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Original Research Paper

Effect of the addition of protected sodium butyrate to the feed on *Salmonella* spp. infection dynamics in fattening pigs

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ABSTRACT

Organic acids (OA) are seen as an alternative to antibiotics to reduce the burden of enteropathogens. Two replicates of a field trial were carried out to assess the effect of the addition of protected sodium butyrate (PSB) to the feed (dose of 3 kg/T) along the fattening period on the dynamics of *Salmonella* spp. infection in pigs. In each trial, around 50 pigs were assigned to a treatment group (TG) and 50 kept as controls (CG). Pigs were serologically monitored monthly and on-farm fecal samples and fecal and mesenteric lymph nodes (MLN) samples at slaughter were collected. In the first replicate, pigs became probably infected with *Salmonella* before the addition of PSB to the feed, but despite of that an overall lower proportion of shedders along the end of fattening period and lower seroprevalence before slaughter (50% *vs*. 89.6%; $P < 0.001$) was observed in the TG. In the second replicate, few pigs shed *Salmonella* during the trial, which precluded the finding of significant differences between groups for both *Salmonella* infection and shedding, but the seroprevalence at slaughter in the CG was again significantly higher than in the TG (31.1% *vs*. 13.7%, respectively; $P = 0.02$) and it was related to a higher proportion of shedders and MLN-positive pigs. When results from both trials were analyzed together, a significant increasing risk of shedding in the CG was observed at 90 days of fattening and at slaughter, and an overall significant decreasing trend in OD% values and thus in seroprevalence was also observed when pigs approached to slaughter. In conclusion, the dietary administration of this PSB during the whole fattening period was able to reduce significantly the seroprevalence in the TG, which may reflect a positive effect on the control of *Salmonella* at the end of the fattening period.

1. Introduction

Human salmonellosis is considered the second most important foodborne infection in the European Union (EFSA and ECDC, 2016), and pigs and products thereof are considered one of the most important sources of infection for humans (De Knegt et al., 2015). In the pig industry, salmonellosis and other bacterial enteric diseases have been traditionally prevented with the use of antimicrobials, which may have favored the selection for antimicrobial resistance (AR) (Davies and Davies, 2010).

Drug-resistance in non-typhoidal *Salmonella* is climbing and it is now considered a matter of concern (CDC, 2013). As examples, high resistance to aminoglycosides, antimicrobials critically important for human medicine, was first discovered in Spain in 2005 in

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an *E. coli* isolate of pig origin (Gonzalez-Zorn et al., 2005) and further detected in *Salmonella* isolates (Folster et al., 2009). Likewise, new plasmid-mediated resistance to colistin, a last-resource antibiotic for Gram-negative human infections, was first found in the *Enterobacteriaceae* family in 2015 (Liu et al., 2016). Aminoglycosides and polymyxins have been commonly used in intensive pig husbandry systems for preventing enteric diseases (EMA, 2014; EMA, 2016).

The concerns about the emergence of AR have prompted European Health Authorities to reconsider their use for meat production, triggering new EU regulations on the use of antibiotics in veterinary medicine. Thus, the use of antibiotics as growth promoters was banned in 2006 (Regulation −EC- no. 1831/2003), and guidelines for the prudent use of antimicrobials in veterinary medicine, among which included avoiding their use as prophylactics, were published (Anon, 2015). In 2015 oral colistin was banned for its use as prophylactic and the period of administration was reduced to a maximum of 7 days (EMA, 2016).

The emergence of AR has reinforced the search for alternative products for the control of enteric infections in pigs. Organic acids (OA) or their salts might be beneficial for the productive performance of fattening pigs (Partanen and Mroz, 1999), but they are also known for their *in vitro* capacity to inhibit the growth and proliferation of Gram-negative pathogens. They would enter into the bacterial cell in their non-dissociated form and decrease the intracellular pH when dissociating, thus disrupting DNA synthesis. They also seem to favor commensal lactic bacteria by reducing extracellular pH and avoid the expression of some *Salmonella* invasion genes (Van Immerseel et al., 2005; Gantois et al., 2006). Therefore, OA are seen as an alternative to antibiotics to reduce the burden of enteropathogens *in vivo*.

