# Attenuated *Salmonella enterica* serovar Typhimurium lacking the ZnuABC transporter: An efficacious orally-administered mucosal vaccine against salmonellosis in pigs

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ABSTRACT

We have recently demonstrated that an attenuated strain of *Salmonella enterica* serovar Typhimurium unable to synthesize the zinc transporter ZnuABC (*S.* Typhimurium *ΔznuABC*), is able to protect mice against systemic and enteric salmonellosis and is safe in pigs. Here, we have tested the protective effects of *S.* Typhimurium *ΔznuABC* in pigs. Resistance to challenge with the fully virulent strain *S.* Typhimurium ATCC 14028 was assessed in animals vaccinated with *S.* Typhimurium *ΔznuABC* (two dosages tested), in controls vaccinated with a formalin-inactivated virulent strain and in unvaccinated controls. Clinical signs of salmonellosis, faecal shedding and bacterial colonization of organs were used to assess vaccine-induced protection. After the challenge, pigs vaccinated with the attenuated *S.* Typhimurium *ΔznuABC* strain did not display clinical signs of salmonellosis (fever or diarrhoea). The vaccine also reduced intestinal tract colonization and faecal shedding of the fully virulent *Salmonella* strain, as compared to control groups. *S.* Typhimurium *ΔznuABC* represents a promising candidate vaccine against salmonellosis in pigs.

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## 1. Introduction

Salmonellosis is one of the most common food-borne zoonoses. *Salmonella* infections in humans may result in several clinical syndromes, including acute gastroenteritis, fever and bacteraemia [1]. A recent epidemiological report attributed 26.9% (95% CI 26.3–27.6%) of human *Salmonella* cases in the EU to the consumption of pork products [2]. Furthermore, the prevalence of *Salmonella* in pigs slaughtered at abbatoir in Europe is medium-high, with 10.3% of animals carrying this pathogen in their lymph nodes [3].

National *Salmonella* monitoring and control programmes have been developed in recent years in different European countries. However, the validity of serology as the method of choice for determining herd prevalence for nationwide *Salmonella* monitoring

programmes has been questioned [4–6]. Regarding control efforts, to date, these are mainly based on measures such as the use of organic acids in feed and water, good management practices, and biosecurity in pig farms (e.g., visitor restriction, pest control) [7]. In addition, appropriate strategies to reduce pig and carcass cross-contamination still need to be perfected [8]. The use of antibiotics is excluded due to the intracellular persistence of this bacterium and the risk of positive selection of resistant clones [9]. In this scenario, vaccination may represent an attractive alternative for the reduction of both *Salmonella* incidence in swine, and the prevalence of carriers at abattoir.

Our research group has recently demonstrated that a mutant strain of *Salmonella enterica* serovar Typhimurium unable to synthesize the zinc transporter ZnuABC (*S.* Typhimurium *ΔznuABC*) is significantly attenuated and able to protect mice against both systemic and enteric salmonellosis, by inducing a strong antigen-specific immune response [10–12]. Furthermore, *S.* Typhimurium *ΔznuABC* was recently shown to be safe and immunogenic in experimentally infected pigs [13].

The aim of the present study was to assess the ability of a vaccine based on the *S.* Typhimurium *ΔznuABC* mutant, to induce protection in pigs against homologous challenge with virulent *S.* Typhimurium.

## 2. Materials and methods

### 2.1. Salmonella spp. cultures

Virulent *Salmonella* Typhimurium ATCC 14028 and its isogenic *znuABC* mutant (*S.* Typhimurium *ΔznuABC*) (*znuABC::cam*), made resistant to streptomycin by P22-mediated transduction of a *hsdR::spec* allele, were used throughout the study [10]. The introduction of the *hsdR::spec* allele does not modify the lethal dose of the wild type and mutant strains in mice (data not shown). The strains were grown overnight at 37 °C in Brain Heart Infusion broth (Oxoid Ltd., Basingstoke, UK), harvested by centrifugation and then washed twice in ice-cold phosphate buffer solution (PBS) (Sigma–Aldrich, Milan, Italy). To obtain bacterin, *S.* Typhimurium ATCC 14028 was inactivated with formalin and adsorbed to aluminium hydroxide.

### 2.2. Animals

A total of 32 female commercial hybrids (Large White × Duroc) 3–5 months of age were enrolled in the study. All were the offspring of five *Salmonella*-free sows (negative for *Salmonella* by both serological and bacteriological tests). The study animals had not been treated with antibiotics nor previously vaccinated and proved to be *Salmonella*-free by both stool culture and serology. Experiments were authorized by national authority in according to Italian and European regulations (D.L. 116/92, 86/609/EEC, Decreto 225/2009-B) and were carried out under the supervision of certified veterinarians.

