ORIGINAL ARTICLE

The Use of Tannins to Control *Salmonella* Typhimurium Infections in Pigs

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Impacts

- Developing alternative control measures to reduce *Salmonella* Typhimurium infections in pigs.
- Tannins have a pronounced effect against *Salmonella* Typhimurium *in vitro*.
- In pigs, tannins do not limit *Salmonella* colonization and excretion.

Keywords:
Salmonella Typhimurium; *in vivo* trial; pig; tannins

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Summary

The aim of this study was to determine whether a hydrolysable tannin extract of sweet chestnut wood (Globatan®) has an inhibitory effect on *Salmonella* Typhimurium survival both *in vitro* and *in vivo* in pigs. In a first experiment, the minimal inhibitory concentration of Globatan® on 57 *Salmonella* Typhimurium isolates was determined. For all isolates, an MIC of 160–320 μg/ml was found. The second *in vitro* study revealed that *Salmonella* growth was strongly reduced using Globatan® concentrations of 25–50 μg/ml and nearly completely inhibited at a concentration of 100 μg/ml Globatan®. In an *in vivo* trial, two groups of six piglets, each group receiving feed with or without the addition of Globatan® (3 g/kg), were orally inoculated with 10^7 colony forming units of a *Salmonella* Typhimurium strain. Globatan® had no effect on faecal excretion of *Salmonella*, and no differences in colonization of the intestines and internal organs were demonstrated in pigs euthanized at 4 days post-inoculation. In conclusion, the hydrolysable tannin extract used in this study showed strong action against *Salmonella* Typhimurium *in vitro* but not *in vivo*.

Introduction

Salmonellosis is one of the most important bacterial zoonotic diseases in humans. In European countries, *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) is the serovar most frequently isolated from pigs. Together with *Salmonella* Enteritidis, this serotype is most frequently associated with human salmonellosis (Anonymous, 2007). Pig carcass contamination with *Salmonella* Typhimurium can largely be attributed to persistently infected pigs (Botteldoorn et al., 2003; Boyen et al., 2008a).

Tannins are water soluble polyphenolic plants' secondary metabolites that are found in almost every plant part. According to their structure, they are distributed in two groups: condensed and hydrolysable tannins (Haslam, 1989). Tannins are characterized by their ability to form strong complexes with proteins, carbohydrates and minerals, otherwise required by the commensal microflora in the gut, through hydrogen bonding and hydrophobic effects (Scalbert, 1991; Cowan, 1999; Goel et al., 2007). As a result of these characteristics, for a long time, tannins were considered anti-nutrients that reduced digestibility in monogastric species (Schiavone et al., 2008). However, tea and berry polyphenols and various other tannins have been shown to possess certain anti-bacterial, anti-fungal and anti-viral properties (Scalbert, 1991; Cowan, 1999; Akiyama et al., 2001; Funatogawa et al., 2004; Puupponen-Pimiä et al., 2005a). This might be due to enzyme inhibition, membrane disruption and substrate as well as metal ion deprivation (Scalbert, 1991).

Puupponen-Pimiä et al. (2005b) showed that berry tannins inhibit the growth of *Salmonella* serovars Infantis and Typhimurium *in vitro*. Following these and other

promising *in vitro* results (Akiyama et al., 2001; Junaid et al., 2006; Goel et al., 2007; Kouitcheu et al., 2007), subsequent *in vivo* trials were performed to investigate the suitability of tannins as an alternative to control *Salmonella* infections in animals. However, these experiments focused mainly on the beneficial effect of tannins in ruminal (Sotohy et al., 1997; Rochfort et al., 2008), poultry (Kubena et al., 2001; Schiavone et al., 2008) and rat health (Das et al., 2003) and not in pig health.

The aim of this study was to determine the usefulness of sweet chestnut tannins for the control of *Salmonella* infection in pigs. The anti-bacterial effect of these products on different *Salmonella* Typhimurium strains was first determined *in vitro*. Thereafter, the tannin extract was evaluated for its capacity to decrease the colonization of *Salmonella* Typhimurium in pigs in an *in vivo* experiment. To our knowledge, this study presents the first *in vivo* experiment in which the influence of tannins on the colonizing capacity of *Salmonella* Typhimurium in pigs was investigated.

Materials and Methods

Bacterial strains and growth conditions

The minimal inhibitory concentration (MIC) of a hydrolysable tannin extract (Globatan®; Sanlac International nv/Global Nutrition sa, Oosterzele, Belgium) was determined for 57 *Salmonella* Typhimurium isolates from pigs, carcasses or environmental swabs, originating from different farms and slaughterhouses. *Salmonella* Typhimurium strain 112910a phage type 120/ad, isolated from a pig stool sample and characterized previously (Boyen et al., 2008b), was used to determine the growth curves in the presence of Globatan®. Its spontaneous nalidixic acid resistant derivative (*Salmonella* Typhimurium 112910a_nal) was used for the *in vivo* trial, to minimize irrelevant bacterial growth when plating intestinal and faecal samples.

