NJAS - Wageningen Journal of Life Sciences 58 (2011) 149–156

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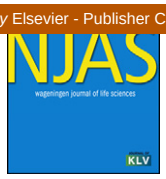

Strategies to augment non-immune system based defence mechanisms against gastrointestinal diseases in pigs

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ARTICLE INFO

Article history:
Received 30 October 2009
Accepted 4 April 2011
Available online 8 May 2011

Keywords:
Probiotics
Prebiotics
Synbiotics
Pigs
Weaning
Gut microbiota

ABSTRACT

Our study addresses the first two weeks of the weaning period of piglets during which stressful physiological and environmental conditions experienced by the animals can promote the proliferation of pathogens in the digestive tract. The aim of the study was to identify new feeding strategies that result in boosting the gastrointestinal tract (GIT) microbiota of piglets and improve growth performance, reducing the negative impact of weaning. In order to identify a new synbiotic combination, 12 new putative probiotic strains of *Bifidobacterium* spp. and three non-digestible oligosaccharides [NDO] were screened in newly weaned piglets. The ability to increase the level of autochthonous bifidobacteria and improve growth performance were assessed. Bifidobacteria strains with a similar ability to develop in the hindgut showed a different effect on piglet performance depending on the dose in which they were provided. Our data support the idea that the presence of fructo-oligosaccharides would stimulate the occurrence of bifidobacteria in the caecum. It was shown that dietary intake of nitrate can generate salivary nitrite, which in turn is acidified in the stomach and could have antimicrobial activity against swallowed pathogens. The efficacy of the resulting synbiotic formula was improved by adding nitrate as antimicrobial. To enhance probiotic survival during gastric transit, a novel technology of microencapsulation was developed and applied to bacteria. The final synbiotic, containing the strain RA 18 of *Bifidobacterium animalis* subsp. *lactis* [10^{11} cfu/day], the prebiotic Actilight® [4% of the diet], and nitrate [150 mg KNO_3/kg feed/day] was tested in organic weaned piglets reared under field conditions. Results show that the strain Ra 18 had a probiotic effect in organic weaned piglets, as it colonized and remained detectable in faecal samples until two weeks after addition. The use of our synbiotic formula improved weight gain, feed efficiency and health status of the weaned piglets.

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1. Introduction

In conventional farrowing systems, the weaning process is considered as a crucial period in the life of the pigs. This means that piglets are simultaneously exposed to a large number of stressors, such as a separation from the sow, an early and critical transition from milk to a diet based on plant polysaccharides, and the end of the lactational immunity. Furthermore, the sudden interruption of the maternal milk supply leads to the progressive withdrawal of the maternal protective IgA that acts locally in the suckling piglet's intestine [1]. These factors combined can disturb the immune function and the intestinal microbiota equilibrium of the piglets [2–4]. Previous research has shown that in a healthy animal a well balanced microbiota helps in efficient digestion and maximum uptake of nutrients, increasing the body's resistance to infections and thus protecting the host against certain enteropathic diseases [5,6].

The effects of the microbiota and its metabolic activities require special consideration when viewed in the context of pig production where animal growth and animal production are the primary objectives [7]. The control of pathogens is of primary importance in livestock production, because of the economically damaging problems linked with infections caused by pathogens in newborn animals [8].

The commensal bacterial populations inhabiting the intestinal tract of pigs have been recognized to play important roles in organ, tissue and immune system development, as well as in providing a variety of nutritional compounds [9]. If the microbiota is compromised, an overgrowth of pathogenic bacteria may occur, giving rise

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doi:10.1016/j.njas.2011.04.001

to gastrointestinal disorders impairing growth performances of the animals, lowering their resistance to endogenous and exogenous opportunistic pathogens, weakening digestive capability causing poorer nutrient uptake and even death [5]. As for animal health and animal production, an important objective is to be able to manipulate the gut microbiota, and qualitative modifications to the diet seem to be a way that could lead to a desiderable microbiota composition.

Including micro-ingredients such as probiotics (live beneficial bacteria) and prebiotics (substrates designed to stimulate groups of desirable bacteria), alone or in combination (synbiotics) in the weaner diet of pigs may help to obtain a gradual change in intestinal microbiota after weaning and may stimulate beneficial bacteria like bifidobacteria and lactobacilli in the large intestine [10–13]. This nutritional approach may lead to a repopulation of normal gut microbiota so that the animal's appetite and ability to efficiently utilize feedstuff returns and the problem of post-weaning stress or growth may be solved [14].

Probiotics are a heterogeneous group of microbes currently used as biotherapeutic agents that are known to have beneficial effects on the digestive ecosystem and to confer resistance to infections. The commonly used probiotics are strains of intestinal origin and belong to the lactic acid producing bacteria (LAB – e.g., *Lactobacillus* spp., *Bifidobacterium* spp. and *Streptococcus* spp.) [6,15,16]. The ability of LAB cells to survive in gastric and bile conditions and to adhere to the intestinal epithelium may confer a competitive advantage and is important for bacterial maintenance in the gastrointestinal tract [17]. However, the beneficial effects of probiotic micro-organisms appear when they arrive in the gut alive, biologically active and in high enough numbers to be able to withstand the host's natural barriers against ingested bacteria [18,19]. Therefore, the intake of a probiotic in combination with a suitable prebiotic (synbiotic) can result in synergistic effects, improving the survival of probiotic bacteria in the host's gastrointestinal tract (GIT), enabling the incorporation of the probiotics into the autochthonous bacterial community and stimulating growth activities of both exogenous (probiotics) and endogenous bacteria. Also other approaches have been proposed that increase resistance of bacteria sensitive to adverse conditions. Viability loss of probiotics in acidic bile conditions of GIT has encouraged researchers to find new methods of viability improvement [20]. Microencapsulation and encapsulation methods that previously have been reported as technologies that can provide protection to these sensitive cultures during transit through the host's GIT, have been applied to increase survival and delivery of bacterial cultures [21].