### 2.3. Experimental design

Pigs were divided into four groups, each of which was kept in a separate pen. Groups A, B, C were composed of six animals each; group D was composed of eight animals. Groups A and B were vaccinated by the oral route, with $5 \times 10^8$ and $5 \times 10^7$ CFU of *S.* Typhimurium *ΔznuABC* in a single dose, respectively. Oral inoculation was performed using a gastric catheter, with *Salmonella* strains suspended in 20 ml of sterile 10% sodium bicarbonate buffer. Group C was primed intramuscularly with a bacterin dose of $2 \times 10^9$ CFU and boosted 14 days later with an identical dose of vaccine. Group D was orally inoculated with saline and served as a naïve control group.

Thirty-four days after vaccination, all animals were weighed and then challenged by the oral route with $4 \times 10^8$ CFU of fully virulent *S.* Typhimurium using a gastric catheter. Here too, *Salmonella* strains were suspended in 20 ml of sterile 10% sodium bicarbonate buffer.

Body temperature and bacterial colonization were used as biomarkers or surrogate endpoints to evaluate the efficacy of vaccines. At 2, 7, 10 and 14 days after the challenge, rectal temperature was recorded and blood, serum and faecal samples were collected to study antigen-specific production of IFN-γ, TNF-α production, and to detect faecal excretion of the challenge strain, respectively.

Diarrhoea was assessed visually by a single independent observer, who attributed a score to each of the samples collected from each animal, using the following scale: (0) faeces with normal appearance; (1) moderately softened faeces, (2) softened faeces, (3) semisolid/semiliquid faeces, (4) liquid faeces, and (5) watery faeces.

Twenty-one days after the challenge, pigs were weighed again, and then euthanized using a captive bolt pistol and exsanguination. Samples of tonsils, liver, kidney, spleen, gall bladder, caecal and mesenteric lymph nodes, lung, duodenum, proximal and distal jejunum, ileum, caecum, colon, stomach content and faeces were collected from each pig for the evaluation of bacterial burden.

To assess the persistence of *Salmonella* in the environment, faecal samples from the pen floors of each group of animals were collected following the ISO 6579:2002/Amendment 1:2007 protocol.

### 2.4. Microbiology

The microbiological analysis of faecal samples, organ samples and environmental (pen floor) samples was conducted according to the ISO 6579:2002/Amendment 1:2007 protocol. Briefly, samples were weighed and homogenized in nine parts of Buffered Peptone Water (BPW) (Oxoid Ltd., UK). This initial solution was then used to perform a decimal dilution series carried out by systematically transferring an aliquot of 0.5 ml of each successive dilution in 4.5 ml of BPW. All BPW-diluted samples were incubated at 37 °C for $18 \pm 3$ h. Cultures (0.1 ml) were subsequently seeded on Modified Semisolid Rappaport-Vassiliadis (MSRV) agar plates (Oxoid Ltd., UK) and incubated at 41.5 °C for 24 h for the selective enrichment of *Salmonella*. A loopful of growth from each MSRV plate was streaked onto Xylose-Lysine-Desoxycholate Agar (Oxoid Ltd., UK) and Brilliant Green Agar (Oxoid Ltd., UK) plates and hence incubated at 37 °C overnight. Five suspect *Salmonella* colonies were subjected to biochemical identification by the BBL Enterotube II system (BD, Franklin Lakes, NJ USA) and to serological identification using *Salmonella* group-specific antisera. This semiquantitative approach allowed us to determine the likely concentration of *Salmonella* in a sample. Distinction between challenge and vaccine strains was carried out by plating bacteria on agar plates containing streptomycin and then transferring individual colonies on agar plates without antibiotics or containing chloramphenicol, where only *S.* Typhimurium *ΔznuABC* can grow. Further confirmation of strain identification was subsequently obtained by analysing at least five colonies from each plate by PCR, exploiting the chromosomal differences between the two strains. Briefly, chromosomal DNA extracted by ZR Fungal/Bacterial DNA Kit (Zymo Research, California, USA) was used as a template for PCR with forward primer oli132 (CGGTAAGAGGGAACGAATCT) and reverse primer oli131 (TCATCAGACCTGGGCGATTT). Amplification of the chromosomal region carrying the modified (deleted) *znuABC* operon yielded a 1384 bp DNA fragment, whereas amplification of the wild type sequence resulted in a 2828 bp DNA fragment.

