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ORIGINAL ARTICLE

Reduction of subclinical *Salmonella* infection in fattening pigs after dietary supplementation with a ß-galactomannan oligosaccharide

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Abstract

Aims: To assess the efficacy of a β-galactomannan oligosaccharide (ß-GMOS) for the control of *Salmonella* infection in fattening pigs.
Methods and Results: Three different doses (0·5, 3 and 2 kg ß-GMOS per ton of feed) were used during the entire period of growing in three similar and independent field trials carried out in a small fattening unit (≈100 pigs). Treatment was randomly assigned to half of the pens. Individual serum samples (20–25 per group) were collected at different times during the fattening period and a similar number of faecal samples during the fattening period and at slaughter. In addition, mesenteric lymph nodes were collected at slaughter. Herdcheck® Swine *Salmonella* ELISA was used for serological analyses, the ISO 6579:2002/Amd 1 : 2007 for bacteriology and the PFGE for molecular characterization of *Salmonella* strains. The addition of ≥2 kg t^{-1} of ß-GMOS to the pig diet during the entire fattening period was associated with a reduction in *Salmonella* prevalence, shedding and seroconversion.
Conclusions: Feed supplementation with ß-GMOS may be a useful complementary tool for the control of salmonellosis in fattening pigs.
Significance and Impact of the Study: ß-GMOS may be a complementary way of reducing *Salmonella* shedding and infection in fattening pigs.

Introduction

In the European Union (EU), human salmonellosis is ranked as the second most important zoonoses transmitted through the consumption of food, after campylobacteriosis (EFSA 2014). This infection has been mostly associated with the consumption of *Salmonella*-contaminated poultry-derived products (eggs, meat, etc.) and in a less extent to other meat products such as pork or pork-derived products (EFSA 2012).

During the last years, the implementation in the EU of national eradication programs for *Salmonella* in poultry has caused a major decline in the incidence of the human salmonellosis related to poultry and products thereof throughout Europe (EFSA 2014), provoking a relative increase in the registered number of pork-related salmonellosis outbreaks, and emphasizing the need for programs to tackle this infection in pigs (Boyen *et al.* 2008).

Salmonella spp. are characterized by their ubiquity and great environmental resistance (Murray 2000), which favours the contact and further infection of domestic animals. Unlike *Salmonella* control programs in poultry, in pigs, vaccines against zoonotic *Salmonella* are not commonly used yet (Farzan and Friendship 2010). Thus, given the problems associated with the identification of asymptomatically *Salmonella*-infected pigs, that is the lack of sensitivity of bacteriological and serological tests (Hurd *et al.* 2004; Mainar-Jaime *et al.* 2008a,b; Vico *et al.* 2010), high standards on farm biosecurity and hygiene are of utmost importance to prevent the contact of this pathogen with the live pig (De Busser *et al.* 2013).

The environmental contamination of the fattening unit is considered a major risk factor of *Salmonella* infection of slaughter pigs (Blaha 2010). Traditionally, the use of antibiotics at subtherapeutic doses, that is as growth promoters (AGP), has helped on the control of enteric diseases at critical periods of animal production such as weaning or the transition from nursery to fattening. However, the use of AGP also allowed for the development of antibiotic resistance (AR) (Witte 1998), which led to the ban of AGP use in Europe (Regulation EC No 1831/2003 on additives for use in animal nutrition). Indeed, the presence of resistant *Salmonella* has increased in recent years, and many human cases would be associated with the consumption of *Salmonella*-contaminated animal-derived foods (Arlet *et al.* 2006). Recent European reports show that AR is common in *Salmonella* isolates from human, animal and food samples (EFSA 2013). The increasing prevalence of AR in *Salmonella* strains highlights the need for the search of new alternatives to AGPs, such as probiotics and prebiotics (Gaggia *et al.* 2010).

Mannan oligosaccharides (MOS) are nondigestible oligosaccharides that have been suggested as a potential alternative to AGPs (Davis *et al.* 2002; Castillo *et al.* 2008). Although MOS may exert a beneficial effect upon health and metabolism, their mode of action seems to differ to that from prebiotics as they do not selectively enrich for beneficial bacterial populations (Gaggia *et al.* 2010; Halas and Nochta 2012). Besides, as they are not direct nutrients, they are considered as nutricines (Adams 2000).

MOS would also have an additional important antimicrobial effect based on their capacity to bind to mannose-specific lectin of gram-negative pathogens that express type-1 fimbriae (Borowsky *et al.* 2009), thus blocking the adhesion of these bacteria to the enterocytes and preventing the subsequent colonization (Becker and Galletti 2008). This binding capacity may, however, be very variable because MOS come from a wide variety of sources (oil palm seeds, coffee beans, yeast, carob seeds, etc.), show a diverse biological composition and are processed in very different ways (Spring 1999; Becker and Galletti 2008). Thus, each product should be well characterized and individually tested to determine its true efficacy for the control of pig salmonellosis.

