The influence of acid tolerance response and diet on the survival of *Salmonella* serotypes through the gastrointestinal tract of pigs

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Abstract
Research findings suggest that acid supplementation of feed and feed characteristics affect the survival of *Salmonella* in pigs although results are not always consistent. The acid tolerance response (ATR) of *Salmonella*, induced by mildly acidic conditions, which can occur both within and outside the host, may influence the survival and transmission of *Salmonellae* in pigs fed certain diets. This study investigates the role of the pH-dependent ATR on relative survival of five *Salmonella* serotypes in gastrointestinal contents of pigs fed regular or acid-supplemented feeds. Stomach, caecal and colon contents were obtained from pigs fed four different diets (6 pigs/diet, pelleted or meal feed with or without potassium diformate, KDF). A five-strain mixture of *Salmonella* serotypes commonly isolated from Irish pigs was inoculated at a level of 10^5 CFU g^{-1}. Survival of acid-adapted (AA) and non acid-adapted (NA) strains was monitored using direct plate counts under simulated gastrointestinal conditions in stomach, caecal and colon contents to evaluate the effect of ATR. Initial results show increased survival of acid-tolerant *Salmonella* strains in the low pH conditions of the stomach with *Salmonella* Typhimurium DT104b demonstrating the highest level of acid resistance. *Salmonella* strains survived longer in caecal and colon contents from pigs fed meal diets compared to those fed pelleted diets but no effect of acid adaptation was observed. KDF supplementation had no apparent effect on survival or ATR of salmonellae in caecal and colon contents from pigs fed this feed additive. Results suggest that there are no marked differences in pH, dry matter (DM) and volatile fatty acid (VFA) concentration of gastrointestinal contents from pigs fed the KDF supplemented diets compared to control diets. Findings of this study indicate that pre-existing acid adaptation of *Salmonella* and feed type affect survival of the organism in the porcine gastrointestinal tract and have implications for control of this pathogen.

Introduction
Research findings suggest that acid supplementation of feed and feed characteristics affect the survival and transmission of *Salmonella* in pigs although these results are not always consistent (Canibe *et al.*, 2001; Février *et al.*, 2001, Mikkelsen *et al.*, 2004). Apart from feeding strategies, gastrointestinal survival of this pathogen may also be influenced by its adaptive acid protection system, the acid tolerance response (ATR), which is triggered by mildly acidic conditions and can occur both within and outside the host (Foster, 1995). The main objective of this study was therefore to determine the influence of pH-dependent ATR on survival characteristics of five commonly isolated *Salmonella* serotypes in gastrointestinal contents obtained from finishing pigs fed different diets. In addition, we evaluated the effect of KDF supplementation on physio-chemical parameters (pH, DM, VFA concentration) of gastrointestinal digesta and its effectiveness in inhibiting survival of these *Salmonella* spp.

Materials and Methods
Feeding trial and collection of gastrointestinal samples: 24 finishing pigs with an average body weight of approximately 75 kg were randomly assigned to one of the four treatment groups (6 pigs/diet). The experimental diets consisted of a non-pelleted meal feed and a pelleted feed with either no supplement (control diet) or supplemented with 0.9% potassium diformate (KDF, Formi®). Pigs were fed each diet for a period of 10 weeks and removed from feed 24 h prior to slaughter. At slaughter, contents of stomach, caecum and colon were collected from the gastrointestinal tract (GIT) of each pig. After collection, samples were immediately stored on dry ice for transportation to the laboratory and then stored at -22°C.
Preparation of gastrointestinal samples: Prior to use, samples were confirmed negative for the presence of *Salmonella* spp. by conventional culture methods on the basis of BS EN ISO 6579:2002. To minimize

exposure to air, all samples were transferred to and prepared in an anaerobic chamber with a mixed gas atmosphere consisting of 80% N_2, 10% H_2 and 10% CO_2 gas (Bug Box M, Ruskinn Technology Ltd.). Since the pH range of the stomach contents was great (pH 2.1 to 6.9) different volumes of individual samples from each treatment group were mixed to achieve a final pH of 3.58±0.06, a gastric pH that is similar to that reported by Mikkelsen *et al.* (2004). For caecal and colon contents equal volumes of all samples from each treatment group were mixed. **Bacterial strains, growth and induction of acid tolerance response:** The bacterial inoculum consisted of a five-strain mixture of nalidixic acid-resistant (at ≤50 μg ml^{-1}) *S.* Typhimurium DT104b, *S.* Typhimurium DT208, *S.* Typhimurium DT193, *S.* Derby and *S.* Bredeney (all porcine isolates). Strains were grown separately overnight at 37°C in Nutrient Broth (Oxoid) that contained 50μg ml^{-1} nalidixic acid supplemented with 1.25% of D-(+)-glucose (Sigma) to induce acid-adaptation (AA strains) or grown in a glucose-free Nutrient Broth to obtain non acid-adapted cultures (NA strains) (modified after Salmelis *et al.*, 2003). After combining the five strains of AA or NA cultures, samples were inoculated with the appropriate dilution to yield a final inoculum level of 10^5 CFU g^{-1}. **Incubation and sampling:** Throughout the survival experiment samples were kept in the anaerobic chamber at a temperature of 39°C to simulate porcine gastrointestinal conditions. Sampling of the stomach contents occurred at 2, 4, 6 and 24 h after inoculation. Sub-samples of caecal and colon contents were taken at 2, 4, 7 and 10 days post-inoculation. The entire experiment was repeated once. **Enumeration and detection of *Salmonella*:** Salmonellae were enumerated using an agar overlay method to promote maximum recovery. For this, sub-samples (3g) were serially diluted in BPW, 100μl of the appropriate dilution was direct plated onto TSA agar (Oxoid), plates were incubated at 25°C for 2.25 h followed by an overlay with XLD agar (Oxoid) containing 50μg ml^{-1} nalidixic acid (Sigma), novobiocin (20 mg/l, Fluka Biochemika) and cefsulodin (12.5 mg/l, Sigma). **Dry matter, pH and volatile fatty acid determinations:** The pH was measured using a portable pH meter (Hanna Instruments, UK). Volatile fatty acids (VFA) determinations were conducted using a method described by Pierce *et al.* (2005). The dry matter (DM) content of the gastrointestinal samples was determined by drying samples at 103°C for 4 h in an oven with forced air circulation (Memmert, Germany).