### 2.5. Production of TNF-α

TNF-α production was measured in sera using a sandwich ELISA (Porcine Quantikine ELISA Kit, R&D Systems, MN, USA) according to the manufacturer's instructions.

### 2.6. Production of IFN-γ

Antigen-specific production of IFN-γ was evaluated after in vitro stimulation of peripheral blood mononuclear cells (PBMC). Aliquots of heparinized blood (1.5 ml) were stimulated with heat-inactivated *Salmonella* ($10^9$ CFU/ml), pokeweed mitogen (PWM) (Sigma–Aldrich, MS, USA) (5 µg/ml) or PBS (Sigma–Aldrich, MS, USA). Supernatants were harvested after a 24 h culture at 37 °C in a humidified 5% $CO_2$ atmosphere. IFN-γ production was evaluated

using a sandwich ELISA (Porcine IFN-γ Quantikine ELISA Kit, R&D Systems, MN, USA) according to the manufacturer's instructions.

### 2.7. Histology

Tissue samples of duodenum, proximal and distal jejunum, ileum, caecum and colon for histological examination were fixed in 10% neutral buffered formalin (pH 7.0) and embedded in paraffin wax. Sections were cut at 4 μm (Leica Microsystems, Wetzlar, Germany), and stained with haematoxylin and eosin. Lymphocytic and eosinophilic infiltration of mucosa, agglutination, blunting and necrosis of villi of all intestinal districts were evaluated.

### 2.8. Statistics

Differences in body temperature were estimated by two-way repeated measures analysis of variance (ANOVA) followed by Tukey multiple comparison tests. Differences in weight gain and TNF-α were estimated by repeated measures analysis of variance (ANOVA) followed by Tukey multiple comparison tests. Differences in diarrhoea severity were estimated by Chi squared test. Differences were considered significant when $P \leq 0.05$.

## 3. Results

### 3.1. Vaccination with S. Typhimurium ΔznuABC considerably attenuates, or even eliminates, the clinical signs and inflammation induced by virulent S. Typhimurium in pigs

Pigs that were vaccinated with *S.* Typhimurium *ΔznuABC* (groups A and B) showed no clinical signs after the challenge infection. Conversely, pigs vaccinated with bacterin and control pigs (groups C and D, respectively) showed prostration and other manifestations of infection. Specifically, pigs of groups A and B had a normal temperature (below 39.5 °C) throughout the observation period and no diarrhoea, while pigs of groups C and D showed anorexia, reluctance to move, and a transient increase in body temperature (Fig. 1). In addition, pigs of groups A and B had normal faeces, whereas pigs of groups C and D had watery diarrhoea (Fig. 2). Histological analysis revealed necrosis of villi in the distal jejunum of unvaccinated pigs (group D) (Supplementary Fig. 1b), and to a lesser extent in vaccinated pigs (group A) (Supplementary Fig. 1a). The large intestine was less severely affected in all animals of all groups. Lymphocytic infiltration was commonly detected in all districts of all groups of animals (Supplementary Fig. 2a), more often in the small intestine of pigs in groups C and D (Supplementary Fig. 2b).

After infection with fully virulent *S.* Typhimurium, pigs of different groups showed different degrees of weight gain, with an average increase of 7.7 kg, 6.1 kg, 5.9 kg, and 3.5 kg, in groups A, B, C and D, respectively (Supplementary Fig. 3). While these results were not statistically significant due to large within-group variability, the animals in groups C and D showed a tendency to gain less weight than the animals vaccinated with *S.* Typhimurium *ΔznuABC*.

Serum TNF-α was measured to assess the inflammatory response induced by challenge with wild-type *S.* Typhimurium. As shown in Fig. 3, pigs vaccinated with *S.* Typhimurium *ΔznuABC* (groups A and B) produced less TNF-α than pigs vaccinated with inactivated *S.* Typhimurium (group C) or unvaccinated pigs (group D).