For the *in vitro* assays, 5 μl of a stationary phase culture was inoculated in 5 ml minimal essential medium (MEM; Gibco Life Technologies, Paisley, Scotland), because tannins induced precipitation in Luria-Bertani broth (LB; Sigma-Aldrich, Steinheim, Germany), and incubated overnight at 37°C without aeration.

For the oral inoculation of pigs in the *in vivo* experiment, *Salmonella* Typhimurium 112910a_nal was grown overnight in 5 ml LB at 37°C with aeration. The bacteria were washed twice in phosphate-buffered saline (PBS; 1361 *g*, 10 min, 4°C) and diluted in PBS to the appropriate concentration of 2×10^7 colony forming units (CFU) per millilitre. The number of viable *Salmonella* bacteria per ml inoculum was determined by plating 10-fold dilutions on brilliant green agar (BGA; Oxoid, Basingstoke, UK) supplemented with 20 μg/ml nalidixic acid (BGA^{NAL}).

Tannin product

In this study, we used Globatan® (Sanluc International nv/Global Nutrition sa, Oosterzele, Belgium), a tannin extract produced from sweet chestnut wood (*Castanea sativa*) and composed of hydrolysable tannins, esters of gallic acid and sugar (75–78%). The other compounds of Globatan® are carbohydrates (18%), inorganic salts (2%) and water (5%).

Minimal inhibitory concentrations of tannins

McFarland 0.5 suspensions were produced from overnight cultures, and 5 μl of these suspensions was each time inoculated in 195 μl MEM, containing the various concentrations of Globatan® being tested: 0, 10, 20, 40, 80, 160, 320 or 640 μg/ml. These suspensions were incubated in a 96-well plate overnight at 37°C. Afterwards, bacterial growth was investigated by determining the presence of a bacterial pellet at the V-shaped bottom of the wells, using a Microtiter mirror (Dynatech, Billingshurst, England). The MIC was determined as the lowest concentration of Globatan® at which no visible bacterial growth occurred.

Effect of tannins on growth of *Salmonella* Typhimurium

Growth curves were prepared in MEM supplemented with Globatan® at concentrations of 0, 10, 25, 50, 100, 500 or 1000 μg/ml. The pH value of all solutions was adjusted to pH 6 with HCl (Castillo et al., 2007). After making a 1 : 1000 dilution of *Salmonella* Typhimurium strain 112910a in these media, the suspensions were statically incubated at 37°C. The number of CFU in these suspensions was determined after 0 h, 2 h, 4 h, 6 h, 8 h and 24 h. This step was carried out by preparing 10-fold serial dilutions of 20 μl of the bacterial suspensions for each time interval. Subsequently, six 20 μl samples of each dilution were plated on BGA and incubated overnight at 37°C, after which the colonies were counted.

In vivo trial

This study was approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University (EC 2006/15). The *in vivo* experiment with Globatan® was performed in 6-week-old piglets (commercial closed line based on Landrace), from a serologically negative breeding herd, and these piglets were negative for *Salmonella* at faecal sampling. Fifteen piglets arrived at the facility 14 days before they were inoculated and were divided at random into three groups: two groups of 6 inoculated pigs, of which one group (group 1) received feed supplemented with Globatan® (3 g/kg feed, according to the

guidelines of the manufacturer) and the other (group 2) received unsupplemented feed, and a negative control group of three pigs (group 3) fed normal feed. The piglets were housed in pairs in separate pens at 25°C under natural day-night rhythm with *ad libitum* access to feed and water. At 12 days on this feed regime, all animals of groups 1 and 2 were orally inoculated with approximately 2×10^7 CFU of *Salmonella* Typhimurium 112910a_nal in 1 ml PBS (Boyen et al., 2008c).

For three executive days post-inoculation (pi), fresh faecal samples were collected from each pig for bacteriological analysis. On day 4 pi, all 12 *Salmonella* inoculated piglets and the three control pigs were humanely euthanized. Samples of tonsils, liver, spleen, mesenterial lymph nodes, ileocaecal lymph nodes, colonic lymph nodes, jejunum, ileum, caecum, colon and contents of jejunum, ileum, caecum and colon were taken for bacteriological analysis.

All tissue and content samples were stored at −70°C until further processing. The samples were thawed, weighed and 10% (w/v) suspensions were made in buffered peptone water (BPW; Oxoid, Basingstoke, UK) after which the material was homogenized with a stomacher. The homogenized samples were examined for the presence of the *Salmonella* bacteria by plating 10-fold dilutions on BGA^{NAL}. If negative at direct plating, the samples were pre-enriched overnight in BPW at 37°C, enriched overnight at 37°C in tetrathionate broth and then plated on BGA^{NAL}. Samples that were negative after direct plating but positive after enrichment were presumed to contain 83 CFU per gram tissue (detection limit for direct plating). Samples that remained negative were presumed to have 0 CFU per gram.

