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Original Article

A highly-safe live auxotrophic vaccine protecting against disease caused by non-typhoidal *Salmonella* Typhimurium in mice

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Abstract *Background: Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) has become an important intestinal pathogen worldwide and is responsible for lethal invasive infections in populations at risk. There is at present an unmet need for preventive vaccines.
Methods: IRTA GN-3728 genome was sequenced by Illumina and D-glutamate and D-glutamate/D-alanine knockout-auxotrophs were constructed. They were characterized using electron microscopy, growth/viability curves, reversion analysis, and motility/agglutination assays. Their potential as vaccine candidates were explored using two BALB/c mouse models for *Salmonella* infections: a systemic and an intestinal inflammation. Clinical signs/body weight and survival were monitored, mucosal lactoferrin and specific/cross-reactive IgA/IgG were quantified by enzyme-linked-immunosorbent assays and bacterial shedding/burden in fecal/tissues were evaluated.

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Results: The D-glutamate auxotroph, IRTA Δ*murI*, is highly attenuated, immunogenic and fully protective against systemic infection. The IRTA Δ*murI* Δ*alr* Δ*dadX* double auxotroph, constructed to reinforce vaccine safety, showed a higher level of attenuation and was 100% effective against systemic disease. In the intestinal model, it proved to be safe, yielding a low-degree of mucosal inflammation, short-term shedding and undetectable invasiveness in the long-term, while eliciting cross-reactive fecal IgA/serum IgG against clinically relevant multidrug-resistant (MDR) *S.* Typhimurium strains. It also conferred protection against homologous oral challenge, and protected mice from local and extra-intestinal dissemination caused by one MDR strain responsible for an international outbreak of highly severe human infections. Additionally, oral vaccination promoted extended survival after lethal heterologous infection.
Conclusion: This study yielded a very safe *S.* Typhimurium vaccine candidate that could be further refined for mucosal application against disease in humans.

Introduction

Non-typhoidal *Salmonella* (NTS) are of considerable concern to public health, animals and the food industry worldwide.[1] *Salmonella enterica* serovar Typhimurium, including its monophasic variant, and *S. enterica* serovar Enteritidis are among the top five serotypes worldwide,[2,3] and have become major causes of serious invasive infections (iNTS) associated with higher case fatality. Children with malnutrition, severe anaemia, malaria or human immunodeficiency virus (HIV) and HIV-infected adults in the sub-Saharan Africa, as well as elderly people and immunocompromised individuals worldwide are at risk. The economic and human health burden is considerable, even in high-income areas.[4—9]

The major *S.* Typhimurium strains associated with gastroenteritis mostly belong to sequence types (ST) 19 and ST34, although other STs has also been detected[10,11]; while a distinct ST313 has emerged in sub-Saharan Africa adapted to cause iNTS in human beings.[5,12—14] The emergence of a new ST34 variant has also been reported to be associated with invasive disease in Asian regions.[15]

On-going human epidemics of *S.* Typhimurium have largely been caused by multidrug-resistant (MDR) strains.[16] MDR is usually associated with poorer clinical outcomes and higher risk of invasive infections and case fatality rates.[17—20] As an example, the MDR monophasic ST34 variant of *S.* Typhimurium has recently caused an international outbreak linked to consumption of chocolate products with an exceptionally high proportion of cases requiring hospitalization.[21] Vaccines are well-recognized tools for fighting resistance,[22] but there are currently no licensed NTS vaccines for human medicine.[23,24] Proof-of-principle studies with live-attenuated *S.* Typhimurium mutants have been performed in mice,[25—29] but only two have reached clinical phases. Despite well tolerated and immunogenic, they presented safety issues, with undesirable bacterial shedding.[30,31] The development of live vaccines from bacteria auxotrophic for D-amino acids present in the cell wall is regarded a promising prophylactic strategy.[32,33] In this study we constructed and characterized auxotrophic mutants of *S.* Typhimurium and explored their usefulness in systemic and intestinal BALB/c mice infection models.

Methods

Bacterial strains, growth conditions and DNA methods

All *Escherichia coli* and *Salmonella* strains used in the study (Table S1) were grown in Luria—Bertani broth (LB: 10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} sodium chloride) or on LB agar at 37 °C unless otherwise stated. Motility medium was prepared by dissolving 0.3% of Eiken agar in LB. Ampicillin and kanamycin antibiotics were used at concentration of 100—200 μg/mL. L-(+)-arabinose, D-glutamate and D-alanine were used at 10 mM unless otherwise specified.

Construction of glutamate racemase and alanine racemases *S.* Typhimurium deletion mutants

The genome of the *S.* Typhimurium IRTA GN-3728 strain was sequenced by Illumina MiSeq PE300, assembled and annotated using RAST (Rapid Annotation using Subsystem Technology). Genome analysis was carried out to identify genes encoding glutamate —*murI*— and alanine —*dadX* and *alr*— racemases. In-frame deletions were produced via λ-Red recombination.[34] Primers used to generate knockouts contained 40-nt extensions homologous to the 5′ and 3′ ends of the target region to be deleted (Table S1). PCR and sequencing were used to verify all replacements and deletions. Unless stated otherwise, the deletion mutants lacking the *aph(3)-IIIa* gene were used throughout the study.

Determination of D-glutamate and D-alanine requirements

Bacteria from log-phase cultures were inoculated on LB agar supplemented with different concentrations of D-glutamate (0.005—10 mM) for IRTA Δ*murI* or on 10 mM D-glutamate LB agar with different concentrations of D-

alanine (0.005—10 mM) for IRTA Δ*murI* Δ*alr* Δ*dadX*. Plates were examined after incubation at 37 °C.

Growth and viability curves

Growth and viability were determined as previously described.[32] Samples were taken every 60 min to determine culture turbidity (OD_{600}) and colony-forming units (CFU) by plating in appropriate medium. All cultures were done in triplicate.

Surface motility assays, phase switching and slide agglutination tests

Motility LB plates were supplemented as necessary and stab-inoculated with each strain. For phase inversion hyperimmune antiserum (SG2 antiserum d + i + e,h, Bio-Rad Laboratories) was added to plates prior to be inoculated. Motility was observed sporadically throughout 24-h of incubation at 37 °C. Cultures were agglutinated with H-typing antiserum (*Salmonella* monovalent H antiserum:i and:2, Bio-Rad Laboratories). The presence or absence of agglutination was determined visually.

Electron microscopy

Samples were prepared for scanning and transmission electron microscopy (SEM and TEM, respectively) as previously described.[32]

Reversion analysis

Bacterial cultures obtained in quadruplicate from LB plates (supplemented as necessary) were re-suspended in LB and spread on LB agar and supplemented LB agar, in parallel, to quantify the phenotypic reversion rate. Plates were examined after 6 days of incubation at 37 °C. Reversion experiments were conducted in duplicate.

Control of phenotypic stability

Overnight bacteria were used to inoculate tubes containing LB ($n = 6$) and TSB (Tryptic Soy Broth, $n = 6$) which were incubated at 37 °C under agitation and examined over time.