Furthermore, it has recently been shown that compounds such as nitrates, naturally present in food like vegetables and fruits [22], become powerful antimicrobial agents in acid conditions prevailing in the stomach. Nitrates therefore increase the resistance to pathogens, whereas acids alone would only have a bacteriostatic effect [23]. The mechanism by which dietary nitrates exploit bactericidal effects is still unclear. It seems to be related to the production of nitrite in acidic conditions to reactive nitrogen compounds including nitrous acid, peroxynitrite, nitrogen dioxide and mostly nitric oxide [24]. Approximately 25% of dietary nitrate is recirculated by a process known as enterosalivary circulation. Nitrites are formed from nitrates in the oral cavity by nitrate reductase expressed by micro-organisms in the mouth and then re-secreted into the upper intestinal tract [22,23,25]. Nitrates can have antimicrobial effects not only on intestinal pathogens like *Salmonella*, *yersinia* and *Escherichia coli* strains, but also on *Helicobacter pylori* [26], a bacterium that is held responsible for ulcers in the mucous of the stomach. Also ulcers in the stomach represent a problem from the commercial point of view of livestock production, due to a reduction in the feed conversion ratio and in animal growth [27]. Furthermore, the recirculation of nitrates by the organism into enterosalivary circulation would also suggest a beneficial function of nitrates for the animals [22].

This study aimed to develop a new suitable and orally administered natural treatment that would promote the gut's microbiota balance and improve growth performances of weaning piglets as an alternative to antibiotic growth promoters. To this end a number of *in vivo* experiments were carried out to evaluate the effects of dietary supplementation with probiotics, prebiotics, synbiotics and nitrate on gut's microecology, and health and growth performances of piglets during weaning.

2. Materials and methods

2.1. Study design

The experimental design included two phases: (1) an initial screening of probiotics, prebiotics and dietary nitrate to develop the treatments and the doses to be used, and subsequently (2) a field study evaluating the resulting formula.

2.1.1. Screening of probiotics and prebiotics

The probiotic ability was assessed of 12 different *Bifidobacterium* strains selected from the species *B. breve*, *B. suis*, *B. animalis* subsp. *lactis* and *B. choerinum*. The probiotic effect of 3 strains of each *Bifidobacterium* species was investigated on 60 pigs, using a dose of 10^{10} colony-forming units (cfu) per pig (Table 1). Each strain was fed to a group of 5 pigs; the control group comprised 4 pigs.

Three additives from a total population of endogenous lactic acid bacteria were assessed for their prebiotic effects. The non-digestible oligosaccharides (NDOs) were (1) Actilight® a fructo-oligosaccharide (FOS) from sugar beet (sbFOS), (2) Raftiline® a FOS from chicory inulin (ciFOS), and (3) Vivnal tGos®, a galacto-oligosaccharide (GOS) from milk whey. The prebiotics were tested at two doses: 1% and 4% (w/w) of the diet. Each dose was fed to a group of 10 piglets; the control group comprised 4 pigs.

2.1.2. Probiotic + prebiotic testing

The most active probiotics resulting from the above screening tests were strain Ra 18 of *Bifidobacterium animalis* subsp. *lactis* and strain Su 891 of *B. choerinum*. The interactions with the most active prebiotic (sbFOS) were independently evaluated for their dosage-related effects in two factorial experiments, each with 2 replications. Each experiment, included 4 doses of the candidate probiotic *Bifidobacterium* strain: 0, 10^7 cfu, 10^9 cfu, and 10^{11} cfu per pig per day with or without sbFOS at 4% (w/w) of the diet, resulting in 16 treatment combinations per test. Each experimental unit consisted of 4 pigs.

Microbiological analyses were done on samples of ceacum contents.

2.1.3. Nitrate experiment

The impact was assessed of two doses of nitrate, supplied as potassium salt, on normal stomach and upper intestine microbiota and on ulceration levels in the stomach of piglets challenged with *Salmonella enterica* serovar *typhimurium*. The piglets were challenged with 1.5 ml orally supplied broth containing 10^9 cfu *Salmonella enterica* serovar *typhimurium*. Control piglets received a placebo consisting of broth only.

A total of 6×16 piglets were randomly assigned to one of the following six treatments: (1) basal diet; (2) basal diet + 15 mg potassium nitrate (KNO_3) per kg feed; (3) basal diet + 150 mg KNO_3 per kg feed; (4) basal diet + salmonella; (5) basal diet + 15 mg KNO_3 per kg feed + salmonella; (6) basal diet + 150 mg KNO_3 per kg feed + salmonella. A good weaning diet was formulated also for these tests. Half the number of pigs were sacrificed on day 7 (+2 after being challenged), and the other half on day 25 (+20

Table 1
Effects of diet, challenge and days since challenge on average values of bacteria in the stomach and in the jejunum contents of piglets fed diets with a high nitrate (Ni) content.

