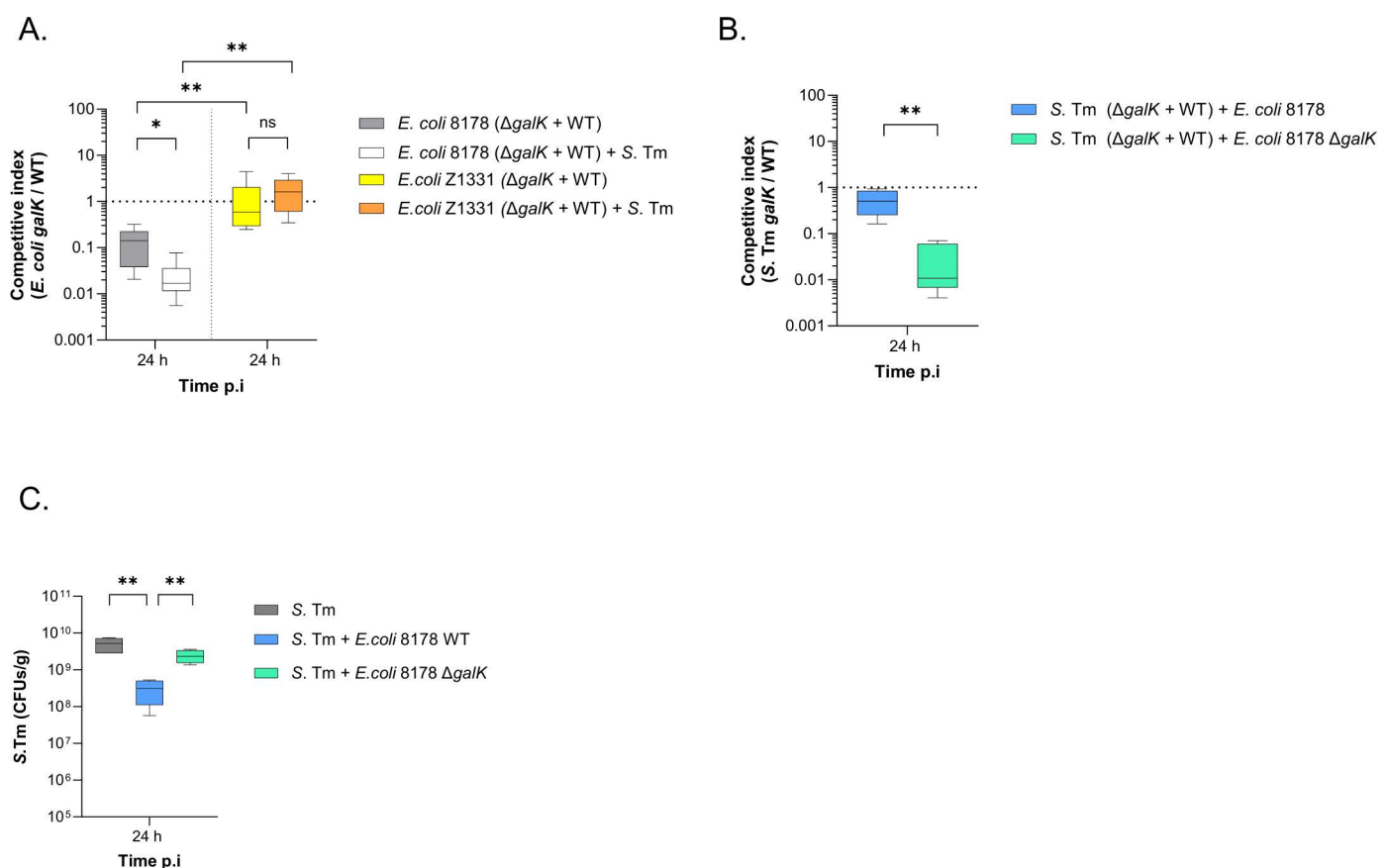


Fig 3. Galactose utilization by *E. coli* 8178 is strain-specific and facilitates the early inhibition of *S. Tm* growth in the gut. **(A)** Fitness of the *galK*-deficient *E. coli* strain *in vivo*. Streptomycin-pretreated 129S6/SvEvTac mice were infected with both the *E. coli* WT and Δ galK strains, either in the presence or absence of the WT *S. Tm* (+ *S. Tm*). The competitive index of the Δ galK mutant in *E. coli* 8178 (left) and Z1331 (right) at 24 h post-infection (p.i.) are plotted. **(B)** Effect of *E. coli* 8178 on the fitness of *galK*-deficient *S. Tm* *in vivo*. Streptomycin-pretreated 129S6/SvEvTac mice were infected with both the *S. Tm* WT and Δ galK strains, in the presence of either the WT or *galK*-deficient *E. coli* 8178. The competitive indexes at 24 h p.i. of the *S. Tm* Δ galK mutant are plotted across these two conditions. **(C)** Competitiveness of the *E. coli* 8178 Δ galK mutant against *S. Tm*. The *S. Tm* load at 24 h p.i. is plotted and compared between *S. Tm* mono-infected and *S. Tm* + *E. coli* co-infected mice. The strains tested are indicated. **(A–B)** Dotted line: Fitness expected for a fitness-neutral mutation. **(A–C)** All experiments are presented in a box-and-whiskers plot, showing the minimum to maximum values, median, and interquartile range (25th to 75th percentiles). The bar plots show the median. Two-tailed Mann–Whitney U tests to compare 2 groups in each panel. $P \geq 0.05$ not significant (ns), $p < 0.05$ (*), $p < 0.01$ (**). A minimum of 5 mice ($n \geq 5$) were used for each experimental group. CFU: colony forming units.

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the *E. coli* 8178 $\Delta galK$ mutant showed an even greater fitness disadvantage compared to the condition without *S. Tm*, underscoring the importance of galactose for *E. coli* 8178 growth, especially when *S. Tm* is present (Fig 3A). In contrast, the fitness of the *E. coli* Z1331 $\Delta galK$ mutant remained neutral and unchanged in the presence of *S. Tm*. To further confirm the capacity of *E. coli* 8178 to actively utilize galactose, we measured and compared *S. Tm* galactose-associated fitness in the presence