Garlic derivative propyl propane thiosulfonate is effective against broiler enteropathogens in vivo

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ABSTRACT Two experiments were carried out to study the effects of dietary supplementation with the garlic (*Allium sativum*)-derived product propyl propane thiosulfonate (PTS-O) on the intestinal $\log_{10}$ number of copies of enteropathogens in broiler chickens, together with their intestinal morphology and growth performance. The additive had no significant effect on feed intake at any dose assayed. In experiment 1 (1 to 21 d of age), the BW of chickens fed on 45 mg of PTS-O/kg of diet was higher ($P < 0.01$) than that of controls. Birds fed on diets containing 45 and 90 mg of PTS-O/kg of diet had improved ($P < 0.01$) feed:gain ratios compared with the controls at 21 d of age. Ileal villus height, width and surface area, mucosal thickness, and muscular layer thickness were considerably greater ($P < 0.01$) than control values in chickens fed 90 mg of PTS-O/kg of diet. The *Clostridium perfringens* $\log_{10}$ number of counts was not significantly affected at any dose assayed. The inclusion of PTS-O at both concentrations (45 and 90 mg/kg of diet) resulted in lower ($P < 0.01$) $\log_{10}$ number of copies of ileal *Salmonella* spp. and crop enterobacteria and *Escherichia coli*. The inclusion of 90 mg of PTS-O/kg of diet also resulted in lower ($P < 0.01$) enterobacteria and *E. coli* $\log_{10}$ numbers of copies in the ileal and cecal contents, respectively. The number of copies of *Campylobacter jejuni* was not significantly affected. In experiment 2 (15 to 28 d of age), lower ($P < 0.01$) $\log_{10}$ number of copies of *Salmonella* spp. and *C. jejuni* were determined in the ileal contents of chickens fed on diets containing 135 mg of PTS-O/kg of diet. The addition of 90 mg of PTS-O/kg of diet lowered ($P < 0.01$) only the number of copies of ileal *Salmonella* spp. This investigation confirmed previous in vitro data and showed that PTS-O lowered the intestinal numbers of enteropathogens and improved the ileal histological structure and productive parameters of broilers.

Key words: garlic derivative, *Salmonella* subspecies, *Campylobacter jejuni*, *Clostridium perfringens*, intestinal histological structure

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INTRODUCTION

The intestinal flora of broiler chickens plays an important role in growth performance and health of birds (Bjerrum et al., 2006), and its influence is particularly relevant when chickens are young and their microbiota is still in the process of development (Gong et al., 2008). The gastrointestinal tract of chickens on hatching is not well adapted to the digestion and absorption of many feed components (Sell, 1996; Uni et al., 1999). Hence, the interaction of intestinal growth, digestive functions, and diet is critical during the posthatching period when birds switch to enteral nutrition (Uni et al., 1999).

Several enteric pathogens associated with the intestinal microbiota of broilers are known to cause millions of losses to the sector in addition to human and animal health problems. In particular, enterobacteria, and more specifically *Salmonella* spp. and *Campylobacter* spp., are not only widespread pathogens in the food industry, but also have been frequently identified as the etiological agents of food-borne outbreaks (Eeckhaut et al., 2008). Zhao et al. (2001) reported the occurrence of 1.4 million cases of human salmonellosis every year in the United States alone. At the end of 2003, the EU issued a regulation (Commission of the European Communities. No. 2160/2003) relating to salmonellosis and other food-borne zoonoses, obliging member states to undertake certain measures to monitor and reduce the risk of transmission of *Salmonella* spp. According to the Centers for Disease Control and Prevention, there are approximately 2.4 million cases of human campylobacteriosis infection each year in the United States. The commonest causes of these illnesses are the handling of raw poultry meat and the consumption of undercooked poultry and poultry products (Mead et al., 1999; CDC, 2008).

Antibiotic growth promoters (**AGP**) have been used as broiler feed additives for years to prevent disease

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and improve performance efficiency (Dibner and Richards, 2005). However, due to the emergence of microbes resistant to the antibiotics used in therapy, in January 2006, the European Commission (**EC**) banned the use of AGP in animal production (EC Regulation No. 1831/2003; http://eur-lex.europa.eu/en/index.htm). Since this ban it has become necessary to search for natural alternatives with effects similar to AGP but without their reported drawbacks. Some plant extracts have shown potential to modulate the intestinal microflora and improve the performance efficiency of broilers (Cross et al., 2011). Garlic (*Allium sativum*) has been used for centuries for its health-giving properties, and garlic and garlic products have shown a broad antibiotic spectrum against both gram-positive and gram-negative bacteria (Harris et al., 2001). In addition, they have been found to be effective against many common pathogenic intestinal bacteria responsible for diarrhea in humans and animals (Amagase et al., 2001; Tatara et al., 2008). Garlic-derived products have been reported to be effective even against those strains that have become resistant to antibiotics; in particular, garlic extract and allicin have been shown to exert bacteriostatic effects on some vancomycin-resistant enterococci (Harris et al., 2001). Due to their antimicrobial activity, garlic-derived compounds might represent a useful alternative to AGP.