	Diet[a]			SEM	Challenge salmonella		SEM	Days since challenge		SEM
	C	Ni15	Ni150		No	Yes		2	20	
Stomach										
E. coli[b]	2.90	3.21	3.44	0.34	3.04	3.32	0.28	2.96	3.40	0.29
LAB	5.99	6.09	6.19	0.34	6.19	5.99	0.28	6.97	5.20	0.28[c]
Yeasts	5.29	5.31	5.45	0.24	5.40	5.30	0.20	5.57	5.13	0.20
Clostridia	5.61	5.54	5.96	0.31	5.84	5.56	0.25	6.39	5.02	0.25[c]
Bifidobacterium spp.	6.41	6.18	6.38	0.12	6.38	6.26	0.10	6.53	6.11	0.09
Jejunum										
E. coli[b]	5.82	5.90	6.01	0.32	6.32	5.50	0.26[d]	5.96	5.86	0.26
LAB	6.55	7.17	6.51	0.21[f]	6.93	6.56	0.17	7.14	6.35	0.17[c]
Yeasts	5.79	5.87	5.82	0.22	5.82	5.83	0.18	5.78	5.88	0.18
Clostridia	7.05	7.13	7.17	0.20	7.31	6.92	0.17[e]	7.16	7.07	0.16
Bifidobacterium spp.	6.82	7.07	6.46	0.19	6.65	6.91	0.16	6.86	6.70	0.16

Note: There were no statistically significant interactions ($p < 0.05$) between factors.
[a] C = control – 0 mg KNO_3 per kg feed; Ni15 – 15 mg KNO_3 per kg feed; Ni150 – 150 mg KNO_3 per kg feed.
[b] Only 32 piglets had detectable values: 10 for C, 12 for Ni15; 10 for Ni150; 20 for unchallenged, 12 for challenged piglets.
[c] Statistically significant effect of duration of challenge ($p < 0.01$).
[d] Statistically significant effect of challenge ($p < 0.05$).
[e] Effect of challenge statistically different at $p = 0.10$.
[f] Contrast Ni15 vs Ni150 statistically significant at $p < 0.05$.

after being challenged). Immediately after slaughter, the stomach was removed for counting the ulcers and for measuring pH of its contents. The stomach content, the gastric and caudal part of the jejunum contents were removed aseptically and prepared for microbial analysis.

Microbiological analyses were done on samples of stomach and caudal part of jejunum contents.

2.2. Animals

In the first phase of the study the piglets (Landrace × Large White) were weaned at 21 days and were assigned to the different treatments (probiotics, prebiotics, etc.), using the criteria of having similar initial live weights and being equally representative for the litter of origin.

Throughout the first three tests, except for the first 2 days of the probiotic screening and the probiotic + prebiotic testing, the pigs were penned individually, fed *ad libitum*, and housed in two identical rooms with controlled temperature. The daily feed intake was registered, and any residual feed was removed and weighed. Every day, 1 ml of a skimmed milk solution with or without the prefixed dose of each *Bifidobacterium* strain was added to the feed trough. For the first 2 days of the probiotic and probiotic + prebiotic tests, the piglets of the dietary groups were housed together in one or two pens and orally administered the probiotic solution.

The experiment with dietary nitrate was performed in four consecutive batches. The pigs were housed two by two in pens with slatted floors. From the start until day 5, the pigs were offered the experimental diets in their pen. Thereafter the pigs were challenged and individually penned in the same type of pens. The challenge organism was orally administered in the form of 1.5 ml broth containing 10^9 cfu of *Salmonella enterica* serovar *typhimurium*. Control pigs received a placebo consisting of broth only. To calculate the daily live weight gain, the piglets were weighed at the start and at the end of the experiment, which lasted 2 weeks on average. In line with the protocol approved by the Ethical Committee of the University of Bologna, the piglets were anaesthetized with sodium thiopenthal (10 mg per kg body weight) then sacrificed (euthanasia) by an intracardiac injection with Tanax® (0.5 ml per kg body weight).

In the *probiotic*, *prebiotic*, and *probiotic + prebiotic tests*, the piglets were sacrificed over a 3-day period, and were taken from the treatment in such a way that the day of sacrifice was distributed equally over the 3 days, enabling to calculate an average day of sacrifice and thus minimizing differences between treatments.

In the *nitrate* experiments, half the number of pigs in each experimental batch were sacrificed on day 7 (+2 after challenge), and half on day 25 (+20 after challenge). All animals were sacrificed at the same time after their last meal. Feed was available until 2 h and water until 1 h prior to sacrifice.

2.3. Series synbiotic formula

The suitability, tolerance and positive effects of the new synbiotic combination formula that resulted from the previous experiments were tested on an organic farm under field conditions. The synbiotic formula was composed of:

1. The putative probiotic strain Ra 18 belonging to the species *B. animalis* subsp. *lactis* (microencapsulated with a protocol developed by Probiotical srl) at a daily dose of 390×10^9 cfu per animal;
2. The NDO extracted from sugar beet, Actilight®, at a daily dose of 4% of the diet;
3. Potassium nitrate at a daily dose of 150 mg per kg feed.

Two independent feeding experiments including a total of 58 40-days-old piglets (Large White × Suffolk) were started at the first day of weaning. The time of adaptation to the experimental conditions was 3 days, after which the animals were fed the diet normally used on the farm supplemented or not with the experimental formula. The feed was supplied once a day, but the animals were not fed individually. Each experiment (Trials 1 and 2) comprised a total of 20 piglets distributed over two groups: a group fed the synbiotic formula and a control group fed the basal diet. Each group was divided in two replicates based on similar levels of mean body weight. The replicates (five piglets each) were housed in separate pens. The formula was supplied only for 2 weeks, but in all experiments the animals were observed over a period of 1 month. Faecal samples for microbiological analysis were collected at three different moments: (1) T_0, when the experiment started, (2) T_1, 15 days later, and (3) T_2, in the wash-out period, after two weeks from ending the period of observation.

Body weights were determined every 2 weeks starting at day 1 until the last day of the experiment to calculate body weight gain. Feed intake was recorded every two weeks by offering a weighed quantity of feed and weighing their residues. The feed conver-

sion ratio was calculated by dividing feed intake by body weight gain.

