

Efficiency evaluation of a novel orally administered subunit vaccine to reduce the prevalence of *Salmonella enterica* in swine under field conditions

Avaliação da eficiência de uma nova vacina de subunidade, administrada por via oral, para reduzir a prevalência de *Salmonella enterica* em suínos, em condições de campo

Caroline Reichen^{1*}; Arlei Coldebella²; Diogenes Dezen³; Mariana Meneguzzi⁴; Caroline Pissetti⁵; Jalusa Deon Kich²

Highlights

A subunit vaccine for the control of any *Salmonella enterica* serovar was assessed.

The vaccine phagocytic capacity was assessed.

Isolation and quantification of *Salmonella* spp. were compared.

The vaccine generated an increase in the phagocytic activity of peripheral monocytes.

Vaccination failed to reduce the number of carriers and shedders of *Salmonella* spp.

Abstract

Salmonella enterica can be carried by pigs and can reach the final product and the consumer. Thus, *Salmonella* reduction strategies along the swine production chain should be studied. In this sense, the objective of the study was to evaluate a subunit vaccine based on secondary antigens administered orally against natural infection in swine farms in Brazil. A field trial study was conducted to estimate the effect of the vaccination on the *Salmonella* spp. seroprevalence, presence in lymph nodes, and fecal content in commercial pig herds in a vertical integration system belonging to an agroindustry. Furthermore, nMPN, qPCR, and phagocytic activity were performed. There were no significant differences in seroprevalence

¹ Master of the Programa de Pós-Graduação em Produção e Sanidade Animal, PPGPSA, Instituto Federal Catarinense, IFC, Campus Concórdia, Concórdia, SC, Brazil. E-mail: caroline.reichen@ufpr.br

² Researchers, EMBRAPA Suínos e Aves, Concórdia, SC, Brazil. E-mail: arlei.coldebella@embrapa.br; jalusa.kich@embrapa.br

³ Prof. Dr. Instituto Federal Catarinense, IFC, Campus Concórdia, Concórdia, SC, Brazil. E-mail: diogenes.dezen@ifc.edu.br

⁴ PhD Candidate. Veterinary Population Medicine Department. University of Minnesota, College of Veterinary Medicine, St. Paul, MN, USA. E-mail: meneg009@umn.edu

⁵ Veterinary, Centro de Diagnóstico de Sanidade Animal, CEDISA, Concórdia, SC, Brazil. E-mail: carolpissetti@gmail.com

* Author for correspondence

between groups. The mMPN count of *Salmonella* spp. in feces was higher in vaccinated group (VG), ranging from 0 to 2.46 log mMPN/g, while in control group (CG) it ranged from 0.0 to 4 log mMPN/g, showing a significant group effect ($p < 0.05$), being confirmed in the qPCR. The activity of phagocytic monocytes was not altered by vaccination on farms. Thus, the oral subunit vaccination strategy at this stage of development did not reduce the spread and amplification of the infection in farms that would impact the prevalence of pigs carrying and shedding *Salmonella* spp. until slaughter.

Key words: *Salmonella*. Field trial. Vaccine. Swine. Subunit vaccine. Phagocytic monocytes.

Resumo

A *Salmonella enterica* pode ser transmitida por suínos e pode chegar ao produto e ao consumidor. Assim, estratégias de redução de *Salmonella* ao longo da cadeia produtiva suína devem ser estudadas. Nesse sentido, o objetivo do estudo foi avaliar uma vacina de subunidade baseada em antígenos secundários, administrada por via oral contra infecção natural em granjas de suínos no Brasil. Um estudo experimental de campo foi conduzido para estimar o efeito da vacinação sobre soroprevalência *Salmonella* spp., presença em linfonodos e conteúdo fecal em rebanhos suínos comerciais em um sistema de integração vertical, pertencente a uma agroindústria. Além disso, foi realizada nMPN, qPCR e atividade fagocítica. Não houve diferenças significativas na soroprevalência entre os grupos. A contagem de mMPN de *Salmonella* spp. nas fezes foi maior no GV (grupo vacinado), variando de 0 a 2,46 log mMPN/g, enquanto no GC (grupo controle) variou de 0,0 a 4 log mMPN/g, mostrando efeito de grupo significativo ($p < 0,05$), sendo confirmado no qPCR. A atividade dos monócitos fagocíticos não foi alterada pela vacinação nas granjas. Assim, a estratégia de vacinação por subunidades orais nesta fase de desenvolvimento não reduziu a propagação e amplificação da infecção nas explorações que teriam impacto na prevalência de suínos portadores e excretados de *Salmonella* spp. até o abate.

Palavras-chave: *Salmonella*. Ensaio de campo. Vacina. Suínos. Vacina de subunidade. Monócitos fagocíticos.