The antimicrobial effects of organosulphurate compounds industrially obtained by decomposition of initial compounds naturally present in garlic have been previously described by Ruiz et al. (2010) in vitro. These compounds showed antimicrobial activity against every bacterial group in pig feces studied, although enterobacteria and coliforms were the most affected populations. Furthermore, these products exerted a bactericidal effect against *Escherichia coli* and *Salmonella* Typhimurium strains. Nevertheless, there is no information in the literature concerning the use of these compounds on the intestinal microbiota of poultry. Accordingly, the aim of the present study was to investigate the antimicrobial effects of propyl propane thiosulfonate (**PTS-O**) dietary supplementation in vivo, particularly against three of the main enteropathogens linked to poultry production, namely *Salmonella* spp., *Campylobacter jejuni*, and *Clostridium perfringens*. Finally, because a connection has been reported between growth performance, gut morphology, and decrease of pathogen populations by using other feed additives (Yang et al., 2008; Baurhoo et al., 2009), these parameters were also studied in the current experiments.

MATERIALS AND METHODS

Dietary Supplements

Proallium-SO-DMC was the commercial preparation used in this study. This product was provided by DMC Research Center S.L. (Granada, Spain). It contained 11.3% of the active compound PTS-O as determined by HPLC according to the procedure described by Iberl et al. (1990). The PTS-O was incorporated in an inert commercial alimentary support (cyclodextrin) to produce Proallium-SO-DMC (Ruiz et al., 2010).

Birds, Diets, and Housing

The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the Spanish Council for Scientific Research (Madrid, Spain), and the animals were cared for in accordance with the Spanish Ministry of Agriculture guidelines (RD 1201/2005).

Experiment 1. A total of 144 male one-day-old Cobb broiler chickens were randomly assigned to 1 of 3 dietary treatments. Each treatment involved 8 replicates with 6 birds each. Birds were weighed on arrival and raised in heated wire-floored batteries, and received a lighting regimen of 23L:1D. Balanced commercial diets (Table 1) free of any feed antibiotics, and formulated to match the requirements for growing birds of this age and genotype, were used. Diets were fed ad libitum for 21 d. The dietary treatments were control (commercial diet with no additive), and PTS-O-45 and PTS-O-90 (commercial diet supplemented with 45 and 90 mg of PTS-O/kg of diet, respectively). The doses of PTS-O used were chosen according to results previously found in vitro (Ruiz et al., 2010). The aim of this experiment was to study the effect of dietary PTS-O addition on the pathogen (*Salmonella* spp., *Campylobacter jejuni*, and *Clostridium perfringens*) or potential pathogen (enterobacteria, *E. coli*) intestinal bacterial numbers, together with the ileal histological structure and productive parameters of broilers.

Experiment 2. A total of 30 male 15-d-old Cobb broiler chickens were purchased from a local farm. The chickens were randomly assigned to 1 of 3 dietary treatments. Treatments involved 2 replicates with 5 birds each. The birds were weighed on arrival and placed in heated wire-floored batteries. They were fed ad libitum for 14 d and received a lighting regimen of 23L:1D. Dietary treatments were control (no additive), and PTS-O-90 and PTS-O-135 (commercial diet supplemented with 90 and 135 mg of PTS-O/kg of diet, respectively). The PTS-O-90 was found to be the most effective in experiment 1 (90 mg/kg), and PTS-O-135 was assayed to determine if a higher dose would be more effective or not. This experiment was also run to check if the results obtained in experiment 1 on pathogen bacteria would be repeated in conditions closer to practical broiler production. Accordingly, the birds were bought in this case from a local producer at 15 d of age so that their initial "natural" intestinal microbiota was already established, and its composition was as close as possible to that in practical rearing conditions.