Microbiological analyses were done on samples of faeces.

2.4. Microbial analyses

After collection, each sample (caecum content, stomach or jejunum content, faeces,) was weighed (1 g) accurately into a 10 ml plastic tube and suspended homogenously in 9 ml glycerol broth to avoid qualitative and quantitative changes in the microbiota. The samples were then stored at −120 °C until analysis. Additional serial 10-fold dilutions of the samples were made in phosphate-buffered saline (PBS, Oxoid) (pH 7.2) for microbial counting. One millilitre of the appropriate dilutions was plated in triplicate onto different solid selective media employed for the quantification of the different bacterial species. The lactic acid bacteria (LAB), *Bifidobacterium* spp., clostridia and yeasts were counted using the plate count method, employing solid selective media.

The *E. coli* contents were determined with Chromocult Coliformen Agar (Merck), after incubation in aerobiosis, at 37 °C for 24 h. *E. coli* typically appears as blue/purple colonies whereas coliforms appear as red/rose colonies. Testing for indole production gave further confirmation of the micro-organism identity.

LAB were quantified by plating on De Man Rogosa Sharpe (MRS) agar (Merck) after incubation in anaerobiosis, at 37 °C for 72 h. No further tests were conducted to identify specific micro-organisms, and the total microbial counts were referred to as LAB.

Yeasts (quantified only in the nitrate experiment) were cultured on malt extract-chloramphenicol (MC) agar (38 °C, aerobic growth for 2 days). The MC agar contained (per litre) yeast extract (3 g, Merck), malt extract (3 g, Merck), peptone from meat peptic digest (5 g, Merck) D (+) glucose (10 g, Merck), and agar (15 g, Merck). After autoclaving, 10 ml of 0.5% chloramphenicol in 96% ethanol was added. *Clostridia* (quantified only in the nitrate experiment) were enumerated as colonies appearing on reinforced clostridium agar (RCM, Merck) (37 °C, anaerobic growth for 2 days).

Bifidobacteria were selectively enumerated using modified trypticase–phytone–yeast extract (mTPY) agar [28,29] to which Mupirocin (100 mg per litre), glacial acetic acid (1 ml per litre), colistin (25 mg per litre) and nystatin, (50,000 U per litre) were added. The antibiotics used were purchased from Applichem GmbH (Darmstadt, Germany), except for nystatin, which was supplied by Sigma. The plates were incubated in anaerobiosis, at 37 °C for 72 h. Colonies grown on mTPY agar were randomly checked for morphology, and Gram stained.

2.5. Genomic DNA extraction from biological specimens

In each trial, genomic DNA was isolated from 200 mg of each biological specimen (faeces, caecum, stomach and caudal part of jejunum content) using the Qiamp DNA stool mini kit (Qiagen West Sussex, UK). The recommended lysis temperature was increased to 95 °C in order to improve bacterial cell rupture. The DNA was stored at −20 °C until analysis.

2.6. Direct semi-quantitative Genus-PCR

A culture-independent PCR protocol was used only for confirming the enumeration of *Bifidobacterium* spp. as described above [30]. Briefly, after extraction from the caecum content, 10 μl of the eluted genomic DNA were serially diluted 10-fold in sterile double distilled H_2O. For all samples 2 μl of each dilution was amplified as described above using the primer set gBifid [31]. The *Bifidobacterium* spp. titre in each caecal sample was determined by considering the sensitivity of this procedure. The sensitivity corresponds to the number of *Bifidobacterium* spp. cells present in the highest DNA dilution of each sample able to give a positive amplification signal.

2.7. Bacteria quantification by real-time PCR (qPCR)

In the final phase of the study, i.e., the synbiotic test, total bifidobacteria and lactobacilli were quantified using the following group-specific primers: RecA-F 5′-CGTYTCBCAGCCGGAYAAC-3′ and RecA-R: 5′-CCARVGCRCCGGTCATC-3′ [32] for total *Bifidobacterium* spp., F-Lac 5′-GCAGCAGTAGGGAATCTTCCA-3′ and R-Lac 5′-GCATTYCACCGCTACACATG-3′ [33] for total *Lactobacillus* spp. To quantify bacterial cells of *B. animalis* subsp. *lactis* and *E. coli* at species level, the species-specific primers used were: *B. lactis*-F: 5′-CCCTTTCCACGGGTCCC-3′, *B. lactis*-R: 5′-AAGGGAAACCGTGTCTCCAC-3′ [34] and *E. coli*-F: 5′-GTTAATACCTTTGCTCATTGA-3′, *E. coli*-R: 5′-ACCAGGGTATCTAATCCTGTT-3′ [34]. For species-specific quantification of *B. animalis* subsp. *lactis* and *E. coli*, Europium-probes were used too: *B. lactis*: 5′(HEX)-AAATTGACGGGGGCCCGCACAAGC-(DABCYL)-3′ ([34] and *E. coli*: 5′(FAM)-CGTGCCAGCAGCCGCGGTA-(DABCYL)-3′ [34]. The oligonucleotides and probes (MWG Biotech AG, Ebersberg, Germany) were adapted from published specific primers or probes using the Primer Express Software (Applied Biosystems, Foster City, CA, USA). Primers were also checked for their specificity using the database similarity search programme nucleotide-nucleotide BLAST [35].