The effect of dietary MOS on *Salmonella* infection and/or shedding has been the subject of many studies in both poultry (Fernández *et al.* 2000; Spring *et al.* 2000) and pigs (Letellier *et al.* 2000; White *et al.* 2002; Burkey *et al.* 2004; Wenner *et al.* 2013). Results either on animal performance or on antimicrobial effect are often contradictory, likely due to the differences among products mentioned above and the dose used (Gaggia *et al.* 2010). In addition, the antimicrobial effect of dietary MOS in pigs has been mostly assessed through studies on weaning or nursery animals, which may not have fully developed their immune system, for short periods of time (around 4 weeks) (Letellier *et al.* 2000; Burkey *et al.* 2004; Castillo *et al.* 2008) and often after experimental infections that generally imply infective doses higher than that usually observed under natural environments (Calveyra *et al.* 2012). All these aspects may have contributed to the observation of inconsistent results.

The objective of this study was to assess the efficacy of a novel β-galactomannan oligosaccharide (commercially available as Salmosan®, Industrial Técnica Pecuaria S.A., Barcelona, Spain) developed from the Mediterranean carob bean gum (*Ceratonia siliqua*) for the control of *Salmonella* spp. in fattening pigs. For this purpose, this MOS was administered to animals during the entire period of growing. In addition, the results from this study will allow us to get a better understanding of the epidemiology of *Salmonella* infection in fattening pigs.

Materials and methods

Experimental design

The β-galactomannan oligosaccharide (ß-GMOS) used in these trials consisted of a β-(1–4)-mannose oligosaccharide backbone with branched galactose molecules (1 : 4 galactose/mannose ratio) (Warrand 2006). Three different doses (0·5, 3 and 2 kg ß-GMOS per ton of feed) were used during the entire period of growing in three similar and independent field trials (I, II and III, respectively) carried out between 2010 and 2013 in a small commercial fattening unit (≈100 pigs) where *Salmonella* had been consistently isolated. The unit had eight pens, which contained 12–13 pigs each.

Treatment was randomly assigned to pigs allocated in half of the pens; thus, pigs from four pens received the basal diet mixed with the corresponding dose of β-GMOS (treated group; TG) and pigs from the remaining four pens were fed with the untreated basal diet (control groups; CG). Given the size of the fattening unit, the compound feed (with and without the β-GMOS) was provided in 40-kg bags and manually administered to the animals by the farmer throughout the entire period of fattening. The farmer was unaware of the treatment allocation.

Sampling scheme

All pigs were given in-feed colistin (120 ppm) for around 2 weeks right after arriving to the farm unit. After colistin, the treatment with the β-GMOS was initiated in the TG and serum was collected from all animals from both

Table 1 Microbiological results for *Salmonella* isolation (ISO 6579:2002/Amd 1:2007) for faecal samples at farm (FSF) after 60 and 90 days in the fattening unit and for mesenteric lymph nodes (MLN) and faecal samples at slaughter (FSS) for the three experimental groups of pigs

		Fattening unit				Slaughter			
		FSF at 60 days		FSF at 90 days		MLN		FSS	
Trial*	Group	No. (%) positives†	*P*‡	No. (%) positives†	*P*‡	No. (%) positives†	*P*‡	No. (%) positives†	*P*‡
I	CG	4/19 (21)	0·41	1/20 (5)	0·62	23/42 (54·8)	0·24	–	–
	TG	3/28 (10)		3/27 (8·3)		19/45 (42·2)		–	
II	CG	3/25 (12)	0·60	2/25 (8)	0·48	37/47 (78·7)	<0·01	26/45 (57·8)	<0·01
	TG	1/25 (4)		0/25 (0)		0/39 (0)		1/39 (2·6)	
III	CG	7/26 (26·9)	0·15	0/28 (0)	1	20/49 (40·8)	<0·01	34/49 (69·4)	<0·01
	TG	2/23 (8·7)		1/27 (3·7)		3/51 (5·9)		6/51 (11·8)	

CG: control group; TG: treatment group.

*Trial I: 0·5 kg ß-GMOS/t feed; trial II: 3 kg ß-GMOS/t feed; trial III: 2 kg ß-GMOS/t feed.

†No. and percentage of *Salmonella*-positive/total samples.

‡Statistical significance (*P* values) between TG *vs* CG groups.

groups to check for possible significant differences in seroprevalence against *Salmonella* spp. at the beginning of the trial. At 60 and 90 days of the fattening period, individual serum was collected again from all animals and faecal samples (faecal samples at the farm, FSF) were collected after spontaneous defecation from 20 to 25 pigs per group. In each sampling, pigs from all the pens were sampled, with a minimum of 2 pigs per pen. One week before slaughter, serum was collected from a minimum of 40 pigs in each group (except in the first trial, where sera could not be collected for logistic reasons). Mesenteric lymph nodes (MLN) and faeces from distal colon (Faecal Samples at the Slaughter -FSS-) were collected at slaughter (after approximately 120 days of fattening) for a similar number of pigs, except for the first trial where only MLN were collected. Because of the inherent difficulties of collecting faeces from rectum, slight variations in the number of sampled pigs with regard to the planned number of pigs to be sampled occurred. The exact number of animals analysed for each type of sample is shown in Tables 1 and 4.

Bacteriology and strain characterization

Bacteriology was performed on FSF, FSS and MLN samples following the standard International Organization for Standardization (ISO) 6579:2002/Amd 1 : 2007 method (ISO 2007). Serotyping was further carried out at the National Reference Laboratory for Animal Salmonellosis (Madrid, Spain) on one representative bacterial colony from culture-positive samples and following the White-Kauffmann-Le Minor scheme (Guibourdenche *et al.* 2010).