Euthanasia and Sample Collection

Data on live BW and feed intake (**FI**) were recorded at the beginning and end of both experiments. These

Table 1. Composition (g/kg) of the diet used in both experiments

Item	Value
Component	
Maize	462
Soy flour	310
Wheat	150
Vitamin + mineral mix[1]	30
Animal fat	20
Calcium carbonate	16.0
Calcium phosphate	3.1
Sodium chloride	4.5
Chromium oxide	2.0
Methionine	2.2
Lysine	0.2
Calculated composition	
ME (cal/g)	2,912
CP	193.0
Crude fiber	33.7
Fat	44.6
Calcium	7.1
Phosphorous	6.2
Methionine + cysteine	8.6
Lysine	1.2

[1]The mineral-vitamin mix contained (per 30 kg): vitamin A, 7,500,000 IU; vitamin D_3, 1,500,000 IU; vitamin E, 25 g; vitamin B_2, 2 g; vitamin B_{12}, 10 mg; vitamin B_6, 67 mg; calcium pantothenate, 7.5 g; nicotinic acid, 10 g; folic acid, 25 mg; vitamin K_3, 1 g; choline chloride, 250 g; Fe, 4 g; Cu, 750 mg; Co, 50 ng; Zn, 38 g; Mn, 42 g; I, 680 mg; Se, 45 mg; coccidiostat (nistatin + nicarbacin), 0.50 kg; BHT, 250 mg.

data were used to calculate final BW, FI, and feed:gain ratio (**F/G**).

Experiment 1. At 21 d of age, 3 birds per replicate (i.e., 18 per treatment) were randomly selected and killed by intra-thoracic injection of 0.2 mL/bird of the euthanasic T-61 (Intervet, Salamanca, Spain). The pH of the crop content of each bird was immediately measured with a Crison pH meter (Crison Instruments, S.A, Alella, Spain). Immediately afterward, samples from the crop, ileal (considered as the section between the Meckel's diverticulum and the ileo-cecal junction), and cecal contents of each bird were collected in plastic tubes, stored at −20°C, and freeze-dried (Ruiz and Rubio, 2009). Samples of about 1 cm taken at the midpoint of the ileum of 3 birds fed on the control or experimental diets were removed for histological analysis. Only samples from the chickens fed on the control or PTS-O-90 diets were subjected to histological analysis because there was a more pronounced effect on the number of enteropathogen copies in these chickens than there was in those fed PTS-O-45. The samples were flushed twice with PBS to remove luminal digesta and immersed in formalin (10% neutral buffered formaldehyde) for fixation. After 24 h in 10% neutral buffered formaldehyde, the tissue samples were carefully cleaned of any remaining digesta with deionized water, and then transferred to a fresh solution of 10% neutral buffered formaldehyde (Sigma, Alcobendas, Spain).

Experiment 2. At 28 d of age, 10 birds (n = 10 per treatment) were weighed and killed by intra-thoracic injection of 0.2 mL/bird of the euthanasic T-61 (Intervet, Salamanca, Spain). For the microbiota analysis, the ileal content of each bird was collected into a plastic tube, stored at −20°C, and freeze-dried (Ruiz and Rubio, 2009).

Real-Time PCR Analysis

Samples for microbial analysis were transferred into 2-mL bead beater vials containing 3 zirconia beads and reduced by physical disruption for 1 min in a Mini-Bead Beater (BioSpec Products, Bartlesville, UK) to small particles to facilitate DNA extraction. Total DNA was isolated from freeze-dried intestinal content samples (40 mg) using the QIAamp DNA stool kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions except that to increase its effectiveness the lysis temperature was raised to 95°C, and an additional step with lysozyme (10 mg/mL, 37°C, 30 min) incubation was included. Eluted DNA was treated with RNase and its concentration assessed spectrophotometrically with a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Purified DNA samples were stored at −20°C until use.

Bacterial numbers in intestinal samples were determined in duplicate by quantitative PCR (**q-PCR**) assays conducted in 96-well polypropylene plates using an iQ5 Cycler Multicolor PCR detection system (BioRad Laboratories, Hercules, CA). The 16S rRNA gene-targeted primers used are shown in Table 2. The composition of the reaction mixture and PCR conditions were those described in the quoted references. A plasmid standard containing the target region was generated using DNA extracted from pooled fecal samples of rats fed an AIN-93G diet. The amplified product was run on a 2% agarose gel, purified with an MBL-Agarose QuickClean kit (Dominion MBL, Spain), cloned using the TOPOTA cloning kit (Invitrogen, Paisley, UK), and transformed into *Escherichia coli* One Shot Top 10 cells (Invitrogen). Plasmids were eluted and the sequences obtained by the sequencing service of the Instituto de Parasitología y Biomedicina López-Neyra (CSIC, Granada, Spain), before being submitted to the ribosomal RNA database (http://blast.ncbi.nlm.nih.gov) to confirm the suitability of the primers. The concentration of the resulting products was determined spectrophotometrically and copy numbers calculated in terms of product size. For quantification of target DNA copy number a standard curve was generated with serial 10-fold dilutions of the extracted product, using at least 6 nonzero standard concentrations per assay. The bacterial concentration in each sample was measured as $\log_{10}$ copy number by interpolating the C_t values obtained from the fecal samples into the standard calibration curves. Each plate included duplicate reactions per DNA sample and the appropriate set of standards.