ELISA assays

The levels of specific antibodies were measured by ELISA against formalin-inactivated bacteria, prepared by incubation with 1% (v/v) paraformaldehyde.[32] HRP-labelled anti-mouse secondary antibodies were used for total IgG (Sigma—Aldrich) or IgA (Bethyl Laboratories Inc.). Fecal lactoferrin was quantified by ELISA and expressed as ng lactoferrin/g of feces.[35]

Animal ethics statement

Female BALB/c mice of age 6—9 weeks were used with the approval of the Ethics and Clinical Research Committee of CFT-XXIAC (15002/2018/09) and performed in full compliance with the European Union recommendations and the guidelines (Directive 2010/63/EU) and with the current national legislation (RD 53/2013) on the protection of animals used for scientific purposes. All mice were bred and maintained under specific pathogen-free conditions in the Centro Tecnológico de Formación de la Xerencia de Xestión Integrada A Coruña (CTF-XXIAC), Servicio Galego de Saúde.

Mouse infection models

The systemic infection was initiated by intraperitonal (IP) injection with 0.1 mL of *S.* Typhimurium strains. Clinical signs of disease were monitored daily and survival was recorded for 7 days. The intestinal infection was induced in mice pretreated with 20 mg of streptomycin (STR) by orogastric (OG) gavage 24-h before infection with 0.2 mL of bacteria by the same route.[36] Fresh fecal pellets were collected for ELISA tests or for bacterial quantification (CFU/g) on *Salmonella* chromogenic agar (Condalab) or supplemented LB agar. Illeum, large intestine/colon, cecum, mesenteric lymph nodes (MLNs), spleen and liver were recovered, homogenized, and plated to determine CFU/g organ. Mice were monitored daily to detect clinical signs of disease.

Inoculation of mice with attenuated derivatives and sampling

S. Typhimurium derivatives were inoculated by IP (IRTA Δ*murI* or IRTA Δ*murI* Δ*alr* Δ*dadX*) or OG (IRTA Δ*murI* Δ*alr*::*aph(3)-IIIa* Δ*dadX*, also named IRTA ΔΔΔ::*aph(3)-IIIa*). Immunizations consisted of one, two or three doses. Mice were weighted and monitored to detect any change in behaviour or disease symptoms. Blood and vaginal lavage fluids were collected as previously described.[32] Fecal samples (0.1 g) were collected and dissolved in cooled sterile 0.9% NaCl with protein inhibitors (Inhibitor Protein Cocktail, Sigma Aldrich) for ELISA tests. Fecal shedding was quantified by plating in LB with 10 mM D-glutamate, 10 mM D-alanine and 200 μg/mL of kanamycin. CFU counts were performed after 24-h of incubation at 37 °C.

Statistical analysis

Statistical analysis was performed and graphics were generated in GraphPad Prism 6.01 (GraphPad Software Inc., San Diego, CA, USA). Mann—Whitney *U* test, Log-rank Mantel-Cox test or unpaired *t* test were applied to compare respectively antibody titres, percent survival or means of body weight between two groups of mice. A value of $P < 0.05$ was considered statistically significant.

Results

Construction and characterization of a glutamate racemase deficient derivative of *S.* Typhimurium

Genome sequencing of *S.* Typhimurium IRTA GN-3728 revealed the presence of a single gene annotated as *murI* and encoding a putative glutamate racemase enzyme (EC 5.1.1.3). No additional genes were identified as being

directly implicated in D-glutamate formation. To obtain D-glutamate auxotrophs, the *murI* gene was removed (Fig. S1, Table S1). IRTA Δ*murI* failed to grow in LB and an estimated minimal concentration of 5 mM D-glutamate was required to restore growth in LB agar (Fig. S2). The growth rate of IRTA Δ*murI* in LB containing 5 mM D-glutamate was similar to that of the wild-type strain (Fig. 1A). However, a decrease in viability was observed for IRTA Δ*murI* when starved of D-glutamate (Fig. 1B). SEM analysis revealed damaged bacteria and cell shape alterations in the Δ*murI* mutant at suboptimal concentrations of D-glutamate (Fig. S3), and loss of the envelope integrity, as observed by TEM, in the absence of D-glutamate (Fig. 2). Also, the motility phenotype and ability to express both types of flagellin of the wild-type strain were unaltered in IRTA Δ*murI* (data not shown).

S. Typhimurium IRTA Δ*murI* exhibits reduced virulence in BALB/c mice

When BALB/c mice were infected IP with the wild-type strain, 100% mice succumbed to infection at all the doses tested (Fig. 3A) being the minimal lethal dose for which 100% of the mice died (LD_{100}) 2.2×10^4 CFU. Interestingly, mice receiving 4.5×10^7 CFU of IRTA Δ*murI* were able to clear the infection and remained healthy (Fig. 3B). The LD_{100} for IRTA Δ*murI* was of 7×10^7 CFU, which is more than 3-log higher than that of the parental strain.

S. Typhimurium IRTA Δ*murI* is immunogenic, confers protection against lethal challenge but displays undesirable phenotypic reversion rates

High serum IgG levels were observed in mice IP injected with IRTA Δ*murI* (Fig. 4A) being the prime-immunization with a dose as low as 2.8×10^6 CFU sufficient to induce IgGs as early as on day 7 (Fig. 4B). After the challenge with the wild-type strain all vaccinated mice survived, while the 100% control mice succumbed to infection ($P < 0.0005$, Log-rank test) (Fig. 4C). We then evaluated whether loss of D-glutamate auxotrophy could occur in this mutant by analyzing the phenotypic reversion rates. CFUs were not detected on non-supplemented LB agar for bacteria concentrations equal to or less than 2×10^8 CFU/mL. By contrast, prototrophic clones were recovered on LB agar at concentrations of 8×10^8 CFU/mL and above (data not shown). Due to the safety requirements in live vaccines, IRTA Δ*murI* was engineered by generating a double auxotroph with additional dependence on D-alanine for growth to decrease the frequency of phenotypic reversion.

Construction and characterization of a double auxotroph for D-glutamate plus D-alanine

Two genes were identified in the genome of IRTA GN-3728—*dadX* and *alr*— presumably coding for two isoforms

