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# Effect of dietary addition of nitrate on growth, salivary and gastric function, immune response, and excretion of *Salmonella enterica* serovar Typhimurium, in weaning pigs challenged with this microbe strain

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**ABSTRACT:** Two dietary additions of nitrate (15 mg/kg or 150 mg/kg, supplied by potassium salt) were tested in a total 96 weaning pigs challenged or not with *Salmonella enterica* serovar typhimurium (ST). The oral challenge was done on d 5 and pigs were sacrificed on d 7 or d 25.

The effect of challenge never interacted significantly with the dietary treatment. Feed intake, growth, body temperature, salivary excretion, and faecal excretion of ST and gastric function were not affected by the nitrate supplementation. With nitrate additions, total IgA in blood serum tended to be higher before and after the challenge ( $P < 0.10$ ). Nitrite in saliva – but not nitrate – increased with the increasing supplementation at d 5, but not at d 19. The nitrate additions did not negatively affect the weaning performance, but also did not contrast the effect of ST infection.

**Key words:** Weaning pigs, Salmonella, Nitrate, Nitrite.

**INTRODUCTION** – Diets with significant amounts of nitrate/nitrite stimulate in human the recirculation of nitrate/nitrite from the stomach to saliva for longer than the maximum gastric retention (McKnight *et al.*, 1997). Bacteria in the oral cavity reduce salivary nitrate, which has no antimicrobial activity at low pH, to nitrite, that is antimicrobial under acid conditions. *In vitro* antimicrobial activity of the stomach acid increases up to 100 fold when the nitrite concentration is similar to that found in human saliva after consumption of diets rich in vegetables (which contribute >75% of the nitrate in the diet) (Xu *et al.*, 2001). Particularly nitrites are highly effective against food borne intestinal pathogens (such as *Salmonella*, *Yersinia* and *E. coli*). In mammals various effects of high doses of nitrates in the diet (or in the water) were documented (Bruning-Fann *et al.*, 1993), but the effect of moderate nitrate levels in the weaning pig production is not studied. The goal was to verify if the dietary moderate supplementation of nitrates could affect growth performance, salivary and gastric function, immune response, and excretion of *Salmonella enterica* serovar typhimurium (ST) in young pigs, challenged or not with this pathogen strain.

**MATERIAL AND METHODS** – A 2-Factorial experiment was carried out to test the effects of two different doses of nitrate (0 – **Control**; 15 mg/kg – **Ni15**; 150 mg/kg – **Ni150**), supplied by potassium salt, in weaning pigs

challenged or not with ST. The low dose of nitrate was fixed equal to the limit value for nitrites in the feed, according to UE legislation (15mg nitrites/kg feed). The high supplementation was decided on the base of the following data. Considering the results from studies on human health, 150mg/kg of nitrates correspond to 15 mg of nitrite, by hypothesizing a 25% nitrate entero-salivary circulation (Spiegelhalter *et al.*, 1976; Tannenbaum *et al.* 1976) and a 40% salivary nitrate reduced to nitrite by nitrate reductase expressed by microorganisms in the mouth. The latter value is slightly higher than the one that can be calculated when nitrate salts are supplied in human (about 33%, McKnight *et al.*, 1997). However it was used as a conservative value and expecting a higher activity in the mouth of the pig, that lives in a dirtier environment, as compared to human.

The experiment was done in 4 consecutive batches. Per each batch, pigs weaned at 3 – 4 weeks were randomly assigned on the base of live weight and of the litter to one of. Total pigs used were: 4 batches x 6 litters x 4 pigs = 96 pigs. From start to d 5, the subjects received the experimental diets in box of 2 individuals, with slattened flour. Then the pigs were challenged and individually penned in the same type of cages. The challenge organism was orally supplied with 1.5 ml broth containing  $1 \times 10^9$  CFU ST. Control pigs received a placebo (only broth). Individual samples of blood serum were collected at start, before the challenge and at the sacrifice. Saliva was sampled on d 5 and d 19. Feces were sampled on d 5, d 7 and d 19.

Half of the pigs were sacrificed on d 7 (+ 2 after challenge), and half on d 25 (+20 after challenge). All the subjects were sacrificed at the same time from the last meal. Piglets had access to feed and water respectively until 2 and 1 hour before the sacrifice. The pigs were then anaesthetized with sodium thiopental (10 mg/kg body weight) and euthanized by intracardiac injection of Tanax<sup>®</sup>. Individual samples of fundic gastric mucosa, caudal part of the jejunum, and ileocecal lymph nodes were collected. PH's in stomach, jejunum, colon and cecum, and the weight of the full and empty stomach and cecum were measured. The procedures were approved by the Ethic-Scientific Committee for Experiments on Animals of the University of Bologna.