These findings suggest that vaccination with *S.* Typhimurium *ΔznuABC* reduces both the clinical effects and the proinflammatory response in pigs challenged with virulent *S.* Typhimurium.
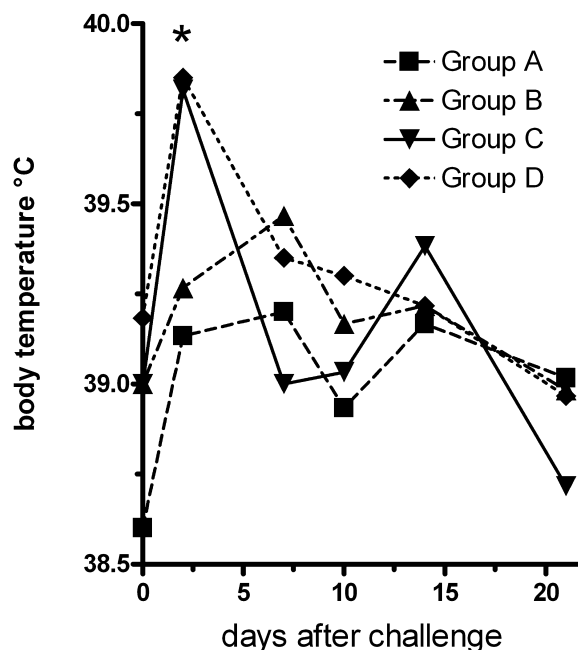


**Fig. 1.** *S.* Typhimurium *ΔznuABC* vaccination prevents a rise in body temperature after challenge infection. Body temperature at different time points after a challenge infection with virulent *S.* Typhimurium in pigs vaccinated with either $5 \times 10^8$ CFU (group A) or $5 \times 10^7$ CFU (group B) of attenuated *S.* Typhimurium *ΔznuABC*; in pigs vaccinated with inactivated *S.* Typhimurium (group C); and in unvaccinated controls (group D). In the upper panel, symbols represent group means and standard errors. In the lower panels, symbols represent individual animals, by group. Data refer to one out of two separate experiments performed with comparable results. The differences between group A and group D were statistically significant ($^*P \leq 0.05$).

### 3.2. Vaccination with S. Typhimurium ΔznuABC reduces faecal shedding after homologous challenge infection with virulent S. Typhimurium

As shown in Fig. 4, as early as 2 days after challenge infection, the levels of *S.* Typhimurium faecal shedding in vaccinated pigs (groups A, B and C) were lower than those of control pigs (group D). Remarkably, at 7 and at 10 days after challenge, pigs of groups A and B shed considerably less bacteria than those of groups C and D. At 14 and 21 days after challenge infection, shedding levels were low overall. At day 14, however, virulent *Salmonella* counts in faecal samples from group A, were still lower than those from group D. Similarly, at day 21 bacterial counts in faecal samples from group B were still lower than those from group D.

These findings suggest that vaccination with *S.* Typhimurium *ΔznuABC* significantly reduces faecal shedding following infection with a virulent strain of *S.* Typhimurium.

### 3.3. Vaccination with S. Typhimurium ΔznuABC reduces virulent S. Typhimurium colonization of organs

As shown in Figs. 5 and 6, the colonization of organs with virulent *S.* Typhimurium in naïve animals (group D) was considerable, with particularly high levels of *Salmonella* in the caecum and in the tonsils, moderate colonization of the jejunum, the ileum and the colon, and low bacterial counts in the stomach and duodenum samples. In contrast, colonization of all organs examined was markedly reduced in pigs from groups A and B. Remarkably, most organ samples taken from pigs in group B were virtually negative. Moreover, the bacteria isolated from the organs of pigs from group A were *S.* Typhimurium *ΔznuABC*, i.e. the vaccine strain (data not shown), suggesting that pigs were strongly protected against the challenge infection but had not completely cleared the vaccine strain. Pigs
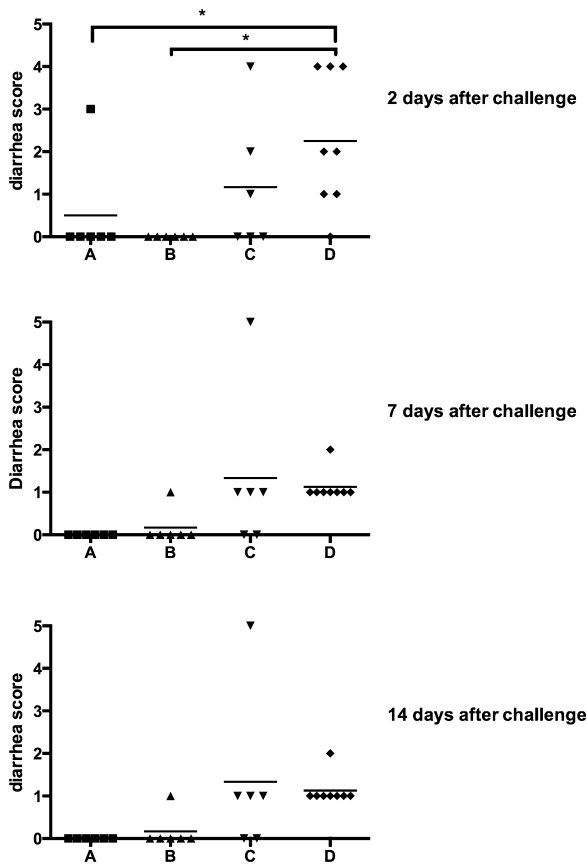
**Fig. 2.** *S.* Typhimurium *ΔznuABC* vaccination reduces diarrhoea in *Salmonella* infected pigs. Diarrhoea scores at different time points after a challenge infection with virulent *S.* Typhimurium in pigs vaccinated with either $5 \times 10^8$ CFU (group A) or $5 \times 10^7$ CFU (group B) of attenuated *S.* Typhimurium *ΔznuABC*; in pigs vaccinated with inactivated *S.* Typhimurium (group C); and in unvaccinated controls (group D). Symbols represent individual animals and bars indicate group means. Data refer to one out of two separate experiments performed with comparable results (*$P \leq 0.05$).

