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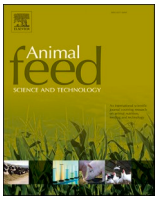

Low sanitary housing conditions increase protein fermentation in piglets but do not aggravate the effects of protein fermentation on intestinal health

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ABSTRACT

Protein fermentation has been identified as a risk factor for the occurrence of post-weaning diarrhea. Associations between low protein digestion – provoking protein fermentation - and low sanitary housing conditions suggest that protein fermentation, low sanitary conditions, and post-weaning diarrhea are interlinked.

To this end, an experiment (2×2 factorial treatment arrangement) was performed with 160 weaned female piglets (TN70 x Tempo) divided over 5 batches (4 piglets per pen, 2 pens/treatment/batch). The piglets were kept under high (HSC) or low (LSC) sanitary conditions and were fed a diet low (LiP) or high (HiP) in indigestible protein for 14 days. Fecal consistency was scored daily per piglet and after one week, digesta samples were collected to analyze protein digestibility, colonic flow of protein-derived metabolites, and digesta mean retention time in 2 piglets per pen. Furthermore, we measured intestinal permeability for Fluorescein isothiocyanate-dextran (FITC, 4 kDa) and Tetramethylrhodamine isothiocyanate–dextran (TRITC, 40 kDa), and jejunal absorption capacity for lysine, methionine, and glucose, using the everted gut sac technique.

Piglets kept under LSC had 9% units lower apparent total tract protein digestibility than piglets kept under HSC ($P < 0.001$), mainly explained by 7% units lower apparent ileal protein digestibility in LSC piglets ($P < 0.001$). Since no relevant correlations were observed between digesta mean retention time and ileal N digestibility and jejunal absorption capacity did not differ between treatment groups, these factors were excluded as underlying causes. The colonic flow of protein-derived metabolites was higher for LSC piglets than for HSC piglets (NH_3: +31%, BCFA: +40%, $P < 0.001$), and higher for HiP piglets than for LiP piglets (NH_3: +76%, BCFA: +42% for LSC, +25% for HSC, $P < 0.001$, SC x Diet $P < 0.05$). Colonic N disappearance was 5.5% units higher for HiP piglets than for LiP piglets ($P < 0.01$), but was not affected by SC. Fecal consistency scores were reduced for both HiP (−1.5, $P < 0.001$) and LSC (−0.5, $P < 0.001$) piglets compared with LiP and HSC, but no SC x diet interaction was observed. Jejunal permeability was slightly

Abbreviations: AID, apparent ileal digestibility; ATTD, apparent total tract digestibility; DM, dry matter; FITC, fluorescein isothiocyanate; HiP, high indigestible protein; HSC, high sanitary conditions; LiP, low indigestible protein; LSC, low sanitary conditions; N, nitrogen; NSP, non-starch polysaccharides; SCFA, short chain fatty acids; TiO_2, titanium dioxide; TRITC, tetramethylrhodamine isothiocyanate; VFA, volatile fatty acids.

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increased for HiP piglets ($P < 0.05$). No negative effects of feeding HiP on colonic permeability were observed.

Low sanitary conditions decrease both ileal and fecal protein digestion to a similar extent and stimulate colonic protein fermentation. It is unlikely that digesta transport or jejunal absorption rates are causal to this effect. There are no indications that housing under low sanitary conditions aggravates the effects of protein fermentation on intestinal health.

1. Introduction

Post-weaning diarrhea (PWD) is a common disorder in weaned piglets, characterized by watery feces and growth retardation (Heo et al., 2013; Rhouma et al., 2017). At weaning, piglets are exposed to multiple stressors, such as maternal separation, adaptation to a new environment, mixing with non-littermate piglets, and dietary changes (Heo et al., 2013; Pluske et al., 1997). These stressors negatively affect post-weaning feed intake and make piglets more susceptible to viral and bacterial infections, which can result in an increased enterocyte turnover and villus atrophy. Villus atrophy and crypt hyperplasia may affect intestinal function and negatively affect digestion and absorption of nutrients in the small intestine of the pig (Heo et al., 2013). Less absorption of nutrients increases its outflow into the large intestine, where, along with endogenous losses, these are prone to fermentation by the resident microbiota. During protein fermentation, metabolites, including biogenic amines, branched-chain fatty acids (BCFA), ammonia (NH_3), hydrogen sulfide, indolic and phenolic compounds, and nitric oxide, are produced. Some of these metabolites are potentially toxic, and may contribute to or aggravate PWD in pigs (as reviewed by Gilbert et al., 2018, Rist et al., 2013).

Restricting the protein flow into the large intestine by altering dietary protein content, source or both, reduces the formation of protein-derived metabolites and this has been associated with decreased incidence of PWD (Heo et al., 2008; Heo et al., 2009; Heo et al., 2010; Wellock et al., 2006). In contrast, feeding a highly indigestible protein diet does not always increase PWD (Htoo et al., 2007; Nyachoti et al., 2006), suggesting that the effects of undigested proteins flowing into the hindgut may depend on the environmental conditions under which the piglets are kept. Pigs kept under low sanitary conditions are exposed to pathogenic and nonpathogenic agents, which both enhance the production of pro-inflammatory cytokines that stimulate the immune system (Johnson, 1997; Williams et al., 1997). Chronic, low-grade activation of the immune system induced by low sanitary conditions can compromise intestinal function in pigs. Piglets kept under low sanitary conditions experience diarrhea more often and have a reduced intestinal villus length and crypt depth (Jayaraman et al., 2017; Pastorelli et al., 2012; Zhao et al., 2007). In addition, piglets kept under low sanitary conditions have increased concentrations of serum haptoglobin, most likely due to enhanced cytokine production (Kampman-van de Hoek et al., 2016; Nordgreen et al., 2018). The produced cytokines may increase intestinal permeability (Al-Sadi et al., 2013; Al-Sadi et al., 2014), and consequently increasing the risk of pathogen invasion. Suboptimal conditions of the intestinal tract caused by hygienic pressure may increase the likelihood of pathogenic bacterial overgrowth elicited by protein fermentation (Johnson, 1997; Pastorelli et al., 2012). But even in the absence of clinical diseases, pigs kept under low sanitary conditions will have impaired growth and a reduced apparent total tract nitrogen digestibility (ATTD of N) of 1–7% units (Kampman-van de Hoek et al., 2016; Le Floc'h et al., 2014; Van der Meer et al., 2016; Van der Meer et al., 2020). It is unknown whether the reduction in ATTD of N is due to decreased N-disappearance in the small or large intestine.

The objectives of our study were to identify the effects of sanitary conditions on apparent ileal and total tract protein digestibility in piglets fed diets containing low or high indigestible proteins and its subsequent effects of protein fermentation on fecal consistency score and intestinal permeability. We hypothesized that low sanitary conditions reduce both ileal and total tract apparent N digestibility (AID of N, ATTD of N) in piglets, particularly when fed diets with high indigestible protein contents. This reduced AID of N would increase protein flow into the colon, increasing the flow of protein-derived metabolites that subsequently increase PWD and intestinal permeability, particularly in piglets kept under low sanitary conditions.