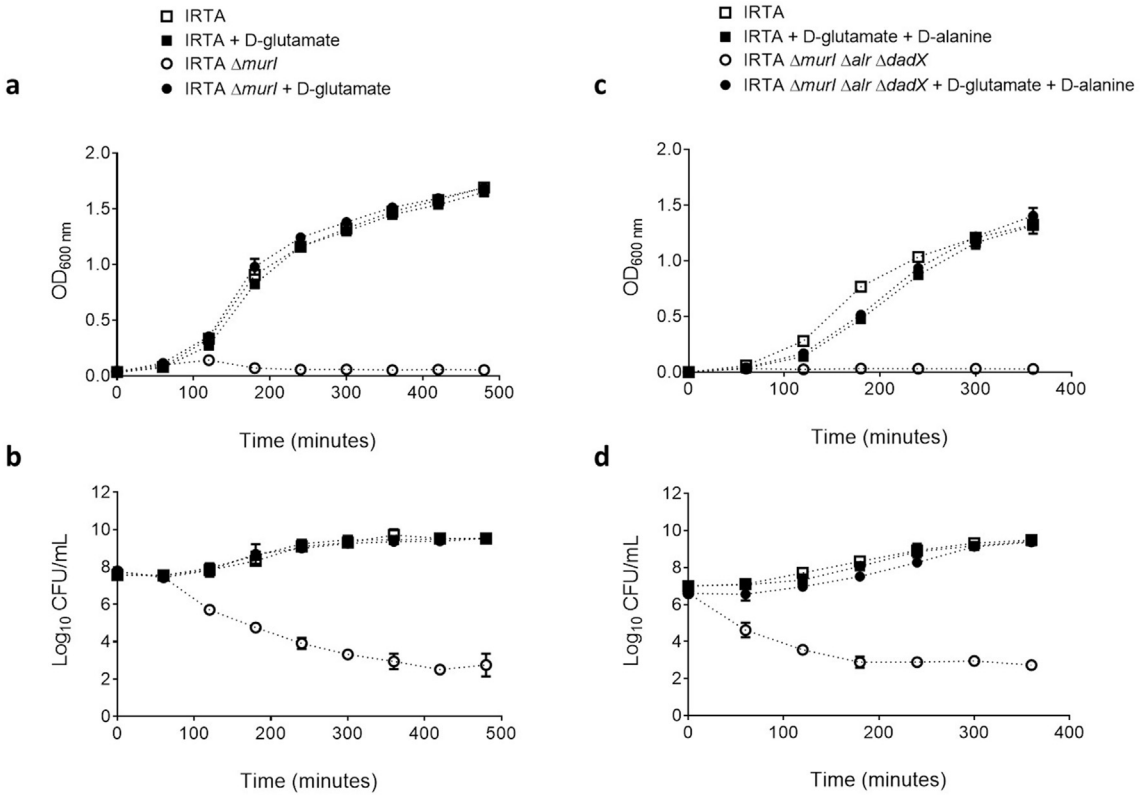

Figure 1. Growth and viability of *S.* Typhimurium IRTA GN-3728 Δ*murI* and IRTA GN-3728 Δ*murI* Δ*alr* Δ*dadX*. (a, c) Growth was monitored by measuring OD_{600} values at 1-h intervals. (b, d) Viability (Log_{10} CFU/mL) was determined by plating cultured bacteria on supplemented LB as required for each mutant strain. All cultures were prepared in triplicate.

of the alanine racemase enzyme (EC 5.1.1.1). Deletion of *dadX* yielded IRTA Δ*murI* Δ*dadX*, which could be recovered in the absence of D-alanine. The *alr* locus was then removed to produce IRTA Δ*murI* Δ*alr* Δ*dadX* (Fig. S1) that required 5 mM D-alanine for recovery and growth in LB agar (Fig. S2). Growth rates were equivalent for the triple mutant and wild-type strains in LB with D-glutamate plus D-alanine at 10 mM (Fig. 1C) but IRTA Δ*murI* Δ*alr* Δ*dadX* showed gradual loss of viability over time in LB (Fig. 1D). The motile phenotype of IRTA Δ*murI* Δ*alr* Δ*dadX* and its ability to switch between flagella were also confirmed as previously (data not shown).

S. Typhimurium IRTA Δ*murI* Δ*alr* Δ*dadX* exhibits a non-reverting and stable auxotrophic phenotype

In the reversion analysis, no CFUs were recovered from LB agar at concentrations equal to or lower than 7.5×10^9 CFU/mL. By contrast, when higher concentrations (1.5×10^{10} CFU/mL and above) were plated several colonies were recovered. The prototrophic clones were subcultured on fresh LB plates that were clean of any growth after optimal incubation. Moreover, the stability of the double auxotrophy was confirmed during prolonged incubation, as no growth occurred after 87 days of cultivation in LB and TSB media (data not shown).

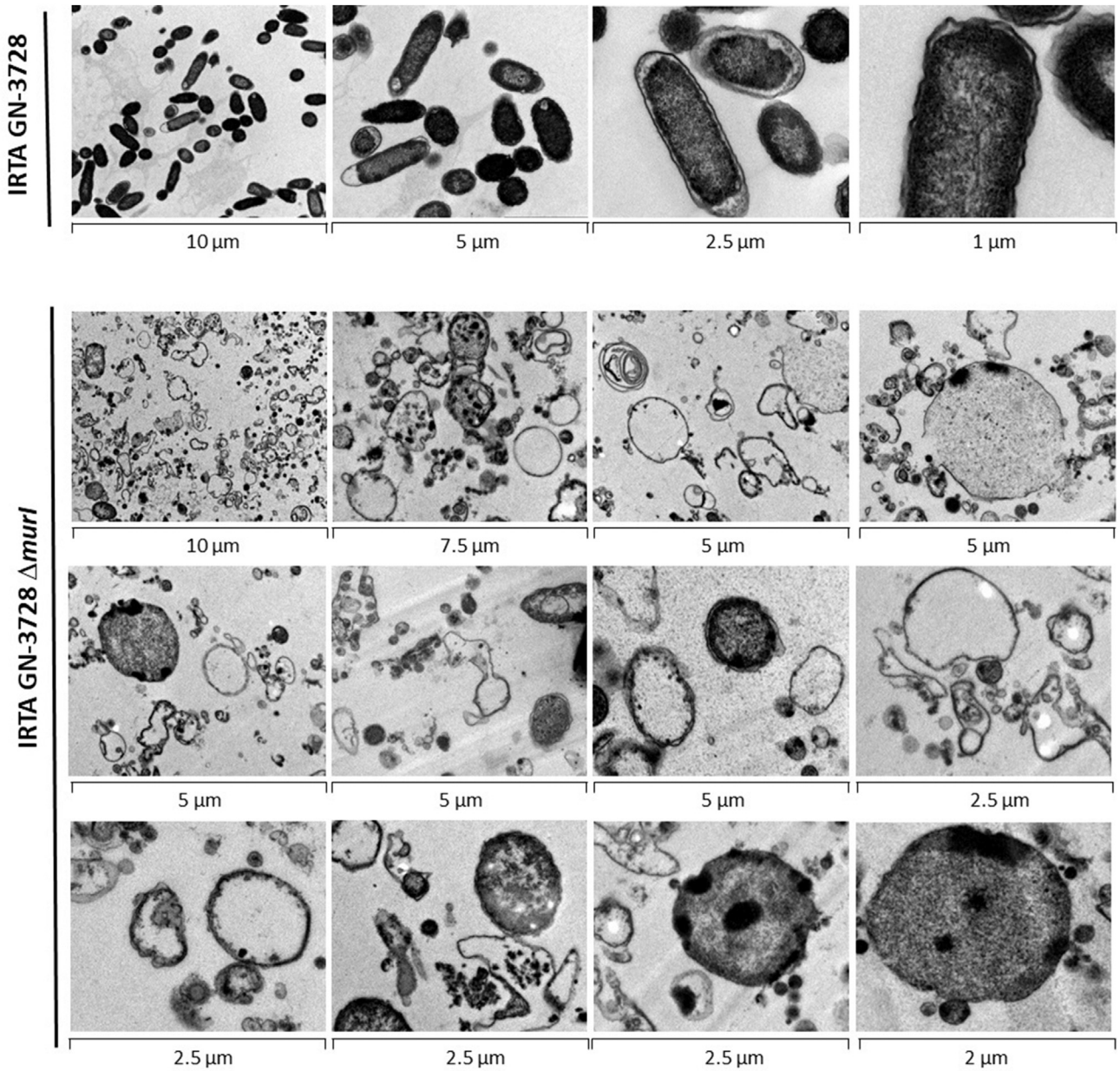

Figure 2. TEM images of morphological alterations shown by *S.* Typhimurium IRTA GN-3728 Δ*murI* under D-glutamate starvation. Micrographs of the wild-type strain are included as controls.