Results on the effectiveness of OA for the control of pig salmonellosis are variable (van der Wolf et al., 2001a; Papenbrock et al., 2005; Creus et al., 2007; De Busser et al., 2009; Martín-Peláez et al., 2010; Calveyra et al., 2012; Walia et al., 2016), which is likely associated to different study designs (i.e. piglets *vs*. fattening pigs; natural *vs*. experimental infection; different administration periods, etc.) or the use of different acids, blends, or doses. A critical review of on-farm intervention strategies against *Salmonella* carried out in 2009 found that only in three (37.5%) out of the eight publications analyzed, a beneficial effect, either a reduction on fecal prevalence or seroprevalence, was detected when OA were used (Friendship et al., 2009). In addition, a systematic review on interventions for *Salmonella* reduction on grow-finish pigs found a significant heterogeneity in results when OA were used, precluding the presentation of a single summary estimate, which was likely due to the small number of studies available on grow-finish pigs (Wilhelm et al., 2012).

There is a clear need for more research on the use of OA for *Salmonella* reduction in fattening pigs to get a better idea of the effectiveness of the different types of OA available in the market. In particular, on new forms of OA (i.e. encapsulated or protected OA) that may act on the more distal part of the gastrointestinal tract (Piva et al., 2007). For this purpose, a field trial was carried out to assess the effect of the addition of protected sodium butyrate (PSB) to the feed on *Salmonella* infection dynamics in fattening pigs from an area of high *Salmonella* infection prevalence.

2. Material and methods

2.1. Experimental design

Two replicates of a field trial were carried out in June 2014 and in August 2015 in a small (≈100 pigs/8 pens) commercial *Salmonella*-infected fattening unit located in NE of Spain. The two replicates were separated a minimum of one year to prevent carry-over effects from the first replicate to the second. Feed with the PSB was administered to animals from 4 randomly selected pens (treatment group −TG-) and the remaining 4 pens were fed with the same basal diet without PSB (control group −CG-). All the pens were within the same barn. The compound feed was provided in 40-kg bags and manually administered to the animals by the farmer, who was unaware of the treatment allocation. A single dose of 3 kg of PSB (GUSTOR BP70, Norel S.A., Madrid, Spain) per ton of feed was used. The treatment began 15 days after pigs entered into the fattening unit and after finishing the in-feed antibiotic treatment (10 weeks-old pigs) and was administered until slaughter (approximately 3.5 months later).

2.2. Sampling scheme

Serum samples from all pigs were collected after 30, 60 and 90 days on the fattening unit, and within the last week before slaughter, to check for the presence of antibodies against *Salmonella* spp. On-farm fecal (OF) samples (a minimum of 25 g of feces) were collected along with the blood from approximately 25 pigs per group. Since fecal material was collected only after spontaneous defecation to reduce the risk of environmental contamination of the sample, the final number of sampled pigs varied between 21 and 29 (Table 1), but at least 3 pigs from each pen were included in each sampling to make sure the sampling of all the pens. The TG and CG were transported to the slaughterhouse separately. At slaughter, fecal (SF) and mesenteric lymph nodes (MLN) samples were collected from all pigs after evisceration.

2.3. Laboratory analysis

Sera were kept at −20 °C until serological analyses were carried out. The Herdcheck Swine *Salmonella* ELISA (IDEXX Laboratories, Westbrook, ME, US) was used for detection of antibodies (IgG) against *Salmonella* spp. Given the low specificity of the ELISA test (Vico et al., 2010), and as suggested in previous studies (Nollet et al., 2005), a cut-off value of OD% ≥40 was considered for seroprevalence estimates. *Salmonella* spp. isolation was performed on OF, SF and MLN samples following the standard ISO 6579:2002/Amd 1:2007 method.

Table 1
Microbiological results for *Salmonella* isolation for pig on-farm fecal samples (OF) after 30 (30d), 60 (60d), 90 (90d) days in the fattening unit and for mesenteric lymph nodes (MLN) and fecal samples at slaughter (SF) for the control (CG) and treatment (TG) groups in trials 1 and 1.