2. Material and methods

A project license was granted by the Central Committee for Animal Experimentation (The Hague, the Netherlands) after approval by the Animal Care and Use Committee of Wageningen University & Research (AVD1040020209705, Wageningen, The Netherlands). This experiment was approved by the Animal Welfare Body of Wageningen University (2020. W-0012.001).

2.1. Experimental design

The experiment was conducted as a randomized complete block design, with 5 independent blocks (batches) and 4 treatments. The 4 treatments were tested in a 2 x 2 factorial arrangement: low or high dietary indigestible protein contents (LiP and HiP) at high or low sanitary conditions (HSC and LSC). In total, 160 female piglets (TN70 x Tempo; Topigs, Helvoirt, The Netherlands) were selected from a commercial farm in the Netherlands, in five batches of 32 piglets each. Each batch consisted of 2 weeks. Immediately after weaning (mean age ± SEM; 29 ± 0.3 d, mean body weight ± SEM; 9.1 ± 0.21 kg), piglets were transported to the research facilities of Wageningen University & Research. Upon arrival, piglets were allocated to 8 pens based on their initial body weight and litter origin, to minimize variation in body weight between pens and to exclude littermates in the same treatment.

2.2. Housing

Per batch, piglets were divided over four climate-controlled respiration chambers with two pens (of 220 cm x 110 cm each) per chamber and four piglets per pen. Both pens in a chamber received the same treatment. The floor of each pen was 2/3 solid and 1/3 slatted. Between pens, piglets were unable to have physical contact. The rooms of HSC and LSC were separated and each had its own entrance and barrier measures. A strict hygiene protocol was applied for people entering the HSC rooms (showering, changing clothes, use of a hairnet, gloves, and face mask). In addition, animal caretakers did not enter HSC after visiting the LSC rooms at the same feeding time. No hygiene protocol was used in the LSC rooms.

The HSC rooms were cleaned prior to the arrival of the piglets by high-pressure washing followed by disinfection with a mixture of hydrogen peroxide and peracetic acid gas for 2 h. Feces were removed per pen twice daily. In LSC, the rooms were cleaned before the arrival of new piglets, but not disinfected. In addition, pooled feces collected from pens of weaned piglets (<25 kg) and sows on five commercial farms were spread on the floor twice a week to enhance antigenic pressure. Finally, in LSC nylon bags filled with ground straw were placed at the top of a chain to create environmental dust when piglets were playing with the chain. In HSC, chains without nylon bags were present.

Feces from each commercial farm were collected separately from multiple departments per farm (30.7 kg feces average per farm). Feces from each department were individually subsampled for pathogen analysis, and subsequently pooled, supplemented with 0.9% NaCl solution (2:1 w/w) and glycerol (12.6% w/w), and stored at − 20 °C pending batch homogenization. The samples taken for pathogen analyses were analyzed for a selection of pathogens (*E. Coli*, heat-stable protein A, heat-labile protein; *Campylobacter jejuni*, CD-toxin; *Clostridium perfringens*, aLiPha-Toxin; *Lawsonia intracellularis,* Surface antigen A). In short, 200 mg of feces per department was used for DNA extraction following previous protocols (Geervliet et al., 2022) using the Tissue LEV Total RNA purification kit on an automated Maxwell 16 (Promega). The resultant DNA was diluted to 1 ng/μl and used for RT-qPCR for toxin gene identification (see Table 1 for primers). Amplification consisted of initial denaturation and hot start at 95 °C (20 s), 60 °C (30 s), and 72 °C (30 s) using a CFX96TM thermocycler (Bio-Rad Laboratories) including a final melt curve from 60 to 95 °C in 0.5 °C increments to validate amplicon size specificity. The gene copy numbers were calculated by internal positive standards for each toxin. Fecal subsamples with a high pathogenic load (3 out of 31 samples), assessed by comparing with previously diarrhea-associated levels, were excluded (Figure S2), to minimize the risk of introducing diseases. The remaining fecal samples were pooled into one large batch, homogenized, divided over buckets containing 2.6 kg of diluted pooled feces each, and stored at − 20 °C. Four buckets with pooled feces were thawed overnight per timepoint before spreading into the LSC pens (days 1, 5, 9, and 12).

For the first three days, heat lamps were provided and the temperature was controlled at 26 °C. After the first three days, the temperature was gradually adjusted to reach 28 °C and the heat lamps were removed. The piglets were exposed to 16 h of light (0800–2400 h). In the second week, the temperature gradually decreased to 24 °C on day 14. Relative humidity was maintained at 65%.

2.3. Diets and feeding

After arrival at the research facilities of Wageningen University & Research, the piglets were allocated to the treatments and immediately switched to one of the test diets (low indigestible protein; LiP or high indigestible protein; HiP, Table 2). Two diets (pellets) containing casein with high protein digestibility (LiP) or sunflower seed meal with low digestibility (HiP) as the main protein source (CVB, 2018), were formulated, creating a difference in the contents of indigestible protein (26 vs 42 g/kg as-fed). To equalize non-starch polysaccharides (NSP) contents between diets, sunflower hulls were added to the LiP diet. In both diets, titanium dioxide (TiO_2; 2 g/kg) was supplemented as indigestible marker. All diets met the nutritional requirement estimates of weaned piglets (CVB, 2020). To habituate the piglets to the experimental diets a 50:50 (w/w) mixture of both diets was introduced on the commercial farm, 2 days before transport to the research facilities.

The piglets had ad libitum access to the experimental diets for the first three days after arrival at the experimental facilities, to allow them to adapt and to record feed intake. After the first three days within each batch, the group with the lowest feed intake was selected as reference group. Subsequently, the other groups were pair-fed: their daily feed allowance was set to the feed intake of the reference group of the day before, with a minimum of 150 g/piglet/day. Piglets were fed twice a day until 36 h before dissection on day 8. From 36 h before dissection, the piglets were fed every six hours and for the last six hours, the piglets were fed hourly 1/12 of the daily portion, to approach steady-state conditions in the gastrointestinal tract and allow for sufficient digesta in the ileum at dissection. The

Table 1
List of primers to selectively quantify toxin genes from pathogens in the fecal samples before pooling of feces used to create low sanitary housing conditions.

