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Salmonella Typhimurium lacking the Znuabc transporter is attenuated and immunogenic in pigs

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ABSTRACT

Meat contamination by Salmonella spp. is emerging as a major cause of human enteric infections in industrialized countries. The attempts to reduce human cases of salmonellosis encompass pre- and post-harvest interventions. In this context, vaccination of pigs may represent an effective instrument in eliminating/reducing *Salmonella* burden through the food chain. We have previously demonstrated that *Salmonella* Typhimurium lacking the ZnuABC transporter (*S.* Typhimurium Δ*znuABC*) is a promising candidate live vaccine in different mouse models of *Salmonella* Typhimurium infection. In this study, we confirmed in pigs the attenuation of *S.* Typhimurium Δ*znuABC*. Moreover, we evaluated the safety and immunogenicity of *S.* Typhimurium Δ*znuABC* administered to pigs by the oral route. We monitored clinical conditions of animals and we conducted a microbiological culture and a quantification of the humoral and cellular immune response, respectively, on fecal and blood samples of pigs. After vaccination with attenuated *S.* Typhimurium Δ*znuABC*, pigs showed a modest degree of hyperthermia. In addition, fecal shedding of *S.* Typhimurium Δ*znuABC* could not be detected 28 days after the inoculum. Furthermore, vaccination with *S.* Typhimurium Δ*znuABC* elicited a distinct production of anti-*Salmonella* antibodies and IFN-γ. Taken together, these results suggest that *S.* Typhimurium Δ*znuABC* is attenuated and immunogenic in pigs. Although the vaccine dosages do not guarantee complete safety there is ample margin to set up better conditions of use, suggesting that *S.* Typhimurium Δ*znuABC* could be a promising attenuated strain to be used as live mucosal vaccine for oral delivery.

1. Introduction

Nontyphoidal salmonellosis infections pose a significant threat to human health and are responsible for high economic losses worldwide [1]. Moreover, many *Salmonella* strains are resistant to a number of antimicrobial agents, narrowing the therapeutic alternatives in cases of severe human infection [2,3]. Pork is the main source of *Salmonella* infection for humans, accounting for 26.9% of the human cases officially reported in the EU [4]. The application of strict hygiene practices and rational husbandry management have been effective in Scandinavian countries, where the prevalence of *Salmonella* in animals and carcasses at slaughter is nearly zero. However, this approach is hardly feasible in countries where high prevalence of infection is observed. In these settings, vaccination is considered as a major tool to minimize *Salmonella* contamination at the early stages of meat production. Although live vaccines provide better protection against *Salmonella* infections compared to inactivated ones [5–8], only a live attenuated vaccine for *Salmonella* Typhimurium is commercially available in Europe at the moment [5]. Nevertheless, several strains showed promising results vaccine in experimental settings [6–8]. Recently, we found that a *Salmonella enterica* serovar Typhimurium mutant strain, deleted of the whole *znuABC* operon (*S.* Typhimurium Δ*znuABC*) encoding for a high affinity zinc importer necessary for metal recruitment within the infected host, is significantly attenuated in mice [9–11] and able to protect mice against both systemic and enteric salmonellosis [12,13]. Based on these results, compared the virulence of *S.* Typhimurium Δ*znuABC* with those of wild type *Salmonella* Typhimurium ATCC 14028 in pigs (Exp. 1). In a second experiment (Exp. 2), we established the safety and immunogenicity of *S.* Typhimurium Δ*znuABC* administered to pigs as live vaccine. The results reported here demonstrate that *S.* Typhimurium

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Δ*znuABC* is attenuated in pigs. Moreover, administered by the oral route, *S.* Typhimurium Δ*znuABC* elicits a short-lasting and immunogenic infection that does not affect the animal health status and production performances in nearly all animals.

2. Materials and methods

2.1. Salmonella spp. cultures

The virulent *Salmonella* Typhimurium ATCC 14028 and its isogenic *znuABC* mutant strain produced according to the method previously reported [9], were used throughout the study. Strains were grown overnight at 37 °C in Brain Heart Infusion (Oxoid Ltd., UK), harvested by centrifugation and then washed twice in ice-cold phosphate buffer solution (PBS) (Sigma–Aldrich, Italy). A bacterin from *S.* Typhimurium ATCC 14028 was obtained by inactivating the bacteria with formalin and absorbing them on aluminum hydroxide.