in group C showed essentially the same degree of colonization as pigs in group D. These findings show that vaccination with *S.* Typhimurium *ΔznuABC* reduces wild-type *Salmonella* colonization of the intestinal tract.
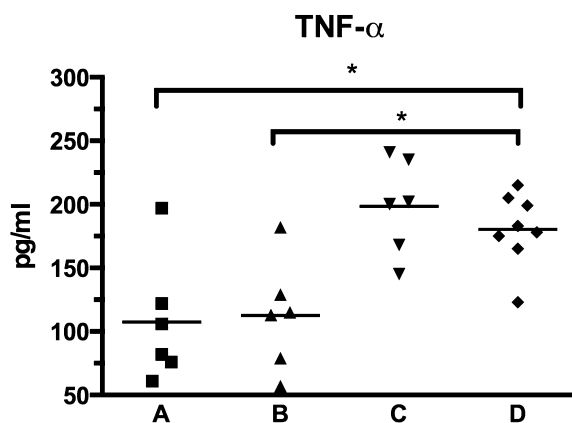


**Fig. 3.** *S.* Typhimurium *ΔznuABC* vaccination reduces inflammation in *Salmonella* infected pigs. TNF-α production 2 days after a challenge infection with virulent *S.* Typhimurium in pigs vaccinated with either $5 \times 10^8$ CFU (group A) or $5 \times 10^7$ CFU (group B) of attenuated *S.* Typhimurium *ΔznuABC;* in pigs vaccinated with inactivated *S.* Typhimurium (group C); and in unvaccinated controls (group D). Symbols represent individual animals and bars indicate group means (*$P \leq 0.05$).
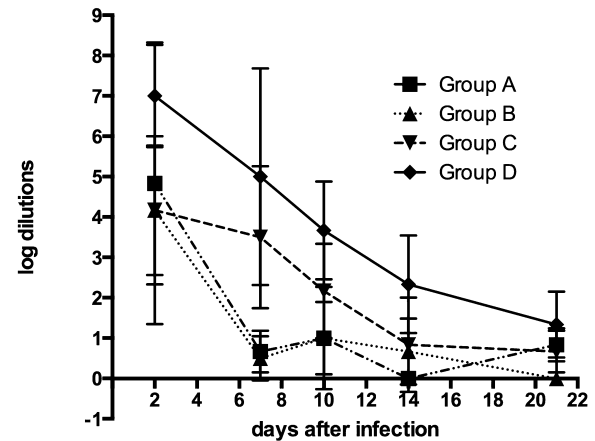


**Fig. 4.** *S.* Typhimurium *ΔznuABC* vaccination reduces *Salmonella* faecal shedding in infected pigs. *Salmonella* faecal shedding, at different time points after a challenge infection with virulent *S.* Typhimurium, in pigs vaccinated with either $5 \times 10^8$ CFU (group A) or $5 \times 10^7$ CFU (group B) of attenuated *S.* Typhimurium *ΔznuABC*; in pigs vaccinated with inactivated *S.* Typhimurium (group C); and in unvaccinated controls (group D). Symbols represent group means; bars represent 95% confidence intervals. Data refer to one out of two separate experiments performed with comparable results.

### 3.4. Protection induced by S. Typhimurium ΔznuABC correlates with antigen-specific production of IFN-γ

As early as 2 days after challenge infection, peripheral blood leukocytes from pigs in group A produced IFN-γ upon in vitro stimulation with heat-inactivated *S.* Typhimurium (Supplementary Fig. 4). Leukocytes from pigs in groups B and C produced the same amount of IFN-γ as cells obtained from animals in group A only 7 days after challenge infection. In contrast, pigs in group D mounted a *Salmonella*-specific immune response involving IFN-γ production only 21 days after infection. These findings suggest that vaccination is able to prime a *Salmonella*-specific immune response, which is subsequently recalled upon challenge.