Histological Analysis

Fixed samples were dehydrated and embedded in paraffin wax. Three slides were prepared from each sample, and one containing a minimum of 2 sections

Table 2. Sequences of the primers used for the quantitative determination of microbial groups in the intestines of the broiler chickens in both experiments

Bacterial group	Primer	Reference
Clostridium perfringens	F: (ATGCAAGTCGAGCGAGTG) R: (TATGCGGTATTAATCTCTCCTTT)	Rinttilä et al., 2004
Campylobacter jejuni	VS15: (GAATGAAATTTTAGAATGGGG) VS16: (GATATGTATGATTTTATCCTGC)	Yang et al., 2004
Salmonella spp.	invA-1: (TTGTTACGGCTATTTTGACCA) invA-2: (CTGACTGCTACCTTGCTGATG)	Cortez et al., 2006
Enterobacteria	F5 (ATGGCTGTCGTCAGCTCGT) R5 (CCTACTTCTTTTGCAACCCACTC)	Castillo et al., 2006
Escherichia coli	F5 (GTTAATACCTTTGCTCATTGA) R5 (ACCAGGGTATCTAATCCTGTT)	Malinen et al., 2003

cut at 4 μm, at least 50 μm apart. The slides were stained with hematoxylin and eosin. All measurements were made with a light microscope with the help of an image analysis system (Cell^{A} Imagen Software, Olympus, Hamburg, Germany) equipped with a monitor. Five well-oriented villi and crypts were selected on each slide to determine villus height and width, and crypt depth. The villus height was determined as the distance from the tip to the bottom of the villi, and crypt depth as the distance between its mouth and its base. Villus surface area was calculated as (3.1416 × villus width) × villus height. Mucosal thickness was determined as the distance between the mucosal epithelium and the muscular layer, and the muscularis as the inner circular and outer longitudinal layers of smooth-muscle cells (Rubio et al., 2010).

Statistical Analysis

Data were analyzed as a one-way ANOVA using the GLM procedure of SAS (SAS Institute, 2003), with the pen serving as the experimental unit for performance parameters, and the individual chicken as the experimental unit for histological and microbiological parameters. Treatment means were separated using Bonferroni's multiple comparison tests. Statistical significance was declared at a probability of $P < 0.05$. All microbiological counts were subject to base-10 logarithm transformation before analysis.

RESULTS

Performance and Crop pH Values

In experiment 1, BW of birds fed PTS-O-45 (Figure 1) was higher ($P < 0.05$) than that of controls at 21 d of age. No significant effect of diets was observed on FI, but F/G of birds fed diets PTS-O-45 and PTS-O-90 was significantly ($P < 0.05$) lower (better) than controls. Crop pH values were 5.87 ± 0.12, 5.89 ± 0.11, and 5.91 ± 0.10 for birds fed on the control, PTS-O-45, and PTS-O-90 diets, respectively. In experiment 2, no significant differences were found at 28 d of age between the controls and the PTS-O-fed birds in final BW (1,044 ± 68, 1,091 ± 181, and 1,060 ± 142 g), FI (6,912 ± 116, 6,663 ± 116, and 6,745 ± 280 g), or F/G (2.30 ± 0.03, 2.12 ± 0.04, and 2.17 ± 0.09) of birds fed on the control, PTS-O-90, and PTS-O-135 diets, respectively.

Real-Time PCR Analysis

Experiment 1. The pathogens here studied could not be properly quantified in the crop and cecal samples due to very low numbers. The results of pathogen quantification in the ileal contents are presented in Table 3. The PTS-O addition had no effect on the *C. perfringens* or *C. jejuni* $\log_{10}$ number of copies, although a significant ($P < 0.01$) decrease in *Salmonella* spp. was determined in birds fed the PTS-O-90 diet. Among po-