Gene	*Target*	*Forward*	*Reverse*
16 S	*All bacteria*	CGGTGAATACGTTCYCGG	GGWTACCTTGTTACGACTT
Sta	*Escherichia coli*	TCCCCTCTTTTAGTCAGTCAACTG	TAACATGGAGCACAGGCAGG
LTb	*Escherichia coli*	ACGGAGCTCCCCAGTCTATT	TGTTGCGCCGCTCTTAAATG
cdtB	*Campylobacter jejuni*	CGCAGCCACAGAAAGCAAAT	GCTCCTACATCAACGCGAGA
α-toxin	*Clostridium perfringens*	TTACTGCCGTTGATAGCGCA	CCTGGGTTGTCCATTTCCCA
LsaA	*Lawsonia intracellularis*	TGCTTATAAATTGCTCGCCGC	CCACCTGTTGATGCACCGAT

Table 2
Ingredients, calculated, and analyzed chemical composition of the experimental diets (LiP; low indigestible protein and HiP; high indigestible protein) fed to weaned piglets (9.1 ± 0.21 kg) kept under high (HSC) or low (LSC) sanitary conditions.

	Diet	
Items	LiP	HiP
Ingredient (g/kg of feed, as-fed)		
Sunflower seed meal	-	437.0
Casein	171.0	30.0
Sunflower hulls	249.0	-
Sunflower oil	25.0	30.0
Gelatinized corn	100.0	100.0
Barley	150.0	150.0
Gelatinized corn starch	114.5	114.5
Sugar	100.0	100.0
Maltodextrin (DE 15–20[1])	56.3	-
Vitamin and mineral mix[2]	5.0	5.0
L-lysine hydrogen chloride	-	3.5
Monocalcium phosphate	12.0	11.5
Sodium hydrogen carbonate	-	2.0
Calcium formate	10.7	10.0
Sodium choride	4.5	4.5
Titanium dioxide	2.0	2.0
Calculated chemical composition (g/kg)[3]		
Net Energy (MJ/kg)	8.6	9.3
Crude protein (CP)[4]	191.0	207.0
Apparent total tract digestible CP	165.0	165.0
Indigestible CP[5]	26.0	42.0
Apparent ileal digestible Lysine	12.5	9.4
Apparent ileal digestible Methionine	4.9	4.0
Apparent ileal digestible Threonine	6.9	5.9
Apparent ileal digestible Tryptophan	2.1	2.0
Apparent ileal digestible Arginine	6.8	15.9
Apparent ileal digestible Histidine	5.5	5.2
Apparent ileal digestible Isoleucine	8.8	8.3
Apparent ileal digestible Phenylalanine	9.4	9.5
Apparent ileal digestible Leucine	17.5	14.1
Apparent ileal digestible Valine	11.4	10.0
Crude fat	45.0	45.0
Crude fiber	135.0	87.0
Non-starch polysaccharides + lignin	216.0	216.0
Total calcium	6.6	6.6
Apparent total tract digestible phosphorus	3.3	3.3
Analyzed chemical composition (g/kg)		
Gross energy	17.8	17.1
Dry matter	909.0	908.0
CP	186.0	208.0
Crude ash	36.3	63.4
Non-starch polysaccharides	126.0	116.0
Titanium	1.15	1.16

[1]Dextrose-equivalent [2]Supplied per kg diet: 10,000 IU retinyl acetate, 2000 IU cholecalciferol, 40 mg DL-α-tocopherol, 1.5 mg menadione, 1.0 mg thiamin, 4.0 mg riboflavin, 1.5 mg pyridoxin-HCl, 20 μg cyanocobalamin, 30 mg niacin, 15 mg D-pantothenic acid, 150 mg choline chloride, 0.4 mg folic acid, 0.05 mg biotin, 331 mg $FeSO_4 \cdot H_2O$, 80 mg $CuSO_4 \cdot 5\ H_2O$, 49 mg MnO, 194 mg $ZnSO_4 \cdot H_2O$, 1.0 mg KI, 0.56 mg Na_2SeO_3. [3]CVB, 2020. [4]N conversion factor = 6.25. [5]Crude protein – apparent total tract digestible crude protein.

daily feed allowance during frequent feeding was based on the feed intake of the reference group on the day before the start of frequent feeding with a minimum of 150 g/piglet/day. In addition, during frequent feeding, a wooden board was placed in the middle of each pen at each feeding time and the piglets were fed in pairs. In the second week, after dissection of half the piglets, the piglets were pair-fed again based on the feed intake of the reference group of the last feeding time before frequent feeding or the minimal feed supply of 150 g/piglet/day. Water was available ad libitum throughout the entire study period.

2.4. Feces, digesta and tissue sampling

Rectal fecal samples were collected from each piglet daily, except for day 8, and feces were scored for consistency from 1 (liquid) to 5 (solid). On day 8, half of the piglets (1 of the 2 pairs fed together with the highest feed intake; 2 piglets per pen) were euthanized. One hour after the last meal, piglets were injected with pentobarbital (Euthasol 20%®; 24 mg/kg BW) into the ear vein and bled.

Approximately 10 min before euthanasia, piglets were sedated via an intramuscular injection of zolazepam/tiletamine (Zoletil 100®; Virbac, Barneveld, The Netherlands) and xylazine (5:2 ratio, 0.1 mL/kg BW).

After opening the abdominal cavity, the digestive tract was ligated at 5 positions to prevent digesta from flowing between gastrointestinal segments. Clamps were placed in front of the stomach, at the beginning of the small intestine, at the ileocecal valve, between the cecum and colon, and at the end of the large intestine. The stomach and small intestine were separated from the cecum and large intestine and the length of the small intestine and the colon was recorded. The stomach and ileum (defined as the last 2 m of the small intestine) were separated from the small intestine. The colon was divided into the proximal colon (before the midpoint) and distal colon (after the midpoint). The contents of each segment (stomach, small intestine excluding ileum, ileum, cecum, proximal and distal colon, and rectum) were collected quantitatively by gentle squeezing, weighed, homogenized, and stored at − 20 °C. Feces were collected when piglets defecated between sedation and euthanasia.

Midway the small intestine, 20 cm was cleared from intestinal content by gently squeezing it away, followed by collection of the segments to measure intestinal permeability and glucose, lysine, and methionine absorption using the everted gut sac technique. Also midway the colon, 20 cm was cleared from intestinal content, followed by collection of the segments to measure intestinal permeability.