of either the WT or *galK*-deficient *E. coli* 8178 strain. We hypothesized that co-inoculation with an *E. coli* strain incapable of using galactose *in vivo* would deplete other carbohydrates (besides galactose) even more efficiently than the WT *E. coli* 8178 and thereby accentuate the reliance of *S. Tm* on galactose. This in turn, should further exacerbate the competitive attenuation of the *S. Tm galK* mutant compared to WT *S. Tm*. Consistent with this hypothesis, *S. Tm* exhibited a significantly greater dependence on galactose when co-inoculated with a *galK*-deficient *E. coli* 8178 strain. This was shown by a *S. Tm galK* competitive index of approximately 0.01, which is 26 times lower than in the presence of the WT *E. coli* 8178 strain (C.I=0.26) (Figs 3B and S3B). Together, these findings demonstrate that galactose metabolism is essential for the gut luminal growth of *E. coli* 8178, suggesting that it may be selectively exploited by this strain during intestinal colonization.

Based on these findings, we hypothesized that *E. coli* 8178 exploits galactose and consequently attenuates the early gut establishment of *S. Tm*. To test this, we assessed the ability of the *E. coli* 8178 $\Delta galK$ mutant to restrict *S. Tm* growth in streptomycin-pretreated 129S6/SvEvTac mice. In contrast to the *E. coli* 8178 WT, *S. Tm* colonization was partially restored in mice co-infected with the *E. coli* 8178 *galK* mutant (Figs 3C and S3C). Similar observations were made when substituting our *Salmonella* model organism SL1344 by a different strain (ATCC 14028) (S3D Fig). This effect was not due to variations in colonization efficacy, as both the *E. coli* 8178 WT and *galK* mutant colonize the gut to a similar extent when competing against *S. Tm* (S3E Fig). While our complementation attempts were unsuccessful, this phenotype remained consistent across multiple *galK*-deficient *E. coli* 8178 clones. We also employed a whole genome sequencing approach that ruled out the presence of background mutations in genes other than the *galK* locus. Combined, this indicates that utilization of galactose by *E. coli* 8178 limits *S. Tm* establishment through competitive nutrient exploitation.