		Fattening unit									Slaughter					
		OF at 30d			OF at 60d			OF at 90d			SF			MLN		
Replicate	Group	No.	No. + (%)	*P*	No.	No. + (%)	*P*	No.	No. + (%)	*P*	No.	No. + (%)	*P*	No.	No. + (%)	*P*
1	CG	29	19 (65.5)	0.07	21	5 (23.8)	0.2	21	7 (33.3)	0.02	48	36 (75)	0.06	48	33 (68.7)	0.06
	TG	28	12 (42.8)		21	2 (9.5)		22	1 (4.5)		50	29 (58)		50	42 (84)	
2	CG	25	0 (0)	0.5	25	1 (4)	0.5	25	2 (8)	0.5	42	4 (9.3)	0.35	42	3 (7.1)	0.4
	TG	25	0 (0)		25	0 (0)		25	1 (4)		48	3 (6.2)		48	2 (4.1)	
Both*	CG	54	19 (35.2)	0.07	46	6 (13)	0.12	46	9 (19.6)	0.02	90	40 (44.4)	0.05	90	36 (40)	0.14
	TG	53	12 (22.6)		46	2 (4.3)		47	2 (4.2)		98	32 (32.6)		98	44 (44.9)	
	OR (95% OR)		NE			NE			5.6 (1.1–26.9)			2 (0.95 − 4.3)			NE	

*Mantel-Haenszel corrected; NE: not estimated; One-tailed *P*-value.

2.4. Statistical analyses

Fisher exact test was used to assess statistical differences between the CG and the TG regarding *Salmonella* seroprevalence (cut-off value ≥40%) and proportion of shedders at different time points, and infection prevalence (proportion of MLN-positive pigs) at slaughter in each trial individually. Wilcoxon test was used when comparing paired samples within a trial. Differences between the CG and the TG for both replicates together were estimated after adjusting by trial through the Mantel–Haenszel test for repeated tests of independence. A one-tailed *P*-value ≤0.05 was considered for significance as only positive effects of PSB were expected (i.e. a reduction of prevalence, seroprevalence or shedding). When differences were statistically significant the probability (measured as *Odds Ratio* −OR-) of a pig shedding *Salmonella* or becoming *Salmonella* infected/seropositive for the CG compared to the TG was also calculated. In addition, logistic regression analysis was used to assess the overall relationship between being a seropositive pig (OD% ≥40) and shedding and infection at slaughter.

The effects of sampling time (within-subject factor), and treatment (between-subject factor), and the corresponding interactions among them, on OD% values during the fattening period were assessed by general linear models repeated measures analysis of variance (ANOVA) for each trial separately and further for both trials together. Log transformation of OD% values was carried out before analysis. All statistical analyses were performed using STATA software (STATA, StataCorp, L.P., USA).

3. Results

3.1. Trial 1

In the first replicate both groups showed a high proportion of pigs shedding *Salmonella* spp. two weeks after beginning of treatment (30 days on fattening), although in the TG it was already somewhat lower (42.8% *vs*. 65.5% in the CG; $P = 0.07$). This difference was also observed after 90 days (33.3 *vs*. 4.5, respectively; $P = 0.02$) and at slaughter (75% *vs*. 58%, respectively; $P = 0.06$). Interestingly, at slaughter some more infected pigs (MLN +) were found in the TG compared to the CG (84% *vs*. 68.7%, respectively; $P = 0.06$) (Table 1).

Table 2
Seroprevalence of *Salmonella*, estimated as the proportion of pigs showing OD% values ≥40, after 30 (30d), 60 (60d), 90 (90d) days in the fattening unit and before slaughter, for the control (CG) and treatment (TG) groups in trials 1 and 2.