2.5. Measurements and analyses

Digesta were thawed and homogenized and samples of the stomach, small intestine excluding ileum, ileum, cecum, and colon (pooled sample of proximal and distal colon, based on the fresh weight proportions, w/w) were freeze-dried and analyzed for Ti to determine mean retention time (MRT) based on Ti pool sizes, and assuming steady-state conditions (De Vries and Gerrits, 2018). In the digesta samples of the ileum and rectum, N was analyzed in freeze-dried samples (International Organization for Standardization ISO, 2008). Titanium was determined in the freeze-dried samples after hydrolyzation with concentrated sulfuric acid in the presence of a copper catalyst at 420 °C and subsequent addition of peroxide. The resulting orange/yellow colored complex was spectroscopically determined at 408 nm. Rectum contents were analyzed for total NSP measured as neutral sugars and uronic acids (Englyst et al., 1994). Non-starch polysaccharides were extracted from the digesta samples (Englyst et al., 1994). First, starch was gelatinized and enzymatically degraded. Subsequently, NSP were precipitated using acidified ethanol. Neutral sugar composition was analyzed according to the method of Englyst et al. (1994). In short, after pre-treatment with 72% (w/w) H_2SO_4 for 30 min at 35 °C, samples were hydrolyzed with 2 M H_2SO_4 at 100 °C for 1 h. Constituent monosaccharides were derivatized into their corresponding alditol acetates and analyzed using gas chromatography (Trace Ultra GC, Thermo Fisher Scientific, Waltham, MA). Inositol and allose were used as internal standards. Uronic acid content was analyzed according to the colorimetric m-hydroxydiphenyl assay (Blumenkrantz and Asboe-Hansen, 1973), using a spectrophotometer (Evolution 201, Thermo Scientific, Waltham, MA, USA). Galacturonic acid was used for calibration. Due to the small amounts of digesta samples from the rectum at dissection, rectal samples from both piglets from one pen were pooled, together with the rectal samples from both piglets collected the day before dissection. Analyses for N, Ti, and NSP were performed in duplicate.

Digesta samples of the proximal and distal colon were thawed and homogenized, and dry matter was measured (International Organization for Standardization ISO, 1999) and subsequently, the samples were split in two and either acidified with trichloroacetic acid or phosphoric acid. Samples were again stored at − 20 °C. Ammonia concentration was measured colorimetrically at a wavelength of 623 nm using a UV spectrophotometer in the thawed trichloroacetic-acidified sample, as described by Searle (1984). For volatile fatty acid (VFA) analyses, the phosphoric acidified samples were thawed and, after shaking the sample for 30 min in a mechanical shaker, centrifuged for 10 min at 20,817 g. Then, the sample solutions were mixed with an internal standard (31.784 mM 2-methyl valeric acid), centrifuged (20,817 g for 5 min), and introduced in the gas chromatograph (HIP-FFAP; 30 m x 0.32 mm x 0.25 μm from Agilent J&W, USA). After separation of the fatty acids, VFA were detected by a flame ionization detector.

2.6. Everted sac technique

Segments (20 cm) of the mid-small intestine and the mid-colon were harvested to measure permeability and absorption capacity by the everted gut sac technique (Wilson and Wiseman, 1954). Briefly, the outer muscular layer and serosa were carefully removed manually. Segments were flushed with phosphate-buffered saline and inverted, filled with Ringer solution (2.25 g/L NaCl, 0.105 g/L KCl, 0.06 g/L $CaCl_2$, 0.05 g/L $NaHCO_3$, 2 mM HEPES) and closed. Sacs were incubated for 60 min at 39 °C in aerated Ringer solution containing 2 mM HEPES, 5 mM glucose, 30 μg/mL fluorescein isothiocyanate (FITC)-dextran (4 kD), 30 μg/mL tetramethylrhodamine isothiocyanate (TRITC)-dextran (40 kD), 8 mM L-Lysine acetate and 8 mM L-Methionine. After incubation, sac contents were collected and stored pending analyses at − 20 °C, and the length and circumference of the intestinal segment was measured. Sac contents were thawed at room temperature and analyzed for FITC and TRITC by a fluorometer (SpectraMax® M3 Multi-Mode Microplate Reader) and glucose was analyzed using a commercial assay kit (D-Glucose Assay Kit, glucose oxidase/peroxidase; GOPOD; Megazyme, Wicklow, Ireland). Free lysine and methionine were determined in sac contents according to International Organization for Standardization ISO (2005), where amino acids were separated by ion-exchange chromatography, and determined by post-column reaction with ninhydrin, using photometric detection at 570 nm.

2.7. Calculations

Apparent ileal and total tract N and total tract NSP digestibility were calculated using the following equation:

Digestibility coefficient N or $NSP = (1 - \frac{Ti\ diet\ x\ N\ or\ NSP\ digesta}{Ti\ digesta\ x\ N\ or\ NSP\ diet})$ x 100%,

where Ti diet is the titanium concentration in the diet (g/kg DM), N or NSP digesta is the N or NSP concentration in the digesta (g/kg DM), Ti digesta is the titanium concentration in the digesta (g/kg DM), and N or NSP diet is the N or NSP concentration in the diet (g/kg DM).

Apparent ileal and total tract DM digestibility was calculated using the following equation:

Digestibility $coefficient\ DM = (1 - \frac{Ti\ diet}{Ti\ digesta})$ x 100%,

where Ti diet is the titanium concentration in the diet (g/kg DM), and Ti digesta is the titanium concentration in the digesta (g/kg DM).

Ileal N Flow was calculated using the following equation:

Ileal N Flow (g/day) = N digesta x $\frac{Ti\ diet}{Ti\ digesta}$ x feed intake during the last 6 h before dissection,

where N digesta is the N concentration in the ileal digesta (g/kg DM), Ti diet is the titanium concentration in the diet (g/kg DM), Ti digesta is the titanium concentration in the ileal digesta (g/kg DM) and feed intake is the feed intake per day (kg DM), based on the last 6 h before dissection.

The mean retention time (MRT; hour) of Ti in stomach, small intestine−ileum, ileum, cecum, and colon was calculated using the following equation, assuming steady-state conditions (De Vries and Gerrits, 2018):

$MRT(h) = H\ x\ \frac{[Ti]\ x\ W}{I}$, where MRT is the mean retention time in hours in the compartment of the gastrointestinal tract, H is the time period (h) of marker ingestion (6 h for small intestine, 24 h for colon), [Ti] is the marker concentration in the digesta (g/kg DM), W is the weight of digesta in the corresponding segment (kg DM) and I is the marker intake in the 6 (small intestine) or 24 h (colon) preceding dissection (in g).

From 36 h before dissection onwards, the piglets were fed every 6 h with 1/4 of the daily portion. For the last six hours, the piglets were fed hourly. To increase the amount of digesta in the ileum at dissection, the piglets were then fed with 1/12 of the daily portion. Assuming only the portions of the last 6 h were in the small intestine, the marker intake rate of the last six hours was used to calculate MRT in the stomach and small intestine. Assuming that the feed ingested in the last six hours did not reach the large intestine (Martens et al., 2019; Schop et al., 2020), marker intake rate from 30 till 6 h before dissection was used to calculate the MRT in the cecum and colon.

The colonic flow of NH_3, BCFA, valeric acid and short chain fatty acids (SCFA) was calculated using the following equation:

Colonic metabolite $flow$ (μmol/hour) $= \frac{[metabolite]\ x\ W}{MRT}$, where [metabolite] is the colonic concentration of the metabolite of interest (μmol/g), W the weight of digesta in the colon (g), and MRT the digesta mean retention time in the colon (in h).