S. Typhimurium IRTA Δ*murI* Δ*alr* Δ*dadX* is highly attenuated in BALB/c mice while maintaining immunogenicity and the ability to confer protection against lethal infection

The virulence of the IRTA Δ*murI* Δ*alr* Δ*dadX* strain was assessed (Fig. 3C) and the LD_{100} was 2.9×10^8 CFU. Interestingly, 100% of the mice injected with a dose as high as 1.49×10^8 CFU overcame the infection, whereas equivalent dose of IRTA Δ*murI* was 100% lethal. Thus, the simultaneous deletion of glutamate and alanine racemase-coding genes enlarged attenuation in *S.* Typhimurium, but did not affect protective immunity. All vaccinated mice showed statistical increase in serum IgG levels on day 8 after the prime immunization that increased gradually until day 27 (Fig. 4D and E). Moreover, immunized mice were 100% protected after a lethal challenge, while all unvaccinated mice succumbed to infection ($P < 0.0005$, Log-rank test) (Fig. 4D and F).

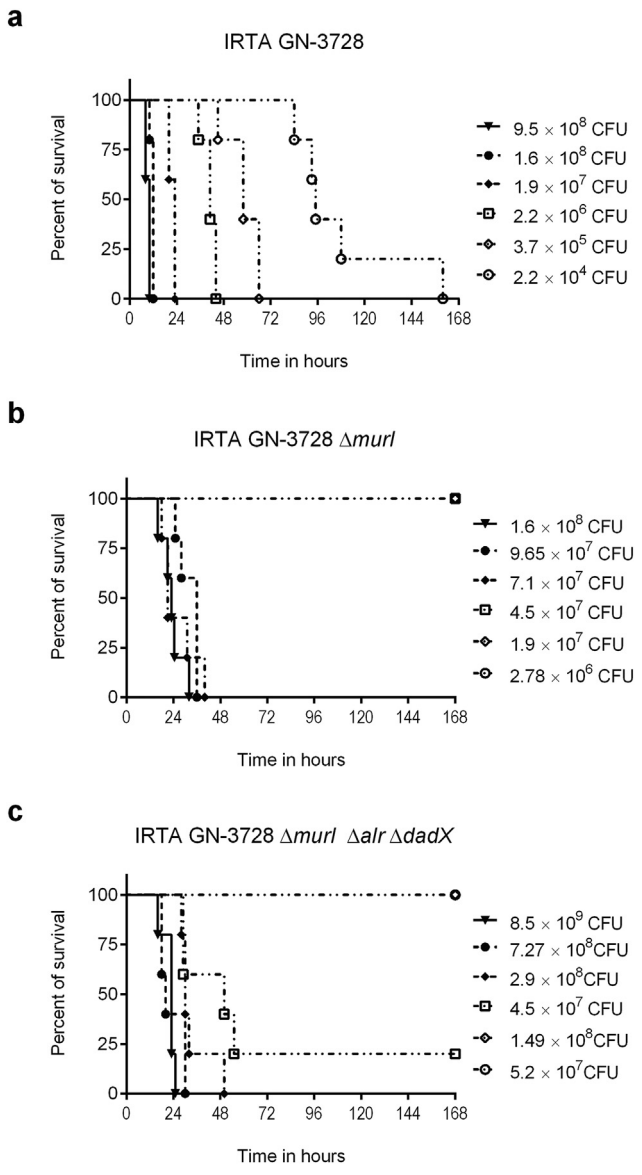

Figure 3. Survival of BALB/c mice infected by the IP route with the indicated CFUs of (a) IRTA GN-3728, (b) IRTA GN-3728 Δ*murI* and (c) IRTA GN-3728 Δ*murI* Δ*alr* Δ*dadX*. Each experimental group consisted of 5 mice. Mice were monitored for signs of disease and mortality over 7 days.

Oral intestinal model: the double D-glutamate plus D-alanine auxotroph of *S.* Typhimurium is attenuated in BALB/c mice

Using an intestinal model, the triple mutant showed a limited ability to persist in the long-term, considering fecal cultures, in contrast to that observed with the wild-type (Table 1). Interestingly, infection with the wild-type strain yielded significantly higher fecal lactoferrin concentrations than the triple mutant that were comparable to those in the mock group receiving saline (Table 1). Moreover, no bacteria were detected in mice tissues after infection with the mutant strain on day post-infection (DPI) 35, while high counts of the wild-type strain were recovered (Table 2). Also, significant differences were seen for IRTA GN-3728 infected mice in relation to the weights of intestines and MLNs in comparison with mock and triple mutant strain groups (Table 2), and the intestines were swollen and pale only in the first case (data not shown).

Oral vaccination with the double auxotroph is safe, occurs with a transient colonization and stimulates cross-reactive immune responses in BALB/c mice

We studied the double auxotroph as a mucosal vaccine. First, after repeated oral inoculation a transient colonization was observed independently of the dosage (10^7, 10^8 or 10^9 CFU), and no negative effects on the appearance, mouse behavior or body weights were detected (Fig. S4). Specific fecal IgA and serum IgG triggered by the oral route were evaluated and the more prominent antibody response was observed with three doses, each of 10^9 CFU (Fig. S5). No IgA were detected in vaginal lavage fluids for any of the groups of vaccinated mice (data not shown). Interestingly, fecal IgA and serum IgG from mice inoculated with the three doses each of 10^9 CFU were able to specifically bind a variety of relevant *S.* Typhimurium strains (Fig. 5, Table S1).

Oral vaccination confers protection against local colonization, extra-intestinal dissemination and promotes extended survival against lethal challenge in BALB/c mice

After a two-dose immunization schedule of 10^9 CFU, mice were orally infected with IRTA GN-3728 (Fig. 6A). Monitoring of mice body weight revealed statistically significant variation between groups ($P < 0.05$, unpaired *t* test) (Fig. 6B). Indeed, a gradual decrease in body weight was observed in control mice, reaching minimal values that correlated with a higher fecal shedding on DPI 8 than the vaccinated mice ($P < 0.05$, Mann–Whitney *U* test). Significant differences were also identified between groups in the bacterial load from the large intestines on DPI 12 (Fig. 6C). After three doses (Fig. 6D), the body weight between the two mice groups evolved significantly different (Fig. 6E), fecal shedding was more than 1-log lower in vaccinated mice on DPI 6 ($P < 0.05$, Mann–Whitney *U* test), and a significant decrease in the burden was obtained from the large intestines on DPI 8 (Fig. 6F). Longer-term protection was also demonstrated