Statistical analysis

The data were analysed using a linear mixed effect regression model with animal as random factor using S-PLUS 7.0 (TIBCO Software Inc., Palo Alto, CA, USA). Differences with a *P*-value ≤0.05 were considered significant.

Results

Minimal inhibitory concentrations of tannins

For 46 of the 57 tested *Salmonella* Typhimurium strains (81%), the MIC of Globatan® was 160 μg/ml corresponding to 120 μg/ml hydrolysable tannins, and for the remaining 11 strains (19%), the MIC was 320 μg/ml corresponding to 240 μg/ml hydrolysable tannins.

Growth curves

At Globatan® concentrations of 5 and 10 μg/ml, no effect on the growth of *Salmonella* Typhimurium was observed. Bacterial growth was strongly reduced using Globatan® concentrations of 25–50 μg/ml and nearly completely inhibited at a concentration of 100 μg/ml Globatan®. A bactericidal effect was seen at concentrations of 500 and 1000 μg/ml. The growth curves are shown in Fig. 1.

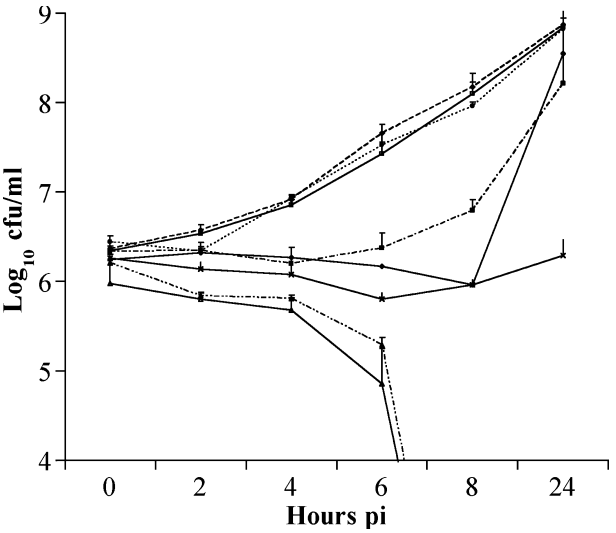

Fig. 1. Growth curves of *Salmonella* Typhimurium in MEM (Minimal Essential Medium) representing the average log(10) of the CFU/ml ± SD of *Salmonella* post-inoculation (pi) for different concentrations of Globatan: (◆) 0 μg/ml, (□) 5 μg/ml, (●) 10 μg/ml, (■) 25 μg/ml, (◇) 50 μg/ml, (x) 100 μg/ml, (△) 500 μg/ml and (▲) 1000 μg/ml.

Tannins do not influence *Salmonella* Typhimurium excretion and colonization in pigs

Salmonella Typhimurium was not isolated from the pigs of the negative control group (group 3). One piglet of the Globatan® group (group 1) died at 3 days pi. High numbers of *Salmonella* were isolated from this piglet's organs, suggesting that this animal succumbed due to septic shock. This pig was not included in further analysis.

The addition of 3 g/kg Globatan® to the feed did not significantly influence ($P > 0.05$) the *Salmonella* Typhimurium excretion during the observation period, compared to the piglets of group 2 which received normal feed without additives. These results are summarized in Fig. 2.

The *Salmonella* Typhimurium organ colonization was determined 4 days after experimental inoculation of the pigs. Although in some organs there was a numerical decrease in *Salmonella* colonization, supplementation of Globatan® did not significantly ($P > 0.05$) reduce the *Salmonella* colonization of the internal organs. These results are summarized in Fig. 3.

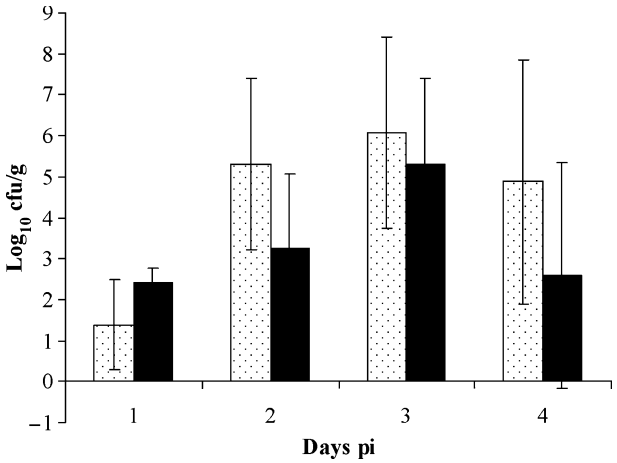

Fig. 2. Faecal excretion of *Salmonella* Typhimurium (average log10 CFU/g ± SD) in six pigs receiving feed without additives (grey bars) and in five pigs receiving Globatan® supplemented feed (black bars).

