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Effects of inulin supplementation to piglets in the suckling period on growth performance, postileal microbial and immunological traits in the suckling period and three weeks after weaning

Bing Li^a, Martine Schroyen^a, Julie Leblois ^{a,b}, José Wavreille^c, Hélène Soyeurt^d, Jérôme Bindelle ^a and Nadia Everaert^a

^aPrecision livestock and nutrition unit, Gembloux Agro-Bio Tech, Teaching and Research Centre, University of Liège, Gembloux, Belgium; ^bResearch Foundation for Industry and Agriculture, Fonds De La Recherche Scientifique - FNRS, Brussels, Belgium; ^cDepartment of Production and Sectors, Walloon Agricultural Research Centre, Gembloux, Belgium; ^dLaboratory of statistics, informatics and modelling applied to bioengineering, agrobiotech department, Gembloux Agro-Bio Tech, Teaching and Research Centre, University of Liège, Gembloux, Belgium

ABSTRACT

The aim of this study was to investigate the effect of inulin (IN) supplementation to suckling piglets at and 3 weeks post-weaning. A total of 72 newborn piglets were used. Twenty-four piglets per group received different amounts of IN during the suckling period: (a) CON: no IN; (b) IN-0.5: 0.5 g IN/d on the 1st week, 1 g IN/d on the 2nd week, 1.5 g IN/d on the 3rd week and 2 g IN/d on the 4th week, or (c) IN-0.75: 0.75 g IN/d on the 1st week, 1.5 g IN/d on the 2nd week, 2.25 g IN/d on the 3rd week and 3 g IN/d on the 4th week. Starting at 28 d of age, piglets were weaned and received a post-weaning diet without inulin during the following 3 weeks. At both 28 d and 49 d of age, piglets were euthanised for sampling. Piglets of group IN-0.5 had the highest body weight starting from the 3rd week ($p < 0.05$), concomitant with the highest villus height and the ratio of villus height/crypt depth in the jejunum and ileum on both sampling days ($p < 0.05$). At 28 d of age, an increased concentration of propionate, *iso*-butyrate or total short chain fatty acids was observed between treatment IN-0.5 and the other groups in the caecum or colon ($p < 0.05$). Moreover, the relative abundance of *Escherichia coli* ($p = 0.05$) and *Enterobacteriaceae* ($p = 0.01$) in colonic digesta were reduced in IN-0.5-treated piglets, and in both IN-supplemented groups, colonic interleukin-8, tumor necrosis factor- α and toll-like receptor-4 mRNA abundance were decreased compared to the CON group ($p < 0.05$). However, at 49 d of age, most of these differences disappeared. In conclusion, treatment IN-0.5 improved during the suckling period of piglets development of intestine, but these beneficial effects were not lasting after weaning, when IN supplementation was terminated. Treatment IN-0.75, however, did not display a prebiotic effect.

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1. Introduction

Weaning is often a stressful period for piglets, resulting in post-weaning diarrhoea, followed by impaired growth performance and a high mortality rate. An approach to overcome these issues is through the dietary administration of prebiotics to modify gut microbiota (Angelakis 2017). Prebiotics are non-digestible food ingredients that selectively stimulate the growth and activity of beneficial bacteria in the large intestine, thereby improving host's health (Gibson et al. 2004). Inulin is an example of a prebiotic that is composed of a mixture of oligomers and polymers in which the degree of polymerisation (DP) varies from 2 to approximately 60 units with an average DP of 12 (Gibson et al. 2004). Inulin is primarily extracted from chicory root and Jerusalem artichoke, which has been found to enhance the abundance of *Bifidobacterium* and *Lactobacillus* in pigs (Tzortzis et al. 2005; Tako et al. 2008; Patterson et al. 2010). In addition, when inulin is fermented in the large intestine, the production of short-chain fatty acids (SCFA) is increased. Increased SCFA leads to a lower pH in the large intestine, reducing the growth of some pathogenic bacteria (Wang and Gibson 1993). In addition, SCFA has a trophic effect on the intestinal epithelium, which has been shown to ameliorate the development of intestinal mucosal architecture (Hosseini et al. 2011; Wang et al. 2012). The intestine contains gut-associated lymphoid tissues (Pabst et al. 2008); hence, the altered SCFA profile (e.g. butyrate) and microbiota (e.g. *Bifidobacterium* or *Lactobacillus*) might also influence the immune response by modulating the mRNA abundance of cytokines as well as other immune mediators (O'Mahony et al. 2005; Place et al. 2005; Lomax and Calder 2008; Uematsu et al. 2008; Shukla et al. 2016).

Many studies reported various beneficial effects of inulin in the diet of weaned pigs (O'Shea et al. 2012; Grela et al. 2013); however, little is known about the function of inulin in suckling piglets. In pigs, the maturation and colonisation of the intestine starts from birth onwards. Intestinal development is unfortunately disturbed during the weaning period (Everaert et al. 2017). Interestingly, a dose-response effect of prebiotics has been found in human and chickens, where an excessive amount resulted in a poor growth performance (Roberfroid et al. 1998; Rao 1999; Tuohy et al. 2003; Xu et al. 2003). Unfortunately, these time frame and dose-dependent effects remain uncharacterised in pigs. Hence, identifying the most beneficial dose and time window of inulin administration is crucial to optimise intestinal health management and feeding strategies. It was hypothesised that the administration of inulin during the suckling period might programme the development of the intestine in suckling piglets in a dose-dependent way. In this study, different amounts of inulin were force-fed to piglets only during the suckling period to investigate the effect of differing amounts of inulin on body weight (BW), gut morphology, SCFA profile of the large intestine, selected colonic bacteria and mRNA abundance of inflammatory markers in suckling piglets. Growth parameters of piglets were followed for 3 weeks after weaning and additional intestinal health parameters were measured at this point to evaluate whether the early programming of inulin resulted in lasting effect beyond the treatment period.