Discussion

Here we report that galactose is a key metabolite in mitigating early gut establishment of *S. Tm* by the mouse commensal *E. coli* 8178. This observation is strain-dependent, as a different *E. coli* isolate, Z1331, did not exhibit this effect.

Characterization of the *S. Tm* growth mitigation was achieved using a previously published, quality-controlled, and rationally designed WISH-barcoded *S. Tm* mutant pool representing the metabolic capacity of *Salmonella* [8]. This allowed to highlight how the presence of an additional strain affects *S. Tm* metabolic requirements during gut-luminal growth. Simultaneous testing of multiple mutants within a single animal can efficiently identify important metabolic pathways, reducing costs and the number of animals needed for a comprehensive analysis [41]. There are two main methodologies for assessing mutant fitness in high-throughput scenarios: randomly barcoded transposon insertion sequencing (RB-TnSeq, [42]) and rationally designed libraries, as used in our study. The latter has technical advantages, utilizing smaller strain libraries for robust mutant representation in niches like the mouse gut (which is often characterized by *S. Tm* population bottlenecks) and enabling simpler bioinformatics, as each mutant is WISH-barcoded for a specific gene inactivation. Testing the *S. Tm* mutant pool in competition with different *E. coli* isolates allowed us to report the strain-specific exploitation of galactose as a novel strategy employed by commensal *E. coli* to mitigate *S. Tm*'s early gut invasion.

Freter's nutrient niche theory suggests that for a bacterium to successfully colonize a niche, it must be the most efficient at metabolizing at least one specific nutrient within that environment [43,44]. In recent years, galactitol has been identified as an important substrate for *Enterobacteriaceae*, specifically for *Klebsiella* spp., *E. coli* spp., and *S. Tm* [33,45–47]. As a result, galactitol was shown to drive metabolic competition between *S. Tm* and other *Enterobacteriaceae* strains [33,47]. In the current study, we identified galactose as an additional carbohydrate that mediates colonization resistance against *S. Tm* SL1344. Galactitol is a sugar alcohol and the reduction product of galactose. This circumstance requires bacterial