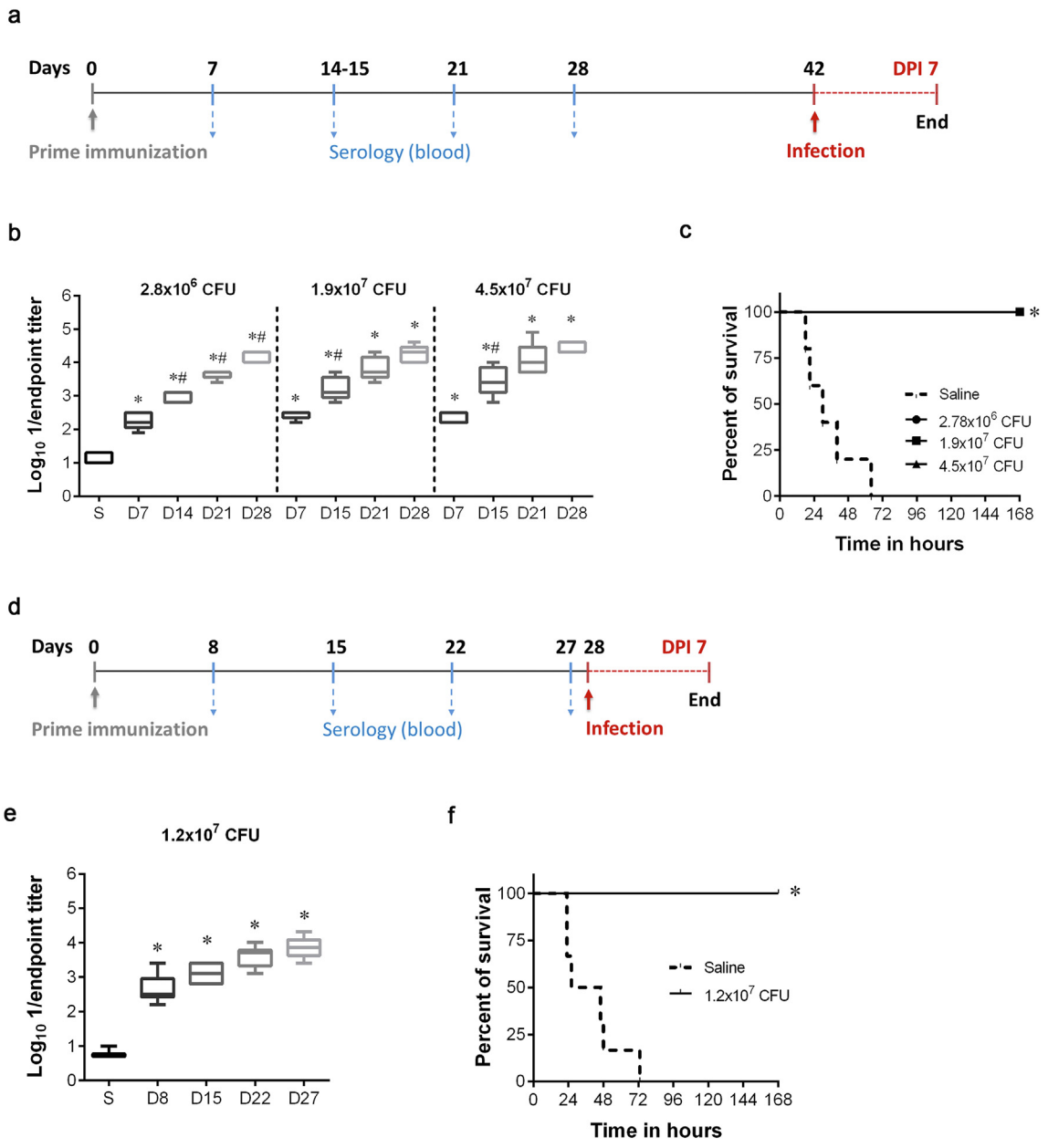

Figure 4. Parenteral inoculation with IRTA GN-3728 Δ*murI* and IRTA GN-3728 Δ*murI* Δ*alr* Δ*dadX* mutants elicits IgG antibodies and conferred protection against lethal systemic infection. Schedules used for vaccination with (a) IRTA GN-3728 Δ*murI* and (d) IRTA GN-3728 Δ*murI* Δ*alr* Δ*dadX*. Log_{10} 1/Endpoint titre of IgG antibodies elicited against IRTA GN-3728 on the indicated post inoculation days after prime immunization with the specified CFUs of (b) IRTA GN-3728 Δ*murI* and (e) IRTA Δ*murI* Δ*alr* Δ*dadX*, relative to control (saline) group. The antibody titres were determined by indirect ELISA. *$P < 0.01$ (Mann—Whitney *U* test), relative to the control (saline) mice. #$P < 0.01$, relative to the preceding condition. Percent survival of mice immunized with (c) IRTA GN-3728 Δ*murI* and (f) IRTA GN-3728 Δ*murI* Δ*alr* Δ*dadX* after challenge with 2×10^6 CFU of IRTA GN-3728. **$P < 0.0005$ (Log-rank Mantel-Cox test), relative to control (saline) mice. Each experimental group consisted of 5—6 mice. Mice were monitored for signs of disease and mortality over 7 days. S, saline; D, day.

after challenging mice on day 75 (Fig. 6G) as reductions in bacterial load from the large intestine and the liver of vaccinated mice were detected (Fig. 6H). Accordingly, fecal IgA and serum IgG remained high in this group on days 55 and 71 (Fig. 6I).

Further evaluation of a three-dose schedule of 10^9 CFU per dose was carried out against heterologous strains. After infection with one of the outbreak isolates (20220258) (Fig. 7A), the vaccinated mice were protected from the pronounced loss of body weight observed in the unimmunized mice (Fig. 7B), and from the bacterial burden in the cecum, large intestine, liver and spleen on DPI 7 (Fig. 7C). Moreover, lethal infection with the highly-virulent ATCC 14028 strain caused dead of 100% of unimmunized mice

Table 1 Fecal bacterial loads and lactoferrin levels in BALB/c mice infected with the wild-type and the double D-glutamate plus D-alanine auxotroph of *S.* Typhimurium.

Days post-infection	Strain	Percentage of mice with negative fecal cultures (number negative/total)	Log_{10} CFU/g feces of positive feces (mean ± S.D.)	ng of lactoferrin/g feces (mean ± S.D.)
DPI 2	Mock	n.d.	n.d.	54.67 ± 32.77
	IRTA ΔΔΔ::*aph(3)-IIIa*	0 (0/5)	5.66 ± 2.25	69.14 ± 15.85
	IRTA GN-3728	0 (0/5)	8.43 ± 0.29[b]	146.13 ± 48.81[a,b]
DPI 6	Mock	n.d.	n.d.	33.47 ± 5.77
	IRTA ΔΔΔ::*aph(3)-IIIa*	60 (3/5)	3.70 ± 0.43	38.91 ± 14.56
	IRTA GN-3728	0 (0/5)	8.90 ± 0.62[b]	223.23 ± 154.47[b]
	Mock	n.d.	n.d.	43.62 ± 8.14
DPI 9	IRTA ΔΔΔ::*aph(3)-IIIa*	100 (5/5)	–	26.04 ± 8.48[a]
	IRTA GN-3728	0 (0/5)	8.32 ± 0.25	131.22 ± 68.29[b]
	Mock	n.d.	n.d.	34.48 ± 15.42
DPI 20	IRTA ΔΔΔ::*aph(3)-IIIa*	100 (5/5)	–	66.47 ± 55.40
	IRTA GN-3728	0 (0/5)	9.05 ± 0.83	243.01 ± 152.24[b]
	Mock	n.d.	n.d.	59.49 ± 16.58
DPI 34	IRTA ΔΔΔ::*aph(3)-IIIa*	100 (5/5)	–	64.44 ± 26.61
	IRTA GN-3728	0 (0/5)	8.27 ± 1.02	102.05 ± 22.24[a,b]

IRTA ΔΔΔ::*aph(3)-IIIa*, triple mutant vaccine strain with resistance to kanamycin; S.D., standard deviation; n.d., not done; –, not applicable; $P < 0.05$: [a] relative to mock group receiving saline; [b]relative to IRTA ΔΔΔ::*aph(3)-IIIa* -infected group.

between DPI 3 and DPI 7, while the first death in the vaccinated group occurred on DPI 6 (Fig. 7D). Accordingly, evident signs of illness were observed in control mice during the first week after challenge. By contrast, most vaccinated mice exhibited normal behavior and appearance at the same time and showed significantly prolonged survival ($P = 0.0044$, Log-rank Mantel-Cox test) (Fig. 7E).