2. Materials and methods

The animal experiment was approved by the University of Liège ethical committee (ethical protocol 1640, Belgium), and all procedures were in compliance with Belgian and European

regulations. The experiment with suckling piglets was performed at the Walloon Agricultural Research Center (Gembloux, Belgium), and the weaned piglets were transferred to facilities at Gembloux Agro-Bio Tech (Gembloux, Belgium) to continue the experiment.

2.1. Animals, diets and experimental design

Seventy-two newborn piglets, 6 piglets (3 males and 3 females) per litter from 12 litters (Piétrain × Landrace, birth weight: 1.29 ± 0.02 kg) were used in this study. Piglets were selected based on their initial BW. Average litter size was 12 piglets with a birth weight of 1.26 ± 0.02 kg. Piglets not meeting the BW criterion stayed with the sow, but were not used in this study. Male piglets were castrated. Treatments were assigned with regard to the sow's consanguinity (all sows came from the same sire line), parity (four sows per treatment, two sows of parity 1 and two sows of parity 2 per treatment) and BW (BW of sows were balanced). From the selected piglets, 6 per litter ($n = 24$ per group) were assigned to 1 of 3 inulin treatment groups during the suckling period. The three treatments included (a) CON: no inulin, (b) IN-0.5: inulin supplementation at 0.5 g/d on the 1st week, 1 g/d on the 2nd week, 1.5 g/d on the 3rd week and 2 g/d on the 4th week, or (c) IN-0.75: inulin supplementation at 0.75 g/d on the 1st week, 1.5 g/d on the 2nd week, 2.25 g/d on the 3rd week and 3 g/d on the 4th week. As it was assumed that the inulin treatment would affect the microbiota of the piglets, and in order to avoid cross-contamination of microbiota between treatments, only one treatment per litter was applied. Inulin was provided as Fibruline Instant by COSUCRA (Warcoing, Belgium). This inulin is composed of linear chains of fructose units with one terminal glucose unit with a degree of DP ranging from 2 to 60 units and an average DP of about 10. Three deionised aqueous solutions of inulin (0% inulin, 20% inulin or 30% inulin) were prepared and orally administered to the piglets by a syringe via mouth twice per day at 09:00 h and 15:00 h. A total of 2.5 ml/d was given during the 1st week, 5 ml/d during the 2nd week, 7.5 ml/d during the 3rd week and 10 ml/d during the 4th week. Thus, piglets from the CON group received 0% inulin solution, the IN-0.5 group received the 20% inulin solution, while the IN-0.75 group received the 30% inulin solution. At weaning (28 d of age), 24 piglets ($n = 8$ per group) were sacrificed and the remaining piglets were weaned in pairs and allocated into 24 pens (1 male and 1 female sibling per pen). Forced inulin feeding ceased at weaning and a weaning diet devoid of inulin and meeting NRC (2012) recommendations for growth was supplied to all the piglets (Table 1). Diet and water were provided *ad libitum* throughout the whole experimental period. No other supplements such as prebiotics or probiotics, organic acids, or essential oils were added to the weaning diet; no antibiotics were given to the sows or piglets during the whole experiment. Rearing environments were kept constant with ambient temperature maintained at 28°C before weaning and 22°C after weaning.

2.2. Sample collection

The BW of piglets was recorded weekly starting at birth and feed intake was recorded weekly from weaning onwards. Two piglets per litter at 28 d of age ($n = 8$ per group) and 1 piglet per pen at 49 d of age ($n = 8$ per group) were anaesthetised by isoflurane, and then euthanised by exsanguination. The gastrointestinal tract was removed within 5 min after exsanguination. Five-centimeter jejunal and ileal segments were taken and stored in 4% paraformaldehyde for

Table 1. Composition and nutrient content of weaning diet.

Ingredient [g/kg]	
Barley	300.0
Wheat	134.5
Maize	50.0
Oat flaked	50.0
Soybean	150.0
Soybean meal	140.2
Potato protein	20.0
Bread meal	50.0
Soybean oil	6.7
Bonilac [#]	46.7
Limestone	10.6
Monocalciumphosphate	10.0
NaCl	5.4
DL-Methionine	1.6
L-Lysine HCl	4.7
L-Threonine	1.1
Sweet whey powder	2.0
Vitamin D ₃	0.8
Lys/Trp mix*	0.2
Vitamin E	3.2
Premix ^{&}	4.0
Phytase	3.3
Titanium dioxide	5.0
Nutrient composition analysed [g/kg]	
Crude protein	226.0
Ether extract	61.7
Neutral detergent fibre	122.0
Acid detergent fibre	41.3
Gross energy [MJ/kg]	19.3

[#]Bonilac: 90% sweet whey powder with 10% coconut oil; *combination of 18.5% lysine and 5% tryptophan with grains (wheat/maize) and wheat bran carrier; [&]provided per kg of feed: 3.8 mg Cu, 20.0 mg Fe, 15.5mg Zn, 6.0 mg Mn, 0.5 mg Se, 0.1 mg I, 1625 IU vitamin A, 1.0 mg vitamin B₂, 7.5 mg niacin, 2.3 mg pantothenic acid, 400 IU vitamin D₃, 12.5 mg vitamin E, 0.2 mg vitamin K₃.

gut morphology analyses. Digesta from the caecum and colon were collected and snap frozen in liquid nitrogen and then stored at -80°C until further SCFA and microbiota analysis. The colon tissue was collected and rinsed with ice-cold sterile Dulbecco's Modified Eagle Medium (Sigma, St Louis, Missouri, USA) and dissected using a 6-mm-diameter biopsy punch (Vtrade, Belgium). The biopsies were placed in 6-well round-bottom cell culture plate containing William's E medium (Sigma) supplemented with fetal bovine serum (Sigma), glucose (Sigma), alanine-glutamine (Sigma), gentamycin (Sigma), penicillin and streptomycin (Sigma). All samples were incubated at 37°C and 5% CO₂ for 2 h (Smith et al. 2011; Bahar et al. 2012; Leonard et al. 2012). Tissue was collected and snap frozen in liquid nitrogen until stored at -80°C prior to RNA extraction.