2.2. Animals

Twenty-eight commercial hybrid pigs aging ~80 days were used in the comparative virulence experiment (Exp. 1). Animals were split in two groups of 10 (Groups A and B) and a group of 8 (Group C). Groups A and B were intragastrically administered with 20 ml of sodium bicarbonate buffer containing 5×10^9 CFU of *S.* Typhimurium Δ*znuABC* (Group A) or 5×10^9 CFU of *S.* Typhimurium ATCC 14028 (Group B). Group C received only sterile sodium bicarbonate buffer and served as control group. Collection of fecal samples of each pig (0, 1, 3 and 7 days) and registration of rectal temperature (0, 1, 2 and 7 days) were performed. For the safety and immunogenicity experiment (Exp. 2), hybrid pigs born by cesarean section and aging 80–100 days were used. Animals were split into 4 groups of 6 (Groups A, B and C) and 8 (Group D) animals/group. Groups A and B were intragastrically vaccinated, respectively, with a suspension of 5×10^8 (Group A) and 5×10^7 (Group B) CFU of *S.* Typhimurium *ΔznuABC* in 20 ml of sodium bicarbonate buffer. Group C was intramuscularly (upper part of the neck, 16G needle, 40 mm length) vaccinated with 2×10^9 CFU of inactivated *S.* Typhimurium ATCC 14028. Group C received an equal booster dose 14 days later. Group D was administered 20 ml of sterile sodium bicarbonate buffer and was left as naïve controls. Individual blood and fecal samples were collected before and after immunization (on day 0, 1, 2, 7, 8, 9, 14, 21, 28 and 35 days). Body temperature was registered on the same dates. On day 0 and at 5 weeks, each animal was weighed to evaluate the growth rate. All the pigs used throughout the study (Exp. 1 and Exp. 2) were proved to be *Salmonella*-free by culture of feces of each animal, and individual sera were checked to exclude *Salmonella* antibodies. Each group was maintained in separate isolation units under natural day–night rhythm with access to feed (FAMAVIT, Italy) and water *ad libitum*.

All the experiments were authorized by national authority and were conducted according to national regulation (D.L.116/92)

2.3. Fecal shedding of S. Typhimurium

Fecal samples of each pig were collected to assess the elimination of bacteria. The microbiological analysis was conducted according to the ISO 6579:2002/Amendment 1:2007 protocol. This is a semi-quantitative approach that allowed determining the concentration of *Salmonella* in a sample within a tenfold band (detection limit 1 CFU/g feces). Suspect *Salmonella* colonies were subjected to biochemical identification by BBL Enterotube II (BD Franklin Lakes, USA) and serological identification using *Salmonella* group-specific antisera (Remel, Lenexa, USA).

2.4. Persistence of S. Typhimurium ΔznuABC in the environment and pig feces

Environmental swabs collected from the pen floor of each group were qualitatively cultured following the protocol indicated in section 2.3 to assess the persistence of *Salmonella* in the environment. In another set of experiments, we compared the viability of *S.* Typhimurium *ΔznuABC* and *S.* Typhimurium ATCC 14028 in feces. Approximately 2 kg of fresh pig feces were collected from a group of sows serologically negative for *Salmonella*. Prior to use, the feces were confirmed negative for the presence of *Salmonella* by culture. A quantity of 27 g of feces was placed into each of 22 sterile 50 ml plastic tubes for inoculation. Three ml of sterile saline containing *S.* Typhimurium *ΔznuABC* or *S.* Typhimurium ATCC 14028 were added to 20 tubes (10 tubes for each strain), to yield a final concentration of 5×10^6 CFU/g of feces. Finally, 3 ml of sterile saline were added to 2 tubes, to be used as controls. Tubes were incubated at 20 °C for three weeks. Sampling for culture was performed at 0, 2, 7, 14 and 21 days post-inoculation. After gently mixing, an aliquot of 1 g of feces was taken from each tube for enumeration of *Salmonella* microorganisms using a miniaturized MPN (Most Probable Number) method based on ISO 6579:2002 [14].