Results *(Statistical analysis is currently ongoing)*

Survival of *Salmonella* in stomach contents. Enhanced survival in stomach contents was observed for AA strains as shown in Figure 1. The survival rate was strongly affected by exposure time resulting in higher reductions in cell numbers after 24 h compared to 6 h of incubation. No NA strains were recovered in three of the treatment groups after 24 h of exposure whereas AA strains from all treatment groups were able to survive throughout this time.

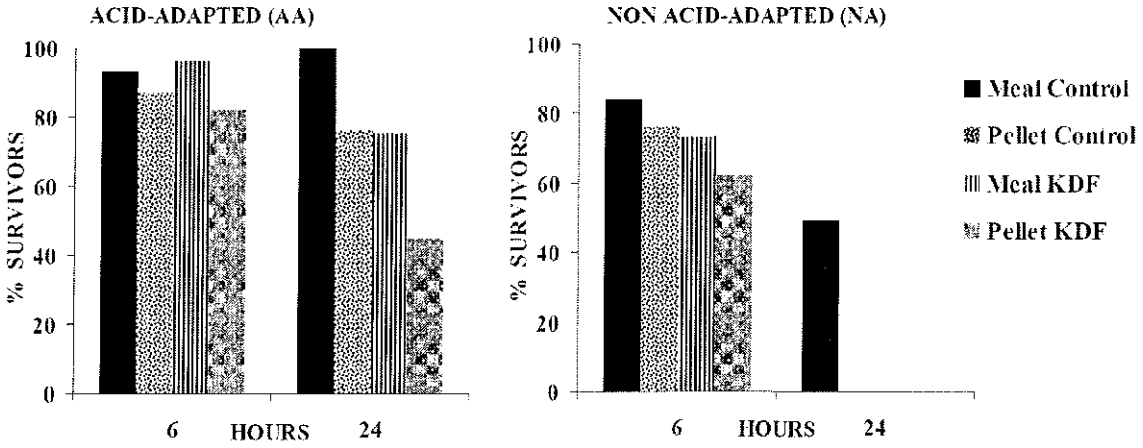

Figure 1. Survival of AA and NA *Salmonella* strains after 6 h and 24 h in stomach contents obtained from pigs fed different experimental diets.

Survival of *Salmonella* in caceal and colon contents. Similar survival patterns among the different treatments were observed in caecal and colon contents, but evidence of multiplication was only seen in cacal samples (Figure 2). *Salmonella* strains were able to survive for a period of up to 7 days in caecal contents and up to 4 days in colon contents. Overall, higher survival rates were observed in intestinal

contents from pigs fed meal diets (both control and KDF supplemented) compared to pelleted diets. Major differences in survival of acid and non-acid adapted strains and a *Salmonella*-inhibiting effect of KDF supplementation were not evident.

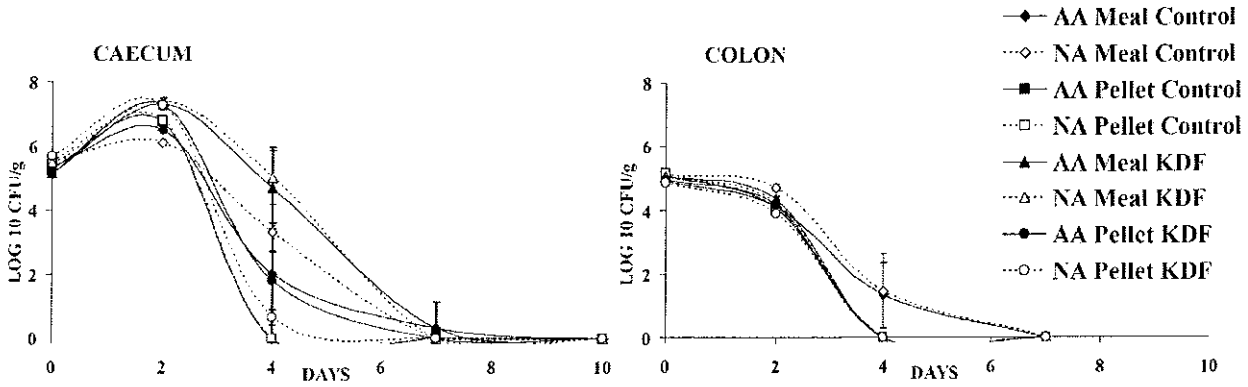

Figure 2. Survival of AA and NA *Salmonella* strains throughout 10 days in caecal and colon contents obtained from pigs fed different experimental diets.