2.3. Gut morphology

Samples were fixed in 4% paraformaldehyde for 48 h and stored at 70% ethanol before embedded in paraffin. Using a microtome, paraffin-embedded samples were sliced into 5- μm sections and then stained with hematoxylin and eosin. Villus height and crypt depth of 15 well-oriented villi and associated crypts per animal were measured at 4 \times magnification using

an OLYMPUS BX51 microscope and soft imaging system (Olympus Corporation, Hamburg, Germany).

2.4. SCFA analysis

A mixture of caecal or colonic digesta and water 1:4 (wt/wt) was homogenised for 30 s and centrifuged at 13,000 g for 15 min. After centrifugation, 1 ml of supernatant was collected and the pH was adjusted to pH 2–3 using 1N H₂SO₄. The supernatant was filtered through a sterile acetate filter. Acetate, propionate, butyrate, valerate, *iso*-butyrate and *iso*-valerate concentrations were analysed by high-performance liquid chromatography, using a Waters system fitted with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) combined with a UV detector (210 nm), using sulphuric acid (5 mM) as mobile phase and a flow rate of 0.6 ml/min. In addition, the dry matter of caecal and colonic digesta were determined after drying at 105°C for 24 h (method 967.03, AOAC 1990)

2.5. Semiquantitative determination of colonic selected bacteria groups

Bacterial content was determined as described by Barszcz et al. (2016). Total DNA of colonic digesta was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations but adding a bead-beating step. Samples were quantified and tested for purity using a NanoDrop 2000 (Thermo Scientific, USA) and integrity was tested on a 1% agarose gel. The polymerase chain reaction (PCR) mixture contained 2 µl of isolated DNA (100 ng), 10 µl of SYBR Premix EX Taq™ II, 0.4 µl of ROX Reference Dye II (50 ×) (SYBR Premix EX Taq™ II (Tli RNaseH plus), Takara, Japan), 1 µl of each primer and 5.6 µl of nuclease-free water. The PCR protocol was performed using an ABI StepOnePlus (Applied Bio systems, USA) system with the following programme: 30 s heating at 95°C, then followed by 40 cycles of denaturation (5 s at 95°C), annealing (30 s at 60°C) and extension (45 s at 72°C). A melting curve analysis was performed to check the specificity of the primers and a standard curve was determined to ensure amplification efficiencies between 90% and 110%. Primer sequences and efficiencies are shown in Table 2. The $2^{-\Delta\Delta C_t}$ method was performed to analyse the selected bacteria, using the control group as 1.0 level after normalisation, where a pooled sample created by mixing all the samples of DNA was used as the calibrator sample (Bustin et al. 2009).

2.6. Colonic mRNA abundance

Total RNA of colonic tissue was extracted using InnuPREP RNA Mini Kit (Analytik JenaAG, Germany) and samples were quantified and tested for purity using a NanoDrop 2000 (Thermo Scientific, USA) (A260/A280 ≈ 2.0). The RNA integrity was tested on a 1% agarose gel (28S:18S area). Unfortunately, due to an operational error, two samples for the colonic mRNA abundance in groups IN-0.5 and IN-0.75 were lost respectively, which made eight samples in the CON group and six samples in the IN-0.5 and IN-0.75 groups. For each sample, 0.95 µg RNA was synthesised as single-stranded cDNA with PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time) kit (Takara, Japan) following the manufacturer's recommendations. The PCR mixture

Table 2. Primer information for quantitative RT-PCR assays.

Bacteria/Gene	5'-primer-3'*	Accession number/ Reference	Efficiency [%]
<i>β-actin</i>	F:GGACTTCGAGCAGGAGATGG R:GCACCGTGTGGCGTAGAGG	U07786	96
<i>PPIA</i> [#]	F:TAACCCACCGTCTTCTT R:TGCCATCCAACCACTCAG	AF14571	101
<i>Interleukin-6</i>	F:CCTCTCCGGACAAAAGTAA R:TCTGCCAGTACCTCCTTGCT	NM_001252429.1	99
<i>Interleukin-8</i>	F:GCTCTGTGAGGCTGCAGTTC R:AAGGTGGGAATGCGTATTATGC	NM_213867	104
<i>Interleukin-10</i>	F:CTGCCTCCCACTTTCTCTTG R:TCAAAGGGGCTCCCTAGTTT	NM_214041.1	100
<i>Toll-like receptor-4</i>	F:CCTGACAACATCCCCACATCA R:TGCTCTGGATAGTGGTAAAAGC	UM_001113039	95
<i>TNF-α</i> [†]	F:ACTGCACTTCGAGGTTATCGG R:GGCGACGGCTTATCTGA	NM_214022	99
Universal	F:CGTGCCAGCCGCGGTAATACG R:GGGTTCGCTCGTTGCGGGACTTAACCCAACAT	Amit-Romach et al. (2004)	97
<i>Lactobacillus</i>	F:CATCCAGTGCAAACCTAAGAG R:GATCCGCTTGCCCTCGCA	Amit-Romach et al. (2004)	101
<i>Bifidobacterium</i>	F:CGGGTGCTCCCACTTTCATG R:GATTCTGGCTCAGGATGAACG	Barroso et al. (2015)	105
<i>Clostridium</i>	F:AAAGGAAGATTAATACCGCATAA R:ATCTTGCGACCGTACTCCCC	Amit-Romach et al. (2004)	92
<i>Clostridium clusters IV</i>	F:GCACAAGCAGTGGAGT R:CTTCCTCCGTTTTGTCAA	Pieper et al. (2012)	96
<i>Clostridium clusters XIVa</i>	F:AAATGACGGTACCTGACTAA R:CTTTGAGTTTCATTCTTGCGAA	Pieper et al. (2012)	98
<i>Bacteroidetes</i>	F:CRAACAGGATTAGATACCCT R:GGTAAGGTTCTCGCGTAT	De Gregoris et al. (2011)	98
<i>Firmicutes</i>	F:TGAAACTYAA AGGAATTGACG R:ACCATGCACC ACCTGTC	De Gregoris et al. (2011)	98
<i>Escherichia coli</i>	F:GGGAGTAAAGTTAATACCTTTGCTC R:TTCCGAAGGCACATTCT	Amit-Romach et al. (2008)	91
<i>Enterobacteriaceae</i>	F:ATGGCTGTGTCAGCTCGT R:CCTACTTCTTTGCAACCCACTC	Tegtmeier et al. (2016)	101