Typical colonies were confirmed serologically as *Salmonella* by polyvalent antiserum (*Salmonella* Test Serum; Siemens Healthcare Diagnostics, Italy) and API rapid 20 E (Api Rapid 20E; Biomerieux, Italy). MPN was calculated according to the ISO 7218:2007.

2.5. Serological examination

The serological exams were performed using a commercial indirect ELISA test capable of detecting antibodies against lipopolysaccharide antigens of *Salmonella* serogroups B, C1 and D (Herd-Check Swine *Salmonella* Antibody Test Kit, Idexx Laboratories Inc., Switzerland). The test was carried out according to the producer's instructions and read at an optical density of 450 nm. Results were expressed as sample to positive ratio {S:P ratio = (OD of sample − OD of negative control)/(OD of positive control − OD of negative control)}.

2.6. Statistical analysis

Exp. 1. Differences in body temperature were estimated using 2 ways analysis of variance with a Tuckey's multiple comparison test and considered significant when $^*P$-value ≤ 0.05. Fecal shedding of group A and group B were compared using non parametric Mann–Whitney test.

Exp. 2. Differences between the groups were analyzed by a Two-way ANOVA and Bonferroni corrections were applied.

Statistical analysis was performed using a Graphpad PRISM 6.0 software (GraphPad Software Inc., USA). A P-value ≤ 0.05 was considered significant.

3. Results

3.1. Pathogenicity of S. Typhimurium ΔznuABC

Our hypothesis was that the deletion of the whole *znuABC* operon encoding for a zinc importer necessary for metal recruitment within the infected host was able to reduce virulence of *S.* Typhimurium in pigs. So, in Exp.1, we compared the values of body temperature and the duration of fecal shedding of group A (*S.* Typhimurium Δ*znuABC*) with those of Group B (*S.* Typhimurium ATCC 14028) and Group C (controls). Two days after the inoculation, group A and group B displayed statistically significant higher values of body temperature than controls. Nevertheless the increase body temperature was more evident in group B (Fig. 1A). *S.* Typhimurium

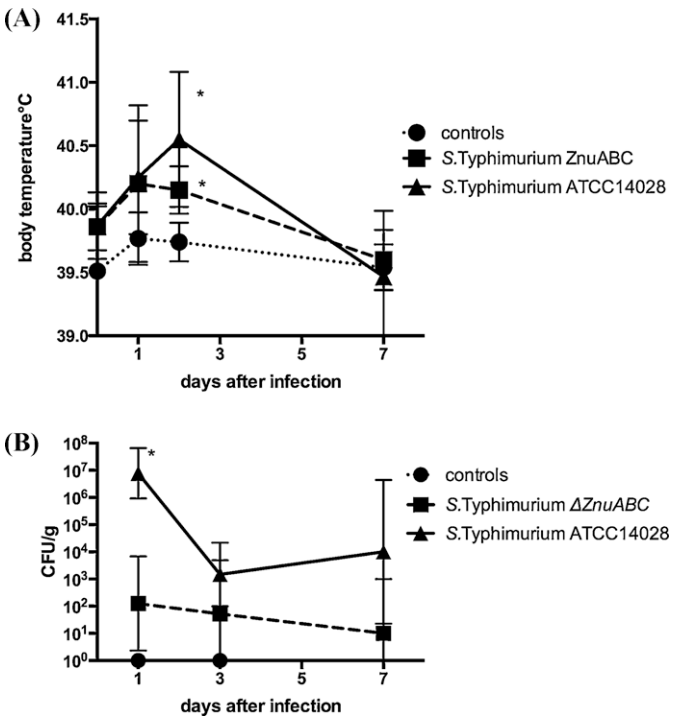

Fig. 1. *S.* Typhimurium *ΔznuABC* shows lower virulence in pigs compared to wild type *S.* Typhimurium ATCC 14028. (A) Mean values and SD bars of body temperature of groups (A–C) at different time points. Results of group A or B were compared with results of group C. Differences were estimated using 2 ways analysis of variance with a Tuckey's multiple comparison test and considered significant when $^{*}P\text{-value} \leq 0.05$. (B) Mean values and SD bars of CFU/g of *S.* Typhimurium *ΔznuABC* (group A) and *S.* Typhimurium ATCC14028 (group B) shed in feces. Results of group A were compared with results of group B. Differences were estimated using non parametric Mann Whitney test and were considered significant when $^{*}P\text{-value} \leq 0.05$.