Standard curves were constructed using the PCR product of the 16 S rDNA gene from *E. coli* (ATCC 26645) for *E. coli* assay; from *L. acidophilus* (DSM 9126), *L. casei* (DSM 20011), *L. lactis* subsp. *lactis* (DSM 20175), and *L. plantarum* (DSM 1055) for *Lactobacillus* spp. assays and from *B. animalis* subsp. *lactis* (DSM 10140), for *B. lactis* assay. A standard curve for *Bifidobacterium* spp. was constructed using the PCR product of the RecA gene from *B. pseudolongum* subsp. *globosum* (DSM 20092), *B. longum* (DSM 20090), *B. animalis* subsp. *lactis* (DSM 10140), and *B. bifidum* (DSM 20082) for *Bifidobacterium* spp. assay. The PCR product was purified with the commercial kit NucleoSpin Extract II (Macherey-Nagel GmbH & Co. KG. Duren, Germany) and the concentration measured at 260 nm (Biophotometer, Eppendorf). Products obtained were also sequenced (BMR, Padova, Italy) and number of copies calculated. Serial dilutions of the products were performed in a range from 10^9 to 10^{12} copies of the gene for calibration and construction of the proper standard curve. The functions describing the relationship between Ct (threshold cycle) and x (log copy number) for the different assays were: $Ct = -3.807x + 45.812$; $r^2 = 0.998$ for total *Bifidobacterium* spp., $Ct = -3.821x + 34.846$; $r^2 = 0.993$ for *Lactobacillus* spp., $Ct = -3.74x + 49.338$; $r^2 = 0.996$ for *E. coli*, and $Ct = -3.644x + 51.025$; $r^2 = 0.995$ for *B. animalis* subsp. *lactis*.

Real-time PCR was performed with the Step One Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using optical grade 48-well plates. The reaction was performed on a total volume of 25 μl using the TaqMan or the SYBR Green chemistry (Applied Biosystems). Each reaction included 2.5 μl 10× FAST SYBR Green Master Mix or 10× TaqMan Gene Expression Master Mix (Applied Biosystems), 0.25 μl of each primer (200 or 400 nmol) and 2.5 μl of ultra pure DNA samples (1 ng) and when necessary 0.25 μl of the specified probes (100 or 250 nmol). Generally, the reaction conditions for amplification of DNA were 95 °C for 30 s, 40 cycles of 95 °C for 3 s, and 62 °C for 20 s. Few modifications were set for the different assays. To determine the specificity of amplification, analysis of product melting curve was performed after the last cycle of each amplification.

2.8. Statistical analyses

All the data were processed using SAS software (SAS version 9) with the procedure GLM. Data from the first phase (probiotics, prebiotics, probiotics + prebiotics testing) were analysed using analysis of variance with the GLM procedure of SAS with a factor design that included: probiotic screening, diet ($n = 6$), litter and day of sacrifice; prebiotics screening, prebiotic ($n = 3$), level of supplementation (1% or 4%), their interaction and litter; probiotics + prebiotics screening, probiotic (yes or not), level of probiotic supplementation (0; 10^7; 10^9, 10^{11}), their interaction, replicate and litter within replicate.

The data from the nitrate experiment were analysed using analysis of variance for the following three factorial models: (1) data *in vivo* before challenge: diet and block (batch); (2) data post challenge: model A + challenge (yes/no) and the interaction; (3) data obtained at different days of sacrifice: model B + day of sacrifice (2 or 20 days after starting the challenge) and the interaction. Orthogonal pre-planned comparisons were tested for the effect of diet: nitrate vs no nitrate addition; between different levels of nitrate addition. The results relating to 7 pigs of the first batch were excluded from the statistical analysis because of the very low feed intake before the challenge (feed intake in the total period lower than 1% of the initial body weight).

In the series synbiotic formula, statistical analyses on qPCR were carried out using ANCOVA models. Differences in microbial counts and body weight between starting time and end of treatment, and between starting time and end of wash out were used as dependent variables. Treatment and sex were used as fixed factors, whereas initial body weight and initial microbial counts for each bacterial group were used as covariates. For the analysis, microbial counts were used as log and for *B. animalis* subsp. *lactis* starting value was excluded from the model as it was negative for all analysed subjects. The effects of different variables included in the models were evaluated using the *F*-test. For the treatments used in the experiments, adjusted media (least square media) were provided. Differences between these values were calculated and used to compare groups. Differences were regarded statistically significant at $p \leq 0.05$.

3. Results and discussion

The hindgut of the pig harbours a dense and metabolically active microbiota comprised primarily of bacteria that have a profound influence on nutritional, immunological and physiological processes in the host. Recently, more attention has been paid to studies of the intestinal bacterial community, notably because of the urgent need to replace antibiotics as growth promoters in animal production [36].

Synbiotics are recognized means of modulating gut microbiota composition and activities. The term synbiotic refers indirectly to a synergy, and that is what some authors have suggested that it should exclusively refer to: products in which the prebiotic compound selectively favours the probiotic [37].

Here we describe the development of a synbiotic formula and the study of its effect on the faecal microbiota, on growth performance and health status of organic recently weaned piglets.

Given that the performance of probiotic strains can vary, assessing *in vivo* their efficacy could be the most appropriate approach to characterize the individual strains. Laboratory testing can provide useful information for the selection of putative effective probiotic strains, but the performance in the gut and the effect on the intestinal microflora can only be accurately determined *in vivo*. Recent data also suggest that it is the condition of the individual host that influences the outcome of the probiotic effect and different researches highlight how the gut environment tends to modify, and thus affects the viability of ingested bacteria [38].

The results related to the first phase of this study were discussed in detail in a previous paper [30]. The study aimed at identifying putative probiotic and prebiotic candidates, and 12 bifidobacteria strains of different ecological origin and three NDOs were screened. For identifying the best probiotic and prebiotic candidate we evaluated the ability of the strains and of the NDOs to modify the piglet gut microecology.