		Fattening unit											
		30d			60d			90d			Before slaughter		
Replicates	Group	No.	No. + (%)	*P*	No.	No. + (%)	*P*	No.	No. + (%)	*P*	No.	No. + (%)	*P*
1	CG	50	9 (18)	0.5	50	48 (96)	0.08	48	44 (91.6)	0.41	48	43 (89.6)	< 0.001
	TG	51	10 (19.6)		51	43 (84.3)		51	45 (88.2)		50	25 (50)	
2	CG	52	13 (25)	0.5	47	6 (12.7)	0.17	45	5 (11.1)	0.43	45	14 (31.1)	0.03
	TG	52	14 (26.9)		50	11 (22)		50	4 (8)		51	7 (13.7)	
Both*	CG	102	22 (21.5)	0.44	97	54 (55.6)	0.46	93	49 (52.6)	0.30	93	57 (61.2)	< 0.001
	TG	103	24 (23.3)		101	54 (53.4)		101	49 (48.5)		101	32 (31.6)	
	OR (95%OR)		NE			NE			NE			4.9 (2.4–10.1)	

*Mantel-Haenszel corrected; NE: not estimated; One-tailed *P*-value.

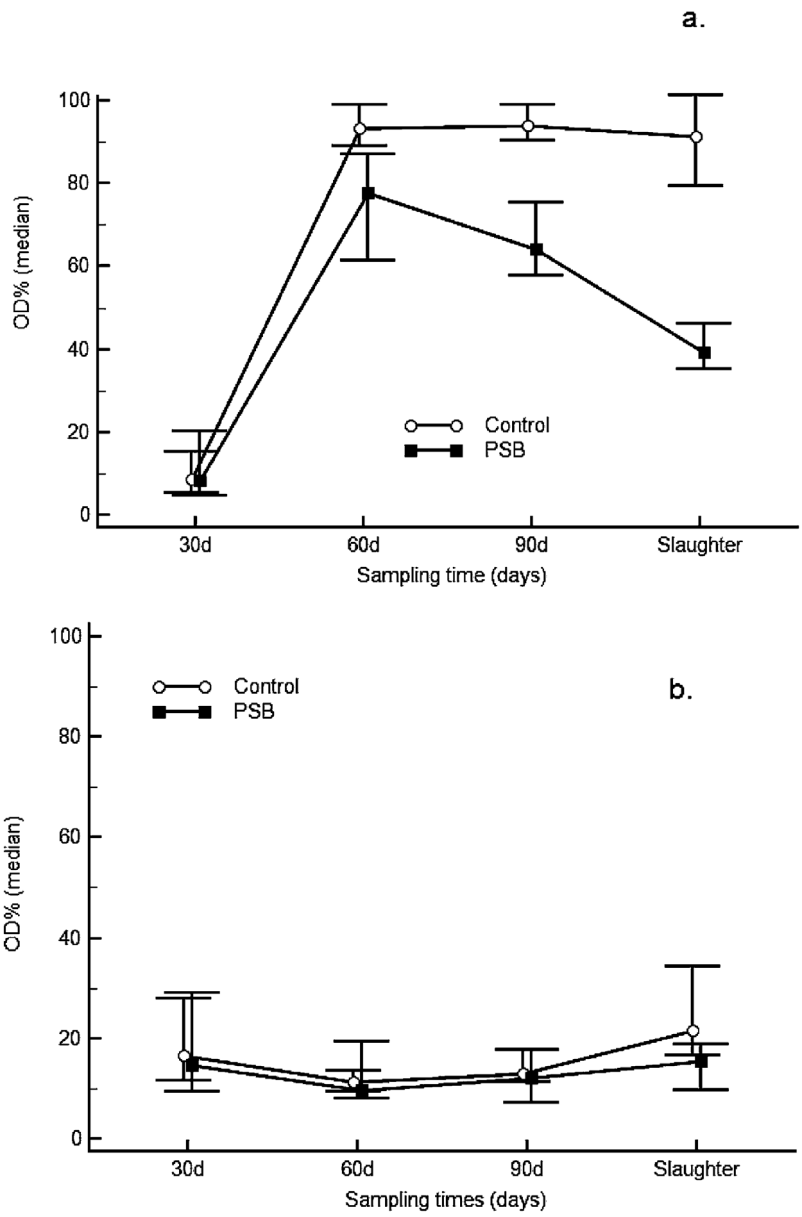

Fig. 1. Median optical density percentage (OD%) values and their corresponding 95% confidence intervals at the different sampling times for the control (Control) and treatment (PSB) groups for trials 1 (a.) and 2 (b.).