ΔznuABC was fecally shed in lower amount than *S.* Typhimurium ATCC 14028 (Fig. 1B). At 1 day after the inoculation this difference reached statistically significance. These results confirmed our hypothesis a *S.* Typhimurium deleted of whole *znuABC* operon has a reduced virulence in pigs.

3.2. Effect of S. Typhimurium ΔznuABC oral administration on body temperature

Then, in Exp. 2, we decided to explore how the oral vaccination with *S.* Typhimurium Δ*znuABC* (our candidate vaccine strain mutated in *znuABC*) modifies the physiologic values of body temperature in pigs, considered to be between 38 and 40 °C [15]. At 1 day after vaccination (DAV), three animals of group B and one animal of group A displayed a body temperature ranging from 40.3 °C and 40.7 °C (Fig. 2 and Fig. S1A). At 2 DAV, three animals of group A and one animal of group B had a body temperature varying from 40.1 °C and 41 °C (Fig. 2 and Fig. S1B). At the following time points (7, 14, 21, 28, 35 DAV), the body temperature of both groups of pigs was comparable with controls. Group C, vaccinated with 2×10^9 CFU of bacterin, and group D (controls) did not show any alteration of temperature at any time points considered (Fig. 2 and Fig. S1C and D). These data indicate that the oral administration of *S.* Typhimurium Δ*znuABC* at currently used inoculation doses increases body temperature of a limited number of pigs.

3.3. Influence of vaccination on growth rate

Next, we evaluated if vaccination with either *S.* Typhimurium Δ*znuABC* or inactivated *S.* Typhimurium ATCC 14028 interferes with the growth of animals. At 35 DAV, one animal of group B and one of group C did not gain weight, a decrease of body weight was registered in a pig of group A. Our observations suggest pigs of groups vaccinated either with *S.* Typhimurium Δ*znuABC* or inactivated *S.* Typhimurium ATCC 14028 showed an heterogeneous growth rate. However, no statistically significant difference was noted between the growth rate of vaccinated pigs and controls (Fig. 3 and Fig. S2).

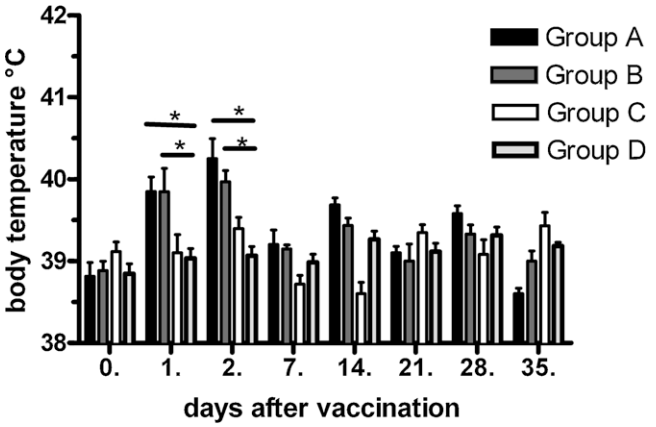

Fig. 2. Group A and group B display a rise of body temperature after vaccination with *S.* Typhimurium *ΔznuABC*. Mean values and SD bars of body temperature of each group at different time points. Two-way ANOVA and Bonferroni corrections were applied. $^{*}P\text{-value} \leq 0.05$.

3.4. Fecal shedding of S. Typhimurium ΔznuABC

At 1 DAV, stools of group A contained approximately 10^5 CFU/g of *S.* Typhimurium Δ*znuABC* and stools of group B contained less than 10^4 CFU/g (Fig. 4). Twenty-four hours later, the concentration decreased by ~2 logs in feces of pigs from both groups. At 7 DAV, group A continued to excrete approximately 10^2 CFU/g. However, at 7 DAV, shedding of group B dropped to less than 10 CFU/g, with 3 animals in this group that had not detectable *S.* Typhimurium Δ*znuABC* in feces. At 21 DAV, one animal of group B shed less than 10 CFU/g of *S.* Typhimurium Δ*znuABC*. At 14 DAV, two pigs of group A shed less than 10 CFU/g and at 21 DAV, two other pigs excreted 10