[#]PPIA, peptidylprolyl isomerase A; [†]TNF-α, tumor necrosis factor-α; *F: forward; R: reverse.

contained 2 µl of cDNA, 10 µl of SYBR Premix EX Taq™ II, 0.4 µl of ROX Reference Dye II (50×) (SYBR Premix EX Taq™ II (Tli RNaseH plus), Takara, Japan), 1 µl of each primer and 5.6 µl of nuclease-free water. The PCR protocol was performed using an ABI StepOnePlus (Applied Bio systems, USA) system with the following programme: 30 s heating at 95°C, then followed by 40 cycles of denaturation (5 s at 95°C), annealing (30 s at 60°C) and extension (30 s at 72°C). The mRNA abundance of interleukin (*IL*)-6, *IL*-8, *IL*-10, tumor necrosis factor-α (*TNF-α*) and toll-like receptor-4 (*TLR4*) were determined. Three reference genes *β-actin* (*ACTB*), *β2*-microglobulin (*B2M*) and peptidylprolyl isomerase A (*PPIA*) were tested and their stability was determined using the GeNorm software. The two most stable reference genes were *ACTB* (suckling period: *M*-value = 0.058 and after weaning: *M*-value = 0.600) and *PPIA* (suckling period: *M*-value = 0.057 and after weaning: *M*-value = 0.637); thus, they were used as the reference genes in this study. A melting curve analysis was performed to check the specificity of the primers and a standard curve was determined to ensure amplification efficiencies between 90% and 110%. Primer sequences and efficiencies are shown in Table 2. The $2^{-\Delta\Delta Ct}$ method was performed to analyse the selected genes, using the control group as 1.0 level after normalisation, where a pooled sample created

by mixing all the samples of cDNA served as the calibrator sample (Bustin et al. 2009; Everaert et al. 2013).

2.7. Statistical analysis

Piglet BW was analysed with an analysis of variance method using SAS software (SAS 9.2, Inc, USA) with mixed procedure. A repeated measures model was used to study the effect of treatment and age for BW. Treatment and age were considered as fixed effects. Litter was considered as a random effect. The piglet was considered as the repeated effect. Two piglets per group died, and another two piglets of the IN-0.5 group had a low BW (6 kg at 49 d of age). Hence, 64 piglets ($n = 22$ for CON; $n = 20$ for group IN-0.5 and $n = 22$ for group IN-0.75) remained in the experiment during the suckling period to use for BW analysis. As 24 piglets were sacrificed at 28 d of age, 40 piglets remained on the experiment after weaning ($n = 14$ for CON; $n = 12$ for IN-0.5 and $n = 14$ for IN-0.75). The data of gut morphology, SCFA, colonic microbiota and mRNA abundance were subjected to an analysis of variance method using SPSS software (IBM SPSS Statistics 21) with general linear model procedure, comparing the CON group to the other two inulin-treated groups. Significant differences among group means were determined by Duncan's multiple range test. Outliers were excluded from the analysis when their value differed more than $\text{mean} \pm 3$ standard deviation (Howell 1998). The results were presented as means \pm standard error of mean (SEM) and were considered statistically significant at $p \leq 0.05$.

3. Results

3.1. Growth performance

There was no difference in the feed intake of sows between treatments during the suckling period (data not shown). The BW of the various groups was characterised by a significant interaction between treatment and age ($p < 0.01$). On the 3rd week, BW of piglets in the IN-0.5 group was higher than that in the IN-0.75 group, with the CON group having an intermediate value ($p = 0.04$) (Figure 1). Starting from the 4th week until the end of the experiment, a higher BW was observed in piglets of group IN-0.5 than in piglets of groups CON and IN-0.75 ($p < 0.05$). Piglets in the IN-0.75 group never differed from the CON group. No effect of treatment was observed on feed intake of piglets during the post-weaning period (data not shown).

3.2. Gut morphology

At 28 d of age, the IN-0.5 group showed the highest jejunal and ileal villus height compared to the CON and IN-0.75 groups ($p < 0.01$), with the IN-0.75 group having a higher ileal villus height compared to the CON group ($p < 0.01$) (Table 3). The results of crypt depth in both jejunum and ileum were not affected by dietary treatment. Compared to the CON group, only the IN-0.5 group increased the ratio of villus height/crypt depth (V/C) in the jejunum ($p < 0.01$), whereas in the ileum, a higher ratio of V/C occurred in both inulin-groups ($p < 0.01$).

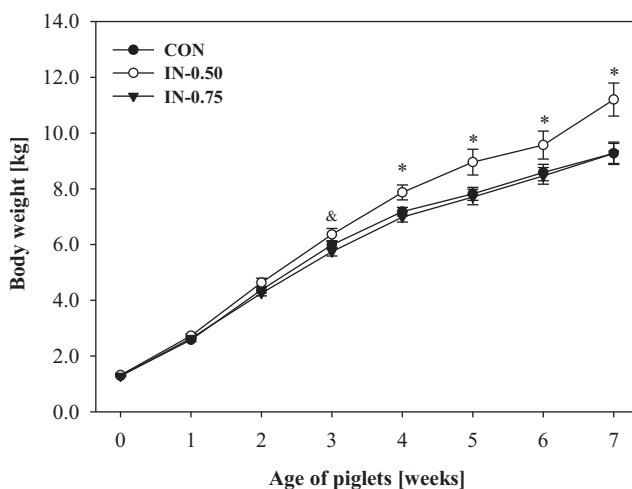


Figure 1. Effect of inulin on the body weight of pigs from 0 to 7 weeks of age.

Values are means \pm SEM, $n = 20\text{--}22$ during the suckling period and $n = 12\text{--}14$ after weaning; [&]Group IN-0.5 significantly different vs. group IN-0.75 ($p \leq 0.05$); *Group IN-0.5 significantly different vs. groups CON and IN-0.75.