The transport of FITC-dextran, TRITC-dextran, lysine, methionine, and glucose across the intestinal wall was calculated as follows:

Transport (nmol or $pmol/cm^2/min) = \frac{Concentration\ intestinal\ sac\ x\ volume\ added}{Mucosal\ surface\ area}$ / 60, where concentration intestinal sac is the concentration of the compound of interest in nmol or pmol/mL, volume added is the amount of buffer added to the intestinal sac in mL, and mucosal surface area is the length of the intestinal segment (cm) x circumference (cm).

2.8. Statistical analyses

For all statistical analyses, R for Windows 3.6.0 was used.

Data from feed intake, AID, ATTD, ileal N flow, MRT, metabolites, permeability, and absorption were analyzed using a generalized linear mixed model (gaussian family, link = identity), as follows:

$Y_{ijk} = \mu + S_i + D_j + B_k + (S\ x\ D)_{ij} + (S\ x\ B)_{ik} + (D\ x\ B)_{jk} + (S\ x\ D\ x\ B)_{ijk} + e_{ijkl}$ where Y_{ijk} = dependent variable, μ = overall mean, S_i = sanitary condition (HSC or LSC), D_j = diet (LiP or HiP), B_k = batch effect (1,2,3,4 or 5), $(S\ x\ D)_{ij}$ = interaction effect between SC and diet, $(S\ x\ B)_{ik}$ = interaction effect between SC and batch, $(D\ x\ B)_{jk}$ = interaction effect between diet and batch, $(S\ x\ D\ x\ B)_{ijk}$ = interaction effect between SC, diet and batch, and e_{ijkl} = residual error. If an interaction between batch, SC or diet was not significant, the interaction was omitted from the model. Pen (consisting of one pair of two piglets) was considered as experimental unit. Colonic NH_3 flow was transformed using the cube root transformation.

Data from fecal scores were analyzed using a generalized linear mixed model (poisson family, link = log), as follows:

$$Y_{ijkl} = \mu + S_i + D_j + B_k + T_l + (S\ x\ D)_{ij} + (S\ x\ B)_{ik} + (D\ x\ B)_{jk} + (S\ x\ T)_{il} + (D\ x\ T)_{jl} + (S\ x\ D\ x\ T)_{ijl} + P_m + e_{ijklm}$$

where Y_{ijkl} = dependent variable, μ = overall mean, S_i = sanitary condition (HSC or LSC), D_j = diet (LiP or HiP), B_k = batch effect (1,2,3,4 or 5), T_l = day, $(S\ x\ D)_{ij}$ = interaction effect between SC and diet, $(S\ x\ B)_{ik}$ = interaction effect between SC and batch, $(D\ x\ B)_{jk}$ = interaction effect between diet and batch, $(S\ x\ T)_{il}$ = interaction effect between SC and day, $(D\ x\ T)_{jl}$ = interaction effect between diet and day, $(S\ x\ D\ x\ T)_{ijl}$ = interaction effect between SC, diet and day, P_m = random pen effect and e_{ijklm} = residual error. If an interaction between batch, day, SC or diet was not significant, the interaction was omitted from the model. Pig was considered as experimental unit.

Normality of the residuals of the response variables was checked graphically with quantil-quantil plots and with the Shapiro-Wilk test. If the residuals were not normally distributed, statistical analyses were performed on transformed data. Data are reported as backtransformed least-square means ± SEM and differences were considered significant if $P < 0.05$. Differences at $P < 0.10$ were considered a trend.

Pearson correlation coefficients were used to evaluate relations among response parameters. For the Pearson correlation

coefficients, pen was considered as experimental unit.

3. Results

3.1. Feed intake

With the exception of the first 3 days, piglets were pair-fed relative to a reference that was assigned within a batch. Therefore, with the exception of days 1–3, the significant differences between sanitary conditions and batches reported in Table 3 are caused by unexpected feed refusals and unintended feeding errors, as detailed below.

On the ad libitum feeding days (d 1–3), the average daily feed intake was 59% higher for HSC piglets than for LSC piglets ($P < 0.001$) and 28% higher for LiP piglets than for HiP piglets ($P < 0.01$). In all batches except batch 3, LSC-HiP had the lowest feed intake and was selected as reference group for the paired feeding days. In batch 3, LSC-LiP was selected as reference group, after extending the ad libitum feeding days by 1 day due to inconsistent observations on feed intake. During paired feeding, occasionally the feed intake of LSC piglets was lower than the minimum daily feed allowance of 150 g/piglet/day, leading to some feed refusals in LSC piglets.

The feed intake differed among batches. The average daily feed intake per piglet in the first week varied among batches between 123 and 153 g. In batch 5, the piglets were unintentionally fed with 1/8 of the daily portion, instead of 1/12 in the last 6 h prior to the dissection. This resulted in 229 g more feed refusals for LSC piglets compared with HSC piglets. The average daily feed intake per piglet in the second week varied among batches between 243 g and 381 g.

3.2. Digestibility

As anticipated, AID of N was 17% units lower for HiP piglets than for LiP piglets ($P < 0.001$) and ATTD of N was 13% units lower for HiP piglets than for LiP piglets ($P < 0.001$, Table 4). Furthermore, AID of N was 7% units lower for LSC piglets than for HSC piglets ($P < 0.001$). ATTD of N was 9% units lower for LSC piglets than for HSC piglets ($P < 0.001$). Colonic disappearance of N (ATTD-AID) was 5.5% units higher for HiP piglets than LiP piglets ($P < 0.01$), but was not affected by SC.

AID of DM was 5.5% units lower for HiP piglets than for LiP piglets ($P < 0.01$) and ATTD of DM was 3% units higher for HiP piglets than for LiP piglets ($P < 0.01$). In addition, ATTD of NSP was 23% units higher for HiP piglets than for LiP piglets ($P < 0.001$). For LSC piglets, AID of DM, ATTD of DM, and ATTD of NSP were lower than for HSC piglets (AID of DM: −1% units when fed the LiP diet, −8% units when fed the HiP diet, SC $P < 0.01$, SC x diet interaction $P = 0.06$, ATTD of DM: −6% units when fed the LiP diet, −10% units when fed the HiP diet, SC $P < 0.001$, SC x diet interaction $P = 0.07$, ATTD of NSP: −7% units, $P < 0.001$). Apparent total tract digestibility of DM varied among batches from 60% to 65% ($P < 0.05$). Colonic N disappearance was lower in batch 5 (66%) than in batch 1–4 (71–72%, $P = 0.05$).

Ileal N flow was 60% higher for HiP piglets than for LiP piglets ($P < 0.001$, Table 4). Ileal N flow did not differ between LSC and HSC piglets.