To establish the relationship between *Salmonella* isolates from MLN and faeces (either FSF or FSS) and thus assess the effect of the β-GMOS on both the infection and further shedding, *Salmonella* strains showing the same serotype in both types of samples were further submitted to pulsed-field gel electrophoresis (PFGE). In addition, a representative number of strains of *Salmonella* serotypes showing the higher prevalence in this farm, that is Typhimurium, its monophasic variant 4,[5],12:i:- and Rissen, were also analysed to assess their possible origin. Genotyping was performed according to the Pulse-Net protocol (Ribot *et al.* 2006). Details on the procedure followed are described elsewhere (Andrés *et al.* 2013).

Antimicrobial resistance (AR) tests were also performed on isolates submitted to PFGE analyses in order to further characterize them. Susceptibility to a panel of 10 antimicrobials, that is ampicillin, chloramphenicol, streptomycin, sulfisoxazole, trimethoprim, tetracycline, gentamicin, nalidixic acid, ciprofloxacin and cefotaxime, was determined by the Kirby-Bauer disc diffusion method (Murray *et al.* 2007). *Escherichia coli* ATCC 25922 and both *Salmonella* Typhimurium ATCC 14028 and DT104 reference strains were used as controls. *Salmonella* strains were classified as resistant, intermediate or susceptible according to the Clinical and Laboratory Standard Institute recommendations (CLSI 2012). To establish the multi-AR profiles, drugs were grouped into the following classes: aminopenicillins (A), phenicols (C), aminoglycosides (S), sulphonamides and dihydrofolate reductase inhibitors (Su), tetracyclines (T), cephalosporins (Cf) and quinolones (Na).

Serology

Collected sera were kept frozen (−20°C) until serological analyses were carried out through the Herdcheck® Swine *Salmonella* ELISA (IDEXX Laboratories, Westbrook, ME). Analyses were performed following manufacturer's

Table 2 Results of the random-effect logistic regression* on the effect of a β-galactomannan on *Salmonella* infection and shedding at slaughter

	Logistic regression parameters					
	Infection (MLN)†			Shedding (faecal samples)‡		
Factor	*P*	OR	95%CI (OR)	*P*	OR	95%CI (OR)
Doses						
0 kg t^{-1} feed		1			1	
0·5 kg t^{-1} feed	0·27	0·60	0·24, 1·50	–	–	–
2 kg t^{-1} feed	<0·01	0·09	0·04, 0·18	<0·01	0·06	0·02, 0·13
3 kg t^{-1} feed	<0·01	0·007	0·001, 0·05	<0·01	0·019	0·002, 0·15
Trial§						
I		1		–	–	–
II	0·06	3·05	0·94, 9·88		1	
III	0·21	0·57	0·23, 1·38	0·30	1·65	0·63, 4·32

OR: odds ratio; 95%CI: 95% confidence interval.

*Pen as random factor and trial as confounding factor.

†An animal was artificially considered positive within the treatment group for trial II to allow for model convergence.

‡No faecal samples were collected for trial I.

§Trial I: 0·5 kg ß-GMOS t^{-1} feed; trial II: 3 kg ß-GMOS t^{-1} feed; trial III: 2 kg ß-GMOS t^{-1} feed.

instructions, and different cut-off values (10, 20 and 40%) were considered.

Statistical analyses

Univariable chi-squared analysis was used to assess statistical differences in seroprevalence and shedding between the CG and the TG at the different point times considered, and in prevalence at slaughter. When low values or zeros were present, the two-tailed Fisher exact test was used (EPI INFO 3.5.3., CDC, Atlanta, Georgia). A random-effects logistic regression (re-LG) with pen as the random factor was further performed to measure the overall effect that the use of the β-GMOS may have on the prevalence of infection and shedding at slaughter. The variable 'Trial' was forced into the regression model as potential confounding factor (STATA/IC 12.1. StataCorp. LP, College Station, TX).

Results

No relevant health-related problems were detected in any of the groups during the development of the field trials. Mortality rate was within the normal range (2–3%) for this farm for both groups for the three trials. No significant differences in seroprevalence were observed between the CG and the TG at the beginning of any of the three trials; thus, groups were considered comparable.

Bacteriological results

The results on *Salmonella* spp. shedding (as indicated by faecal positive samples) and infection prevalence (MLN positive samples) for both the CG and the TG are presented in Table 1. The prevalence of *Salmonella* spp. infection in the CGs was above 40% in the three field trials and significantly higher in trial II (78·7%) compared to trials I and III (54·8 and 40·8%, respectively; $P < 0·05$).

In trial I, where the lowest dose of the β-GMOS (0·5 kg t^{-1}) was used, no significant differences were observed between TG and CG at any sampling time. However, at higher doses (2 and 3 kg t^{-1}), both the prevalence of infection (i.e. proportion of pigs with *Salmonella* in MLN) and shedding at slaughter were significantly lower in the TG than in the CG.

When the overall effect of the β-GMOS on *Salmonella* infection and shedding was estimated by the re-LG logistic regression using combined results from all the trials at slaughter, and after adjusting by trial and taking into account the pig allocation to pens (random factor), doses of 2 or 3 kg t^{-1} of β-GMOS decreased significantly ($P < 0·01$) the prevalence of infection and shedding (Table 2). A higher reduction was observed when a higher dose was used.