Table 3. Effect of inulin on the villus height, crypt depth and villus height/crypt depth ratio (V/C ratio) in the jejunum and ileum of pigs at 28 d and 49 d of age.

	28 d of age					49 d of age				
	CON	IN-0.50	IN-0.75	SEM	<i>p</i> -Value	CON	IN-0.50	IN-0.75	SEM	<i>p</i> -Value
Villus height [μm]										
Jejunum	403 ^b	527 ^a	398 ^b	13.9	< 0.01	436 ^b	480 ^a	440 ^b	6.74	< 0.01
Ileum	315 ^c	416 ^a	355 ^b	11.5	< 0.01	373 ^{ab}	403 ^a	346 ^b	7.87	< 0.01
Crypt depth [μm]										
Jejunum	263	225	231	9.10	0.19	282	282	291	3.67	0.50
Ileum	187	204	177	5.07	0.08	262 ^a	226 ^b	215 ^b	6.67	< 0.01
V/C ratio										
Jejunum	1.57 ^b	2.35 ^a	1.77 ^b	0.09	< 0.01	1.55 ^b	1.70 ^a	1.51 ^b	0.03	< 0.01
Ileum	1.70 ^b	2.06 ^a	2.03 ^a	0.06	< 0.01	1.43 ^b	1.78 ^a	1.63 ^a	0.05	< 0.01

Values are means, 6–8 pigs per group after removing outliers, and 15 units per pig; ^{a-c} means in the same row not sharing a common superscript are significantly different ($p \leq 0.05$).

At 49 d of age, the IN-0.5 group still had a higher villus height in the jejunum compared to the other two groups ($p < 0.01$). However, the ileal villus height in the IN-0.5 group was only higher when compared to the IN-0.75 group ($p < 0.01$). No difference in crypt depth was observed in the jejunum ($p = 0.50$); however, piglets from inulin-treated groups showed a lower ileal crypt compared to piglets from the CON group ($p < 0.01$). The V/C ratio followed a similar trend at 49 d of age as was observed at 28 d of age, in both jejunum ($p < 0.01$) and ileum ($p < 0.01$).

3.3. SCFA analysis

The digesta dry matter of caecum and colon was determined and there was no effect of treatment observed (Table 4). In addition, the concentrations of SCFA in the caecal and colonic digesta were not affected by inulin supplementation when adjusted for caecal and colonic digesta dry matter at either 28 or 49 d of age (data not shown).

Table 4. Effect of inulin on the profile of short chain fatty acids (SCFA) [mmol/l fresh intestinal contents] in the caecal and colonic digesta of pigs at 28 d and 49 d of age.

	28 d of age					49 d of age				
	CON	IN-0.50	IN-0.75	SEM	<i>p</i> -Value	CON	IN-0.50	IN-0.75	SEM	<i>p</i> -Value
Caecum										
Total SCFA	155.35	158.11	146.48	2.82	0.21	163.00	155.72	148.93	3.48	0.26
Acetate	79.05	80.78	78.04	2.76	0.93	73.61	72.28	66.37	3.14	0.64
Propionate	26.99 ^b	30.34 ^a	25.40 ^b	0.72	0.01	36.35	40.32	31.56	2.04	0.22
Butyrate	17.74	17.48	14.83	0.74	0.20	17.85	13.25	11.78	1.35	0.16
<i>iso</i> -butyrate	3.45 ^b	4.62 ^a	3.52 ^b	0.21	0.03	1.53	1.60	0.70	0.21	0.12
Valerate	8.73	10.43	8.97	0.43	0.21	15.98	19.05	15.46	0.98	0.29
Iso-valerate	3.65	4.56	4.45	0.22	0.21	1.16	1.74	2.37	0.07	0.41
Dry matter [%]	19.17	19.44	19.85	0.55	0.85	10.59	11.00	10.20	0.39	0.72
Colon										
Total SCFA	104.92 ^b	131.49 ^a	95.07 ^b	5.93	0.03	148.72 ^a	144.44 ^a	122.55 ^b	4.63	0.03
Acetate	57.40	59.45	46.89	3.54	0.32	78.12	73.42	69.04	2.00	0.18
Propionate	21.32 ^{ab}	26.46 ^a	17.14 ^b	1.32	0.01	31.99	31.68	26.75	1.50	0.29
Butyrate	13.54	11.33	11.37	0.67	0.30	13.44	15.48	11.29	0.99	0.23
<i>iso</i> -butyrate	2.13 ^b	2.81 ^a	2.01 ^b	0.14	0.03	1.58	1.60	1.41	0.08	0.61
Valerate	6.30	7.61	6.25	0.55	0.55	8.02	8.50	6.69	0.39	0.15
Iso-valerate	3.39	3.46	2.76	0.19	0.29	1.29	1.57	1.47	0.14	0.75
Dry matter [%]	20.30	20.62	20.93	0.54	0.90	11.34	12.19	11.16	0.39	0.54

Values are means, $n = 6-8$ per group after removing outliers; ^{a-b}means in the same row not sharing a common superscript are significantly different.

However, based on the digesta fresh matter, the SCFA profile differed at 28 d of age. The caecal digesta of piglets of group IN-0.5 had a higher concentration of propionate ($p = 0.01$) and *iso*-butyrate ($p = 0.03$) compared to the piglets receiving the CON and IN-0.75 diets. In the colonic digesta, feeding diet IN-0.5 increased the concentration of total SCFA ($p = 0.03$) and *iso*-butyrate ($p = 0.03$) compared to the other two groups. The IN-0.5 group also showed an increased concentration of propionate compared to the IN-0.75 group ($p = 0.01$), with the CON group having an intermediate value. At 49 d of age, the only observed difference was related to total SCFA in the colonic digesta, which was higher in the CON and IN-0.5 groups compared to the IN-0.75 group ($p = 0.03$).

