Dietary Nitrate: Effects on the health of weaning pigs and Antimicrobial activity on seven probiotic *Bifidobacterium* spp. strains

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Abstract

The potential role of nitrite as an antimicrobial substance in the stomach may be of some importance in the ecology of the gastrointestinal tract and in host physiology. It has been shown that nitrite, under the acidic conditions of the stomach, may kill gut pathogens like Salmonella enteritidis, Escherichia coli, Salmonella typhimurium, and Yersinia enterocolitica, whereas acid alone has only a bacteriostatic effect. An in vivo study was conducted in order to assess the effects of dietary nitrate on microbiota and on the health of the gut (particularly in the stomach and small intestine). 96 weaning pigs were fed a diet containing high nitrate levels (15 mg and 150 mg) and then challenged with Salmonella enterica serovar typhimurium.

Differences in composition of the gut microbiota were assessed by analysing samples from the pigs: To date analysis of 48 pigs has been completed.. Preliminary results demonstrated no effect on the population densities of microbial groups either from the challenge or from nitrate intake. However, increasing the time from challenge decreased either the counts of LAB in the stomach and jejunum or of clostridia in the stomach.

Bifidobacteria also decreased in the stomach contents as nitrate supplementation increased. Supplementing the feedstuff with high dietary nitrate intake and then challenging with Salmonella did not affect the gastric pH or the degree of ulceration in the pigs.

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The synergistic bactericidal effects of pH, nitrite and thiocyanate on seven probiotic Bifidobacterium spp. strains were also investigated in an in vitro study.

The results of the in vitro study demonstrated that an inhibitory effect exists on the seven probiotic bifidobacteria investigated with an exposure longer than 2 hours and pH values < 5.0. Addition of thiocyanate also increased the susceptibility of the tested strains. In this in vitro study, the most resistant strains at all conditions were B. animalis subsp. lactis Ra 18 and P32 and B. choerinum Su 877, Su 837 and Su 891.

Introduction

Some beneficial effects of dietary nitrate on the physiology of the intestinal tract have been shown (McKnight G.M., et al. 1997). Recent studies also suggest a new non-immune host defence mechanism involving nitrite, which prevents pathogens from entering the bowel.

The mechanisms of this bactericidal effect are still unclear. However, the effect seems to be due to the production in acidic stomach conditions of some reactive nitrogen compounds like nitrous acid, peroxynitrite, nitrogen dioxide (NO_2) and frequently nitric oxide (NO) from nitrite (Xu J, et al., 2001).

The potential role of nitrite as an antimicrobial substance in the stomach may have some importance for the ecology of the gastrointestinal tract and host physiology, modifying normal microbiota and/or ingested probiotic viability (Xu J, et al., 2001).

The aims of these studies were to:

- assess the impact of high nitrate levels in the diet of weaning pigs on ulceration levels, on antimicrobial activity of the stomach acid against gastrointestinal diseases and on population levels of normal stomach and upper intestine microflora;
- quantify the resistance of probiotic bifidobacteria cultures to simulated gastric juice containing different concentrations of nitrite and isothiocyanate.

Materials and methods

Experimental design: *In vivo* study

A 2-factorial experiment was conducted to test the effect of three different doses of nitrate (supplied as a potassium salt) on normal stomach and upper intestine microbiota, on ulceration levels in the stomach and on gastric pH in pigs challenged or not with *Salmonella enterica* serovar *typhimurium* (orally supplied by 1.5 ml of broth containing 1 $x10^9$ CFU).

A total of 96 pigs (Landrace x Large White), weaned at 21 days, were randomly assigned (16 pigs each) to one of the following treatments: (1) base diet; (2) base diet + 15 mg/kg nitrates; (3) base diet + 150 mg/kg nitrates; (4) base diet + *Salmonella*; (5) base diet + 15 mg/kg nitrates + *Salmonella*; (6) base diet + 150 mg/kg nitrates + *Salmonella*.

The animals were sacrificed and stomachs removed for quantification of the ulcerae. The gastric contents were then rapidly harvested and pH was measured.

Microbiological analyses are currently in progress to enumerate five microbial groups in the stomach and in the jejunum contents: Lactic Acid Bacteria (LAB),

Bifidobacterium spp., *Enterobacteriaceae*, *Clostridium* spp, and yeasts. Quantitative detection of *Bifidobacterium* spp. was performed by a dilution PCR method.

Experimental design: *In vitro* study

Concentrations of nitrite characteristic of those found in saliva were tested on seven probiotic bifidobacteria of various origins (Tables 2,3).