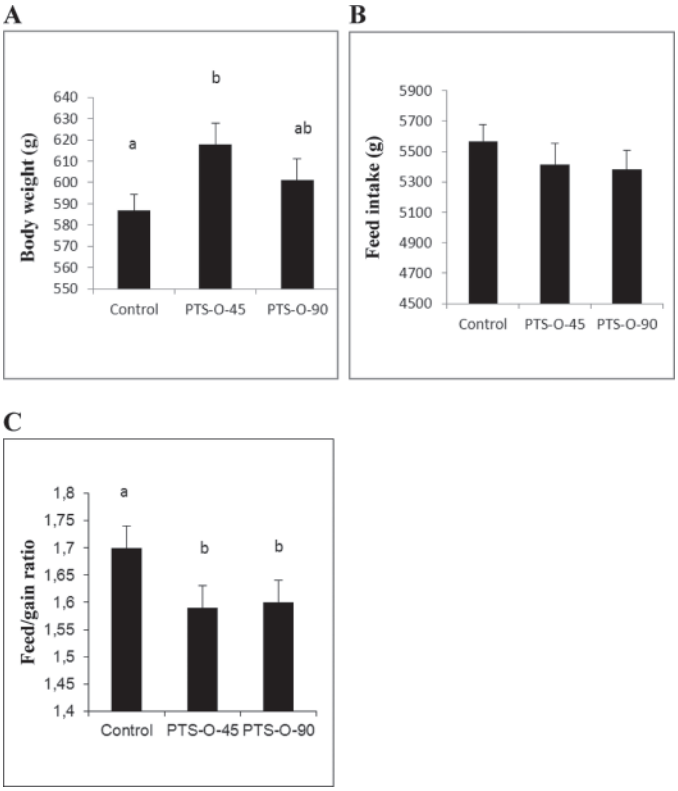

Figure 1. Effects of dietary inclusion of propyl propane thiosulfonate (PTS-O) on final BW (A), feed intake (B), and feed:gain ratio (C) of chickens in experiment 1. [a,b]Bars with different letters were significantly different ($P < 0.01$). Values are means (8 replicates of 6 birds each) with their SD in bars.

Table 3. Effect of propyl propane thiosulfonate (PTS-O) dietary addition on the $\log_{10}$ number of copies per milligram of intestinal content of *Salmonella* spp., *Campylobacter jejuni*, and *Clostridium perfringens* in the ilea of birds in experiment 1[1,2]

Item	Control	PTS-O-45	PTS-O-90	Pooled SD
Salmonella spp.	3.48[a]	3.48[a]	2.77[b]	0.49
C. jejuni	4.12	4.42	3.83	0.59
C. perfringens	2.93	3.32	3.03	0.31

[a,b]Means with different superscripts in each row were significantly different ($P < 0.01$).

[1]For details on the measurements and calculations of the different parameters, see Materials and Methods.

[2]Control = commercial diet with no additive; PTS-O-45 and PTS-O-90 = commercial diet supplemented with 45 and 90 mg of PTS-O, respectively.

tentially pathogenic bacteria, significantly ($P < 0.01$) lower $\log_{10}$ number of copies of enterobacteria and *E. coli* populations were found respect to controls in the crop of both PTS-O-fed groups (Table 4). Lower ($P < 0.01$) number of copies of enterobacteria and *E. coli* were respectively determined in the ileal and cecal contents of birds fed PTS-O-90 compared with the control group. No significant effect was observed with regard to enterobacteria and *E. coli* $\log_{10}$ number of copies in birds fed PTS-O-45 in the ileum and ceca, respectively.

Experiment 2. Birds fed on PTS-O-135 had lower ($P < 0.01$) *Salmonella* spp. and *C. jejuni* number of copies than controls. The values for *C. jejuni* or *C. perfringens* number of copies, although numerically lower than controls, did not reach significant differences in birds fed the PTS-O-90 diet. No significant effect was observed in *C. perfringens* $\log_{10}$ number of copies (Table 5) at any dose assayed.

Morphology of the Ileal Mucosa

Crypt depth and the villus height/crypt depth ratio were not significantly influenced by supplementation with 90 mg of PTS-O/kg of diet (Table 6) in experiment 1. Significant differences in histometrical parameters of small intestinal samples were found, however, for villus height, villus width, villus surface area, mucosal thickness, and muscular layer thickness, which were all greater ($P < 0.01$) in birds fed the PTS-O-90 diet than in the controls (Table 6 and Figure 2).

DISCUSSION

Improving feed efficiency is a major factor in reducing the costs of poultry production (de Verdal et al., 2011). In the current experiments, we observed a significantly positive effect of dietary PTS-O addition on the productive parameters of broilers. Thus, F/G decreased for both PTS-O doses assayed in experiment 1 (Figure 1), and tended to be lower for the PTS-O fed birds in experiment 2. Our results are in line with those of Javandel et al. (2008), who reported that garlic meal at high doses led to lower feed conversion ratios in broilers, and Jagdish and Pandey (1994), who found similar effects in cockerels. In contrast, Choi et al. (2010) found no differences in F/G with different levels of garlic powder in chickens. Body weight increased with respect to controls in birds fed PTS-O-35 in experiment 1, which is in agreement with data by Shi et al. (1999), who reported positive effects of garlic meal on weight gain in broilers, even though other authors (Dey and Samantha, 1993; Javandel et al., 2008; Choi et al., 2010) found no significant effect of garlic feeding on daily weight gain of broiler chickens. The PTS-O supplementation did not affect FI in any case in the current work, which agrees with the findings of some researchers (Horton et al., 1991; Choi et al., 2010), but not with those by others (Dey and Samantha, 1993; Shi et al., 1999; Javandel et al., 2008) working with garlic powder or garlic meal. These discrepancies may be due to a variety of reasons: i) differences in the actual products used (garlic