Regarding seroprevalence, it was significantly lower in the TG only before slaughter (89.6% in the CG *vs*. 50% in the TG; $P < 0.001$) (Table 2). A significant decrease in seroprevalence was observed in the TG between the third and fourth sampling (88.2 *vs*. 50%, respectively; $P < 0.001$) (Table 2). On the first sampling in this first trial, OD% values were low for both groups (median OD% = 9.2 in CG, and 7.4 in TG), but 30 days later they increased significantly (90.4 and 78.2, respectively). Although the median OD% values were high in the TG, they remained significantly lower than in the CG for the rest of the fattening period (Fig. 1a). The repeated measures ANOVA showed a significant effect of treatment and time on OD% values ($P < 0.01$). A clear decreasing trend of OD% values was observed in the TG as pigs approached to slaughter. At slaughter, median OD% for the TG was significantly lower than that for the CG (39.2 *vs*. 91.3, respectively; $P < 0.001$) (Fig. 1a). Overall, the mortality rate recorded by the farmer in this replicate was within the normal range for this farm in both groups (2–3%).

3.2. Trial 2

In the second trial, very few pigs shed *Salmonella* along the fattening period and no significant differences were observed between groups along the trial (Table 1). Overall seroprevalence was much lower than in the first trial as well as the prevalence of infection at

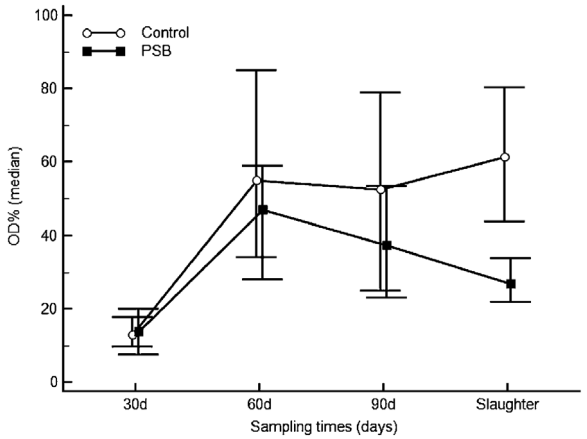

Fig. 2. Median optical density percentage (OD%) values and their corresponding 95% confidence intervals at the different sampling times for the control (Control) and treatment (PSB) groups when results from trial 1 and trial 2 were analyzed together.

slaughter. However, as in the first trial, seroprevalence before slaughter was significantly higher for the CG (31.1% *vs*. 13.7% in the TG; $P = 0.03$) (Table 2).

The median OD% value at slaughter for the CG was significantly higher than that at 30, 60 and 90 days on fattening ($P = 0.06$, $P < 0.001$, $P < 0.001$, respectively), but no differences in OD% values were observed across samplings in the TG. The repeated measures ANOVA did not find any significant effect of treatment on OD% values ($P = 0.34$), but the median OD% value at slaughter was significantly lower in the TG compared to the CG (15.6 *vs*. 21.5, respectively; $P < 0.001$) (Fig. 1b). Mortality in the CG was much higher than that in the TG. Seven (14%) pigs died in the CG during the fattening while only one (2%) was withdrawn from the TG ($P = 0.03$).

3.3. Overall results (Trials 1 and 2)

An overall significant reduction in the number of shedders was observed for the TG after 90 days after adjusting by the effect of the trial. The probability of shedding at this time was around 5.6 times higher for the CG compared to the TG (OR = 5.6, 95%CI: 1.1-26.9; $P = 0.04$). The difference remained somewhat at slaughter (OR = 2, 95%CI: 0.95-4.3; $P = 0.05$). No relationship was observed between treatment and *Salmonella* infection (Table 1).