Determination of the bactericidal (MBC) and bacteriostatic activities (MIC) of acidified nitrite was performed on disposable microwell plates using the method described by Dykhuizen et al.(1996). Nitrite solutions with final concentrations of 0, 0.05, 0.125, 0.2, 0.25, 0.375, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 10mM in the microwells and TPY broth solutions acidified by hydrochloric acid with different final pH values were prepared.

Results: *In vivo* study

Ability of supplementation with nitrate to enhance stomach acidity and impact on ulceration levels in pigs fed with high nitrate concentration

The effects of diet, challenge, and time from challenge on average values of gastric pH and on ulceration levels are presented in Table 1.

Table 1. Effect of diet, challenge and time from challenge on average values of gastric pH and ulceration levels in pigs fed high nitrate concentration

	Treatment			SEM	Challenge		SEM	Sacrifice, days		SEM
	Control	Ni15	Ni150		No	Yes		2	20	
Gastric pH	2.86	3.31	2.88	0.165†	3.04	2.99	0.134***	3.35	2.69	0.133 ***
Ulceration score	1.36	1.24	1.33	0.204 ns	1.29	1.33	0.165 ns	1.26	1.35	0.164 ns

† Between nitrate supplementations, approaching significance (P=0.066); *** P<0.001; ns = P>0.10.

Effect of different nitrate concentrations on the population levels of normal stomach and upper intestine microbiota

The diet did not affect the contents of cultivable LAB, Clostridia and yeasts in either segment, nor did it affect *E. coli* contents in the jejunum. The time from challenge had an important effect on the counts of LAB which decreased with age in the stomach (P<0.001) and in the jejunum (P< 0.05). In addition, *Clostridia* spp. in the stomach were reduced.

With respect to *E. coli* contents in the stomach, only 35 – 40% of the subjects of each diet had a bacteria concentration sufficient to be recoverable with the cultivation, and the values in the positive samples were very low (3.12 log cfu on average, data not reported in table). In the older pigs, a trend of decreasing bifidobacteria count in the stomach (P=0.07) was noted.

Results: *In vitro* study

Resistance of probiotic bifidobacteria cultures to simulated gastric juice containing different concentrations of nitrite and isothiocyanate

Susceptibilities to acidified nitrite solutions expressed as MIC values ranked as follows: Su 905>Su 932/1>Ra 18>P32>Su 837>Su 877 and Su 891 (Table 2).

The MBC was determined at three different times (Tables 2,3). Increasing time of exposure, all strains survived unless nitrite was present in the solution but their susceptibility increased at all pH settings. (Table 2). Susceptibility as MBC_{24h} was: Su 905>Su 932/1>Su 837, Su 891, Ra 18, P 32> Su 877.

The addition of sodium thiocyanate (10mM) resulted in a reduction in the amount of acidified nitrite required to accomplish bacteriostatic and bactericidal activity (Table 3).

Increasing time of exposure, the bacteriocidal effect of thiocyanate was more evident. All strains were killed within 24 hours at a pH of 2.5. MBC_{24h} was different for each strain and the susceptibility at all pH settings ranked as follows: Su 905>Su 932/1>Ra18, P32>Su 837>Su 891>Su 877.

Table 2. Activity of nitrite acidified with HCl at various pH values on select probiotic *Bifidobacterium* spp. strains

			Antimicrobial nitrite concentration (mM) at specified pH							
Strain (Species and origin)	Antimicrobial activity	Exposure time (h)	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0
RA 18	MBC	0.5 h	6	10	>10	>10	>10	>10	>10	>10
B. animalis		2	3.5	4	8	>10	>10	>10	>10	>10
subsp. *lactis*		24	1	1.5	2	4	6	>10	>10	>10
(from rabbit)	MIC	24	0	0	0	0	3	6	>10	>10
P 32	MBC	0.5 h	6	>10	>10	>10	>10	>10	>10	>10
B. animalis		2	3.5	4	8	>10	>10	>10	>10	>10
subsp. *lactis*		24	1	1.5	2	4	6	>10	>10	>10
(from chicken)	MIC	24	0	0	0	0	3.33	7.33	>10	>10
SU 905	MBC	0.5 h	4.5	6	>10	>10	>10	>10	>10	>10
B. suis		2	2.5	3	6	>10	>10	>10	>10	>10
(from pig)		24	0.2	0.75	1	1.5	2	8	8	≥
	MIC	24	0	0	0	0	1.25	3	5	5
Su 932/1	MBC	0.5 h	5	≥8	>10	>10	>10	>10	>10	>10
B. suis		2	3	4	8	>10	>10	>10	>10	>10
(from pig)		24	0.75	1	1.25	1.75	4.5	≥8	>10	>10
	MIC	24	0	0	0	0	1.5	3.5	6	10
Su 837	MBC	0.5 h	6	10	>10	>10	>10	>10	>10	>10
B. choerinum		2	3	4	8	>10	>10	>10	>10	>10
(from pig)		24	1	1.5	≤2	3.5	6	>10	>10	>10
	MIC	24	0	0	0	0	4	7.33	>10	>10
Su 877	MBC	0.5 h	8	>10	>10	>10	>10	>10	>10	>10
B. choerinum		2	4	5	8	>10	>10	>10	>10	>10
(from pig)		24	1	1.5	2	4	6.67	>10	>10	>10
	MIC	24	0	0	0	0	4.67	8.67	>10	>10
Su 891	MBC	0.5 h	6	>10	>10	>10	>10	>10	>10	>10
B. choerinum		2	3.5	4	8	>10	>10	>10	>10	>10
(from pig)		24	1	1.5	2	4	6	>10	>10	>10
	MIC	24	0	0	0	0	4	8.67	>10	>10