Introduction

Salmonella enterica is one of the most reported causes of foodborne illness in humans (European Food Safety Authority [EFSA], 2019). Swine carrying *Salmonella* spp. are reservoirs and responsible for maintenance of bacteria through the swine production chain (Boyen et al., 2008; Griffith et al., 2019). The bacteria can reach the final product and be transmitted to the consumer (Bonardi, 2017; Neto et al., 2021). Brazilian studies have shown that 67% of pigs are carriers of *Salmonella* spp. in mesenteric

lymph nodes (Kich et al., 2011) and up to 96% of animals with positive serology in slaughter (Paim et al., 2019). A decrease in the number of animals carrying and shedding *Salmonella* spp. at slaughter is considered the first step towards achieving product from swine without *Salmonella* spp. (Berends et al., 1996).

One strategy to reduce the number of carrier animals is vaccination. In addition to the risk mitigation in the slaughter environment, it can be useful to prevent cases of clinical salmonellosis in pigs, a disease present in Brazil (Meneguzzi et al., 2021). However,

studies evaluating *Salmonella* vaccination have variable results (Costa et al., 2020; Cruz et al., 2017; Gil et al., 2020; Peeters et al., 2019, 2020; Schwarz et al., 2011).

Salmonella spp. has in its membrane lipopolysaccharides (LPS), a class of glycoconjugates unique to Gram-negative bacteria, and are present in the outer monolayer of the outer membrane along with phospholipids and proteins. In addition, LPS are large antigenic molecules, easily recognized by the immune system and targets of most vaccines. These molecules tend to be specific for a particular serovar and/or serogroup (Bearson et al., 2016), thus becoming a limiting factor due to the variability of serovars on farms (Bersot et al., 2019; Kich et al., 2011; Paim et al., 2019). These limitations drove investments in innovative products to overcome the specificity barrier. A study using DIVA (Differentiating Infected from Vaccinated Animals) vaccines shows encouraging results in experiments with *Salmonella Typhimurium* (Gil et al., 2020), but not yet validated with different serovars and in the field. Experimentally, the use of subunit vaccines provided broad protection for *Salmonella enterica* regardless of serovar (Martinez-Becerra et al., 2018), however, this technology has not yet been validated in pigs. In this sense, this work aimed to evaluate a commercial subunit vaccine based

on secondary antigens administered orally against natural infection in swine farms.

Material and Methods

Study design

A field trial study was conducted to estimate the effect of the vaccination on the *Salmonella* spp. seroprevalence, prevalence of *Salmonella* carriers and shedder pigs in 20 commercial finishing pig herds belonging to a vertical integration system located in Midwest of Santa Catarina state, Brazil. The study was conducted following Ethical Principles in Animal Experimentation adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the Ethics Committee in Animal Experimentation (CEUA/IFC) (Protocol nº 02/2017).

To supply 20 finishing herds, 16 nursery farms were allocated to vaccinated (VG=8) or control group (CG=8). Piglets were from the same origin for all 16 nursery farms. When the pigs achieved an average age of 65 days and the average weight of 25.07 kg they were transported and followed throughout 20 finishing farms. On average, the pigs remained in the finishing herd for 110 days and were slaughtered at 175 days of life, weighting 125.3 kg (Figure 1).