3.3. Mean retention time

Digesta MRT was shorter for the HiP diet than for the LiP diet in the stomach (−58 min, $P < 0.01$), cecum (−85 min, $P < 0.001$), and colon (−185 min, $P < 0.01$). Gastric MRT was 61 min shorter for piglets kept under LSC and fed the LiP diet (SC x Diet, $P = 0.05$). Furthermore, the digesta MRT in the duodenum/jejunum tended to be 7 min shorter for HSC than LSC ($P = 0.07$). Gastric MRT of digesta varied among batches between 120 and 236 min ($P < 0.001$). Digesta MRT in the duodenum/jejunum varied among batches from 50 to 65 min ($P < 0.05$) and MRT in the cecum varied from 109 to 255 min ($P < 0.001$). For the cecum, a batch x diet interaction was observed, caused by a difference in effect size between batches. No relevant correlations were observed between digesta MRT and ATTD of N, DM, or NSP (Table 7).

Table 3
Feed intake (g/piglet/day) of weaned piglets (9.1 ± 0.21 kg) kept under high (HSC) or low (LSC) sanitary conditions and fed a diet low (LiP) or high (HiP) in indigestible proteins, during various phases of the experiment.[1]

	HSC		LSC			P-value[2]			
Items	LiP	HiP	LiP	HiP	SEM	SC	Diet	SC x Diet	Batch
No. of pens[3]	10	10	10	10					
Feed Intake									
Days 1–3, *Ad libitum* access to feed	179	146	118	86	25.7	**< 0.001**	**0.002**	0.97	**<0.001**
Days 4–8, Paired fed within batch	158	153	131	130	14.0	**< 0.001**	0.73	0.78	**0.012**
Days 9–14, Paired fed within batch[4]	323	315	304	341	47.4	0.92	0.27	0.11	**0.038**

[1]Data are presented as LS means ± pooled standard error of the mean (SEM). [2]Model established *P*-values for the fixed effects of diet, SC, and blocking factor batch, and their interactions. Bold *P*-values are $P < 0.05$ and underlined *P*-values are $P \leq 0.10$. [3]Number of replicate pens with 4 (d 1–7) or 2 (d 7–14; from 36 h prior to dissection) piglets per pen. [4]Batch x diet interactions were observed for days 9–14, caused by a diet effect in batch 2 only.

Table 4
Apparent ileal (AID) and total tract digestibility (ATTD) coefficient of nitrogen (N), dry matter (DM), and non-starch polysaccharides (NSP), colonic disappearance of N and DM, ileal N flow (g/day), and digesta mean retention time (MRT; hr:min) in different segments of the gastrointestinal tract of weaned piglets (9.1 ± 0.21 kg) kept under high (HSC) or low (LSC) sanitary conditions and fed a diet low (LiP) or high (HiP) in indigestible proteins.[1]

	HSC		LSC			*P*-value[2]					
Items	LiP	HiP	LiP	HiP	SEM	SC	Diet	SC x Diet[3]	Batch	Batch x Diet[4]	Batch x SC[5]
No. of pens[6]	10	10	10	10							
AID											
N	0.77	0.61	0.71	0.53	0.036	**<0.001**	**<0.001**	0.63	ns	ns	ns
DM	0.52^{a}	0.50^{a}	0.51^{a}	0.42^{b}	0.022	**0.005**	**0.002**	0.063	ns	ns	ns
ATTD											
N	0.80^{a}	0.70^{b}	0.74^{ab}	0.58^{c}	0.031	**<0.001**	**<0.001**	0.11	ns	ns	ns
DM	0.65^{a}	0.70^{b}	0.59^{c}	0.60^{c}	0.018	**<0.001**	**0.004**	0.072	**0.023**	ns	ns
NSP	0.48	0.72	0.42	0.64	0.040	**<0.001**	**<0.001**	0.27	ns	ns	ns
Colonic disappearance[7]											
N	0.03	0.10	0.03	0.07	0.022	0.43	**0.004**	0.38	0.050	ns	ns
DM	0.14	0.21	0.09	0.19	0.022	**0.022**	**<0.001**	0.28	0.51	**<0.001**	ns
Ileal N flow	2.78	4.85	3.29	4.90	0.020	0.16	**<0.001**	0.28	0.25	**<0.001**	**0.004**
Digesta MRT											
Stomach	03:45^{a}	02:07^{b}	02:44^{ab}	02:26^{b}	00:29	0.29	**0.004**	0.051	**<0.001**	ns	ns
Duodenum/jejunum	00:52	00:57	01:01	01:01	00:04	0.065	0.49	0.44	**0.010**	ns	ns
Ileum	00:43	00:48	00:44	00:33	00:05	0.14	0.57	0.095	ns	ns	ns
Oro-ileal	05:09	03:44	04:19	03:45	00:26	0.29	**0.005**	0.16	**0.002**	ns	ns
Cecum	03:42	02:08	03:27	02:12	00:28	0.72	**<0.001**	0.58	**<0.001**	**0.001**	ns
Colon	16:15	13:22	15:14	11:58	01:06	0.21	**0.001**	0.84	ns	ns	ns
Cecal-colonic	19:57	15:31	18:41	14:10	01:18	0.21	**<0.001**	0.97	ns	ns	ns
Oro-colonic	25:05	19:15	23:00	17:55	01:26	0.12	**<0.001**	0.73	ns	ns	ns

[1]Data are presented as LS means ± pooled standard error of the mean (SEM). [2]Model established p-values for the fixed effects of diet, SC, and blocking factor batch, and their interactions. Bold *P*-values are $P < 0.05$ and underlined *P*-values are $P \leq 0.10$, ns= non-significant, excluded from the model. [3]In case of a significant SC x diet interaction, lowercase letters (a, b, c) indicate differences at $P < 0.05$ within each row. [4]Batch x diet interactions for colonic disappearance of DM, ileal N flow, and cecal digesta MRT were caused by a difference in effect size between batches. [5]Batch x SC interactions for Ileal N flow were caused by an opposite effect in batches 2 and 5. [6]Number of replicate pens with 4 (d 1–7) or 2 (d 7–8; from 36 h prior to dissection) piglets per pen. [7]As proportion of intake.

3.4. Protein-derived metabolites

The colonic flow of NH_3 (+31%, $P < 0.001$) and BCFA (+40%, $P < 0.001$) was higher for LSC than for HSC piglets (Table 5). In addition, the colonic flow of NH_3 was 76% higher for HiP piglets than for LiP piglets ($P < 0.001$). The colonic flow of BCFA was 25%

Table 5
Colonic flow of ammonia (NH_3), branched-chain fatty acids (BCFA), short-chain fatty acids (SCFA), and valeric acid in μmol/hour in weaned piglets (9.1 ± 0.21 kg) kept under high (HSC) or low (LSC) sanitary conditions and fed a diet low (LiP) or high (HiP) in indigestible proteins.[1]