Gastric morphology, genes expression and immune globulins were assessed as reported by Bosi *et al.* (2006) and Bosi *et al.* (2007). Nitrate and nitrite in serum blood and saliva were measured by the Nitric Oxide Assay Kit (Assay design, Ann Arbor, MI, USA). To test the presence of ST, 10 g faecal samples were tested by a standard bacteriological method including non selective pre-enrichment (BPW), selective enrichment (Rappaport-Vassiliadis broths, subculture on solid media (Hektoen Enteric Agar and XLD Agar) and serotyping.

The data were analysed by analysis of variance (GLM of SAS) of three factorial models, A) data in vivo before challenge: diet and block (batch); B) data post challenge: model A + challenge (yes/not) and the interaction; C) data obtained at different days of sacrifice: model B + day of sacrifice (2 or 20 days from the challenge) and the interaction. Orthogonal pre-planned comparisons were tested for the effect of the diet: control vs. nitrate additions; between nitrate additions. The results relating to seven pigs of the 1<sup>st</sup> batch were excluded from the statistical analysis, due to the very low feed intake before the challenge (feed intake in the total period lower than 1% of the starting body weight).

**RESULTS AND CONCLUSIONS** – No statistically significant interaction among the tested parameters was found. Nitrate addition did not affect daily feed intake and growth, before and after challenge (Tab.1). The daily live weight gain post challenge was 167 g in challenged pigs compared to 205 g in control pigs ( $P=0.18$ ), on average for the two different times of sacrifice. However the challenge was effective because at sacrifice ileocecal lymph nodes of most of challenged pigs were positive for the presence of ST, even if feces were negative before the challenge. The body temperature rose one day after challenge in stimulated pigs compared to controls, but the diet did not modify the values (Table 2). Then, in general, health was good in most of the pigs, diarrhea was very scarce and this fact confirms that in swine the infection of ST is often asymptomatic. However 2 Salmonella control pigs, 1 Salmonella Ni15 pig, and 1 Control Ni150 pig died apparently not for the Salmonella challenge. Faeces collected respectively at 2 d and 14 d post challenge were positive for the presence of ST in 62 % and 52 % of challenged pig (no difference between diets) and in none of unchallenged subjects.

A trend of increase of total IgA in serum before and after challenge ( $P<0.10$ ) was seen with nitrate supplementations, while total IgA content was not affected significantly by the challenge. No effect was seen also for Ig's in saliva. The production of saliva per unit time (measured before the challenge only) was also not affected by the diet. Nitrate in blood and in saliva was not affected by nitrate supplementation. Nitrite in saliva increased with the supplementation, but only at the 1<sup>st</sup> sampling (+36%, Ni15, and +258%, Ni150,  $P<0.05$ ). At sacrifice no difference between diets was seen for pH's in stomach, jejunum, colon and cecum, and for the weight of the full and empty stomach and cecum. The counts of parietal cells per each oxyntic gland, the gene expression of ATPase and of TNF $\alpha$  in fundic gastric mucosa did not change too. These data indicate that nitrate did not affect the gastric secretory capacity and that, presumably, the gastric inflammatory status did not vary, as indicated by the TNF $\alpha$  expression. The results indicate that a moderate addition of nitrates does not negatively affect growth performance, but also

that the transfer of nitrite in the saliva was not sufficient to improve the health of weaning pigs stimulated with *Salmonella typhimurium*.

Table 1. Effect of diet and day of sacrifice on feed intake and daily live weight gains (DLWG).

	Diet			SEM (1)	Sacrifice, days after challenge		SEM
	Control	Ni15	Ni150		2	20	
Daily feed intake, kg					-	-	
- before	128	132	120	5.3	-	-	
- after challenge	323	332	320	12.4	181	466	10.1***
DLWG before challenge, g	19.4	13.5	11.6	12.3	-	-	
DLWG after challenge, g	189.8	195.5	172.6	24.0	52.8	319.2	19.6***

(1) Diet effect always  $P > 0.10$ . \*\*\*  $P < 0.001$ .

Table 2. Effect of diet and challenge on body temperature and humoral immunity.

	Treatment			SEM	Challenge		SEM
	Control	Ni15	Ni150		No	Yes	
Body temperature, °C							
- before challenge	39.25	39.27	39.31	0.059	-	-	
- 1 d later	39.37	39.28	39.42	0.100	39.11	39.60	0.082***
IgA, serum, µg/ml							
- before challenge	191	234	266	27.7 <sup>†</sup>	-	-	
- 20 d later	478	675	617	82.4 <sup>†</sup>	599	580	68.1

<sup>†</sup> Control vs Nitrate supplementations, approaching significance ( $P < 0.10$ ); \*\*\*  $P < 0.001$ .

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