Discussion

Currently, there is an unmet need for effective vaccines against *S.* Typhimurium which represents a leading causative agent of human gastroenteritis and invasive disease in endemic regions and populations at risk globally. Vaccines based on live bacteria have been extensively considered in

Table 2 Bacterial burden and weight of tissues recovered from BALB/c mice infected with the wild-type and the double auxotroph of *S.* Typhimurium on day post-infection 35.

Organ	Strain	Weight (g) of organ (mean ± S.D.)	Percentage of mice with negative cultures (number negative/total)	Log_{10} CFU/g organ of positive cultures (mean ± S.D.)
Spleen	Mock	n.r	n.d.	n.d.
	IRTA ΔΔΔ::*aph(3)-IIIa*	n.r	100 (5/5)	–
	IRTA GN-3728	n.r	0 (0/5)	3.22 ± 1.41
Liver	Mock	n.r	n.d.	n.d.
	IRTA ΔΔΔ::*aph(3)-IIIa*	n.r	100 (5/5)	–
	IRTA GN-3728	n.r	20 (1/5)	2.75 ± 1.74
MLNs	Mock	0.060 ± 0.014	n.d.	n.d.
	IRTA ΔΔΔ::*aph(3)-IIIa*	0.071 ± 0.022	100 (5/5)	–
	IRTA GN-3728	0.142 ± 0.045[a,b]	0 (0/5)	3.59 ± 0.26
Illeum	Mock	0.066 ± 0.013	n.d.	n.d.
	IRTA ΔΔΔ::*aph(3)-IIIa*	0.082 ± 0.016	100 (5/5)	–
	IRTA GN-3728	0.116 ± 0.026[a,b]	40 (2/5)	4.84 ± 0.47
Cecum	Mock	0.055 ± 0.008	n.d.	n.d.
	IRTA ΔΔΔ::*aph(3)-IIIa*	0.069 ± 0.013	100 (5/5)	–
	IRTA GN-3728	0.157 ± 0.042[a,b]	0 (0/5)	6.45 ± 1.45
Colon	Mock	0.184 ± 0.022	n.d.	n.d.
	IRTA ΔΔΔ::*aph(3)-IIIa*	0.187 ± 0.017	100 (5/5)	–
	IRTA GN-3728	0.289 ± 0.047[a,b]	0 (0/5)	6.56 ± 1.42

IRTA ΔΔΔ::*aph(3)-IIIa*, triple mutant vaccine strain with resistance to kanamycin; S.D., standard deviation; n.d., not done; n.r., not relevant; –, not applicable; $P < 0.05$: [a] relative to mock group receiving saline; [b]relative to IRTA ΔΔΔ::*aph(3)-IIIa* -infected group.

view of their plausible advantages over other formulations,[24,25] however the fine tuning required for balanced safety and protective immunity have recurrently posed a challenge for the development of anti-NTS vaccines.[37] Many live-attenuated *S.* Typhimurium strains have been explored,[23–25] but the prolonged and/or persistent fecal shedding observed in mice and/or human volunteers restrained further developments. In this study, we constructed vaccine candidates with particular emphasis on safety issues. We have demonstrated that deleting the glutamate racemase-coding gene in a *S.* Typhimurium strain (IRTA Δ*murI*) renders this derivative auxotrophic to D-glutamate, and as a consequence exhibited a lower level of virulence than the wild-type strain. Interestingly, a single inoculation with this mutant elicited serum IgG and protected mice against homologous lethal challenge. Live vaccines preserve pathogen-associated molecular patterns to which the host' immune system is exposed in natural infections, such as flagellin, that also acts as efficacious adjuvant.[38] The IRTA Δ*murI* conserves the flagellated phenotype and the ability to exchange the both flagellins produced by the wild-type strain. Interestingly, hyperflagellated NTS strains have been proposed as oral vaccines and some also as reagent strains for a large-scale purification of flagellin to be included as adjuvant in subunit formulations.[39] Unfortunately, we observed that IRTA Δ*murI* could occasionally regain the wild-type phenotype which may pose a risk when used in humans, and we then reinforced the vaccine safety. The triple mutant, IRTA Δ*murI* Δ*alr* Δ*dadX*, showed a double nutritional dependence on D-glutamate and D-alanine and exhibited a stable, non-reverting phenotype under the tested conditions. The additional deletions of alanine racemase-coding genes led to greater attenuation of IRTA Δ*murI* Δ*alr* Δ*dadX* relative to IRTA Δ*murI*, but no undesirable effect on the protective immunity was detected, or in the flagellated phenotype. Oral formulations mimic the natural route of *Salmonella* infection and evoke mucosal immunity. To date, the usefulness of *S.* Typhimurium mutants remain controversial in view of variable but undesirable time frame of shedding, considerable invasive rates and persistent colonization of mice tissues.[25,26,30,31] Here, we demonstrated a more favorable attenuation profile for the double auxotroph since this mutant retained some ability to colonize mice, but caused low local inflammation, and was completely cleared from mice with undetectable invasiveness in the long-term. Some efforts have been made to reduce fecal excretion in a Δ*aroA* prototype, by deletion of *shdA* and/or