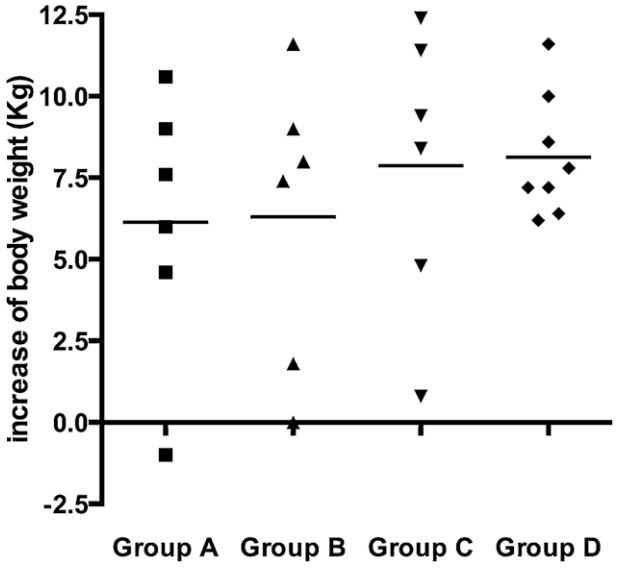

Fig. 3. The weight gain of pigs of vaccinated groups is heterogeneous. Weight gain of animals of different groups. Each symbol represents one animal and bars represent mean value of the group.

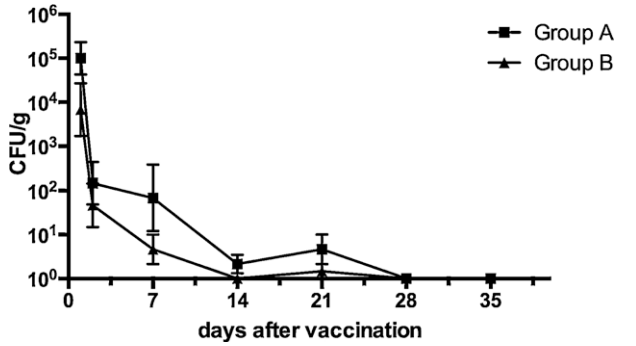

Fig. 4. Fecal shedding of *S.* Typhimurium *ΔznuABC*. Fecal elimination of *S.* Typhimurium *ΔznuABC* by group A and group B after oral administration respectively with a dose of 5×10^8 and 5×10^7 CFU/pig. Mean values with SD bars of CFU/g of each group at different time points.

and 10^2 CFU/g of *S.* Typhimurium Δ*znuABC* each. The shedding of *S.* Typhimurium Δ*znuABC* by animals of group A was not detectable at 28 DAV. These data indicate that at 2 DAV, the fecal shedding of *S.* Typhimurium Δ*znuABC* decreases to approximately10^1–10^2 CFU/g and continues to comparable values till day 28.

3.5. Viability of S. Typhimurium ΔznuABC in the environment and pig feces

As indicator of the viability of *S.* Typhimurium Δ*znuABC*, we assessed the environmental contamination of pig units after vaccination. As expected, *S.* Typhimurium Δ*znuABC* was not detected in pen of controls (group D) and animals of group C, which were vaccinated with the bacterin. Environmental swabs collected from pens of groups A and B revealed that *S.* Typhimurium Δ*znuABC* contaminated the environment till 7 DPI. To corroborate these findings we set out to determine the survival of *S.* Typhimurium Δ*znuABC* in comparison with *S.* Typhimurium ATCC 14028 in a fecal matrix. In this setting, the concentration of *S.* Typhimurium Δ*znuABC* dropped from 4×10^6 CFU/g (Standard Deviation 1.4×10^4) to 844 (Standard Deviation 8.9×10^2) in two days and was under the detection limit at 14 days after inoculation in the majority of the samples. In contrast, *S.* Typhimurium ATCC 14028 was still detectable at a concentration of 1.4×10^6 at 14 days after inoculum (Fig. 5). These data suggest that *S.* Typhimurium Δ*znuABC* does not persist long in conditions resembling the natural environment.