Strain characterization

Of 198 strains of *Salmonella* isolated, only 149 (75%) were serotyped due to budget restrictions. All the nonserotyped strains corresponded to isolates from the CGs from trials II and III, as they were the groups presenting a larger number of isolates. At least 70% of the isolates from each of these two groups were serotyped (Table 3).

Most (53%) of the serotyped isolates corresponded to the monophasic variant of Typhimurium (4,[5],12:i:-),

Table 3 Distribution of *Salmonella* serotypes according to trial, group and sample type

			Serotypes						
Trial*	Group	Sample type	Typhimurium	4,[5],12:i:-	Rissen	Goldcoast	Other serotypes†	Nonserotyped	Total
I	CG	FSF	2		2		2		6
		FSS‡							
		MLN	1	15	3	4			22
	TG	FSF					4		4
		FSS‡							
		MLN	1	16		4			21
II	CG	FSF	5						5
		FSS	7	4	4			11	26
		MLN	3	16	1	1		16	37
	TG	FSF	1						1
		FSS			1				1
		MLN							
III	CG	FSF			8				8
		FSS	3	14			3	14	34
		MLN	1	10	1			8	20
	TG	FSF			4		1		5
		FSS	2	2	1				5
		MLN	1	2					3
Total			26	79	25	9	10	49	198

CG: control group; TG: treatment group; FSF: faecal samples at farm; FSS: faecal samples at slaughter; MLN: mesenteric lymph nodes.
*Trial I: 0·5 kg ß-GMOS t^{-1} feed; trial II: 3 kg ß-GMOS t^{-1} feed; trial III: 2 kg ß-GMOS t^{-1} feed.
†Give (3), Kapemba (3), Derby (2), Muenchen (1), Brandenburg (1).
‡Samples were not collected for trial I.

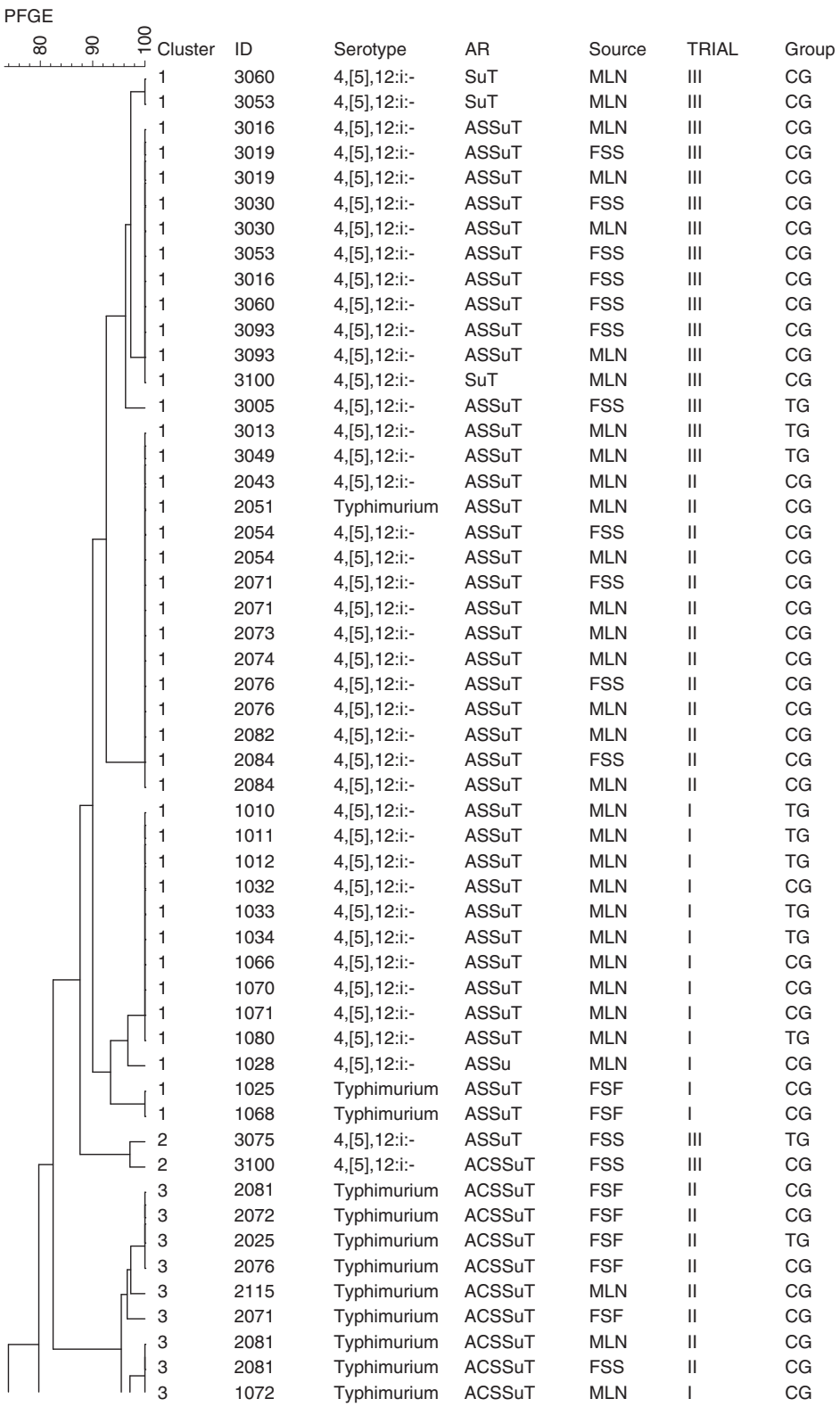
followed by Typhimurium (17·4%), Rissen (16·8%), Goldcoast (6%) and other (6·7%) less prevalent serotypes. The distribution of strain serotypes by trial and type of sample is presented in Table 3. Interestingly, all 4,[5],12:i:- strains were detected in samples collected at the slaughterhouse (either from FSS or from MLN), while Typhimurium and Rissen were detected at the farm and slaughter level.