	HSC		LSC			*P*-value[2]						
Items	LiP	HiP	LiP	HiP	SEM	SC	Diet	SC x Diet[3]	Batch	Batch x Diet[4]	Batch x SC[5]	Batch x Diet x SC
No. of pens[6]	10	10	10	10								
Metabolites												
NH_3	165	293	218	380	35.53	**<0.001**	**<0.001**	0.79	**<0.001**	**0.003**	ns	ns
BCFA	12.3a	15.4^{b}	16.0^{b}	22.7^{c}	1.645	**<0.001**	**<0.001**	**0.021**	**0.001**	ns	ns	**0.045**
Isobutyric acid	5.37a	6.94^{b}	7.10^{b}	10.6^{c}	0.766	**<0.001**	**<0.001**	**0.002**	**<0.001**	ns	ns	**0.043**
Isovaleric acid	6.96a	8.44^{a}	8.90^{a}	12.2^{b}	0.894	**<0.001**	**<0.001**	0.064	**0.017**	ns	ns	0.062
Isocaproic acid[7]	B.D.	B.D.	B.D.	B.D.								
SCFA	562	1196	704	1421	145.0	**0.006**	**<0.001**	0.54	**<0.001**	**0.007**	ns	ns
Acetic acid	422	899	534	1051	107.0	**0.007**	**<0.001**	0.68	**<0.001**	**0.005**	ns	ns
Propionic acid	110	222	132	271	27.95	**0.013**	**<0.001**	0.33	**0.007**	**0.023**	ns	ns
Butyric acid	28.7	74.5	38.0	98.3	11.66	**0.006**	**<0.001**	0.23	**0.040**	0.063	ns	ns
Caproic acid	0.50a	0.25^{b}	0.38^{ab}	0.34^{ab}	0.083	0.80	**0.004**	**0.037**	0.27	ns	**<0.001**	0.069
Valeric acid	12.0	21.0	13.2	26.4	3.501	0.21	**<0.001**	0.43	**0.047**	ns	ns	ns

[1]Data are presented as LS means ± pooled standard error of the mean (SEM). [2]Model established p-values for the fixed effects of diet, SC, and blocking factor batch, and their interactions. Bold *P*-values are $P < 0.05$ and underlined *P*-values are $P \leq 0.10$, ns= non-significant, excluded from the model. [3]In case of a significant SC x Diet interaction lowercase letters (a, b, c) indicate differences at $P < 0.05$ within each row. [4]Batch x diet interactions for all metabolites were caused by a difference in effect size between batches. [5]Batch x SC interaction for caproic acid was caused by opposite effects between batches. [6]Number of replicate pens with 4 (d 1–7) or 2 (d 7–8; from 36 h prior to dissection) piglets per pen.[7]Below detection limit.

higher for HiP piglets than for LiP piglets when kept under HSC and 42% higher when kept under LSC (diet $P < 0.001$, diet x SC $P < 0.05$). Colonic flow of SCFA was 21% higher for LSC piglets than for HSC piglets ($P < 0.01$) and 107% higher for HiP piglets than for LiP piglets ($P < 0.001$). The effect of SC on caproic acid flow differed among batches between 0.296 and 0.431 μmol/hour (SC x batch $P < 0.001$), and were in opposite directions (batch 2,4 and 5: HSC>LSC, batch 1 and 3: LSC>HSC). Valeric acid flow, produced during both carbohydrate and protein fermentation (Ma et al., 2017; Weimer et al., 2015), was unaffected by SC. Valeric acid flow was 88% higher for HiP piglets than for LiP piglets ($P < 0.001$). Colonic flow of all protein-derived metabolites, except caproic acid, was affected by batch. For NH_3 and all SCFA except caproic acid, an interaction was observed between batch x diet, caused by a difference in effect size between batches.

3.5. Amino acid-absorption capacity

Transport of lysine, methionine, and glucose across the intestinal wall was not affected by diet or SC but varied among batches ($P < 0.001$ for lysine, $P < 0.001$ for methionine, $P < 0.05$ for glucose, Table 6). For glucose, a diet x batch interaction was found ($P < 0.01$), and for methionine a tendency for a diet x batch interaction ($P = 0.09$), caused by opposite effects of diets across batches. Lysine and methionine transport ($r = 0.82$, $P < 0.001$) and methionine and glucose transport ($r = 0.73$, $P < 0.001$) were strongly positively correlated (Table 8), lysine and glucose transport was moderately positively correlated ($r = 0.51$, $P < 0.01$).

3.6. Fecal consistency score and intestinal permeability

No SC x diet interaction was observed for fecal consistency scores. Fecal scores and the effect of SC and diet on fecal scores were influenced by day (day $P < 0.001$, diet x day $P < 0.01$, SC x day $P < 0.05$, Fig. 1). From day 5 after weaning, the fecal scores were lower for HiP piglets than for LiP piglets ($P < 0.001$). On days 4, 5, 12, and 13, fecal scores were lower for LSC piglets than for HSC piglets ($P < 0.001$). Fecal dry matter and flows and colonic concentrations in dry digesta of NH_3 (flow $r = -0.68$, concentration $r = -0.82$, both $P < 0.001$) and BCFA (flow $r = -0.57$, concentration $r = -0.69$, both $P < 0.001$) were negatively correlated (Table 8 and S2).

In the jejunum, HiP piglets had a higher passage of FITC-dextran (8%, $P < 0.05$) and TRITC-dextran (8%, $P < 0.05$) across the intestinal wall than LiP piglets (Table 6). The passage of FITC-dextran varied among batches from 1.09 to 2.25 pmol/cm^2/min ($P < 0.001$) and TRITC-dextran varied among batches from 0.10 to 0.14 pmol/cm^2/min ($P < 0.001$). For FITC-dextran, an interaction was found between batch x diet, caused by an opposite diet effect in batch 5 compared with the other batches. For both FITC-dextran and TRITC-dextran, an interaction between batch x SC was found, caused by an opposite SC effect in batch 5 for FITC-dextran, and an opposite SC effect in batch 3 and a lack of effect in batch 5 for TRITC-dextran. In contrast to the jejunum, in the colon, HiP piglets had a lower passage of FITC-dextran (29%, $P < 0.001$) and TRITC-dextran (9%, $P < 0.05$) than LiP piglets. Passage of FITC-dextran varied among batches from 1.09 to 2.25 pmol/cm^2/min ($P < 0.001$) and TRITC-dextran varied among batches from 0.16 to 0.21 pmol/cm^2/min ($P < 0.001$). For both FITC-dextran and TRITC-dextran, an interaction between batch x SC was found, caused by an opposite SC

Table 6
Transport of lysine (nmol/cm^2/min), methionine (nmol/cm^2/min), and glucose (pmol/cm^2/min) across the jejunal wall and intestinal permeability to FITC-dextran and TRITC-dextran (pmol/cm^2/min) in the mid-jejunum and mid-colon of weaned piglets (9.1 ± 0.21 kg) kept under high (HSC) or low (LSC) sanitary conditions and fed a diet low (LiP) or high (HiP) in indigestible proteins.[1]