An overall significant effect of treatment on seroprevalence was also observed for the sampling before slaughter. The probability of being a seropositive pig before slaughter was almost 5 times higher for the CG compared to the TG (OR = 4.9, 95%CI: 2.4-10.1; $P < 0.001$). When OD% values from both trials were analyzed together by repeated measures ANOVA after including replicate as another between-subject factor, a significant effect of treatment ($P < 0.01$), time of sampling ($P < 0.01$), trial ($P < 0.01$), and the interactions between treatment and time ($P < 0.05$) and time and trial ($P < 0.01$) on the OD% values was found. In general, after an initial and sharp upward trend from the first to the second sampling in both groups, a continuous decrease of the OD% values was observed in the TG, while they increased in the CG (Fig. 2).

No overall relationship was detected between being a seropositive pig and shedding (OR = 1.4, 95%CI: 0.65, 3.2; $P = 0.19$) or infection (OR = 0.47, 95%CI: 0.16, 1.36; $P = 0.09$) at slaughter. However, this relationship differed by trial. In the first trial no association was detected between seropositivity and shedding at slaughter ($P = 0.26$), but a negative significant one was observed between being seropositive and infection (OR = 0.16, 95%CI: 0.03–0.74; $P = 0.01$). On the contrary, in the second trial, a significant relationship was observed between seropositivity and shedding at slaughter (OR = 35, 95%CI: 3.9–321; $P < 0.01$), and between seropositivity and infection (OR = 7, 95%CI: 1.1–45.6; $P = 0.05$).

4. Discussion

To assess the effectiveness of OA for *Salmonella* reduction under field conditions is complex. There are many factors (i.e. *Salmonella* exposure and prevalent serotypes, palatability of the feed, diet buffering capacity, presence of concomitant infections, etc.) that may contribute to the variability of results (De Lange et al., 2010; Walia et al., 2016). In this study, two replicates of the same trial were carried out one year apart on the same fattening unit under the same management conditions but disparate results were found between trials. Differences were most likely due to different environmental conditions, i.e. levels of initial exposure to *Salmonella* spp., occurring in the fattening unit for each trial. In the first replicate, a large number of pigs became likely infected within the two weeks before starting the treatment with PSB, as suggested by the high proportion of shedders after 30 days on fattening and the low OD% values observed at that time for both groups. Thus, many pigs from the TG may have developed a carrier state that would not have been affected by the treatment. Although this initial situation supposed a true challenge for any product intended to reduce *Salmonella* infection, it was still possible to assess the impact that the use of the PSB may have on *Salmonella* shedding in pigs supposedly infected previously. In the second replicate, however, few pigs shed *Salmonella* spp. along the fattening period, which

precluded the finding of significant differences between the CG and TG for both *Salmonella* infection and shedding.

The dynamics of shedding in the first trial, as suggested by looking at results from the CG, showed a decrease in the number of shedders after the first sampling (days 60 and 90), which may be attributed to the lower level of stress of the infected pigs due to their adaptation to the farm environment (i.e. after the establishment of group hierarchies). The transport to the slaughter and the lairage period would have acted as stressor factors that would explain the reactivation of the shedding at slaughter along with possible new infections or re-infections at these stages (Hurd et al., 2002; Rostagno et al., 2003; Scherer et al., 2008) (Table 1). Although this pattern did not appear to be altered by the addition of the PSB in the TG, in general a lower number of shedders was detected along the trial for this group. A significant reduction ($P = 0.02$) in the number of shedders in the TG was observed after 90 days on fattening, and a close-to-significant ($P = 0.07$) reduction after 15 days on treatment (first sampling) and at slaughter ($P = 0.06$) (Table 1), suggesting some positive effect of PSB on the reduction of *Salmonella* shedders. The limited number of pigs considered in each group may have prevented to attain significant results.