3.4. Gut microbiota

With regard to the bacterial populations in the colonic digesta (Figure 2), the relative abundance of *Bifidobacterium*, *Lactobacillus*, *Clostridium*, *Clostridium clusters IV*, *Clostridium clusters XIVa*, *Bacteroidetes*, *Firmicutes* and the ratio of *Firmicutes/Bacteroidetes* (F/B ratio) did not differ between the dietary treatments at 28 d of age. However, in the IN-0.5 group, a reduced relative abundance of *Escherichia coli* ($p = 0.05$) and *Enterobacteriaceae* ($p = 0.01$) was observed compared to the CON and IN-0.75 groups. This difference disappeared at 49 d of age.

3.5. Colonic mRNA abundance

The administration of inulin affected the mRNA abundance of *IL8*, *TNF- α* and *TLR4* with lower values in both inulin-groups compared to the CON group ($p < 0.05$) at 28 d

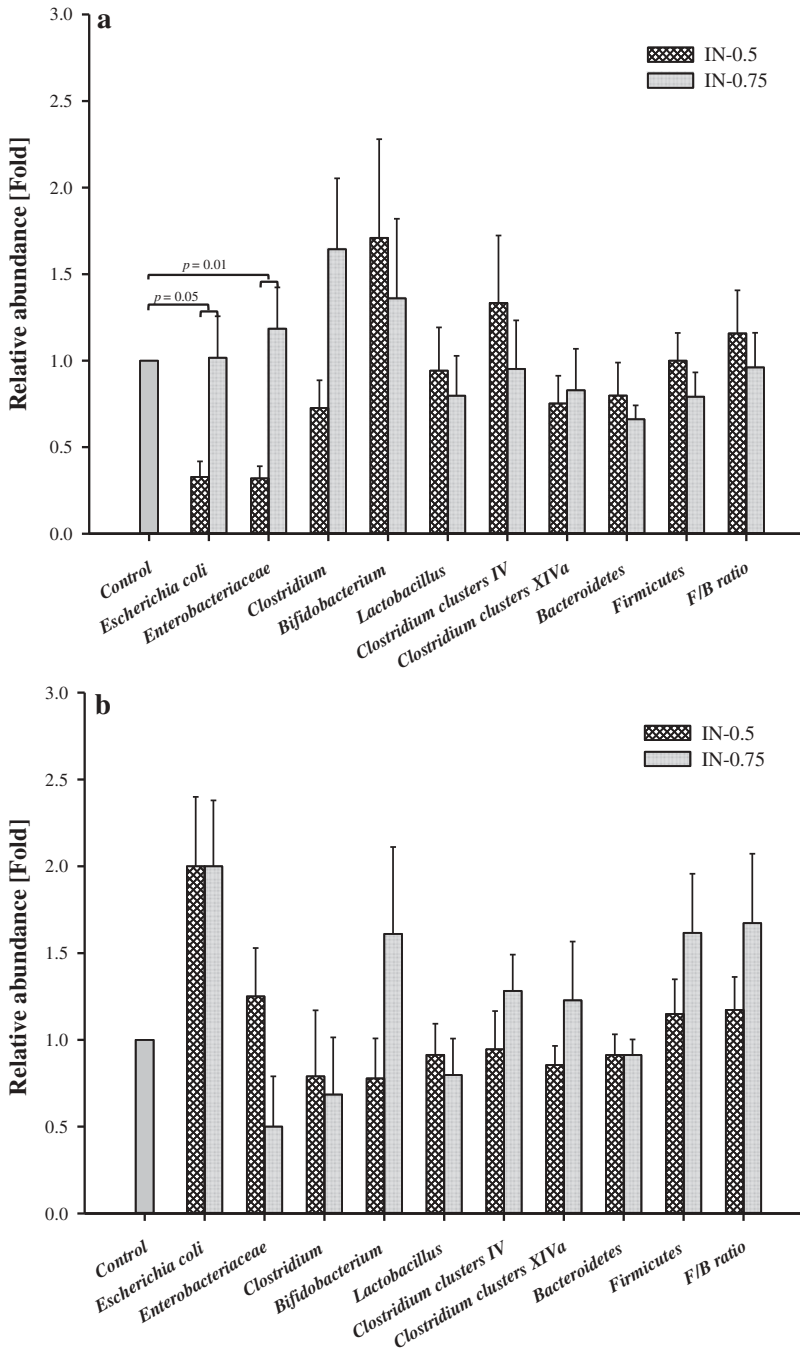


Figure 2. Effect of inulin on the relative abundance of selected bacterial populations in the colonic digesta of pigs on 28 d (a) and 49 d (b) of age.

Values are means \pm SEM, $n = 6-8$ per group after removing outliers.