PFGE analyses were performed on isolates representing the three major serotypes (4,[5],12:i:-, Typhimurium and Rissen) as they were the only serotypes isolated from both faecal (either FSF or FSS) and MLN samples. A total of 19 isolates from trial I, 27 from trial II and 34 from trial III were analysed (Fig. 1). The PFGE analysis of these 80 *Salmonella* isolates showed 26 different *Xba*I patterns grouped into six main clusters with at least 90% of similarity. Overall, clusters matched well with serotypes and AR profiles.

All strains showed resistance to at least two antimicrobial classes (Fig. 1). AR to tetracycline was the most common (97·5%), followed by sulfisoxazole (95%), streptomycin (91·25%) and ampicillin (90%). A total of 10 multi-AR profiles were detected, being the most common ASSuT ($n = 42$; 52·5%) generally associated with the 4,[5],12:i:- serotype and ACSSuT ($n = 23$; 28·75%) mostly associated with Typhimurium (Fig. 1).

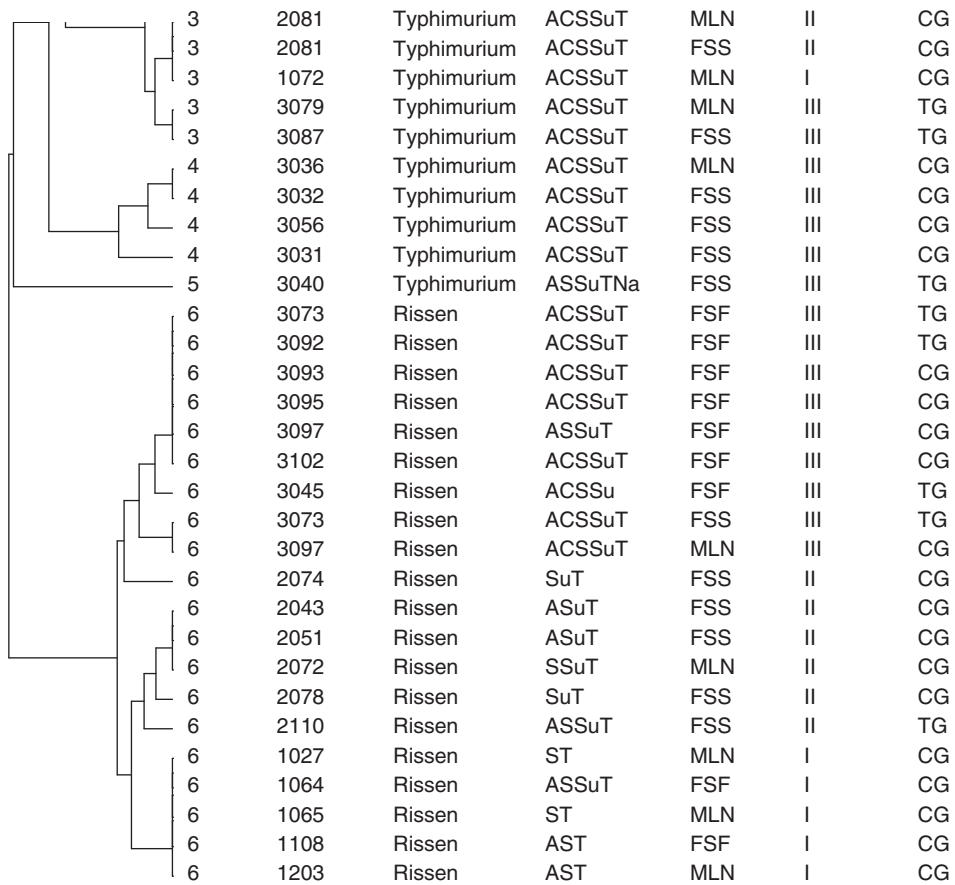
Cluster 1 included mostly 4,[5],12:i:- strains from the three trials but also included three *S* Typhimurium strains, two of them isolated from FSF. Cluster number 3 was composed by *S* Typhimurium strains coming also from all the trials and from samples collected both at the farm and at the slaughter. Cluster number 6 consisted in 20 *S* Rissen strains from the three trials and also collected both at the farm and at the slaughter. Clusters 2 (two strains), 4 (four strains) and 5 (one strain) were smaller clusters where all isolates belonged exclusively to trial III. When analysed by trial, three main clusters were observed for trials I and II, and six for trial III. In all of them, there were samples collected at the farm and at the slaughter within the same cluster. Considering the 90% similarity cut-off, the same strains of *S.* 4,[5],12:i:-, *S* Typhimurium and *S* Rissen were detected in all the trials (Fig. 1).