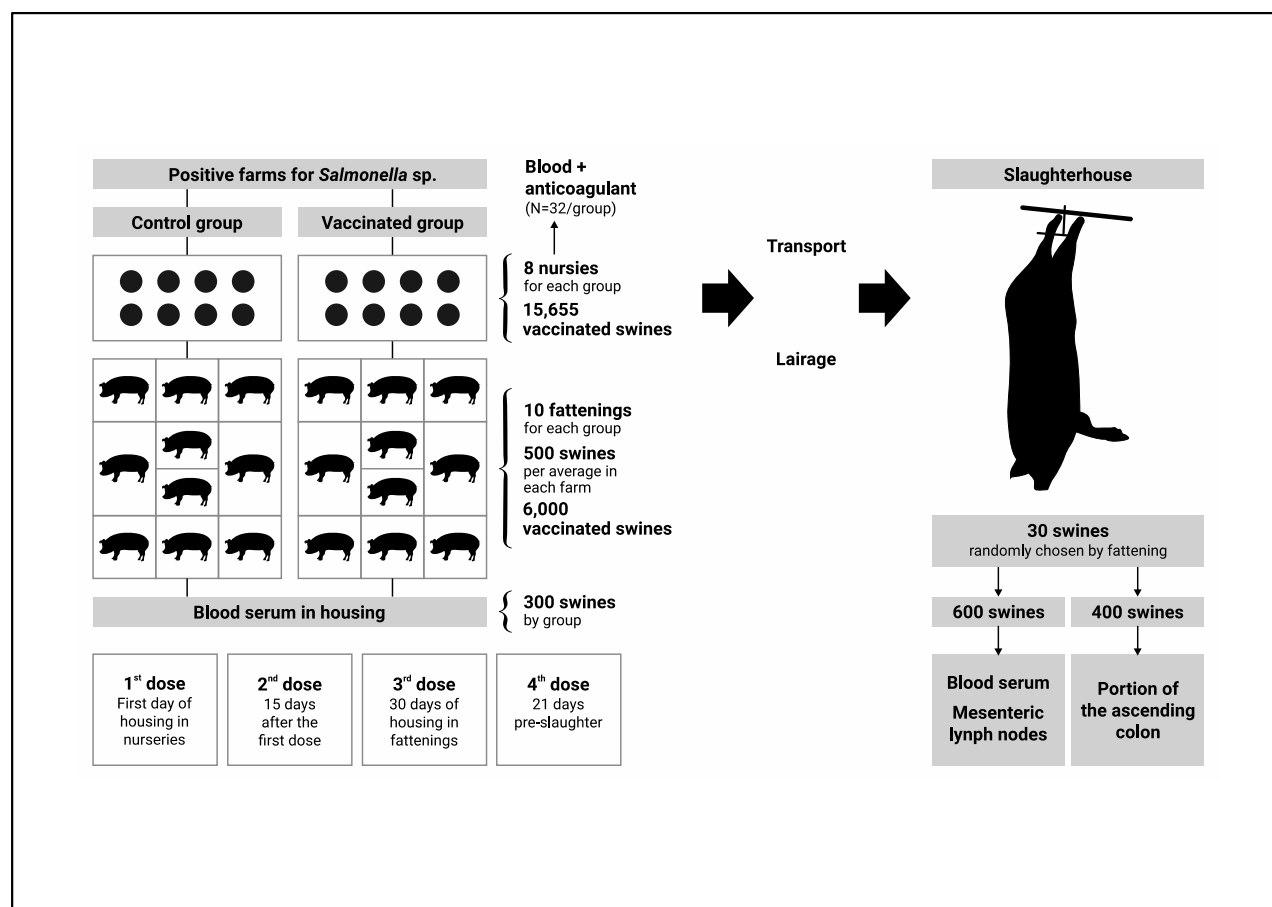


Figure 1. Flowchart of the experimental design.

Blood samples were taken in three different moments over time. The first blood collection was on the third day after the second dose of the vaccine at 30 days old; the second blood collection occurred in 30 animals from each nursery farm at the time of transfer to finisher ($n = 600$) at 45 days old; the third blood sample was conducted at slaughter at 175 days old, from 30 animals ($n = 600$) during bleeding. In addition, blood with anticoagulant (heparin) was collected from 4 randomly selected animals in each group nursery farm ($n = 64$).

In the slaughter line, mesenteric lymph nodes (MLN) and colon fragments were collected from 30 animals from each finishing farm, belonging to the VG ($n = 600$) and 20 pigs from each finishing farm belonging to the control group ($n=400$). Details of the experimental design and vaccination are summarized in Figure 1. All samples were kept in refrigeration until processing in the laboratory of Embrapa Swine and Poultry and CEDISA.

Vaccine and vaccination

A vaccine composed of a subunit based on secondary antigens was used. A common genetic sequence for all species of *Salmonella* spp. was cloned into a plasmid and inserted into *Bacillus subtilis*, which produced peptides incorporated by microparticles, constituting a mucosal vaccine. These secondary antigens and sequence used are particularly patents, and a trade secret (Manufacturer's communication).

It was administered 2 mL of the vaccine per animal, orally, at four ages. The first dose was on the second day at the nursery (on average at 28 days of age). The second dose was at 45 days of age (15 days after the first dose), the third dose was at 95 days of age and the fourth dose was administered 21 days pre-slaughter (age 154). The vaccine was diluted according to the manufacturer's instructions.

Sample processing

Serology

A serological analysis was performed using a commercially available indirect enzyme-linked immunosorbent assay (ELISA, Herd Check Swine *Salmonella*® IDEXX Laboratories, ME, USA) to detect anti-*Salmonella* IgG, following the manufacturer's instructions.

Bacteriology

Salmonella spp. was investigated in MLN and feces from colon, following the ISO 6579:2002 amendment 1:2007

protocol (International Organization for Standardization [ISO], 2007), using Xylose Lysine Deoxycholate agar (XLD) (Merck, Frankfurter, Germany) and Brilliant Green Phenol Red Lactose Sucrose agar (Merck, Frankfurter, Germany) for the selective differential isolation step. Typical colonies were confirmed by their biochemical profile and agglutination with monovalent *Salmonella* antiserum OMNI-O (A-60) (Bio-Rad, Marnes-la-Coquette, France).