## 4. Discussion

Salmonellosis in pig farms entails both an economic cost and a considerable public health concern [14]. Although *S.* Typhimurium infection may cause septicaemia in pigs, it is most often characterized by enterocolitis and diarrhoea. It has been shown, however, that pigs that have suffered from enterocolitis can become chronic carriers. In such cases, *Salmonella* typically persists in mesenteric lymph nodes, tonsils and the gut. The presence of adult carriers within swine herds may therefore play a role in the maintenance of infection, reducing the effectiveness of conventional *Salmonella* control programmes [15]. In this context, vaccination may constitute a powerful and effective tool for *Salmonella* control in pig farms [14]. A recent systematic literature review has indicated that vaccination is associated with reduced *Salmonella* prevalence in swine at or near harvest [16]. Nevertheless, to date, the efficacy of such vaccines is not sufficiently supported by the experimental data [16] and recent studies on the efficacy of commercially available vaccines have yielded inconsistent results [17–19]. In addition, it has been demonstrated that a monovalent vaccine does not induce protection against heterologous infections [17,18]. Our approach could overcome this limitation, since it allows the production of a polyvalent vaccine preparation by combining attenuated strains of the most epidemiologically significant serovars.

There is a pressing need to find novel vaccines that are effective in preventing and eradicating salmonellosis in pigs, with the goal of reducing transmission of this disease to the human host.
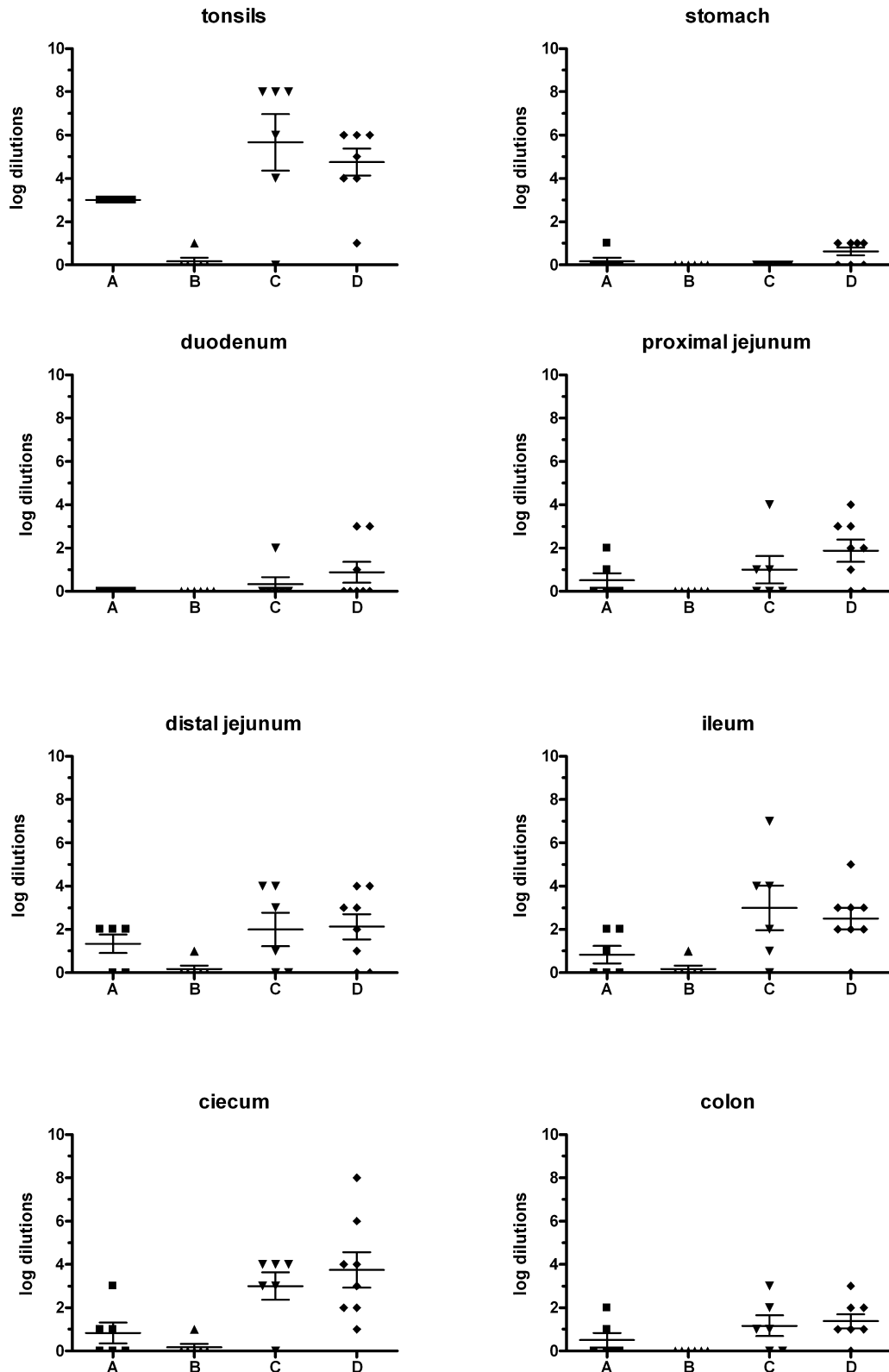
**Fig. 5.** *S.* Typhimurium *ΔznuABC* vaccination reduces *Salmonella* gut colonization in infected pigs. Recovery of *Salmonella* from different parts of the gastrointestinal tract after a challenge infection with virulent *S.* Typhimurium, in pigs vaccinated with either $5 \times 10^8$ CFU (group A) or $5 \times 10^7$ CFU (group B) of attenuated *S.* Typhimurium *ΔznuABC*; in pigs vaccinated with inactivated *S.* Typhimurium (group C); and in unvaccinated controls (group D). Symbols represent individual animals and bars indicate group means and standard errors. Data refer to one out of two separate experiments performed with comparable results.