An overall positive significant association between infection (i.e. *Salmonella* spp. isolated from MLN) and shedding was observed when all pigs from trials II and III were considered together. A carrier pig had >7 times higher risk of shedding *Salmonella* spp. than a noncarrier pig (odds ratio= 7·7; 95%CI: 3·3–18·4; $P < 0·0001$; results not shown). These results were supported by the observation in trial II of four control-infected pigs shedding the same strain with which they were infected (three infected



Cluster	ID	Serotype	AR	Source	TRIAL	Group
1	3060	4,[5],12:i:-	SuT	MLN	III	CG
1	3053	4,[5],12:i:-	SuT	MLN	III	CG
1	3016	4,[5],12:i:-	ASSuT	MLN	III	CG
1	3019	4,[5],12:i:-	ASSuT	FSS	III	CG
1	3019	4,[5],12:i:-	ASSuT	MLN	III	CG
1	3030	4,[5],12:i:-	ASSuT	FSS	III	CG
1	3030	4,[5],12:i:-	ASSuT	MLN	III	CG
1	3053	4,[5],12:i:-	ASSuT	FSS	III	CG
1	3016	4,[5],12:i:-	ASSuT	FSS	III	CG
1	3060	4,[5],12:i:-	ASSuT	FSS	III	CG
1	3093	4,[5],12:i:-	ASSuT	FSS	III	CG
1	3093	4,[5],12:i:-	ASSuT	MLN	III	CG
1	3100	4,[5],12:i:-	SuT	MLN	III	CG
1	3005	4,[5],12:i:-	ASSuT	FSS	III	TG
1	3013	4,[5],12:i:-	ASSuT	MLN	III	TG
1	3049	4,[5],12:i:-	ASSuT	MLN	III	TG
1	2043	4,[5],12:i:-	ASSuT	MLN	II	CG
1	2051	Typhimurium	ASSuT	MLN	II	CG
1	2054	4,[5],12:i:-	ASSuT	FSS	II	CG
1	2054	4,[5],12:i:-	ASSuT	MLN	II	CG
1	2071	4,[5],12:i:-	ASSuT	FSS	II	CG
1	2071	4,[5],12:i:-	ASSuT	MLN	II	CG
1	2073	4,[5],12:i:-	ASSuT	MLN	II	CG
1	2074	4,[5],12:i:-	ASSuT	MLN	II	CG
1	2076	4,[5],12:i:-	ASSuT	FSS	II	CG
1	2076	4,[5],12:i:-	ASSuT	MLN	II	CG
1	2082	4,[5],12:i:-	ASSuT	MLN	II	CG
1	2084	4,[5],12:i:-	ASSuT	FSS	II	CG
1	2084	4,[5],12:i:-	ASSuT	MLN	II	CG
1	1010	4,[5],12:i:-	ASSuT	MLN	I	TG
1	1011	4,[5],12:i:-	ASSuT	MLN	I	TG
1	1012	4,[5],12:i:-	ASSuT	MLN	I	TG
1	1032	4,[5],12:i:-	ASSuT	MLN	I	CG
1	1033	4,[5],12:i:-	ASSuT	MLN	I	TG
1	1034	4,[5],12:i:-	ASSuT	MLN	I	TG
1	1066	4,[5],12:i:-	ASSuT	MLN	I	CG
1	1070	4,[5],12:i:-	ASSuT	MLN	I	CG
1	1071	4,[5],12:i:-	ASSuT	MLN	I	CG
1	1080	4,[5],12:i:-	ASSuT	MLN	I	TG
1	1028	4,[5],12:i:-	ASSu	MLN	I	CG
1	1025	Typhimurium	ASSuT	FSF	I	CG
1	1068	Typhimurium	ASSuT	FSF	I	CG
2	3075	4,[5],12:i:-	ASSuT	FSS	III	TG
2	3100	4,[5],12:i:-	ACSSuT	FSS	III	CG
3	2081	Typhimurium	ACSSuT	FSF	II	CG
3	2072	Typhimurium	ACSSuT	FSF	II	CG
3	2025	Typhimurium	ACSSuT	FSF	II	TG
3	2076	Typhimurium	ACSSuT	FSF	II	CG
3	2115	Typhimurium	ACSSuT	MLN	II	CG
3	2071	Typhimurium	ACSSuT	FSF	II	CG
3	2081	Typhimurium	ACSSuT	MLN	II	CG
3	2081	Typhimurium	ACSSuT	FSS	II	CG
3	1072	Typhimurium	ACSSuT	MLN	I	CG

Figure 1 Dendrogram showing the main *Xba*I patterns (>90% homology) for the *S* Typhimurium, *S.* 4,[5],12:i:- and *S* Rissen strains isolated from the three trials carried out to assess the efficacy of different concentrations of a β-galactomannan oligosaccharide to control *Salmonella* infection in a fattening pig unit.