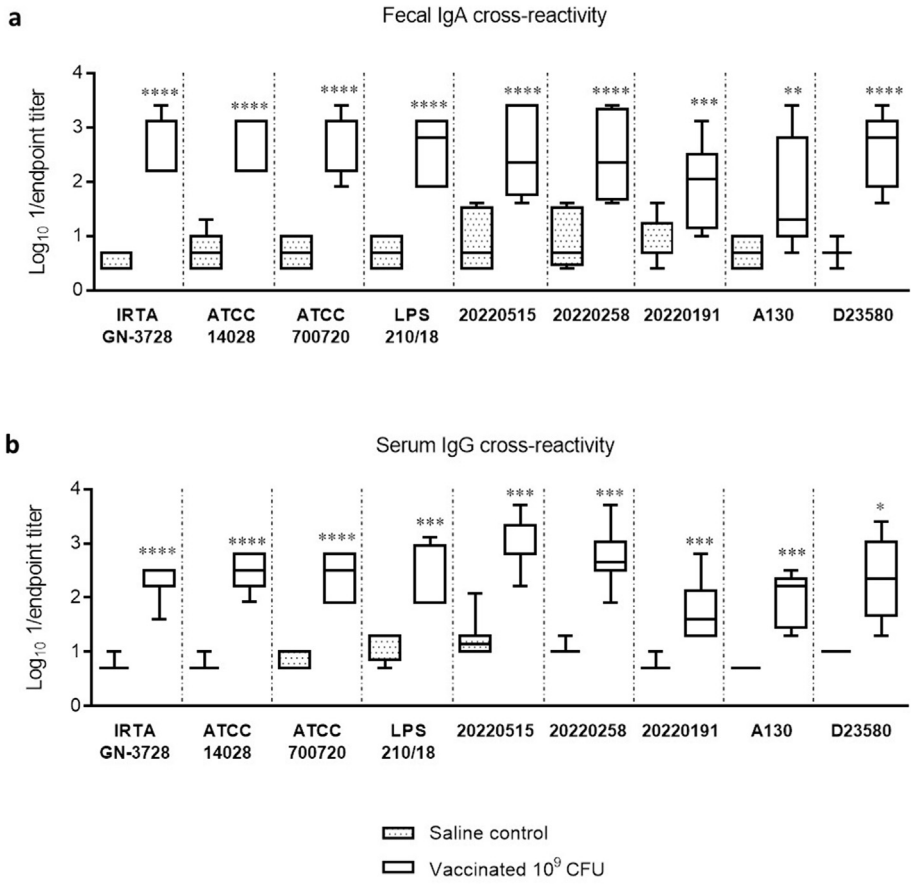

Figure 5. Oral inoculation with the double auxotroph elicits cross-reactive antibodies. Log_{10} 1/Endpoint titre of (a) fecal IgA and (b) serum IgG antibodies produced by BALB/c mice against IRTA GN-3728 and other eight heterologous *S.* Typhimurium strains on the post-vaccination days 34 (for 20220515, 20220258 and 20220191) or 37 (the remaining strains) and in control (saline) mice. The antibody titres were determined by indirect ELISA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, ****$P < 0.001$ (Mann–Whitney U test) relative to control (saline) mice. Each experimental group consisted of 5–8 mice. The *S.* Typhimurium strains used are listed in Table S1.

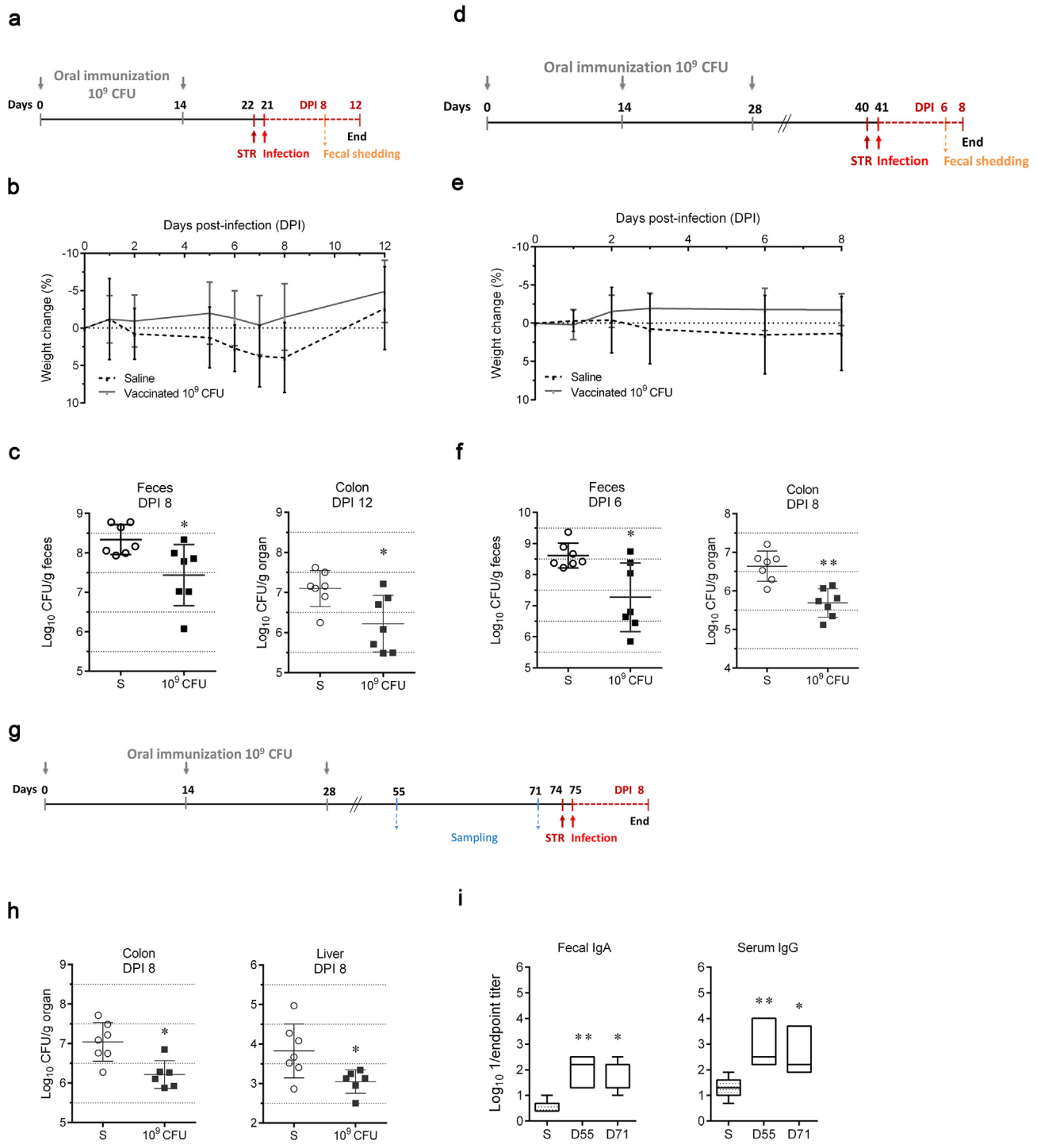

Figure 6. Oral inoculation with the double auxotroph confers partial protection against homologous *Salmonella*. (a, d and g) BALB/c mice were orally inoculated with two or three doses of IRTA GN-3728 Δ*murI* Δ*alr*::*aph(3)-IIIa* Δ*dadX* (dose of 10^9 CFU approximately) and then infected with IRTA GN-3728 (dose of 4×10^6 CFU), by the same route (intestinal model). (b, e) Percentage change in body weight in the inoculated mice relative to control (saline) mice (mean ± s.e.m.). (c, f, h) Bacterial load (Log_{10} CFU/g) from feces and organs determined on the indicated post-infection days in inoculated mice compared to control (saline) mice. $^*P < 0.05$, $^{**}P < 0.005$ (Mann–Whitney *U* test). (i) Log_{10} 1/Endpoint titre of specific fecal IgA and serum IgG antibodies produced by BALB/c mice against IRTA GN-3728 on the indicated days post-inoculation after administration of three doses of IRTA GN-3728 Δ*murI* Δ*alr*::*aph(3)-IIIa* Δ*dadX*. $^*P < 0.005$, $^{**}P < 0.001$ (Mann–Whitney *U* test) relative to the control (saline) mice. Each experimental group consisted of 6–7 mice. STR, streptomycin; DPI, day post-infection; S, saline.