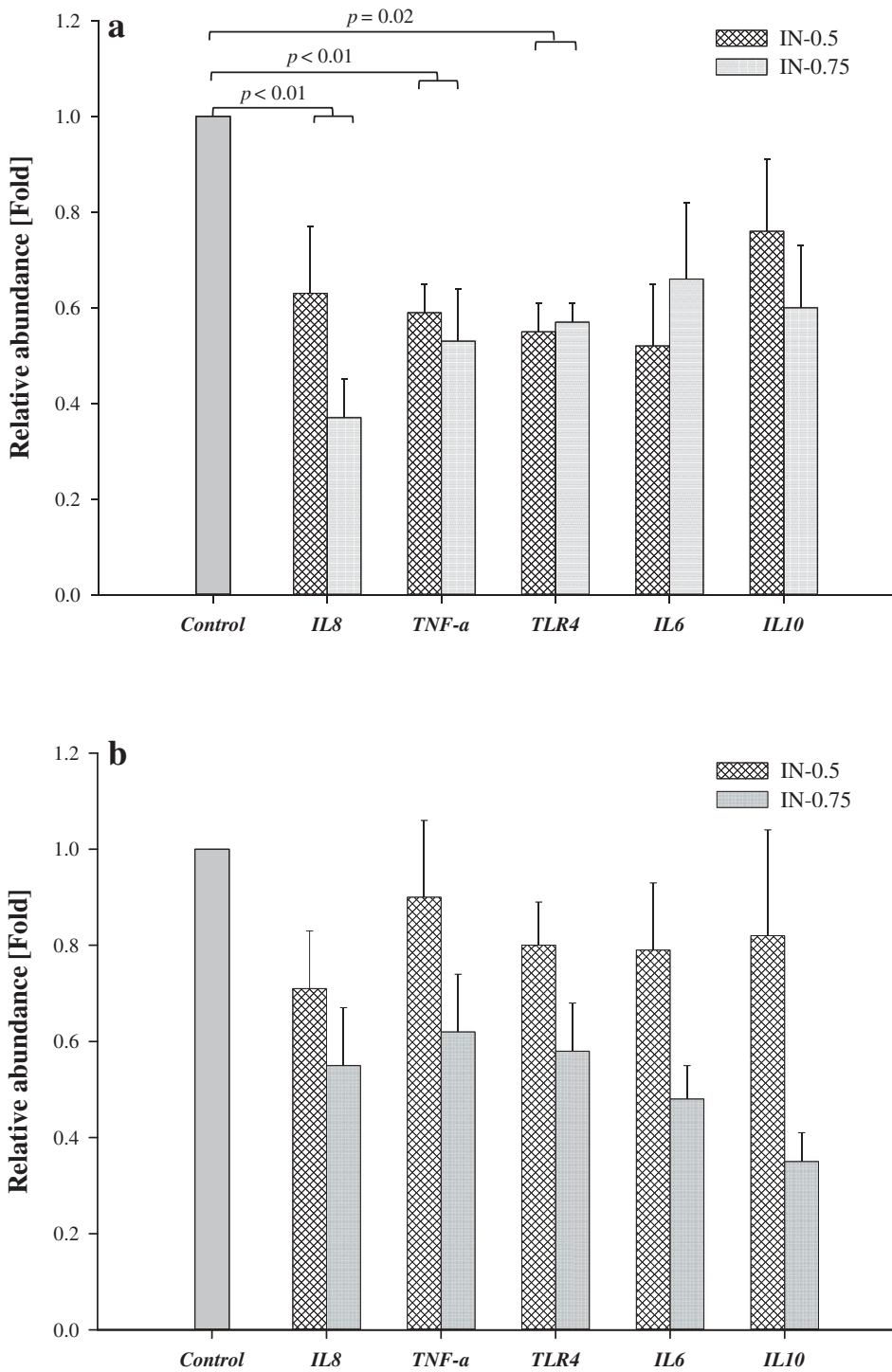


Figure 3. Effect of inulin on the colonic mRNA abundance of interleukin-6 (*IL-6*), interleukin-8 (*IL-8*), interleukin-10 (*IL-10*), tumor necrosis factor- α (*TNF- α*) and toll-like receptor-4 (*TLR4*) of pigs at 28 d (a) and 49 d (b) of age.

Values are means \pm SEM, $n = 5-8$ per group where two samples were lost due to an operational error and one outlier was removed.

of age (Figure 3). Again, these differences disappeared when looking at mRNA abundance at 49 d of age.

4. Discussion

Inulin is responsible for its effects through the alternation of gut microbiota, which starts from the very beginning in life (Edwards and Parrett 2002, 2003; Thompson et al. 2008). Hence, unlike previous studies (Eberhard et al. 2007; Mair et al. 2010; Grela et al. 2013; Sobolewska and Grela 2013; Yan et al. 2013; Barszcz et al. 2016), we examined inulin's effects in suckling piglets rather than post-weaning or during the grower-finisher phase, on BW and intestinal parameters, and further evaluated its effect 3 weeks beyond weaning, when inulin was no longer supplemented.

Our study revealed a significantly increased BW in piglets receiving the IN-0.5 dose during the suckling period. In agreement, Sobolewska and Grela (2013) and Grela et al. (2013) demonstrated an increased daily weight gain in adult pigs that fed an inulin-supplemented diet. Examining the result of gut morphology, the IN-0.5 group showed an increased villus height and an increased V/C ratio in both jejunum and ileum compared to the CON group during the suckling period, suggesting a link between BW and gut morphology. This is not surprising since an increased villus height represents an increased surface area, which will result in an increased nutrient absorption capacity (Samanya and Yamauchi 2002; Pappenheimer and Michel 2003). The relationship between crypt depth and growth performance is not as clear. Deeper crypts can be advantageous because it entails faster villi rebuild from damage to improve absorption. However, faster cell turnover leads to increased energy utilisation, which reduces energy for growth performance (Yason et al. 1987; Xu et al. 2003; Awad et al. 2006). Hence, the V/C ratio is often used to interpret gut morphology. In practice, adding inulin to the diet has frequently been shown to improve the intestinal mucosal architecture in broiler chickens (Rehman et al. 2007; Nabizadeh 2012). In weaned piglets, fructooligosaccharides (FOS) were observed to increase jejunal villus height (Xu et al. 2005).

As a prebiotic, inulin serves as a substrate for SCFA synthesis by different types of bacteria in the large intestine (Gibson et al. 2004). However, dietary administration of inulin has been shown to have different effects on the SCFA profile in different studies (Loh et al. 2006; Eberhard et al. 2007; Varley et al. 2010; Hansen et al. 2011; Paßlack et al. 2012; Barszcz et al. 2016; Grela et al. 2016). In our study, there was no change in the fermentation pattern of SCFA induced by inulin supplementation during the suckling period based on the digesta dry matter. However, based on the digesta fresh matter, propionate and *iso*-butyrate concentrations were elevated after administration of IN-0.5. Propionate is known to have several health effects and to play a crucial role in both intestinal morphology and function (Hosseini et al. 2011). In contrast with other studies in pigs *in vivo* (Loh et al. 2006) or *in vitro* (Ying et al. 2013), inulin supplementation did not lead to a difference in butyrate, which is the most important energy source for enterocytes (Roediger et al. 1995).