oxidation of galactitol before it can be degraded via glycolysis, increasing the metabolic cost of galactitol utilization compared to galactose. Unlike galactitol, which is less common, galactose is relatively abundant in the diet [48–50] and in the cecum contents of mice with a complex microbiota and gnotobiotic models [6,8]. This is also relevant for the caecal contents of streptomycin-pretreated mice, such as those used in the present study, which were shown to exhibit approximately 2 mM galactose [6]. Taking this information into consideration, we propose that galactose is a key nutrient that can mediate *Enterobacteriaceae*-*Enterobacteriaceae* competition in the gut. In line with this, galactose was shown to act as an important nutrient that promotes *S. Tm* growth in the gut [51]. Furthermore, galactose degradation genes in *Enterobacteriaceae* have been identified as part of the core genome, underscoring the significance of galactose as a specific niche for *Enterobacteriaceae* [8]. While these observations support the idea of a galactose-rich diet as mediator for *S. Tm* elimination by commensal *Enterobacteriaceae*, it is important to note that galactose supplementation experiments have only partially, but not fully, rescued *S. Tm* survival against *E. coli* 8178. This might be attributable to the host's relatively high efficiency in absorbing galactose or to the already sufficient levels of galactose in the caecal contents of our mice, making galactose supplementation ineffective. Despite both *E. coli* 8178 and *E. coli* Z1331 having similar *in vitro* growth on galactose as the sole carbon source, only *E. coli* 8178 was able to exploit it and attenuate *S. Tm* growth in the gut. This suggests that the metabolic repertoire of a given bacterial strain alone is insufficient to predict colonization or competitive interactions *in vivo*. The challenge of defining bacterial nutrient preferences *in vivo* stems not only from our limited understanding of the bacterial factors that drive the hierarchical consumption of carbon substrates [52] but also from the highly complex and dynamic environment that bacteria encounter within the gut microbiota [53]. Although both *E. coli* 8178 and Z1331 can metabolize galactose under defined *in vitro* conditions—where D-galactose is the sole and abundant carbon source—translating this into the gastrointestinal setting may reveal differential requirements for galactose metabolism and other growth-fuelling metabolites. Metabolic exploitation can also be influenced by the sequence of the transcriptional regulator which may differ at the strain level. In some *E. coli* serovars, the start codon of the transcriptional regulator LacI for lactose metabolism is GTG, leading to lower expression of the repressor. This results in higher basal expression of the lactose-utilizing genes, giving these strains a metabolic advantage over isogenic strains with the ATG start codon in the *lacI* gene [54]. A similar observation was made in *Clostridioides difficile*, in which the transcriptional regulator for trehalose in some epidemic ribotypes has acquired a mutation that allows them to metabolize trehalose at lower concentrations [55]. Both adaptations provide a competitive advantage in colonization and highlight the extent of strain adaptation required to efficiently utilize a specific niche. Understanding nutrient preferences at the strain-specific level could introduce novel concepts in basic microbiology while also providing optimized strategies for enhancing probiotic efficiency. Taken together, our data indicate that galactose is a crucial nutrient niche for the establishment of *S. Tm* and its exploitation can be affected by the presence of endogenous and specific *E. coli* strains. Similar observations will guide the design of optimized microbiota-based strategies aimed at limiting these nutrients to inhibit or even prevent pathogen invasion.

Methods

Animals and ethic statements

Male and female 8–12 weeks old 129S6/SvEvTac (Jackson Laboratory) mice were randomly assigned to experimental groups and used in this study. The mice were housed under SPF conditions in individually ventilated cages at the EPIC mouse facility, ETH Zurich. All animal experiments were conducted in accordance with Swiss and cantonal regulations and were reviewed and approved by the Tierversuchskommission, Kantonales Veterinäramt Zürich under license ZH158/2019, ZH108/2022, and ZH109/2022, in compliance with the cantonal and Swiss legislation.

Strains, media, and chemicals

All strains, plasmids and oligonucleotides used in this study are listed in S1–S3 Tables. Custom oligonucleotides were synthesized by Microsynth AG (Balgach, Switzerland). *E. coli* 8178 and Z1331 strains originate from previous work [14,35].

The *S. Tm* mutant pool was designed in [8]. Bacterial strains were routinely cultured in lysogeny broth (LB) with or without 1% agar-agar. Mutant selection was carried out using antibiotics: streptomycin (100 µg/ml), carbenicillin (100 µg/ml), kanamycin (50 µg/ml), and chloramphenicol (30 µg/ml). Gene deletions in *E. coli* 8178 and Z1331 were performed using a modified lambda red recombinase one-step inactivation method [56]. In brief, kanamycin resistance cassettes (kanamycin for deletions, were PCR-amplified (*Phusion* DNA Polymerase, Sigma-Aldrich)) with primers containing 50-nucleotide homologous regions flanking the target site. Electroporation of these PCR products into *E. coli* cells expressing lambda red recombinase from the pSIM5 plasmid allowed for mutant generation, which were then selected on antibiotic plates [57]. Gene deletions were confirmed by colony PCR and whole-genome sequencing, with a particular focus on the *E. coli* 8178 $\Delta galK$ mutant (PRJEB85163).