Our findings extend those reported by Schwarz et al. [17] providing evidence that attenuated *S.* Typhimurium can also serve as an effective orally delivered vaccine. The use of attenuated bacteria in vaccine formulations has significant advantages over whole inactivated bacteria because, by inducing a mild and limited infection, they stimulate a robust and protective immune response. In addition, their natural ability to colonize the host makes them suitable for mucosal delivery. The development of mucosal vaccines
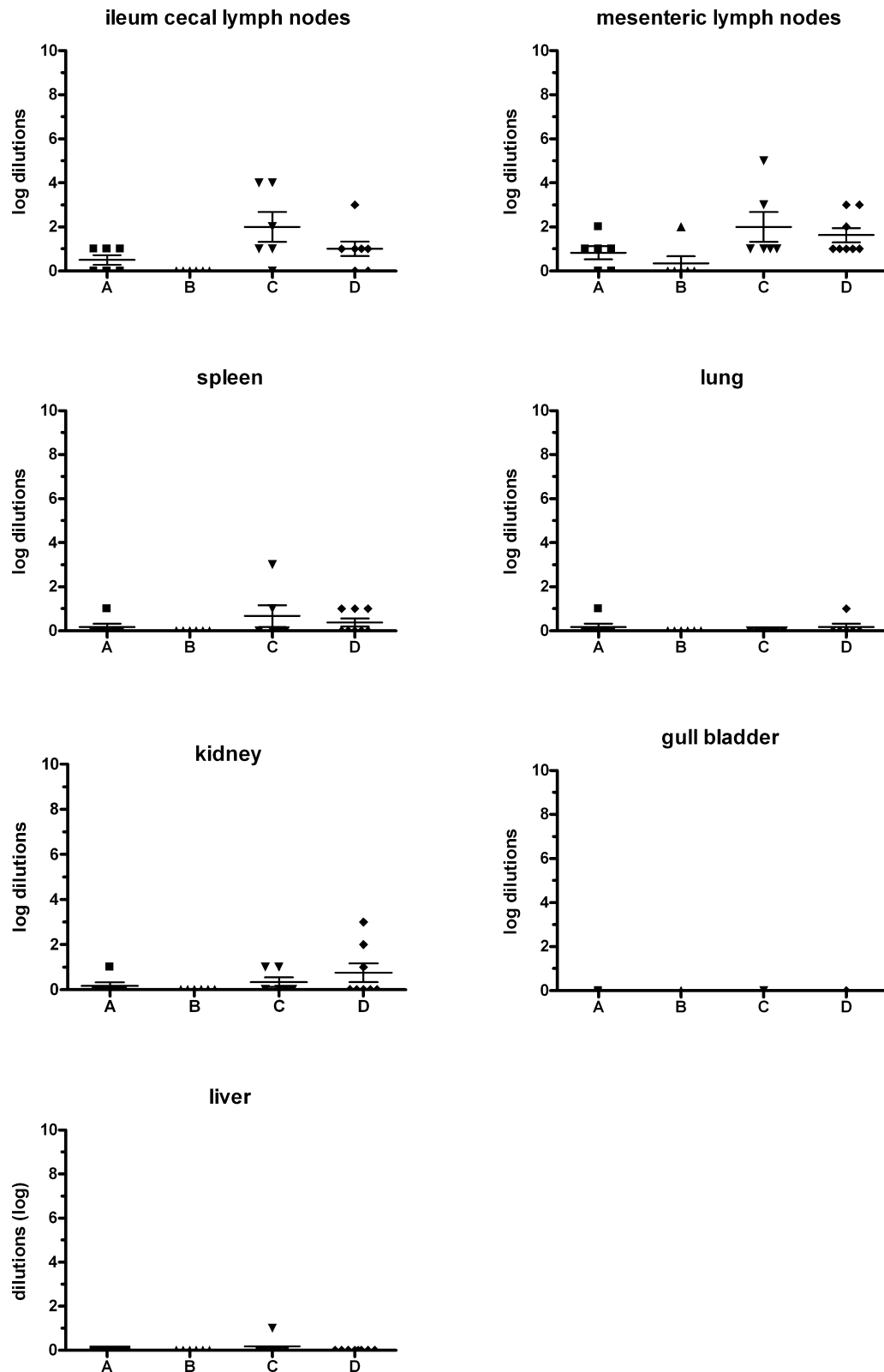
**Fig. 6.** *S.* Typhimurium *ΔznuABC* vaccination reduces *Salmonella* colonization of systemic organs in infected pigs. Recovery of *Salmonella* from different organs after a challenge infection with virulent *S.* Typhimurium, in pigs vaccinated with either $5 \times 10^8$ CFU (group A) or $5 \times 10^7$ CFU (group B) of attenuated *S.* Typhimurium *ΔznuABC*; in pigs vaccinated with inactivated *S.* Typhimurium (group C); and in unvaccinated controls (group D). Symbols represent individual animals and bars indicate group means and standard errors. Data refer to one out of two separate experiments, performed with comparable results.