Miniaturized most probable number (mMPN)

From the positive samples to feces in the qualitative method, the quantification of *Salmonella* spp. was carried out by the mMPN technique, following Jarvis et al. (2010). The colonies compatible with *Salmonella* spp. were previously submitted to biochemical and serological screening. The mMPN was calculated by the MPN Calculator (Ofer Fridman, 2021).

Real-time polymerase chain reaction (qPCR)

Quantification by qPCR was performed on 46 stool samples previously positive, 11 belonging to CG and 35 samples to VG. The fecal DNA was extracted using the QIAamp PowerFecal Pro DNA Kit (Qiagen, Hilden, Germany). DNA samples were quantified with a fluorometer (Qubit 2.0, ThermoFisher Scientific, Massachusetts, USA). The *HillA* gene was amplified using the following forward (5'-CGC TGG CAG AAT GCT ACC TC-3') and reverse (5'-AGC CCC AGT AAT CCT AAA GCT TG-3') primers (Brunelle et al., 2011). In brief, PCR mixtures contained 1 µl of each primer, 6 µL of Syber

Green systems (Life Technologies, Carlsbad, USA), and 2 μ L of template DNA (28 μ L of total volume). qPCRs were performed in 7500 Real-Time PCR (Applied Biosystems, Foster City, USA). The amplification conditions were 95°C for 10 min in the holding stage, followed by 40 cycles of 95°C for 15 sec and 62°C for 30 sec. The reading step of the melting curve was programmed with the following temperatures: 95°C for 15 sec, 58°C for 1 min, 95°C for 30 sec, and 58°C for 15 sec. The quantification curve was created using free feces contaminated with strain of *Salmonella* Typhimurium BRMSA 1830 (Embrapa Suínos e Aves), with $3,15 \times 10^8$ UFC/g. All tests were performed in triplicates with negative control.

Phagocytic activity

Blood samples were collected from the animals three days after the first dose of the vaccine. There were 8 swine for each group, and the animal being the experimental unit. After the extraction of peripheral blood mononuclear cells (PBMC), a procedure was performed, separating the blood cells into Histopaque (Sigma-Aldrich, St. Louis, MO, USA). Whole blood was diluted 1:1 with BPW, and the same volume of Histopaque was added. It was then centrifuged at $400 \times g$ for 30 min. The PBMC layer was collected, and the cells were counted. For 10^6 leukocytes, 1 μ L of pHrodo (Invitrogen, Eugene, OR, USA) was added, and incubated at 37°C for 30 min. The number of cells that phagocytosed, the reagent and the intensity of phagocytosis were measured at a 488 nm wavelength on BD FACSCalibur™ (BD Biosciences, Franklin Lakes, NJ, USA), with excitation of the fluorescence by argon laser.

Statistical analysis

All statistical analyses were performed using the commercial software Statistical Analysis System® [SAS] (2012). To harmonize the groups of animals used in the experiment, the animal's performance, through weight and age at housing and slaughter, were compared by analysis of variance. The mean optical density (OD) per batch was assessed by a repeated-measures analysis. The effects of the groups, collection periods, the interaction between the groups, and the three types of variance and covariance matrix structures (PROC MIXED) were considered. The structure used in the analysis was chosen based on the lowest Akaike Information Criterion (AIC) value. The estimation method used was the maximum likelihood method.

For the seroprevalence, the probability distribution was considered binomial, and its analysis was carried out using the Generalized Estimating Equations (GEE) (Liang & Zeger, 1986). The fixed effects of the groups and collection periods and the interaction between the two factors were tested (GENMOD). The effect of the treatment on the isolation of *Salmonella* spp. in the feces and lymph nodes between vaccinated and control animals, as well as its categorization into positive and negative, was analyzed by the Chi-square test (χ^2). The mMPN data were transformed into $\log(y + 1)$. After the calculation of the means per batch, the data were analyzed through an analysis of variance. The association between *Salmonella* spp. isolated in the feces and the seroprevalence and intensity of the serological reaction measured by the optical density variability (% DO), was evaluated through logistic regression.

For flow cytometry, t-tests or Mann-Whitney tests were performed between the groups, and for graph analysis of all samples, repeated measures analysis of variance (ANOVA) was used.

Results and Discussion

The groups harmonization was tested by weight and age comparison at housing and slaughter time. No statistical differences were observed for the analyzed variables (Table 1).

Table 1
Comparison of weight and age between vaccinated groups (VG) for *Salmonella* spp. and control group (CG)

Variables	Group		p value
	Vaccinated Group	Control Group	
Slaughter age	175,10 ± 0,89	174,90 ± 0,98	0,8816
Housing age	64,70 ± 0,30	65,00 ± 0,00	0,3306
Market weight	124,02 ± 2,33	126,55 ± 1,76	0,3984
Weaning weight	24,08 ± 0,89	26,07 ± 1,01	0,1551

The seropositive swine at time of transfer to finisher were 15% in the CG and 22% in the VG, considering cut off 20% of OD. At the time of bleeding, there was an increase in seropositive animals, 75% and 80% respectively for CG and VG (Figure 2b).