3.6. Vaccination induces antibody specific immune response

The antibody response of pigs after vaccination with *S.* Typhimurium Δ*znuABC* or inactivated *S.* Typhimurium ATCC 14028 was investigated. All vaccinated animals mounted a humoral immune response regardless of the type of vaccine administrated

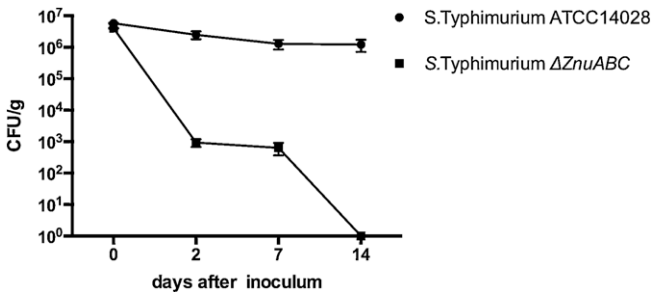

Fig. 5. *S.* Typhimurium *ΔznuABC* has a limited survival in fecal material. Survival of *S.* Typhimurium *ΔznuABC* and *S.* Typhimurium ATCC 14028 in artificial contaminated feces maintained at room temperature.

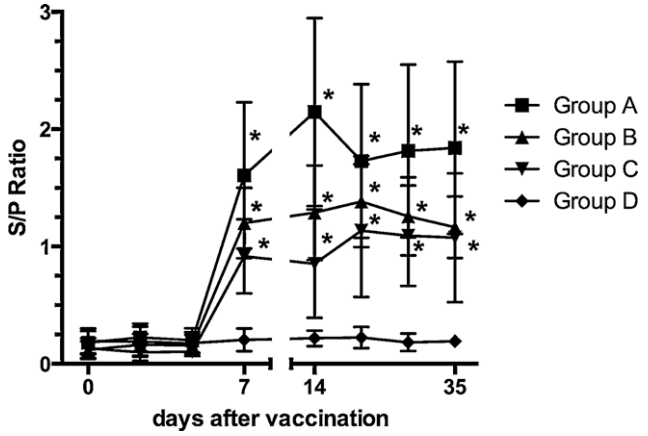

Fig. 6. *S.* Typhimurium *ΔznuABC* and inactivated *S.* Typhimurium ATCC14028 induce a similar pattern of humoral immune response. After vaccination, serum of pigs was collected at several time points; a commercially available ELISA test was used to detect antibodies against *Salmonella.* The X-axis (time) of figure is divided in two segments. Each segment has different intervals coinciding with time points. This solution was adopted to ameliorate the graphical illustration of data. Mean values with SD bars of antibodies titers of each group at different time points. Groups with asterisk are statistically different from group D at the time point considered $^*P$-value ≤ 0.05.

(Fig. 6). Anti-*Salmonella* antibodies raised in group A, group B and group C at 7 DAV and remained high throughout the observational period. At 7, 14, 21, 28 and 35 DAV, antibody titers of groups A, B and C were statistically higher than those of controls. These data demonstrate that administration of *S.* Typhimurium Δ*znuABC* or inactivated *S.* Typhimurium ATCC 14028 is effective in inducing a humoral immune response in pigs.

4. Discussion

The here reported results show that the mutant strain *S.* Typhimurium lacking the ZnuABC transporter inoculated in pigs by oral route shows characteristics of attenuation if compared with virulent *S.* Typhimurium ATCC 14028. Moreover, vaccination with *S.* Typhimurium *ΔznuABC* (our candidate vaccine strain mutated in *znuABC*) caused an increase of body temperature for two days but there was no difference in body temperature between the groups by 7 DAV). A heterogeneous growth rate within the vaccinated groups was noticed. However, mean of the weights of vaccinated groups were comparable with those of controls. *S.* Typhimurium *ΔznuABC* primed a specific humoral and cellular immune response (Supplementary Data). On the whole, these findings demonstrate that *S.* Typhimurium *ΔznuABC* has appreciable characteristics of safety and immunogenicity in pigs.

Vaccination is one of the methods suggested to decrease the burden of *S.* Typhimurium at swine farm level and prevent its dissemination through the pork production chain [5,16,17].