Serological results in this trial supported previous findings. Median OD% values decreased significantly in the TG after 60 days on fattening, being this reduction particularly important after 90 days (Fig. 1a). The proportion of seropositive pigs during the fattening period that showed seronegative results at slaughter was significantly higher in the TG compared to the CG (Table 2). This important drop suggested a lower exposure to *Salmonella* spp. in the TG during the last period of fattening, which would be explained by either a reduction in the proportion of *Salmonella* shedders or in the amount of bacteria shed in the feces in this group. The lack of exposure to *Salmonella* spp. in the TG would have ceased stimulating the pig's immune system and thus antibody levels would tend to decline. The observation of seropositive growers pigs that become seronegative at slaughter has been already described (Dahl et al., 1997; van der Wolf et al., 2001b). However, this quick decrease in OD% values in a relatively short period of time (one month) was really unexpected. The authors are not aware of studies assessing the evolution of the pig immune response once *Salmonella* exposure ceases, and further research is warranted on this subject.

Despite the significantly higher number of *Salmonella*-infected pigs at slaughter in this trial, the lower number of shedding pigs and the decreasing trend in OD% values observed in the TG would suggest that the PSB had a positive effect on reducing the shedding in *Salmonella*-infected pigs.

Regarding the second trial, bacteriology on OF samples indicated a low level of pig infection during the fattening for both groups and no significant differences were found between them. However, the number of dead/withdrawn pigs in the CG was significantly larger than in the TG (14% *vs*. 2%, respectively). Although the causes of death/withdrawal were not recorded, it is likely that it had an impact on the proportion of shedders in this group, as weakened pigs would have been more prone to become infected by *Salmonella* and therefore shed the pathogen, thus increasing the odds of infecting other pigs within the group. *Salmonella* shedding has been previously associated with other concomitant enteric pathogens such as *Lawsonia intracellularis* that may affect the efficacy of PSB (Walia et al., 2016).

Serology in this trial would somewhat reflect the overall low prevalence of infection during most of the fattening period. Indeed, it showed an initial decrease in OD% values (Fig. 1b) for both groups from the first to the third sampling, likely associated with a lack of new infections. However, as expected for an infection that usually builds up as time passes when no specific control measures are taken, in the CG the median OD% value was significantly higher at slaughter compared to previous samplings. This pattern was not seen in the TG, likely due to the treatment with the PSB. The significant lower seroprevalence observed prior to slaughter in the TG would suggest the positive effect of PSB on reducing the likelihood of infection and shedding. Indeed, in this trial a significant positive relationship was observed between being a seropositive pig and shedding and infection at slaughter.

When analyzing both trials together an increased number of sampled pigs were considered in each group and, after adjusting by trial, a significant increasing risk of shedding in the CG was observed at 90 days on fattening and at slaughter. The magnitude of this increase was somewhat limited at slaughter (44.4% in the CT *vs*. 32.6% in the TG), which could likely be due to the large number of pigs that were already infected in the TG at the beginning of the first trial and that ended up shedding after being stressed. Regarding serology, an overall significant decreasing trend in DO% values (Fig. 2) and thus in seroprevalence was observed when pigs approached to slaughter, which may be associated with some prevention of *Salmonella* infection due to the likely lower exposure to the pathogen. This reduction in seroprevalence may also help to improve the herd risk category in the context of a national *Salmonella* control program based on serological results, such as those implemented in Denmark or Germany (Mousing et al., 1997; Merle et al., 2011).

These results are similar to those found in previous studies where OA were administered for at least 4 weeks (Creus et al., 2007; Argüello et al., 2013; De Ridder et al., 2013; Walia et al., 2016), which suggested that long periods of treatment are required before any positive effect is detected. The use of the PSB with the only purpose of reducing *Salmonella* seroprevalence may then be expensive for a commercial pig farm. However, OA might also improve pig performance, especially after exposure to enteric pathogens such as *Salmonella* (Gebru et al., 2010), which may help to reach a cost-benefit trade-off. Since no results on production data (weight gain, feed intake, etc.) were considered in this study, no proper cost-benefit analysis could be performed to assess the economic feasibility of using PSB for a period as long as the whole fattening in a *Salmonella*-infected unit.

It can be concluded that the administration of PSB at 3 kg/T for the whole fattening period was able to reduce seroprevalence before slaughter, which may reflect a positive effect on the control of *Salmonella* at the end of the fattening period.

Conflict of interest

None.

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