The group effect was not significant ($p > 0.05$) in any collection period for two variables. Other cutoff was tested, 10% (Figure 2a) and 40% (Figure 2c), without statistical difference being observed between the groups.

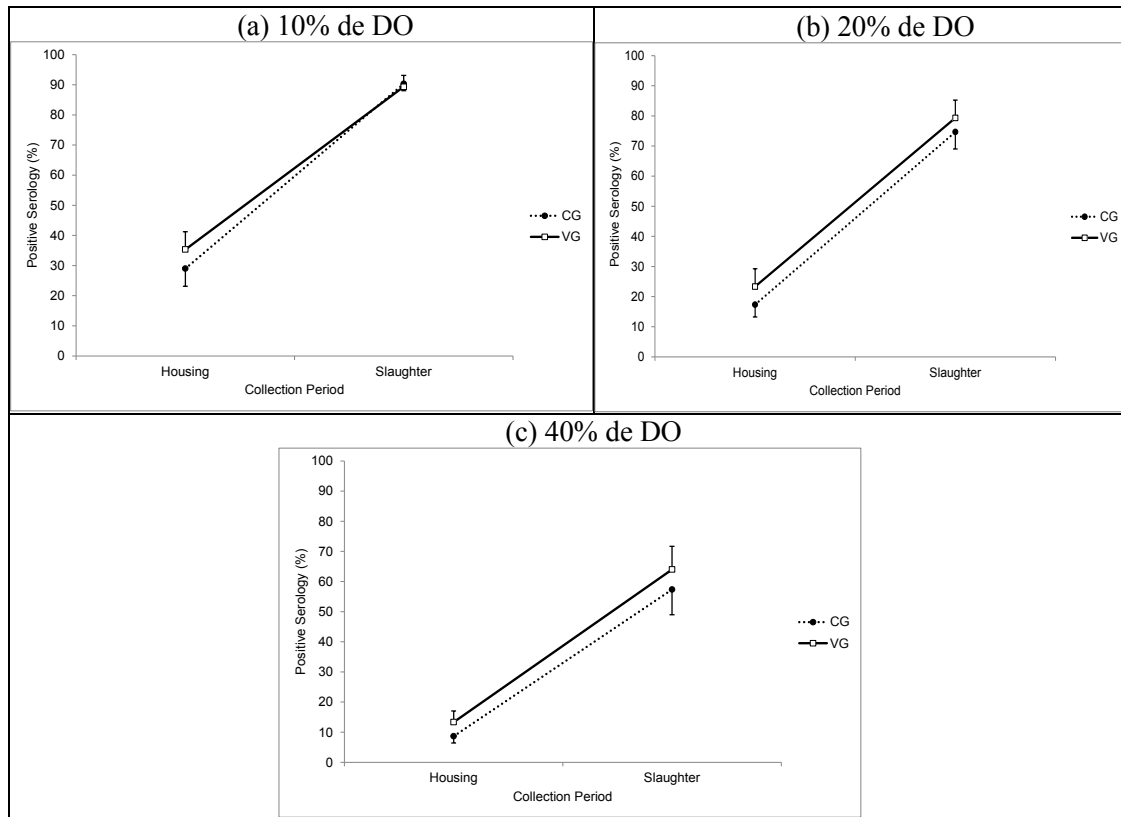


Figure 2. a) Profile of seroprevalence according to the group and the collection period using 10% of cut-off; b) Profile of seroprevalence according to the group and the collection period using 20% of cut-off; c) Profile of seroprevalence according to the group and the collection period using 40% of cut-off.

The VG showed a higher percentage of positive shedders of *Salmonella* spp. In

feces, and the carriers in the MLN at slaughter (Table 2).

Table 2

Percentage of positive *Salmonella* spp. carriers in MLN and shedders in feces and respective test of χ^2 , for the control and vaccinated group

Variables	Group		Pr> χ^2
	Vaccinated Group	Control Group	
Feces	23.62% (47/199)	33.00% (66/200)	0.0367
MLN	30.00% (90/300)	38.33% (115/300)	0.0314

95% CI, 95% confidence interval.

Isolation of *Salmonella* spp. in swine feces as a function of seroprevalence and in relation to optical density in % it was positive and significant ($p < 0.05$). For every 10 units of increase in seroprevalence, using a 40% OD cutoff point, expect a 30.3% increase in

the percentage of isolation of *Salmonella* spp. in the stool (Figure 3). In the case of average optical density, for every 10 units of increase, an estimated 15.6% increase in the percentage of isolation of *Salmonella* spp. in the feces (Figure 4).