As viable and biologically active micro-organisms are required at the target site in the host, it is essential that probiotic strains are able to withstand the host's barriers against exogenous bacteria [1].

Therefore *in vivo* challenges are indispensable to show the capacity of probiotics to survive after the GI transit. The most active potential probiotic strains identified in the probiotic series, belong to the species of *B. animalis* subp. *lactis*, (strain Ra 18) and *B. choerinum* (strain Su 891). They grow in the gut, increasing the numbers of viable bifidobacteria but show different effects on health of treated piglets.

Evaluating the influence of different prebiotic intake, it was observed that supplementation with different FOS increased the frequency of finding pigs that were positive for culturable bifidobacteria in the caecum content, and the effect increased with the dose. However, the average bifidobacteria counts tended to increase only with the SbFOS supplementation, regardless of the supplementation dose. During these two feeding trials all pigs were healthy: there were no signs of diarrhoea, weight loss or loss of appetite.

Many publications that show the beneficial therapeutic effect of probiotics point to the dose–response effect. For a given strain, variations in colonization and persistence rates can be the result of intervention time, differences in administered dose, and the detection methods used [5,39–43].

Thus the dose–response effects of supplementation with more than two doses of previously identified probiotic bacteria, Ra 18 and Su 891, were studied. As was previously found, the general picture of the different synbiotic treatments with *B. animalis* subsp. *lactis* Ra 18 and with *B. choerinum* Su 891, tested in these two trials reveals that the sbFOS supplement had no effect on growth performance, gut pH, or small intestine morphology of the weaned piglets. The SbFOS only showed a tendency towards increased bifidobacteria ($p = 0.09$) in the caecum only when coupled in synbiosis with strain Ra 18. The daily live weight gain (DLWG) of the piglets tended to improve linearly ($p = 0.07$) with increasing doses of *B. animalis* subsp. *lactis* strain Ra 18. High doses of *B. animalis* subsp. *lactis* had also a growth-promoting effect that was not seen with *B. choerinum* supplementation, though strains belonging to this species have been suggested to be particularly well adapted to the gut of preweaning piglets [44].

Therefore the data show that the two strains of bifidobacteria (R18 and Su 891), which have similar ability in improving the count of hindgut bifidobacteria, did not influence piglet growth performance in the same way [30].

The aim of the nitrate series was to evaluate the host response to the treatments with increasing doses of dietary nitrates (0 – control; 15 mg KNO_3 per kg – Ni15; 150 mg KNO_3 per kg – Ni150), and their efficacy in controlling salmonella infections in newly weaned piglets. Indeed, recently there has been a growing interest in the role of nitrates and nitrites in protecting mammals from oral and gastrointestinal diseases and different studies suggest that both nitrate and nitrite play important roles in the prevention of infectious diseases in the gastrointestinal tract [45]. The human enterosalivary recirculation is an intriguing phenomenon since mammals express no enzyme capable of nitrate utilization [46]. Different *in vitro* studies have shown that both nitrite and low pH contributed to the killing of pathogens such as *E. coli*. However, not only enterobacteria but also LAB and bifidobacteria are sensitive to acidified nitrite. Sensitivity to acidified nitrite varies

with different species of lactobacilli, and even within the same species [47].

Piglets require time to adapt to a diet rich in nitrates, but in general, health was good in most of the pigs. Nitrate addition did not affect daily feed intake and growth, before and after challenge [48] in treated animals.

There were few signs of diarrhoea, which confirms that in swine the infection of *Salmonella typhimurium* is often asymptomatic. However, 2 salmonella control pigs, 1 salmonella Ni15 pig, and 1 control Ni 150 pig died. At sacrifice no difference between diets was seen for pH values in the stomach and for the weight of the full and empty stomach. This indicates that nitrate did not affect the gastric secretory capacity and that, presumably, the gastric inflammatory status did not vary.

With a dietary nitrate addition equal to the maximum dose allowed by the EU for feedstuffs, the appearance of the gastric mucosa is nearly always indicative of a healthy condition for pigs of this age and the values noted were lower than those observed in pigs at the slaughterhouse [27]. The dietary addition of nitrate and challenge with salmonella did not affect the degree of ulceration.

The diet did not affect the content of culturable LAB, clostridia and yeasts in both segments, nor did it affect *E. coli* contents in the jejunum. No effect of the challenge was observed on any of the microbial parameters. The time since challenge had an important effect on the counts of LAB: it decreased with age in the stomach ($p < 0.001$) and in the jejunum ($p < 0.05$). Also clostridia in the stomach were reduced. With respect to the *E. coli* content in the stomach, only 35–40% of the subjects of each diet had a bacteria concentration sufficient to be recoverable through culturing and the values in the positive samples were very low (3.12 log cfu on average; data not presented in Table 1). In the older pigs, a trend of decreasing bifidobacterial counts in the stomach ($p = 0.07$) was noticed. The supplementation of the diet with nitrates did not affect the bacterial population density in both segments. On average, supplementation of the diet with nitrates did not affect bifidobacteria concentrations in the two digestive tracts. However, the number of bifidobacteria in the jejunum tended to decrease with increasing nitrate supplementation. In the jejunum contents of challenged piglets the concentration of *E. coli* was significantly ($p < 0.05$) reduced and the clostridia levels tended to decrease. This might have been due to a niche exclusion mechanism and/or to a competition for active sites. All challenged pigs and some unchallenged pigs were positive for salmonella presence in the lymph nodes and in the jejunum contents. The results indicate that a moderate addition of nitrates does not negatively affect growth performance, and also that the transfer of nitrite in the saliva was not sufficient to improve the health of weaning pigs stimulated with *Salmonella typhimurium*. However, in the tissue samples we only investigated the presence of salmonella, whereas it may have been more interesting to determine the population level of the pathogen. Indeed, more than 10^3 salmonella bacteria are required to overcome the immune system and cause acute salmonella infection in the host [49]. It also has to be underlined that we used pathogen challenge doses higher than those normally infecting the pigs.