To assess the safety of *S.* Typhimurium *ΔznuABC* in pigs we took into account several parameters such as body temperature, weight gain, serum concentration of TNF-α and fecal shedding of the vaccine strain.

Some vaccinated animals showed a rise in body temperature at 1 and 2 DAV, however it ended within one week. The administration of *S.* Typhimurium *ΔznuABC* caused stunted growth of some pigs regardless of vaccine dose. Nevertheless, body weight means of the experimental groups were comparable. However conclusive assessment requires further studies enrolling larger groups of animals with an homogeneous initial size and age, since a previous study reported that the administration of *Salmonella* live vaccines decreased the growth rate of vaccinated pigs [18].

The serum concentration of TNF-α in pigs inoculated with *S.* Typhimurium *ΔznuABC* showed a significant increase compared to controls only when the highest vaccinal dose is administered (Fig. S4) These data are consistent with previous observations that report reduced variations of serum concentration of TNF-α even in pigs fed a diet containing virulent *S.* Typhimurium [19].

Isolation of *S.* Typhimurium *ΔznuABC* from environmental swabs yielded from pens of vaccinated pigs ended within two weeks. Furthermore, *S.* Typhimurium *ΔznuABC* evidenced a shorter viability in pig feces when compared with *S.* Typhimurium ATCC 14028. These findings suggest that *S.* Typhimurium *ΔznuABC* express a limited persistence in the environment.

The serum concentration of IgG and the antigen specific *ex vivo* production of IFN-γ (supplementary data) by whole blood samples were chosen as parameters to assess the immunogenicity of *S.* Typhimurium *ΔznuABC*. It is known that B-cell and antibodies play a role in the protection from infection in mouse.pigs and humans [20,21]. In particular, antibodies opsonize *Salmonella* in the early stages of infection, hindering microorganisms to reach their intracellular niche within phagocytes [21,22]. In pigs [23], as well as in humans [24] a humoral immune response is crucial in the early phase of life when an immune response against *Salmonella* is not developed yet. Nevertheless, in pigs maternal antibodies tend to disappear at the time of weaning, leaving piglets susceptible to *Salmonella* infection [25]. On that account, *S.* Typhimurium *ΔznuABC* proved to prime a humoral immune response inducing high serum levels of IgG.

Live vaccines are believed to be more effective than inactivated ones in controlling *S.* Typhimurium infection because of their pronounced ability to stimulate the cellular immune compartment and to determine the production of mucosal IgA [16,25,26].

A Th1-oriented immune response is crucial for the complete clearance of *Salmonella* microorganisms, with activated IFN-γ producing $CD4^+$ T-cells fostering bacteria killing by monocytes [21]. Although the *ex vivo* antigen specific release of IFN-γ by the whole blood samples of animals vaccinated with *S.* Typhimurium *ΔznuABC* or inactivated *Salmonella* vaccine suggests a similar Th1 antigen specific immune response (Fig. S3), we have evidence that after an oral virulent challenge with *S.* Typhimurium ATCC14028, groups vaccinated with *S.* Typhimurium *ΔznuABC* displayed a greater and more prompt IFN-γ production if compared to animals vaccinated with inactivated *Salmonella* vaccine (data in publication). Oral vaccination of pigs with *S.* Typhimurium *ΔznuABC* induced serum IgG and evoked a cellular immune response. These data suggest that *S.* Typhimurium lacking the ZnuABC transporter is a promising strain to be used as live vaccine for mucosal delivery in pigs. Furthermore, live vaccines can be administered through drinking water, offering a welfare safeguarding method of vaccination avoiding stressful handling and adverse reactions frequently observed when parenterally injected [27].

Although we proved *S.* Typhimurium *ΔznuABC* to be immunogenic, vaccinated pigs showed an increase of body temperature and a heterogeneous weight gain that may raise some concerns about its safety. Therefore, the effects in pigs of a reduction of the vaccine dose or the use as vaccines of *S.* Typhimurium strains deleted of ZnuABC and of ZinT [10] and ZupT [28], two other zinc uptake systems, should be investigated. Overall, our data support the safety and immunogenicity of oral administration of *S.* Typhimurium ZnuABC in pigs.

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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.2013.04.032.

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