3	2081	Typhimurium	ACSSuT	MLN	II	CG
3	2081	Typhimurium	ACSSuT	FSS	II	CG
3	1072	Typhimurium	ACSSuT	MLN	I	CG
3	3079	Typhimurium	ACSSuT	MLN	III	TG
3	3087	Typhimurium	ACSSuT	FSS	III	TG
4	3036	Typhimurium	ACSSuT	MLN	III	CG
4	3032	Typhimurium	ACSSuT	FSS	III	CG
4	3056	Typhimurium	ACSSuT	FSS	III	CG
4	3031	Typhimurium	ACSSuT	FSS	III	CG
5	3040	Typhimurium	ASSuTNa	FSS	III	TG
6	3073	Rissen	ACSSuT	FSF	III	TG
6	3092	Rissen	ACSSuT	FSF	III	TG
6	3093	Rissen	ACSSuT	FSF	III	CG
6	3095	Rissen	ACSSuT	FSF	III	CG
6	3097	Rissen	ASSuT	FSF	III	CG
6	3102	Rissen	ACSSuT	FSF	III	CG
6	3045	Rissen	ACSSu	FSF	III	TG
6	3073	Rissen	ACSSuT	FSS	III	TG
6	3097	Rissen	ACSSuT	MLN	III	CG
6	2074	Rissen	SuT	FSS	II	CG
6	2043	Rissen	ASuT	FSS	II	CG
6	2051	Rissen	ASuT	FSS	II	CG
6	2072	Rissen	SSuT	MLN	II	CG
6	2078	Rissen	SuT	FSS	II	CG
6	2110	Rissen	ASSuT	FSS	II	TG
6	1027	Rissen	ST	MLN	I	CG
6	1064	Rissen	ASSuT	FSF	I	CG
6	1065	Rissen	ST	MLN	I	CG
6	1108	Rissen	AST	FSF	I	CG
6	1203	Rissen	AST	MLN	I	CG

Figure 1 (Continued).

with *S.* 4,[5],12:i:- and one with *S* Typhimurium) and in trial III with six *S.* 4,[5],12:i:- infected pigs shedding the same strain at slaughter (Fig. 1).

In addition, five pigs in trial II and two pigs in trial III shed at some point *Salmonella* strains different from those isolated from their MLN (Fig. 1).

Serology

When the highest dose of the β-GMOS (3 kg t^{-1} feed) was used (trial II), a significant lower seroprevalence was observed in the TG compared to the CG at both 60 ($P = 0{\cdot}005$) and 90 days ($P = 0{\cdot}06$) in the fattening unit, and also before slaughter ($P = 0{\cdot}004$), when an OD ≥ 40% was used as the cut-off value. No more differences in seroprevalence were observed at any time and at any cut-off for the other two trials (Table 4). Interestingly, in trial III, a slightly higher seroprevalence was observed for the TG at 90 days and previous to slaughter compared to the CG, but this difference was not statistically significant ($P = 0{\cdot}12$).

Discussion

In this study, we looked at the effect of a dietary MOS, a β-GMOS characterized by a 1 : 4 galactose/mannose ratio, when administered for the entire growing period on a small *Salmonella*-positive pig unit with low biosecurity level (i.e. no bird/rodent control, no changing room, no footbaths or boots for visitors available, etc.). Under these field conditions, we ensured the exposure of all pigs to the same naturally *Salmonella*-contaminated environment, while half of them were also exposed to a constant concentration of the β-GMOS through the feed. Initially, a low dose (0·5 kg t^{-1} of feed) of β-GMOS was used as it had shown some antimicrobial effects in experimental studies on poultry (manufacturer personal communication). In this first trial, infection at MLN was the main outcome of interest and no faecal or serum samples were collected at slaughter. No significant effect was, however, observed on *Salmonella* infection at slaughter, neither on faecal shedding at the farm. These results could be explained by the large differences expected between

Table 4 Serological results (Herdcheck® *Salmonella* ELISA, cut-off %OD ≥ 40) for the three trials after 60 and 90 days in the fattening unit and before slaughter

		60 days			90 days			Before slaughter		
Trial*	Group	*N*	No. (%) positives†	*P*‡	*N*	No. (%) positives†	*P*‡	*N*	No. (%) positives†	*P*‡
I§	CG	55	3 (5·5)	0·98	55	4 (7·3)	0·46	–	–	–
	TG	56	3 (5·4)		53	6 (11·3)		–	–	–
II	CG	55	18 (32·7)	<0·01	53	13 (24·5)	0·06	40	15 (37·5)	<0·01
	TG	56	6 (10·7)		55	6 (10·9)		40	4 (10)	
III	CG	51	1 (1·9)	1	51	0 (0)	0·12	49	0 (0)	0·12
	TG	51	2 (3·9)		50	3 (6)		51	4 (7·8)	

CG: control group; TG: treatment group.

*Trial I: 0·5 kg ß-GMOS t^{-1}; trial II: 3 kg ß-GMOS t^{-1}; trial III: 2 kg ß-GMOS t^{-1}.

†No. and percentage of *Salmonella* seropositive.

‡Statistical significance (*P* values) between TG *vs* CG groups.

§No serum samples were collected for trial I before slaughter.

poultry and pigs regarding intestinal physiology, amount of feed ingested, etc. In contrast, when the dose of β-GMOS was considerably increased (trials II and III), a significant reduction in the proportion of *Salmonella*-infected (i.e. positive in MLN), *Salmonella*-shedding and *Salmonella*-seropositive (trial II) pigs at slaughter was observed in TGs with respect to the corresponding CGs. In fact, the addition of ≥2 kg t^{-1} of β-GMOS during the entire growing period was able to decrease the prevalence of *Salmonella* infection at slaughter to 0% in trial II and to less than 6% in trial III (Table 1). A similar finding was observed regarding *Salmonella* shedding at slaughter, which was reduced to 2·6% in trial II and to 11·8% in trial III (Table 1), while a significant reduction in seroconversion was only evidenced with the highest β-GMOS dose used (Table 4).