The nitrite formation in the saliva was also measured (data published in a different paper [48]). The production of saliva per unit time (measured before the challenge only) was not affected by the diet. Nitrate in blood and in saliva was not affected by nitrate supplementation. Nitrite in saliva increased with nitrate supplementation, but only at the first sampling (+36%, Ni15, and +258%, Ni150, $p < 0.05$) [48].

The level of salivary nitrite is dependent on the nitrate reductase activity in the oral cavity. Yet, it may also be markedly affected by the enterosalivary circulation including salivary flow rate, redox potential in the mouth and stomach, pH values in the stomach, absorption of nitrate in the small intestine, and active transportation from blood to the salivary gland [47]. Studies with rats, pigs and humans have also shown that the tongue of newborns are virtually free from nitrite-producing bacteria, which results in the absence of oral nitrite and stomach nitric oxide (NO) production. It was also hypothesized that this contributes to the poor resistance of neonatal animals to gastroenteritis. Animals are thought to acquire nitrite-producing bacteria both from other animals and/or the feeding environment. However, if pigs were fed with sterile food and if the contact of newborn pigs was restricted to the mother, nitrite population bacteria developed more slowly and remained lower than in pigs kept under conventional intensive rearing conditions. Furthermore, pigs raised in minimum-infection units and under laboratory conditions might acquire a lower density of nitrite producing bacterial species than those raised under conventional rearing conditions or in the wild [25].

At the end of the first phase of this study a new synbiotic treatment was identified, containing the putative probiotic strain Ra 18, belonging to the species *B. animalis* subsp. *lactis* (microencapsulated with a protocol developed by Probiotical srl) at a daily dose per animal of 390×10^9 cfu, and with NDO extracted from sugar beet, Actilight®, at a daily dose of 4% of the diet, nitrate (as potassium salt) at a daily dose of 150 mg per kg feed. In the final phase of this study an organic farm was selected where management practices were applied that included late weaning; an all-in farming system; appropriate environmental conditions with regular cleaning and disinfection of equipment and barns; hygienic conduct of employees; good air quality; appropriate pen design and space allocation within the pen; the use of pathogen-free organic feeds, and clean water supply. In this organic herd the piglets were usually weaned at the age of 40 days. They were allowed in a larger housing area without outdoor access to avoid infestation with parasites.

In this study the piglets were separated in pens with 5 animals each, where they remained until the age of 70 days. After that the animals were moved to the fattening unit where they were kept until slaughter.

The weaning pens were large enough to ensure animal movement, and the presence of a rest area as well as enrichment materials like small chains (enrichment materials are playthings for avoiding stress signs such as tail biting in penned piglets).

Two trials were designed to evaluate tolerability and suitability in use of the new synbiotic formula. Each trial used 20 piglets assigned to two groups: synbiotic and non-synbiotic (control). The tolerability and the efficacy of this formula were measured by an overall assessment of symptoms after 2 weeks of treatment: presence and/or frequency of post weaning diarrhoea, effects on growth performance and feed efficiency, and changes in faecal microbiota.

The results showed a good tolerability of the new formula together with a significant effect on piglets' growth performance. Two weeks after intake the body weight of treated piglets had increased at T_1 and T_2 with an average of 5.62 kg and 8.68 kg, respectively, in the first trial and 3.27 kg and 7.96 kg, respectively, in the second (Tables 2 and 4). Differences between the two groups (synbiotic vs control) were statistically significant ($p = 0.03$ at T_1; $p = 0.01$ at T_2). Good feed efficiency was also observed at the end of the wash-out period in both trials.

Furthermore, synbiotic intake also led to significant changes in faecal microbiota composition of treated piglets (Tables 3 and 5). Bifidobacteria and all other bacterial groups showed the same trend: a significant increase during the treatment and a slight decrease at the end of the wash-out period. After 2 weeks of feeding, all treated piglets showed higher numbers of beneficial bacteria like bifidobacteria and lactobacilli and lower numbers of potential pathogenic bacteria like *E. coli* in their faeces compared with the baseline values.

The results of many studies reported in literature [50–53] point to a synergistc effect of probiotic and prebiotic

Table 2
First trial. Growth performance of the groups of piglets at different growth stages.

Growth stage	Control		Synbiotic	
	Group 1	Group 1B	Group 2A	Group 2B
	Average body weight (kg) ± SD			
T_0	12.1 ± 1.57	10.6 ± 0.56	12.2 ± 0.22	10.7 ± 0.83
T_1	17.2 ± 2.15	15.6 ± 1.80	19.2 ± 1.20	16.1 ± 0.83
T_2	25.5 ± 1.84	23.5 ± 2.11	29.7 ± 2.15	24.1 ± 0.70
	Average daily weight gain (kg) ± SD			
T_1	0.34 ± 0.07	0.33 ± 0.15	0.47 ± 0.07	0.36 ± 0.05
T_2	0.59 ± 0.03	0.56 ± 0.07	0.75 ± 0.08	0.57 ± 0.06
	Average body weight (kg)			
$T_0 - T_1$	5.06	4.98	7.05	5.44
$T_1 - T_2$	8.30	7.84	10.51	8.02
	Feed conversion ratio (kg kg^{-1})			
$T_0 - T_1$	1.87	1.73	1.75	1.75
$T_1 - T_2$	1.82	1.89	1.86	1.80

Table 3
First trial. Quantitative real-time PCR analysis of microbial groups in piglets faecal samples (log_{10} gene copies numbers per g wet weight ± SD).