against enteric pathogens is a challenging task, both because a large number of bacterial pathogens exploit the mucosal surface to penetrate and colonize the host and because classical parenteral inoculation tends to induce an excellent systemic immune response, but may not prime the mucosal immune system [20,21]. Recent studies suggest that *Salmonella* colonization of the gut is promoted by its ability to acquire nutrients, including metal ions, in the harsh host environment [22]. Metals are essential to most

organisms, as they are catalytic or structural cofactors in a wide range of proteins. Given the importance of metals, most bacteria have evolved complex mechanisms to ensure an adequate supply of these elements, while averting their potentially toxic intracellular accumulation. Indeed, it is now widely recognized that the ability of the host to control infectious bacteria requires the activation of metal sequestration mechanisms that remove essential metals from the host's cellular and extracellular compartments [22]. Conversely, the invading pathogens respond to the paucity of metal ions by expressing high-affinity metal-binding sites [23]. Although the competition for iron has been recognized as relevant to host-pathogen interaction for a long time, other divalent metals, such as zinc and manganese, have been shown to play a comparable role [24]. Specifically, our studies have established that the competition for zinc plays a critical role in host–*Salmonella* interaction [10,25]. More recently, we demonstrated that, whereas wild-type *Salmonella* exploits inflammation to overcome competing intestinal microflora and proliferate in the gut, a *Salmonella* strain lacking the necessary transporter (ZnuABC) cannot compete with other microorganisms and is killed by the inflammatory response [26]. These results showed that *Salmonella* uses the Znu-ABC transporter to acquire zinc during calprotectin-mediated zinc chelation and that calprotectin, rather than controlling *Salmonella* infection, confers an advantage on this pathogen over competing microbes. These studies contribute to our understanding of the attenuation mechanism of the *S.* Typhimurium *ΔznuABC* vaccine strain, whose inability to stably persist in the host and cause disease is clearly related to its impaired ability to recruit zinc.

In conclusion, our results show that oral administration of an attenuated *S.* Typhimurium *ΔznuABC* vaccine reduce clinical signs, bacterial colonization and shedding in pigs challenged with a virulent strain of *S.* Typhimurium, corroborating our previous studies on salmonellosis in mice [11,12]. Coupled with our results demonstrating the safety and immunogenicity of *S.* Typhimurium *ΔznuABC* in pigs [13], these findings underscore the potential usefulness of this attenuated strain as a candidate mucosal vaccine in swine and the possible benefits of its use in pig farms.

## Acknowledgements

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.05.105.

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