Physio-chemical parameters of gastrointestinal digesta. VFA concentration and percentage of dry matter were generally low in all gastric samples compared to values measured in caecum (Table 1). Highest values of VFA and DM were observed in colon contents. The pH of the stomach contents prior to adjustment ranged between 2.1 to 6.9 (data not shown). Caecal and colon contents showed a narrow pH range (pH 6.4 to 7.2) with slightly lower pH values in intestinal digesta obtained from pigs fed the meal diet. As compared to control diets, the digesta of pigs fed KDF supplemented diet showed similar physio-chemical parameters.

Table 1. VFA concentration, DM and pH[a] measured in stomach, ceacum and colon contents of pigs fed different experimental diets.

GIT content	Diet type	VFA (mmol/kg)	Dry matter (%)	pH
Stomach	Meal Control	3 (0.7)	3 (2.5)	3.65[b]
	Pellet Control	2 (0.4)	6 (3.0)	3.58[b]
	Meal + KDF	1 (0.3)	4 (1.6)	3.53[b]
	Pellet + KDF	1 (0.0)	3 (0.4)	3.52[b]
Cecum	Meal Control	178 (15.8)	13 (0.6)	6.4 (0.06)
	Pellet Control	172 (4.1)	13 (0.5)	7.1 (0.02)
	Meal + KDF	196 (16.7)	15 (0.7)	6.7 (0.14)
	Pellet + KDF	161 (5.7)	8 (0.3)	7.2 (0.05)
Colon	Meal Control	247 (10.4)	23 (0.6)	6.4 (0.03)
	Pellet Control	200 (7.8)	23 (0.4)	6.8 (0.07)
	Meal + KDF	198 (4.3)	24 (0.3)	6.5 (0.02)
	Pellet + KDF	210 (12.5)	26 (0.3)	6.6 (0.08)

[a] Values shown are the means of six (DM and pH) and three (VFA) determinations. Values in parentheses are the standard deviations of the mean value.

[b] pH was adjusted by mixing stomach content samples to respective value.

Discussion

Findings of this study indicate lowest survival of *Salmonella* in the acidic conditions of the stomach (pH of 3.58±0.06). Under such conditions, AA *Salmonella* survived better than NA organisms. These findings support those of previous research published by Samelis *et al.* (2003), who reported a greater acid

tolerance for AA cultures at a pH of 3.5 in studies with pure cultures. Data from current in vitro experiments that evaluate the ATR in low pH conditions of each of the five strains used in this study (data not shown) indicate strain variability among the different serotypes, with *S.* Typhimurium DT104b showing highest acid tolerance. Due to the low percentage of dry matter present in stomach contents, no evidence of dietary effects were observed and survival of AA and NA *Salmonella* strains in stomach contents was principally pH-dependent. Survival rates were probably also influenced by the presence of undissociated lactic acid and other components, as outlined by Mikkelsen *et al.* (2004).

Results from this study indicate enhanced survival of *Salmonella* in intestinal contents compared to stomach contents. *Salmonella* numbers declined more rapidly in colon than in caecal contents, which is possibly linked to higher concentrations of organic acids, microbial competition and lower nutrient availability in this part of the intestines. Overall, longer survival of *Salmonella* was observed in intestinal contents obtained from pigs fed the meal diet in contrast to findings by Mikkelsen *et al.* (2004), who suggested that feeding a coarsely ground meal feed to pigs reduced the number of coliform bacteria in the stomach and in the lower parts of the intestinal tract compared to feeding a finely ground pelleted feed. Physio-chemical analyses indicated that feeding a meal diet to finishing pigs did not result in major differences in pH, DM or VFAs of the gastrointestinal digesta compared to pigs fed the pelleted diet. In addition, a supplementation of 0.9% of KDF did not markedly influence intestinal survival patterns of *Salmonella* or physio-chemical properties of the gastrointestinal digesta compared to unsupplemented diets. These observations differ from those reported by Février *et al.* (2001), who found a reduction in pH and the number of coliforms in the stomach and the proximal colon after feeding pigs with a KDF-supplemented diet. The fact that no effect of ATR on *Salmonella* survival was evident in caecal and colon contents suggests that this stress response is playing a minor role in intestinal survival of this pathogen.

Conclusion

In summary, findings of this study indicate that pre-existing acid adaptation of *Salmonella* affects survival of the organisms in the low pH conditions of the stomach and physical properties of feed influence its survival in the porcine intestinal tract, which has implications for control of this pathogen.

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