Changes in SCFA profile might indicate shifts in microbial populations. Usually, the abundance of indigenous *Bifidobacterium* and *Lactobacillus* genera are selectively stimulated by prebiotics (Gibson et al. 2004), stimulating mucosal immunity or occupying

adhesion sites (Servin 2004). However, we did not find any alteration of *Bifidobacterium* or *Lactobacillus* by inulin supplementation, which is in line with Eberhard et al. (2007), Varley et al. (2010) and Paßlack et al. (2012), (2015)), but in contrast with other studies in pigs (Tzortzis et al. 2005; Tako et al. 2008; Patterson et al. 2010). The lack of an observable difference might be due to the age of the piglets, as the treatment was applied when the complexity of the microbiota increases. Alternatively, the effect observed might be related to the dose of inulin. Interestingly, in our study was observed that adding IN-0.5 suppressed the relative abundance of less desirable bacteria such as *Escherichia coli*, *Enterobacteriaceae* and also *Clostridium* numerically, though the relative abundance was slightly decreased at weaning. These findings are consistent with previous studies that identified lower abundances of members of the *Clostridium*, *Escherichia coli* or *Enterobacteriaceae* after administration of inulin (Wang and Gibson 1993; Patterson et al. 2010; Varley et al. 2010; Nabizadeh 2012). The *Clostridium clusters IV* and *XIVa* are known as butyrate-producing species, having the capacity to degrade inulin-type fructans, producing butyrate (Hold et al. 2003; Moens and De Vuyst 2017). However, in our study, concomitant with the result of butyrate, no difference was observed in *Clostridium clusters IV* and *XIVa* counts. Koleva et al. (2012) also did not detect differences in *Clostridium clusters IV* and *XIVa* in rats that fed an inulin diet. Furthermore, in pigs, the most dominant phyla are *Firmicutes* and *Bacteroidetes* (Isaacson and Kim 2012). Unfortunately, inulin administration did not affect the abundance of *Firmicutes* and *Bacteroidetes*, as well as F/B ratio in our study.

Pro-inflammatory and anti-inflammatory cytokines can be modulated by inulin either indirectly or directly (Babu et al. 2012; Johnson-Henry et al. 2014; Vogt et al. 2015; Halmes et al. 2017). An indirect impact might refer to stimulation of growth and activity of bacteria and its fermentation products such as SCFA. Inulin can also be detected directly by intestinal immune cells, through receptor ligation of pathogen recognition receptors such as TLR, eventually inducing pro-inflammatory and anti-inflammatory cytokines. Both IL8 and TNF- α are established pro-inflammatory cytokines in piglets (Brix-Christensen et al. 2001). Inulin administration has been found to reduce IL8 and TNF- α at the protein level in pigs, mice and humans (Taranu et al. 2012; Bermudez-Brito et al. 2016; Shukla et al. 2016). This is in agreement with our study where both inulin groups decreased IL8 and TNF- α at the mRNA level in the colon at 28 d of age. Prebiotics such as inulin could act as TLR4 ligands in intestinal immune cells (Capitán-Cañadas et al. 2014) and TLR4 can induce a pro-inflammatory response (Mulla et al. 2009; Płóciennikowska et al. 2015). Together with the decrease of IL8 and TNF- α , the mRNA abundance of TLR4 was also reduced by inulin supplementation. Therefore, the presence of inulin in the intestine might lead to a trend towards an anti-inflammatory situation via suppressing the expression of pro-inflammatory cytokines. In contrast with what would be expected in our study, IL6 and IL10 did not significantly differ between dietary groups (Johnson-Henry et al. 2014; Shukla et al. 2016; Zhang et al. 2018).

Two interesting effects of inulin in our study should be mentioned. Firstly, a dose-response effect of inulin was observed. This observation is in agreement with research in broiler chickens where adding 2.0 and 4.0 g/kg of FOS in the diets resulted in slightly improved growth performance, gut morphology and microbiota in comparison with adding 8.0 g/kg of FOS in the diet (Xu et al. 2003). In previous studies on weaned pigs, 2%~4% inulin in the post-weaning diet has been found to display prebiotic effects, improving host's health (Loh et al. 2006; Tako et al. 2008; Yasuda et al. 2009; Varley et al. 2010). Hence, taking the

daily feed intake and BW of weaned pigs into account, we designed our oral supplementations (IN-0.5) in suckling piglets to imitate 2%~4% inulin in post-weaning diets. An additional IN-0.75 group was also prepared to represent a relatively higher inulin supplementation. Compared to the beneficial effects of the IN-0.5 group, the IN-0.75 group did not demonstrate any changes in BW, gut microbiota, SCFA profile and only a minor change in gut morphology. This might be due to an excess of inulin inducing some side effects due to over-fermentation, such as diarrhoea, flatulence or abdominal bloating (Tuohy et al. 2003). The second important finding highlighted that changes in SCFA profile, gut microbiota and mRNA abundance of inflammatory markers disappeared when inulin supplementation ceased after weaning. Thus, observed effects may be only due to direct effects of inulin administration and fermentation, not modulation of the microbiota. Although the IN-0.50 treatment continued to have beneficial effects on BW and gut morphology, it was probably just an accumulation of the benefits induced by inulin during the suckling period. However, these lasting effects of inulin may protect piglets against weaning stress.

5. Conclusion

In conclusion, the administration of inulin during the suckling period ameliorated BW showed a positive effect on gut morphology of the small intestine and revealed an increase in propionate and iso-butyrate concentrations in the large intestine based on the fresh digesta samples. Moreover, inulin feeding reduced pro-inflammatory cytokines at mRNA level. A dose-response effect of inulin was observed, as only IN-0.5 displayed beneficial effects. These beneficial effects faded out during the post-weaning period when inulin was no longer supplemented, suggesting inulin has a direct effect rather than a lasting programming effect. Nevertheless, these beneficial effects induced by inulin during the suckling period might help piglets to be more resilient to weaning stress. Application of inulin will require investigation of more feasible methods of feeding, such as addition in milk replacer or supplementation in creep feed, taking into account that dose effects exist.

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ORCID

Julie Leblais  <http://orcid.org/0000-0001-5112-0262>

Jérôme Bindelle  <http://orcid.org/0000-0001-6974-4313>

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