Discussion

Our study showed a bacteriostatic effect of Globatan® on *Salmonella* Typhimurium *in vitro* at a concentration of 160–320 μg/ml for all 57 tested strains, corresponding to an actual hydrolysable tannin concentration of 120–240 μg/ml (75%). Several other research papers already communicated the inhibitory effects of various tannin containing plant extracts on *Salmonella* Typhimurium *in vitro* with MIC's ranging from 125 μg up to 150 mg/ml (Baumann and Muller, 1993; Sotohy et al., 1995; Taguri et al., 2004; Junaid et al., 2006; Amarowicz et al., 2007). This large variation in MIC values may at least in part be attributed to the stronger anti-microbial effect of hydrolysable tannins compared to that of condensed tannins (Smith et al., 2005). Globatan® is composed of a hydrolysable tannin mixture which may account for the low MIC values found in this study.

In feed administration, tannins had no significant effect on faecal shedding and on the colonization of porcine organs by *Salmonella* Typhimurium. Possibly, tannins are already degraded by host or gut microflora enzymes or preliminary inactivated by binding various constituents present in the gut, before they reach the distal region of the small intestine, the part of the intestine preferably colonized by *Salmonella* Typhimurium. Indeed tannins can bind a diversity of substrates (Goel et al., 2007). Moreover, Goel et al. (2005) showed in ruminants that tannins can be degraded by certain gastrointestinal bacteria and that hydrolysable tannins like Globatan® are more susceptible to hydrolysis and subsequent inactivation than condensed tannins (Goel et al., 2005). In addition to this, Abia and Fry (2001) showed that the amount of tannins that reaches the gut can differ from the orally administered amount of tannins in rats. Although the functioning of the digestive system of pigs and rats, and subsequently its effect on modification and/or degradation of tannins, can differ, extrapolation of our *in vivo* results must be made very carefully.

To come to a possibly successful trial, more animals could be treated with a higher concentration of Globatan® for a longer time period. However, we must emphasize that administration of high doses of tannins can affect normal digestion in monogastrics like pigs (Smulokowska et al., 2001). Although the inoculation dose used in this experiment is relatively low (Wood and Rose, 1992; Brumme et al., 2007; Carnell et al., 2007; Österberg

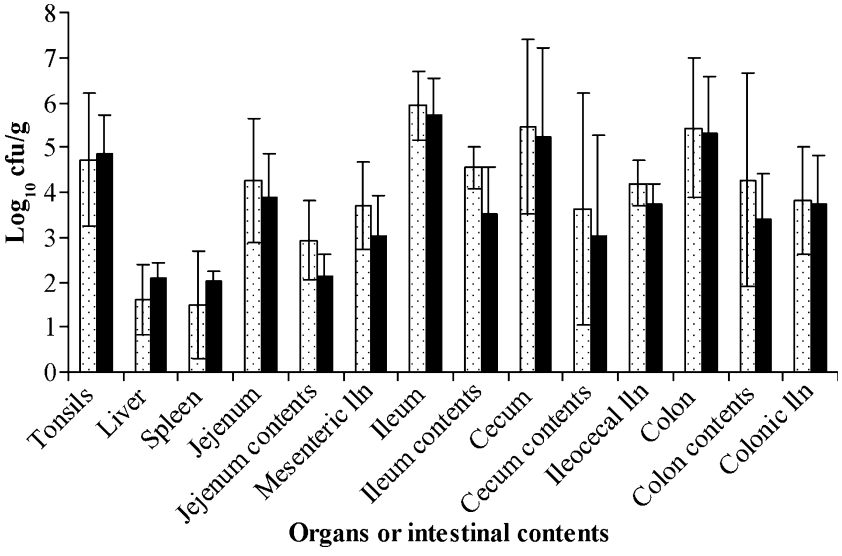

Fig. 3. The average log10 CFU of *Salmonella* Typhimurium per gram organ or intestinal contents ± SD in piglets euthanized at 4 days pi. Grey bars represent piglets receiving feed without additives. Black bars represent piglets receiving feed supplemented with Globatan®.

and Wallgren, 2008; Scherer et al., 2008), it is still higher than in practice. An effect of Globatan® on *Salmonella* Typhimurium might be seen when a lower inoculation dose is used.

In conclusion, it was found that although the sweet chestnut wood tannin extract Globatan® has a marked inhibitory effect on *Salmonella* Typhimurium *in vitro*, this inhibition was not confirmed in an *in vivo* trial.

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