Table 4. Effect of propyl propane thiosulfonate (PTS-O) dietary addition on the $\log_{10}$ number of copies per milligram of intestinal contents of enterobacteria and *Escherichia coli* in the crop, ileal, and cecal contents of birds in experiment 1[1,2]

Item	Control	PTS-O-45	PTS-O-90	Pooled SD
Crop				
Enterobacteria	5.54[a]	5.07[b]	4.4[b]	0.70
E. coli	5.48[a]	4.79[b]	4.02[b]	0.81
Ileum				
Enterobacteria	4.41[a]	4.14[a]	3.59[b]	0.60
E. coli	3.16	3.34	3.03	0.76
Ceca				
Enterobacteria	5.58	5.73	5.62	0.53
E. coli	5.90[a]	5.84[a]	5.41[b]	0.63

[a,b]Means with different superscripts in each row were significantly different ($P < 0.01$).

[1]For details on the measurements and calculations of the different parameters, see Materials and Methods.

[2]Control = commercial diet with no additive; PTS-O-45 and PTS-O-90 = commercial diet supplemented with 45 and 90 mg of PTS-O, respectively.

Table 5. Effect of propyl propane thiosulfonate (PTS-O) on the ileal $\log_{10}$ number of copies per milligram of intestinal content of *Salmonella* spp., *Campylobacter jejuni*, and *Clostridium perfringens* in the ileal content of birds in experiment 2[1,2]

Item	Control	PTS-O-90	PTS-O-135	Pooled SD
Salmonella spp.	3.25[a]	2.21[b]	2.41[b]	0.72
C. jejuni	2.61[a]	2.14[ab]	1.98[b]	0.47
C. perfringens	4.82	4.45	4.61	0.63

[a,b]Means (n = 10) with different superscripts in each row were significantly different ($P < 0.01$).

[1]For details on measurements and calculations of the different parameters, see Materials and Methods.

[2]Control = commercial diet with no additive; PTS-O-45 and PTS-O-90 = commercial diet supplemented with 45 and 90 mg of PTS-O, respectively.

meal, garlic powder, garlic derivatives, and so on); ii) the nature of the additive employed and the concentrations of active components, which differed considerably between experiments; iii) the complicated chemistry of garlic (see Amagase et al., 2001), and the quality of garlic products themselves, which depends very much on the manufacturing process; and iv) inconsistencies in the efficacy of garlic supplements, which may be due to incorrect standardization, overlooking of truly active compounds, and so on. The advantage of the results here reported mainly lies in the fact that a defined, stable, chemically well-characterized compound was used (Ruiz et al., 2010).

The proliferation of pathogens in the intestine often results in inflammatory responses that lead to productivity losses, increased mortality, and increased contamination of poultry products (Jalahtii and Kettunen, 2004; Baurhoo et al., 2009). In addition, it is well established that food safety is closely related to the prevention or elimination of pathogenic bacteria in food products (Loar et al., 2010). Among pathogens that inhabit the broiler intestine, *Salmonella* spp., *C. jejuni*, and *C. perfringens*, are among those with higher economic and health impact. *Clostridium perfringens*, a spore-forming gram-positive anaerobic bacterium that usually forms part of the normal microbiota of productive animals and birds, is known to be the major causative agent of necrotic enteritis in broiler chickens (Van Immerseel et al., 2004), a disease that has reemerged after the ban on the use of AGP in feed (Gholamiandehkordi et al., 2009). Poultry and poultry products are considered to be one of the most significant sources of *Campylobacter* spp. infections (Vellinga and Van Loock, 2002), and a plethora of different approaches have been examined over the past few years to reduce its prevalence in farms, including the use of bacteriocins (Line et al., 2008) and organic acids or their derivatives (Hilmarsson et al., 2006). Nevertheless, it has proved very difficult to find a single, adequate, practical intervention measure able to reduce the colonization of the broiler gut by *Campylobacter* spp. (Lin, 2009). Species belonging to enterobacteria such as *Salmonella* spp. are the main cause of food-borne gastroenteritis in humans. Indeed, chicks can easily be infected with enteric pathogens such as *Salmonella* spp. in the period between 0 and 21 d of age (Tanikawa et al., 2011). Finally, *E. coli* is a pathogen of particular interest to the broiler industry (Loar et al., 2010), and poultry litter, a potential reservoir and transmission vehicle for pathogens and potential pathogens, is a major source of this bacterium (Garrido et al., 2004; Schrader et al., 2004). In summary, gastrointestinal infections can be controlled to some extent by using antimicrobial agents in animals and birds, but as mentioned above, their use is severely dissuaded in many countries owing to the growing awareness of problems related to antimicrobial resistance (Eeckhaut et al., 2008).