Mouse infection experiments

The 8- to 12-week-old mice were orally pretreated with streptomycin (25 mg) 24 h before inoculation [58]. *S. Tm* and *E. coli* cultures were individually grown in LB at 37°C for 4 h and washed twice with a phosphate-buffered saline solution (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄). The *S. Tm* pool was cultured in a similar fashion from pre-mixed cryo stocks. Prior to colonization experiments, *E. coli* strains were electroporated with the pRSF1010 (P3) plasmid from *Salmonella enterica* serovar Typhimurium SL1344 which confers streptomycin resistance [59]. Each mouse received a single oral dose of 50 µl containing approximately 5×10^7 colony forming units (cfu) of an inoculum mixture consisting of equal ratios of the indicated strains. Faecal samples were collected 24 hours post-infection, and animals were euthanized via CO₂ asphyxiation on day 1 post-infection. Faecal samples were then suspended in 1 ml of PBS and homogenized using a TissueLyser (Qiagen). Bacterial loads were quantified by plating the homogenized suspension on selective MacConkey or LB agar plates. In competition assays involving two bacterial strains (WT and mutant), the mutant's bacterial load was directly quantified by plating on antibiotic-containing agar. Conversely, the bacterial load of the WT strain was derived from the total colony count on antibiotic-free plates using the formula: $cfu_{WT} = cfu_{total} - cfu_{mutant}$. The load of each mutant strain was then normalized to its initial inoculum, and this value was used to calculate the normalized competitive index (C.I.) to assess its fitness relative to the WT strain. The C.I. for each mutant was determined by taking the ratio of cfu_{mutant} to cfu_{WT} , divided by the ratio of the mutant and WT strains in the inoculum.

Evaluation of *S. Tm* metabolic mutant fitness via barcode counting

Preparation and analysis of *in vivo* samples containing the *S. Tm* WISH barcoded mutant pool was achieved as previously reported [8]. Specifically, faecal *S. Tm* cells were cultivated in LB medium supplemented with carbenicillin to select for WISH-barcoded strains. Following enrichment, cells were pelleted and stored at -20°C. DNA was extracted from the thawed pellets using the Qiagen Mini DNA kit, following the manufacturer's protocol. Amplification of WISH barcodes via PCR was performed using the DreamTaq MasterMix (Thermo Fisher Scientific) under the following cycling conditions: (1) initial denaturation at 95°C for 3 min, (2) denaturation at 95°C for 30 sec, (3) annealing at 55°C for 30 sec, (4) extension at 72°C for 20 sec, repeated for 25 cycles, followed by (5) a final extension at 72°C for 10 min. The resulting PCR products were purified (Macherey-Nagel) and subsequently indexed by a second PCR with unique dual index primers using the following program: (1) 95°C for 3 min, (2) 95°C for 30 sec, (3) 55°C for 30 sec, (4) 72°C for 20 sec, repeated for 10 cycles, with a (5) final extension at 72°C for 3 min. The indexed products were checked by gel electrophoresis (1% w/v agarose in Tris, acetate, EDTA buffer: 40 mM tris-acetate, 1 mM EDTA), pooled based on band intensity, and purified using AMPure beads (Beckman Coulter) for library preparation. Amplicon sequencing was carried out by BMKGENE (Münster, Germany) using the Novaseq platform, with 150 bp paired-end reads at a target output of 1 G per sample. Following sequencing, reads were demultiplexed and WISH-barcodes were counted using the mBARq software [60]. Misreads or mutations of up to five bases were assigned to the closest matching WISH-tag sequence. WISH-barcode counts for each mouse are provided in S1 Data file. These counts were then used to calculate competitive fitness and Shannon evenness scores across

seven wild-type strains. WISH counts ≤ 10 were excluded from further analysis, setting the detection limit as previously described by [38].