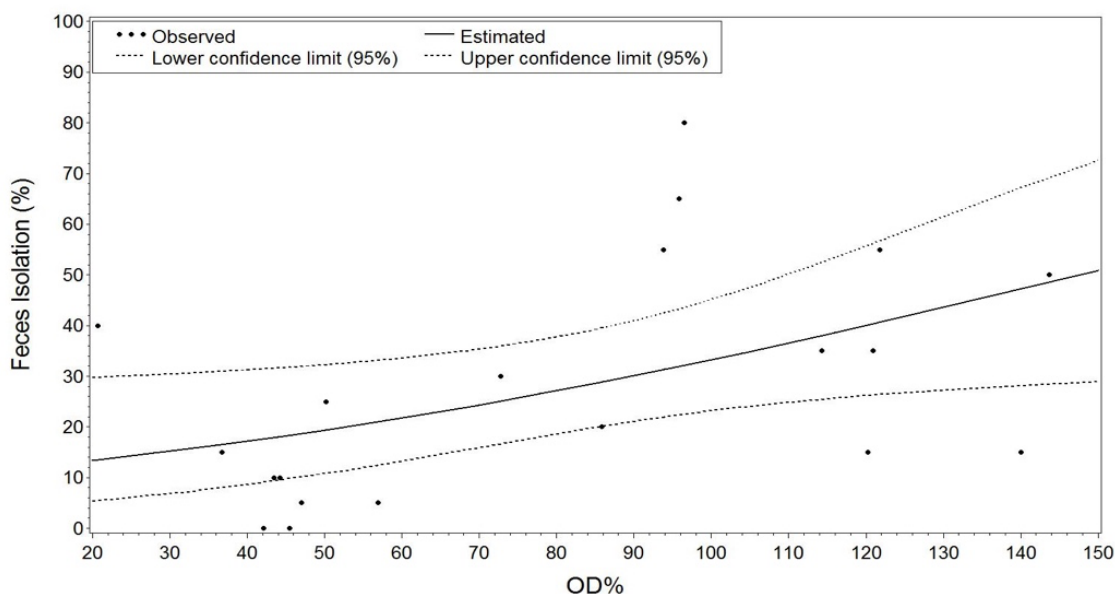


Figure 3. Percentage of isolation of *Salmonella* spp. in feces as a function of mean optical density.

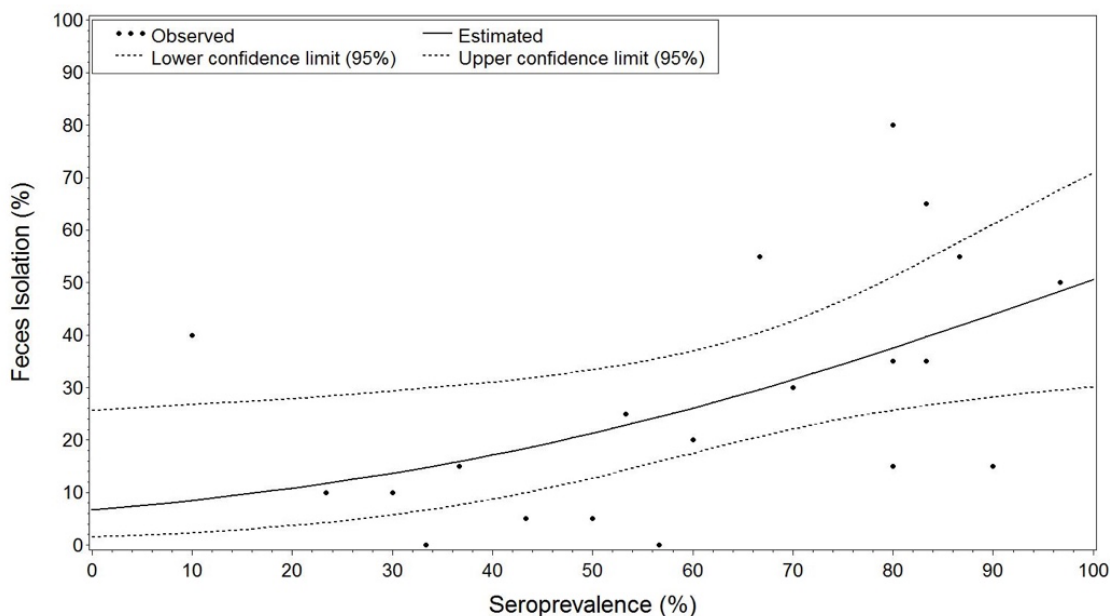


Figure 4. Percentage of isolation of *Salmonella* spp. in feces due to seroprevalence.

The result found in qPCR quantification showed a significant group effect ($p < 0.05$). The variation was from 7.69×10^3 to 2.62×10^5 bacteria/g of feces in the CG, while the VG showed a greater variation, from 5.62×10^3 to 2.15×10^9 , showing a difference of 3 logs between the groups, when considering the general average.