Garlic extracts have been reported to exert antimicrobial activity against these potentially pathogenic populations (Curtis et al., 2004). The antibacterial activity of garlic is widely attributed to its major thiosulfinate, allicin (Cavallito and Bailey, 1944; Ariga and Seki, 2006). Previous studies (Ross et al., 2001) have proved that garlic derivatives inhibit the growth of many enteric bacteria, including pathogenic bacteria. Indeed, allicin and garlic acid extracts have exhibit-

Table 6. Morphology[1] of the ileal sections of 21-d-old broiler chickens fed on control or experimental (propyl propane thiosulfonate; PTS-O-90) diets in experiment 1

Item	Control	PTS-O-90	Pooled SD
Villus height, μm	785[a]	937[b]	89
Crypt depth, μm	96	105	15
Villus height/crypt depth	8.7	8.9	1.9
Villus width, μm	131[a]	276[b]	62
Villus surface area, μm^2	325,940[a]	807,766[b]	183,053
Mucosal thickness, μm	47[a]	66[b]	16
Muscular layer thickness, μm	172[a]	204[b]	39

[a,b]Means (n = 3, with 5 measurements per sample) with different superscripts in each row were significantly different ($P < 0.01$).

[1]For details on measurements and calculations of the different parameters, see Materials and Methods.

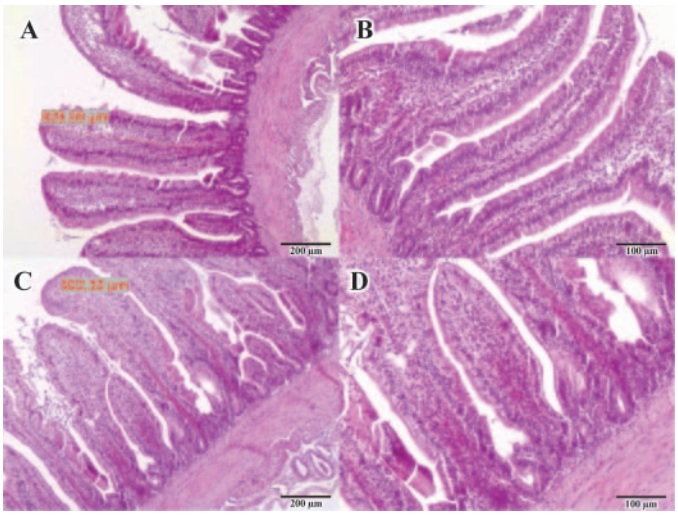

Figure 2. Light microscope photograph showing the histological structure of ileal sections of broiler chickens fed on the control (A and B) or propyl propane thiosulfonate (PTS-O-90; C and D) diets. Villi in PTS-O birds were taller and wider than those of controls. For specific measurements, see Table 6. Bars represent 200 (A and C) or 100 (B and D) µm. Color version available in the online PDF.

ed a wide spectrum of antibacterial activity, including *Escherichia* spp., *Salmonella* spp., and *Clostridium* spp. (Uchida et al., 1975; Cellini et al., 1996). Ruiz et al. (2010) demonstrated a bactericidal effect of PTS-O in vitro against enterobacteria, coliforms, *E. coli*, and *Salmonella* Typhimurium. Our present in vivo results are in accordance with those previous in vitro studies. The results of experiment 1 showed that different concentrations of PTS-O had a significant antimicrobial effect in vivo against *Salmonella* spp., *C. jejuni*, enterobacteria and *E. coli* populations. The effect is likely to be affected by dose because PTS-O-135 was more effective than PTS-O-90 (Table 5), which in turn was more effective than PTS-O-45 (Tables 3 and 4). The main antimicrobial effect of thiosulfinates such as allicin has been reported to be due to their chemical reaction with thiol groups of various enzymes such as the acetyl-CoA-forming system, consisting of acetate kinase and phosphotransacetyl-CoA synthetase (Focke et al., 1990). The RNA polymerase might also be a target for allicin. Allicin at bacteriostatic concentrations inhibits RNA synthesis in *Salmonella* Typhimurium (Feldberg et al., 1988). In *E. coli* this same enzyme contains a single sulfhydryl group, which might react with allicin (Ozolin et al., 1990). The significance of allicin as a biological effector molecule is not only due to its high reactivity with low and high molecular weight thiols, but also to its accessibility, resulting from high membrane permeability (Miron et al., 2000). Finally, the number of *C. perfringens* copies was not affected by any dose of PTS-O assayed. The resistance of *C. perfringens* to antibiotics of different nature and mechanism of action, applied in both human and veterinary care, has previously been described (Martel et al., 2004; Slavić et al., 2011; Voidarou et al., 2011), but the precise mechanism behind this lack of effect is as yet unknown.