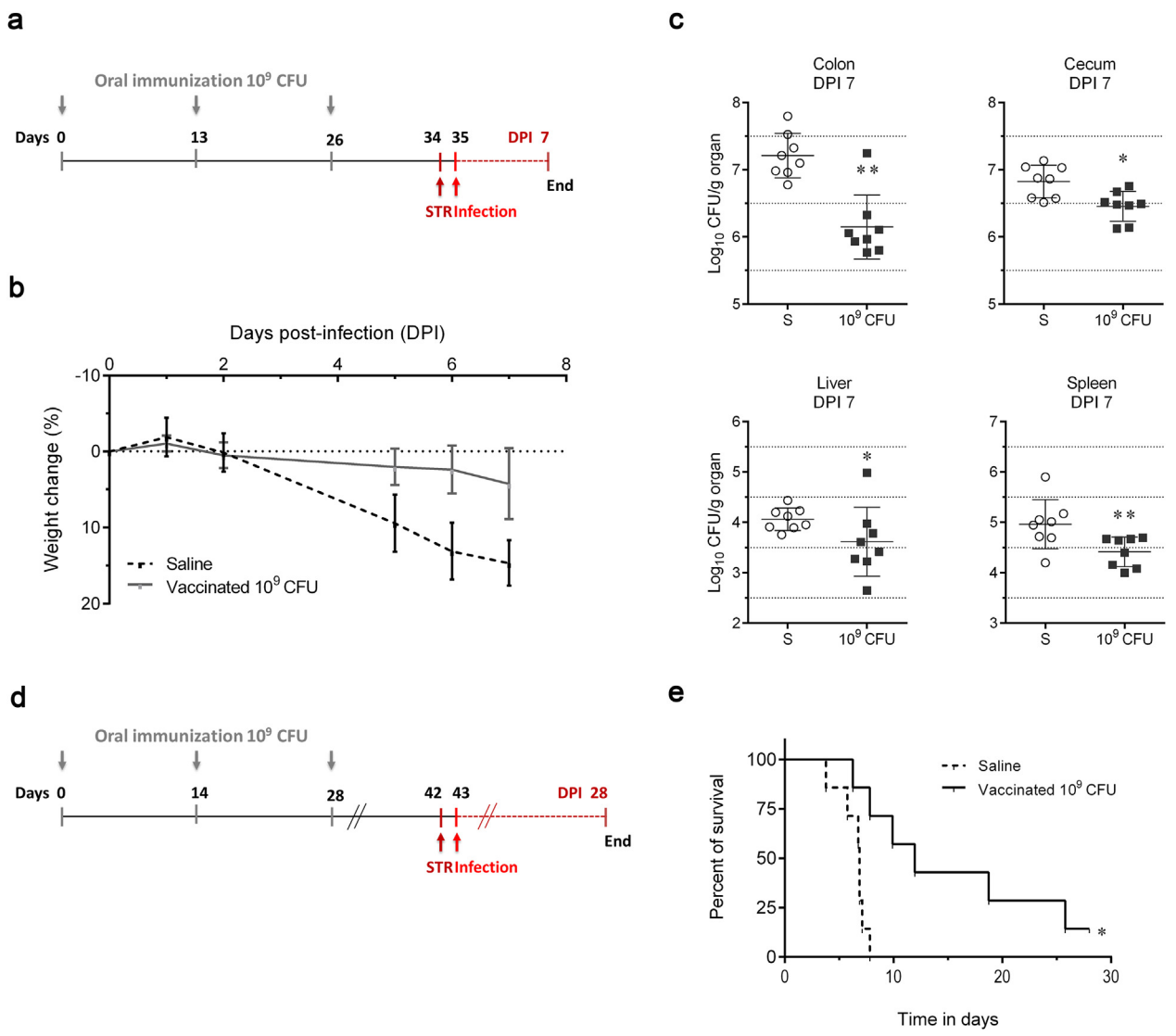

Figure 7. Oral inoculation with the double auxotroph provides protection against heterologous *Salmonella*. BALB/c mice were inoculated three times with IRTA GN-3728 Δ*murI* Δ*alr::aph(3)-IIIa* Δ*dadX* (dose of approximately 10^9 CFU) and infected with (a) 202200258 (dose of 1.5×10^5 CFU) or (d) ATCC 14028 (lethal dose of 3×10^4 CFU) by the same route (intestinal model) as shown. (b) Percentage change in body weight in the inoculated mice relative to control (saline) mice after infection with 202200258 (mean ± s.e.m.). (c) Bacterial load (Log_{10} CFU/g) from organs determined on the post-infection day 7 after infection with 202200258 in inoculated mice relative to that in the control (saline) mice. *$P < 0.05$, **$P < 0.005$ (Mann—Whitney *U* test). (e) Survival of BALB/c mice infected with ATCC 14028. *$P = 0.0044$ according to the Log-rank (Mantel-Cox) test. Mice were monitored for signs of disease and mortality over 28 days. Each experimental group consisted of 7—8 mice. STR, streptomycin; DPI, day post-infection; S, saline.

misL genes, which are directly implicated in shedding. Even though these mutations were able to lower fecal CFUs, bacteria were still detectable on day 14.[40] Further supporting the safety level of the double auxotroph, repeated oral inoculations did not affect mice body weight, and fecal shedding was limited to two days after inoculation. This is comparable with the fecal excretion of the live Ty21a, the only vaccine approved for *Salmonella* in humans.[41] A dedicated balance of attenuation-immunogenicity has been observed for *Salmonella*.[35] Despite the high attenuation level shown by the double auxotroph it was still able to trigger relevant and broad humoral immunity in mice. A single IP inoculation was sufficient to produce IgG in serum and to protect 100% of immunized mice against lethal wild-type infection. In addition, three oral doses of 10^9 CFU promote local IgA together with serum IgG. We also demonstrated moderate, long-lived protection against intestinal infection with the wild-type by preventing loss of weight in the mice, and lowering intestinal colonization. The circulating IgG antibodies were probably also responsible for reducing bacterial loads in the liver. Remarkably, immunized mice were also protected against a representative MDR monophasic *S.* Typhimurium strain that caused a recent international outbreak with increased clinical severity of infection.[21] Vaccination reduced weight loss, partially prevented colonization of intestinal mucosa and

also minimized spreading of bacteria to other tissues, thus limiting the systemic phase of *Salmonella* infection. Interestingly, there was also a notable delay in the time of death after highly lethal infection with ATCC 14028. The ability of other live S. Typhimurium prototypes to confer cross-protection against heterologous strains and/or serotypes has previously been reported.[25,26] Noticeably, the antibodies generated in mice with the S. Typhimurium double auxotroph were able to cross-react with MDR clinical strains that showed extended third-generation cephalosporin resistance, and with epidemic clones of public health concern like those causing extensive foodborne outbreaks or iNTS in sub-Saharan regions.

In conclusion, the double D-glutamate plus D-alanine auxotroph of S. Typhimurium represents a new, promising vaccine candidate which is very safe when administered by the oral route in mice. Notwithstanding the weak virulence of this mutant strain, a moderate and wide protection against disease was achieved. Further studies will be needed to refine protective efficacy of this prototype for mucosal applications in human health.

Data Statement

All relevant data are within the manuscript and its Supporting Information files. Genome of IRTA GN-3728 is available from the corresponding author, upon request.

Author contributions

Conceptualization: P. García, G. Bou; Formal analysis: P. García; Investigation: P. García, M. Moscoso, V. Fuentes-Valverde; Validation: P. García, G. Bou; Writing — original draft: P. García; Writing — review & editing: P. García, M. Moscoso, V. Fuentes-Valverde, M. R. Rodicio, S.Herrera-León and G. Bou.

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Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2022.10.002.