Through the results of flow cytometry, it was possible to evidence the activity of phagocytic monocytes was not altered by vaccination in farms A-B. The same can be seen with figures C and D, which were not statistically significant (Figure 5).

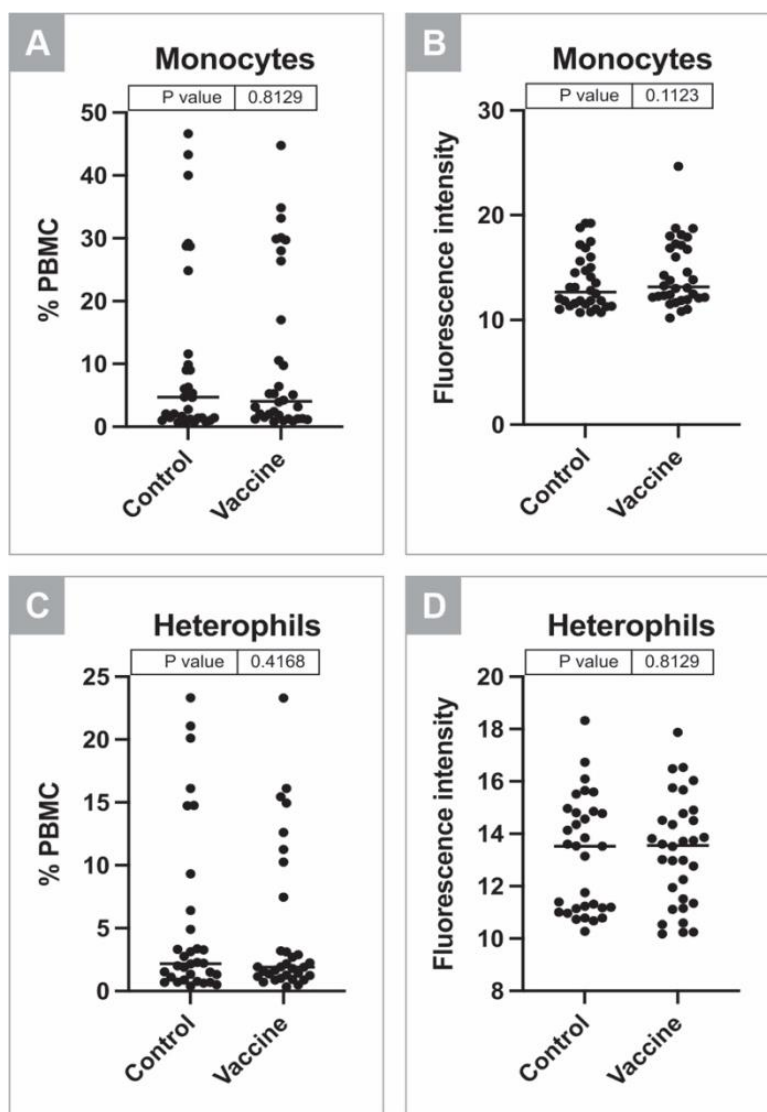


Figure 5. Phagocytic activity of monocytes and heterophils in the control and vaccine group. The figures A and B indicate relative number of monocytes, and C and D, indicate relative number of heterophils and their activity. The lower figures indicate the relative number of heterophils and their activity.

The control of *Salmonella* spp. in pig production it is necessary for both public and animal health, and vaccination is an accepted measure to reduce the number of animals colonized by the bacteria (Wales & Davies, 2017). However, the diversity of existing serovars (Bersot et al., 2019; Kich et al., 2011, 2020) reduces the success of some vaccines due to the antigenic specificity of the agent (Costa et al., 2020; Schwarz et al., 2011), making new studies of vaccines based on other technologies important. The use of a subunit vaccine, using secondary surface molecules common to all serovars, is an interesting alternative and offers excellent safety profiles (Li et al., 2020; Martinez-Becerra et al., 2018).

The vaccine used in this work uses a common genetic sequence for all serovars. This sequence was cloned into a plasmid, and this was inserted into a *Bacillus subtilis*, which produced subunits (peptides) that were incorporated by microparticles, making up the mucosal vaccine. Other vaccines, administered orally, induce a mucosal immune response with production of IgA (Haesebrouck et al., 2004; Roesler et al., 2006) and stimulation of gut-associated lymphoid tissue-GALT (Ogra et al., 2001). With this, it was expected a decrease in the colonization of the bacteria and, consequently, a smaller number of seropositive animals and less excretion of *Salmonella* spp. in swine at the time of slaughter.