***In vitro* galactose utilization assay**

Biolog PM1 MicroPlates (BIOLOG; [61]) were used for metabolic profiling of *S. Tm* and *E. coli* strains according to the manufacturer's protocol. Bacteria were grown overnight in M9 minimal medium with 25 mM pyruvate as the sole carbon source at 37°C with shaking. They were then washed and resuspended in M9 medium without pyruvate to an OD₆₀₀ of 0.056. 100 μ l of this suspension was added to each well of the microplate. Plates were sealed with parafilm and incubated at 37°C. OD₆₀₀ was measured every 10 minutes for 12 hours with orbital shaking at 282 rpm using a BioTek Synergy H1 Microplate spectrophotometer (Agilent).

Statistical analysis

Statistical analyses and data visualization were conducted using GraphPad Prism version 9.2.0 for Windows (GraphPad Software, La Jolla, CA, USA). For comparisons between two groups, the unpaired Mann–Whitney U-test was employed to evaluate statistical significance, based on rank comparison. *P* values < 0.05 were considered to indicate statistical significance.

Materials availability

Mouse lines used in this study can be obtained from Jackson laboratories.

Supporting information

S1 Fig. *E. coli* 8178 and Z1331 loads in co-infected mice. (A). The competitive index between *S. Tm* and *E. coli* from Fig 1B and 1C are shown. The box-and-whiskers plot represent the minimum to maximum values, median, and interquartile range (25th to 75th percentiles). Two-tailed Mann–Whitney U tests to compare 2 groups in each panel. *P* < 0.01 (**). (TIFF)

S2 Fig. Quality-control of the *S. Tm* mutant pool analysis *in vivo*. (A) The Shannon evenness score at 24 h post-infection (p.i) is presented for each condition tested. A score close to 1 signifies an even distribution of the 7 *S. Tm* wild-type strains, reflecting the absence of a bottleneck effect. The median and standard deviation are shown, with the dotted line representing the threshold (0.9) below which samples were excluded from analysis. (B) Fitness of the internal *S. Tm* metabolic mutant controls. The competitive index of the *S. Tm* Δ dcuABC (left) and Δ frd (mutant) at 24 h p.i is plotted for each condition tested. Data are presented in a box-and-whiskers plot, showing the minimum to maximum values, median, and interquartile range (25th to 75th percentiles). The bar plots show the median. Dotted line: competitive index expected for a fitness-neutral mutation. (A–B) A minimum of 6 mice ($n \geq 6$) were used for each condition tested. (TIFF)

S3 Fig. *E. coli* 8178 and Z1331 grow on galactose as the sole carbon source. (A) *In vitro* growth on galactose as the sole carbon source was assessed for *E. coli* 8178 and Z1331 strains. Growth was measured in a Biolog PM1 microplate, with data plotted as Δ OD_{600nm} for 3 hours and 12 hours for each bacterium as indicated. (B) Bacterial loads of the WT and *galK*-deficient *S. Tm* strains in presence of the WT (left) or Δ galK (right) *E. coli* 8178 are plotted as CFU per gram of feces at 24 h p.i (from Fig 3B). (C) Competitive index at 24 h p.i between *S. Tm* and *E. coli* (from Fig 3C). (D) The *S. Tm* ATCC 14028 loads at 24 h p.i. are plotted and compared between *S. Tm* mono-infected and *S. Tm* + *E. coli* 8178 co-infected mice. (E) *E. coli* 8178 loads in *S. Tm* + *E. coli* co-infected mice (from Figs 3C and S3D). (B–E) Data are presented in a box-and-whiskers plot, showing the minimum to maximum values, median, and interquartile range (25th to 75th percentiles).

The bar plots show the median. Two-tailed Mann–Whitney U tests to compare 2 groups in each panel. $P \geq 0.05$ not significant (ns), $p < 0.01$ (**).

(TIFF)

S1 Table. Bacterial strains used in this study.

(XLSX)

S2 Table. Plasmid used in this study.

(XLSX)

S3 Table. Oligonucleotides used in this study.

(XLSX)

S4 Table. List of abbreviations.

(XLSX)

S1 Data. Raw data from this study.

(XLSX)

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