Table 3. Activity of nitrite acidified with HCl at various pH values on select probiotic *Bifidobacterium* spp. strains with the addition of 10mM KSCN in the solution

			Antimicrobial nitrite concn (mM) at pH							
Strain (Species and origin)	Antimicrobial activity	Exposure time (h)	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0
RA 18	MBC	0.5 h	5	8	>10	>10	>10	>10	>10	>10

B. animalis		2	3	3	6	>10	>10	>10	>10	>10
subsp. *lactis*		24	0	0.83	0.83	2	4	6	7,33	>10
(from rabbit)	MIC	24	0	0	0	0	2	2.5	6	10
P 32	MBC	0.5 h	5	8	>10	>10	>10	>10	>10	>10
B. animalis		2	3	3	6	>10	>10	>10	>10	>10
subsp. *lactis*		24	0	0.83	0.83	2	4	6	7.33	>10
(from chicken)	MIC	24	0	0	0	0	2	2.83	6	10
SU 905	MBC	0.5 h	3.5	5	8	>10	>10	>10	>10	>10
B. suis		2	2	2	3.5	10	>10	>10	>10	>10
(from pig)		24	0	0.25	0.25	0.5	1.25	4	4	6
	MIC	24	0	0	0	0	0.75	1	3	3
Su 932/1	MBC	0.5 h	4	7.33	≤10	>10	>10	>10	>10	>10
B. suis		2	3	3	4	10	>10	>10	>10	>10
(from pig)		24	0	0.57	0.57	1.25	2	4	5.33	8
	MIC	24	0	0	0	0	1	2	4	6
Su 837	MBC	0.5 h	5	8	>10	>10	>10	>10	>10	>10
B. choerinum		2	3	3	6	>10	>10	>10	>10	>10
(from pig)		24	0	0.87	0.87	1.67	4	6	10	>10
	MIC	24	0	0	0	0	2.16	3.50	6	10
Su 877	MBC	0.5 h	5	8	>	>10	>10	>10	>10	>10
B. choerinum		2	3.5	3.5	7.33	>10	>10	>10	>10	>10
(from pig)		24	0	1.25	1.25	2.5	4	6	10	>10
	MIC	24	0	0	0	0	2.5	5.33	≥8	10
Su 891	MBC	0.5 h	5	8	>10	>10	>10	>10	>10	>10
B. choerinum		2	3	3	6	>10	>10	>10	>10	>10
(from pig)		24	0	1	1	2.5	4	6	10	>10
	MIC	24	0	0	0	0	2	4	6.67	10

Discussion

In vivo study

A trend of increase in gastric pH can be expected with the addition of dietary nitrate, whether challenged by Salmonella or not. More experimental data are required to explain this observation and why this increase was not observed for a dose ten times higher. The dietary addition of nitrate and challenge with *Salmonella* do not affect the degree of ulceration.

On the average the supplementation of the diet with nitrates did not affect bifidobacteria concentrations in the two digestive tracts. However, the trend of decreases in bifidobacteria and LAB could be related to the suspension of milk intake after weaning and the consequent reduction of substrates favourable for growth of this kind of bacteria.

Salmonella enterica serovar *typhimurium* was found in almost all pigs challenged with this pathogen. Nitrates did not show good resistance to pathogen colonisation, even though some unchallenged pigs also resulted positive for Salmonella in the lymph nodes.

In vitro study

Antibacterial potential of swallowed salivary nitrite at low pH values has already been demonstrated by various studies. Our work confirmed that an inhibitory effect also exists on the seven probiotic bifidobacteria strains investigated for time exposures greater than 2 hours and pH values < 5.0. The addition of thiocyanate increased the

susceptibility of the tested strains. Overall, the most resistant strains were Ra 18, P32, Su 877, Su 837 and Su 891 at all conditions studied.

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