Growth stage	Control	Synbiotic
	E. coli	
T_0	6.22 ± 0.53	6.27 ± 0.53
T_1	10.74 ± 0.52	8.39 ± 0.36
T_2	10.20 ± 0.49	9.16 ± 0.69
	Lactobacilli	
T_0	11.03 ± 0.80	10.98 ± 0.37
T_1	10.70 ± 0.58	12.83 ± 0.52
T_2	10.60 ± 0.54	11.42 ± 0.52
	Bifidobacterium spp.	
T_0	10.52 ± 0.15	10.44 ± 0.19
T_1	10.88 ± 0.18	13.63 ± 0.26
T_2	9.72 ± 0.16	12.29 ± 0.19
	Bifidobacterium animalis subsp. lactis	
T_0	0	0
T_1	0	14.09 ± 0.29
T_2	0	11.24 ± 0.21

combinations on faecal microbiota of experimental animals. This effect was demonstrated by increased total anaerobes, aerobes, lactobacilli and bifidobacteria counts as well as by lower clostridia, *Enterobacteriaceae* and *E. coli* counts. The combination of probiotic and non-digestible carbohydrates may be a way of stabilization and/or improvement of the probiotic effect.

The ability of Ra 18 to pass down, survive and persist in the piglets' GIT was also investigated by analysing the recovery of Ra 18 from faecal samples following oral intake and wash-out phase. As expected, prior administered Ra 18 *B. animalis* subsp. *lactis* was not detected in the faeces of treated and untreated piglets, being a species normally found in the microbiota of rabbits. However, 15 days after administration, the probiotic strain appeared in the faeces of all treated piglets, reaching a high mean level of gene copy numbers per gram faeces. At the end of the wash-out period, at T_2, the strain was still detectable in the faeces of all treated piglets (Tables 3 and 5). These data are in accordance with the results of earlier intervention trials showing that probiotic strains usually disappear within some weeks after their intake is discontinued [54–56].

Table 4
Second trial. Growth performance in the groups of piglets at different growth stages.

Growth stage	Control		Synbiotic	
	Group 1A	Group 1B	Group 2A	Group 2B
	Average body weight (kg) ± SD			
T_0	13.2 ± 1.20	11.3 ± 0.95	13.1 ± 0.69	11.5 ± 0.79
T_1	16.6 ± 1.27	14.2 ± 1.57	16.5 ± 1.51	14.9 ± 1.45
T_2	24.7 ± 1.92	22.0 ± 2.84	24.3 ± 2.20	23.0 ± 2.95
	Average daily weight gain (kg) ± SD			
T_1	0.23 ± 0.07	0.19 ± 0.11	0.22 ± 0.13	0.22 ± 0.10
T_2	0.58 ± 0.05	0.56 ± 0.10	0.56 ± 0.14	0.58 ± 0.11
	Average body weight (kg)			
$T_0 - T_1$	3.42	2.86	3.33	3.36
$T_1 - T_2$	8.11	7.08	7.83	8.10
	Feed conversion ratio (kg kg^{-1})			
$T_0 - T_1$	2.26	2.18	2.26	2.14
$T_1 - T_2$	1.76	1.69	1.75	1.65

Table 5
Second trial. Quantitative real-time PCR analysis of microbial groups in piglets faecal samples (log_{10} gene copies numbers per g wet weight ± SD).

Growth stage	Control	Synbiotic
	E. coli	
T_0	7.09 ± 0.54	7.42 ± 0.50
T_1	10.43 ± 0.34	8.46 ± 0.58
T_2	10.03 ± 0.72	8.95 ± 0.59
	Lactobacilli	
T_0	10.49 ± 0.74	10.40 ± 0.80
T_1	10.16 ± 0.46	12.90 ± 0.40
T_2	9.83 ± 0.39	11.14 ± 0.47
	Bifidobacterium spp.	
T_0	10.47 ± 0.36	10.59 ± 0.27
T_1	10.16 ± 0.29	13.64 ± 0.43
T_2	9.22 ± 0.31	10.54 ± 0.18
	Bifidobacterium animalis subsp. lactis	
T_0	0	0
T_1	0	14.20 ± 0.58
T_2	0	12.41 ± 0.83

The qPCR analysis confirmed that after ingestion, the putative probiotic strain Ra 18 was able to survive the conditions of the piglets' GIT and persist in the colonic ecosystem in high numbers until 2 weeks after cessation of intake. Detection of the strain in all follow-up samples suggests prolonged colonization in the intestine of the treated piglets. Furthermore, microencapsulation of the probiotic strain and the addition of prebiotic SbFOS have been confirmed to satisfactorily increase the survival of the micro-organism during its transit in the gastrointestinal tract of the piglets. That the microencapsulation technique is a valid tool to significantly improve gastroresistance of strains and to enhance their probiotic activity has been confirmed by earlier research [57]. To the best of our knowledge there are no publications of studies where both probiotic bifidobacteria and prebiotic FOS have been studied in synbiotic trials with weaning piglets.

The results from this study also demonstrate that the inclusion of our synbiotic formula into the weaning diet affected piglets' growth rate and feed efficiency. In all experiments we also observed differences in faecal microbiota composition between treated and untreated piglets (Tables 3 and 5) that resulted from dietary supplementation with this new formula. Our formula led to a beneficial intestinal microbiota, dominated by health-promoting bacteria (bifidobacteria and lactobacilli) and to a reduction of pathogenic bacteria like *E. coli*. These results are very promising since studies on faecal samples may underestimate the colonization of colonic mucosa by probiotic strains.

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