The gastrointestinal tract plays a vital role in the digestion and absorption of nutrients required for maintenance and growth. In the current study, PTS-O dietary supplementation improved intestinal histological structure. Similar effects have previously been reported with other additives, such as Cecropin hybrid (Wen and He, 2012), the bacteriocin Albusin B (Wang et al., 2011), and mannan oligosaccahrides (Yang et al., 2008; Baurhoo et al., 2009). An increase in the efficiency of nutrients absorption and performance, associated with increased absorptive area of the gut and lower numbers of intestinal pathogens, may occur due to villi height increases (Stappenbeck et al., 2002; Baurhoo et al., 2009). It has also been shown that microorganisms can interact directly with the lining of the gastrointestinal tract (van Leeuwen et al., 2004). In our study, the morphological structure of the small intestine was affected in birds fed on PTS-O-90 (Table 6 and Figure 2). The height, width, and surface area of the villi, together with the thickness of the mucosa and the muscular layer thickness, all increased considerably compared with control values. It is known that a progressive increase both in absorptive area and in the mucosal capacity for hydrolysis occurs at early ages in poultry, and that FI, intestinal growth, and brush border enzyme development are finely controlled in young chickens to maintain an efficient nutrient supply (Uni et al., 1999). It is also accepted that a lower microbial proliferation in the gut reduces the competition for nutrients between the host and its resident microbiota, and that this reduction in competition has been reported as one of the mechanisms responsible for improved nutrients utilization (Dibner and Buttin, 2002). Kim et al. (2011) proved that lower numbers of certain gut pathogens such as *E. coli* may improve broiler performance. Furthermore, the morphology of the gut wall is altered by bacterial activity in the gastrointestinal tract (Sakata, 1987; Rebolé et al., 2010). Indeed, an overgrowth of some microorganisms in the intestine has been reported to result in mucosal impairment and villus erosion, thus reducing its absorptive potential of nutrients (Pelicano et al., 2005). According to Bourlioux et al. (2003), enterobacteria can cause damage to the intestinal cells, and Fonseca et al. (2010) linked a decrease in the quantity of cecal enterobacteria with increased ileal villus height. Improvements in performance may be due to changes in the small intestinal morphology, including an increased area for nutrients absorption (Onderci et al., 2006). As nutrients are absorbed via the villus surface area, a greater villus surface area results in greater nutrient absorption (Rezaei et al., 2011), and a shortening of the villi and large crypts can lead to poor nutrient absorption, increased secretion in the gastrointestinal tract, diarrhea, reduced disease resistance, and lower overall performance (Xu et al., 2003). Hampson (1986) established in pigs that the intestinal absorption efficiency is correlated with its surface area, which is in turn affected by villus height and mucosa thickness. In chickens, Parsaie et al. (2007) and Liu et al. (2008)

found a relationship between an increase in the height of the intestinal villi and an improved intestinal structure. Also, the morphology of the intestine changed after treatment with antibacterial additives due to increased villus height and mucosal thickness in broilers (Bao et al., 2009). Garlic and its derivatives may have similar effects, as shown in studies carried out in pigs fed aged garlic extract, where increased villus height and width in the small intestine (ileum) were observed (Tatara et al., 2008). The results here reported showed that supplementation of broiler rations with PTS-O had a significantly positive effect on gastrointestinal tract absorptive parameters.

In conclusion, dietary supplementation with the garlic derivative PTS-O at levels studied here (45 to 135 mg/kg of diet) had a beneficial effect by reducing the numbers of pathogens and potentially pathogenic bacteria in intestinal contents, and also by improving the morphological structure of the ileal mucosa and the productive parameters of broiler chickens. Accordingly, this product may represent a valuable alternative in the control of pathogenic bacteria in poultry production. Whereas previously used products are either not properly characterized (garlic powder, aged garlic, dried garlic, and so on) or difficult to obtain and unstable (allicin), PTS-O is a chemically characterized, stable product with defined properties. It may therefore represent a reliable practical alternative to AGP use because it has shown antimicrobial, growth-promoting properties, which are likely to positively influence animal well being and product safety. Due to the potential practical relevance of these results, further research involving experimentally challenged birds is warranted.

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