However, VG showed a higher frequency of detection of *Salmonella* spp. than the CG, with a difference of 8.33% in the MLN samples, and 9.38% in the analyzed stool samples. This result was also observed when compared to fecal quantification, both by

mMPN and qPCR, in which both VG samples had a higher bacterial load when compared to the control. It is known that the destruction of microorganisms phagocytosed by macrophages is due to the production of nitric oxide (NO) and other intermediates due to classical activation (Th1) of macrophages by IFN- γ or LPS (Classen et al., 2009). Nevertheless, several intracellular bacterial pathogens have been shown to induce metabolic alterations in macrophages in a targeted, specific manner that benefits the pathogen. However, the mechanisms about how the altered macrophage metabolism promotes these pathogens growth and how a *Salmonella* spp. reprograms macrophage metabolism are largely unclear (Jiang et al., 2021). In a study carried out with a virulent strain of *Brucella melitensis*, its phagocytosis by mouse peritoneal macrophages was demonstrated in the presence of hyperimmune anti-LPS serum of *B. melitensis*. However, once internalized, the bacterium efficiently multiplied into non-activated macrophages, and its elimination occurred only when macrophage activation by IFN- γ was induced (Eze et al., 2000).

Thus, the results found in this work do not allow us to conclude whether this increase in phagocytic activity resulted in the effective targeting of field strains by macrophages or whether these cells potentiated the multiplication of the pathogen serving as a replication site. The results of isolation in feces, MLN, and mMPN point to the second hypothesis, since the percentage of *Salmonella* spp. detected was higher in the vaccinated group than in the control group.

Antibody testing, performed using the ELISA test, was used as an indicator

of previous exposure to *Salmonella* spp. Considering 20% of OD as the cutoff point, seroprevalence in housing was low, ranging from 15% to 22%; at the time of slaughter, this variation increased to 75% and 80%, which confirms that it is at this stage of production that the bacteria spread between animals and, consequently, increasing the infection, corroborating studies that showed that pigs are infected at some point during the termination period (Berends et al., 1996; Kranker et al., 2003).

As an indicator of the spread of the bacteria in the herd, serology has been used as an indicator of the risk of introducing bacteria into the slaughter environment. Allows for the discrimination between herds in a concise, fast, and low-cost way. However, as some animals can eliminate the infection, but remain seropositive or even infected in the pre-slaughter period and serologically negative, they have a limited ability to determine the infection status of an individual; however, it is useful to determine the level of infection in the herd (Vico & Mainar-Jaime, 2011).

According to Mainar-Jaime et al. (2017), it is possible to associate the intensity values of the serological reaction measured by the optical density variability, the % DO with the % of *Salmonella* spp. shedders, at different ages at termination. They describe that 90-day-old pigs have a 66% chance of spreading *Salmonella* spp. when they arrive for slaughter, with 40% OD. When analyzing the data from the present study, for each 10% increase in OD, it is estimated that there is an increase of 15.6% in the percentage of *Salmonella* spp. isolated in the animal's feces at the time of slaughter (110 days of fattening).

The vaccine tested had no effect on the seroprevalence of the herd at the time of slaughter. It would be expected that the vaccinated herds would have a lower seroprevalence than the control, preferably less than 40%, comparable to those considered at low risk in other control programs (Alban et al., 2002). Although studies show that seropositive individuals do not pose a risk per se; studies show that about 67% of animals that had contact with *Salmonella* spp. become carriers (Letellier et al., 2009) and 75% shedders (Silva et al., 2006). Thus, the seroprevalence of the flock is positively correlated with the level of excretion in feces. This fact was demonstrated in this study, with a 10% increase in herd seroprevalence followed by a 30% increase in the possibility of excretion of *Salmonella* spp. in the bank.

Although the subunit vaccine did not demonstrate a difference when compared to the control group, it is important to have more studies and vaccine varieties to test a field. Other subunit vaccines already classified have good results in other species when tested against specific serovars (Li et al., 2020; Martinez-Becerra et al., 2018), reinforcing the need for studies in field pigs, in a situation where the animals encounter a greater variety of serovars.

Conclusions

In this study, the commercial subunit vaccine administered orally did not show a good result against natural infection in swine farms when compared to the control group.

Acknowledgments

We are grateful to veterinary colleagues, farms, Embrapa Swine and Poultry, Cedisa, and Vetanco for technical, financial, and scientific support, and to slaughterhouse for cooperation in the development of this study.

Ethics approval

The study was conducted following Ethical Principles in Animal Experimentation adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by Ethics Committee in Animal Experimentation (CEUA/IFC) (Protocol nº 02/2017).

Author contributions

Study conception and design were performed by C. Reichen, A. Coldebella, and J.D. Kich. Material preparation, data collection and analysis were performed by C. Reichen, A. Coldebella, D. Dezen, M. Meneguzzi, C. Pissetti and J.D. Kich. The first draft of the manuscript was written by C. Reichen and J.D. Kich and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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