At farm level, no differences in *Salmonella* shedding during the growing period were observed between the CG and the TG for any of the trials. However, factors such as the intermittent shedding of these bacteria over time (Pires *et al.* 2013), the lack of sensitivity of the microbiological technique when performed on faecal samples from asymptomatic pigs (Hurd *et al.* 2004; Mainar-Jaime *et al.* 2008a) and the limited number of animals analysed in each group may have hindered the detection of significant differences in shedding between the CGs and TGs, at both 60 and 90 days of growing. At slaughter level, stress situations such as those associated with the transport of pigs to the slaughterhouse and with the lairage may have favoured the shedding of *Salmonella* from previously infected pigs (Hurd *et al.* 2001a; Boughton *et al.* 2007; Rostagno and Lay 2011), which may have allowed for the detection of significant differences in the proportion of shedders between the CGs and TGs. The highly significant relationship observed between *Salmonella*-infected and *Salmonella*-shedder pigs would support this hypothesis.

The results from the re-LG showed a clear decreasing trend in the prevalence of infection (i.e. *Salmonella* in MLN) at slaughter when the dose of β-GMOS increased from 2 to 3 kg t^{-1} of feed, in trials III and II, respectively. According to these results, the overall efficacy of β-GMOS in these field trials, measured as the preventive fraction (1-odds ratio), could reach from 91% (when a 2 kg t^{-1} dose was used) up to 99·2% (3 kg t^{-1}) when compared to the group composed by all control pigs from the trials. A similar decreasing trend would be expected on shedding, according to results shown in Table 2. Bearing in mind that the proportion of carrier pigs at slaughter could likely be considered a proxy of the *Salmonella* contamination arriving to the slaughter line (Berends *et al.* 1997), the addition of this β-GMOS may have contributed to decrease significantly the risk of *Salmonella* contamination of the carcasses. The PFGE analysis revealed that within a trial, some strains of *S* Typhimurium and *S* Rissen that had been previously isolated from faeces at the farm were later found in MLN from control pigs at the slaughter (Fig. 1), supporting this conclusion. It is worth to mention that a few pigs (5 in trial II and 2 in trial III) shed at some point *Salmonella* strains different from those isolated from their MLN (Fig. 1), which would support the presence of simultaneous infections by different *Salmonella* strains in a single pig, as it has been recently described elsewhere (Garrido *et al.* 2014).

Interestingly, no *S.* 4,[5],12:i:- strains were isolated from faecal samples at the farm despite this serotype was the most prevalent in the three trials. This finding may be related to infections occurring outside the farm. In this regard, it has been observed that the truck and the

lairage of a pig abattoir present a substantial risk for slaughter pigs to become infected with *Salmonella* (Swanenburg *et al.* 2001; Dorr *et al.* 2009) and that *S* Typhimurium may colonize enterocytes very rapidly (<2 h) after oral inoculation (Reed *et al.* 1986; Hurd *et al.* 2001b). Bearing in mind the average time spent by these pigs on the truck (1 h) and waiting for slaughter (12 h), either the truck or the lairage area may have acted as possible sources of infection for this serotype. Regardless where and when the exposure occurred, these results also suggest that the β-GMOS may have had an effect beyond the farm, reducing the risk of infection of the pigs even until right before the slaughter line.

The positive effect of this β-GMOS on the control of *Salmonella* infection was further supported by the significant lower number of pigs seroconverting in the TG compared to the CG in trial II. In trial III, however, no differences were observed, as the proportion of pigs seroconverting was very low in both groups. This low level of seroconversion could be attributed to a late infection, either at the end of the fattening period, or even during transport to the slaughter or at lairage, as at least 7 days are usually required to detect specific antibodies against *Salmonella* spp. (Nielsen *et al.* 1995; Scherer *et al.* 2008). In fact, 85% of the strains from this trial were isolated once the pigs arrived to the slaughter. The lack of seroconversion could also be related to the delayed onset of seroconversion showed by certain *Salmonella* strains that show higher faecal shedding and an increased persistence (Van Parys *et al.* 2013). The unexpected but nonsignificant difference in seroprevalence between CG and TG in this trial (0% *vs* 7·8%, respectively) was most likely due to the intrinsic variability of the ELISA used, as three animals from the CG had high %ODs (>36%) but lower than the 40% required to be deemed test negative.

A matter of concern derived from this study was the high level of AR observed. All *Salmonella* strains analysed showed some type of multi-AR. As the origin of most isolates could not be traced to this farm, particularly the *S.* 4,[5],12:i:- strains, these results would reflect the overall increasing trend in AR for pig-related *Salmonella* strains.

In view of the bacteriological results observed, this β-GMOS seemed to be effective against *Salmonella* infection in fattening pigs when a dose ≥2 kg t^{-1} of feed was used for the entire period of growing. Its use during the whole period of fattening was the consequence of the lack of knowledge regarding the time of higher risk of *Salmonella* exposure in this growing unit. A good knowledge of the dynamics of *Salmonella* infection in the farm may allow for the definition of the best periods of application, thus reducing its cost. Overall, feed supplementation with this β-GMOS could be considered a useful complementary tool for the control of pig salmonellosis, along with good hygiene and farm biosecurity practices.

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Conflict of Interest

All authors declare that no competing financial interests exist.

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