	HSC		LSC			P-value[2]						
Items	LiP	HiP	LiP	HiP	SEM	SC	Diet	SC x Diet	Batch	Batch x Diet[3]	Batch x SC[4]	Batch x Diet x SC
No. of pens[5]	8	8	8	8								
Jejunum												
Lysine	2.47	2.41	2.69	2.59	0.178	0.092	0.52	0.87	**<0.001**	ns	ns	ns
Methionine	3.95	3.79	3.88	4.37	0.420	0.38	0.58	0.26	**<0.001**	0.085	ns	ns
Glucose	42.0	35.4	39.7	42.8	6.558	0.61	0.73	0.33	**0.041**	**0.004**	ns	ns
FITC-Dextran (4 kDa)[6]	1.03	1.11	1.09	1.19	0.085	0.091	**0.029**	0.81	**<0.001**	**<0.001**	**0.002**	**0.033**
TRITC-Dextran (40 kDa)[7]	0.116	0.131	0.125	0.129	0.008	0.45	**0.046**	0.23	**<0.001**	ns	**0.016**	ns
Colon												
FITC-Dextran (4 kDa)[6]	2.17	1.44	2.06	1.58	0.244	0.87	**<0.001**	0.23	**<0.001**	ns	**0.005**	**0.009**
TRITC-Dextran (40 kDa)[7]	0.201	0.170	0.205	0.198	0.0148	0.063	**0.032**	0.17	**<0.001**	ns	**0.011**	**0.013**

[1]Data are presented as LS means ± pooled standard error of the mean (SEM). [2]Model established *P*-values for the fixed effects of diet, SC, and blocking factor batch, and their interactions. Bold *P*-values are $P < 0.05$ and underlined *P*-values are $P \leq 0.10$, ns= non-significant, excluded from the model. [3]Batch x diet interactions for methionine and glucose were caused by opposite diet effects between batches and for FITC-dextran by an opposite diet effect in batch 5. [4]Batch x SC interactions for FITC-dextran and TRITC-dextran in the jejunum were caused by an opposite SC effect in batch 5 for FITC-dextran, and an opposite effect in batch 3 and a lack of a SC effect in batch 5 for TRITC-dextran. Batch x SC interactions for FITC-dextran and TRITC-dextran in the colon were caused by an opposite SC effect in batch 4 and a lack of SC effect in batch 5 for FITC-dextran and an opposite effect in batch 2 for TRITC-dextran. [5]Number of replicate pens with 4 (d 1–7) or 2 (d 7-8; from 36 h prior to dissection) piglets per pen. [6]Fluorescein isothiocyanate-dextran. [7]Tetramethylrhodamine isothiocyanate–dextran.

Table 7
Correlations between apparent ileal digestibility (AID) and apparent total tract digestibility (ATTD) of nitrogen (N), dry matter (DM), and non-starch polysaccharides (NSP), digesta mean retention time (MRT), and concentrations of metabolites (mmol/mmol Ti) in weaned piglets (9.1 ± 0.21 kg) kept under high (HSC) or low (LSC) sanitary conditions and fed a diet low (LiP) or high (HiP) in indigestible proteins.[1]

Items	AID N	ATTD N	AID DM	ATTD DM	ATTD NSP	MRT ST	MRT DJ	MRT CE	MRT COL	Conc NH_3	Conc IBA	Conc IVA	Conc VA	Conc AA	Conc PA	Conc BA
ATTD N	0.80 *															
AID DM	0.76 *	0.65 *														
ATTD DM	0.19	0.52 *	0.43 *													
ATTD NSP	− 0.56 *	− 0.39 *	− 0.26	0.54 *												
MRT ST	0.15	0.05	− 0.00	− 0.22	− 0.26											
MRT SI	0.09	0.10	0.26	0.17	0.12	− 0.28										
MRT CE	0.45 *	0.31	0.12	− 0.17	− 0.42 *	0.07	0.07									
MRT COL	0.40 *	0.40 *	0.32 *	− 0.01	− 0.43 *	0.10	0.09	0.21								
Conc NH_3	− 0.70 *	− 0.81 *	− 0.64 *	− 0.37 *	0.43 *	0.09	0.22	− 0.28	− 0.41 *							
Conc IBA	− 0.60 *	− 0.80 *	− 0.66 *	− 0.63 *	0.17	0.15	0.01	− 0.10	− 0.19	0.88 *						
Conc IVA	− 0.56 *	− 0.77 *	− 0.63 *	− 0.63 *	0.14	0.19	− 0.06	− 0.08	− 0.12	0.84 *	0.99 *					
Conc VA	−0.64*	− 0.51 *	− 0.34 *	0.05	0.50 *	− 0.04	− 0.01	− 0.46 *	− 0.55 *	0.52 *	0.29	0.26				
Conc AA	− 0.83 *	− 0.81 *	− 0.62 *	− 0.19	0.60 *	− 0.05	0.20	− 0.47 *	− 0.54 *	0.86 *	0.66 *	0.60 *	0.71 *			
Conc PA	− 0.82 *	− 0.83 *	− 0.67 *	− 0.26	0.55 *	− 0.00	0.16	− 0.44 *	− 0.49 *	0.91 *	0.74 *	0.69 *	0.63 *	0.97 *		
Conc BA	− 0.87 *	− 0.81 *	− 0.68 *	− 0.17	0.61 *	− 0.09	0.05	− 0.49 *	− 0.49 *	0.76 *	0.61 *	0.58 *	0.62 *	0.91 *	0.93 *	
Conc CA	0.09	0.20	− 0.14	− 0.09	− 0.30	− 0.01	− 0.32 *	0.26	0.09	− 0.19	− 0.06	− 0.03	− 0.08	− 0.24	− 0.17	− 0.08

[1]ST = stomach, SI = duodenum/jejunum, CE = cecum, COL = colon, IBA = isobutyric acid, IVA = isovaleric acid, VA = valeric acid, AA = acetid acid, PA = propionic acid, BA = butyric acid, CA = caproic acid. * = correlation is significant ($P < 0.05$).

Table 8
Correlations between ileal nitrogen nitrogen (N) flow (g/day), fecal freeze dry matter (FDM; g/kg), passage of FITC-dextran (pmol/cm^2/min), TRITC-dextran (pmol/cm^2/min), lysine (nmol/cm^2/min), methionine (nmol/cm^2/min) and glucose (pmol/cm^2/min) across the jejunal wall and colonic flow of protein-derived metabolites (μmol/hour) in weaned piglets (9.1 ± 0.21 kg) kept under high (HSC) or low (LSC) sanitary conditions and fed a diet low (LiP) or high (HiP) in indigestible proteins.[1]

Items	Ileal N Flow	Fecal FDM	FITC jejunum	TRITC jejunum	Lys jejunum	Met jejunum	Glu jejunum	FITC colon	TRITC colon	Flow NH_3	Flow IBA	Flow IVA	Flow VA	Flow AA	Flow PA	Flow BA
Fecal FDM	− 0.64 *															
FITC jejunum	0.23	− 0.12														
TRITC jejunum	0.23	− 0.17	0.56 *													
Lys jejunum	− 0.05	− 0.01	0.64 *	0.18												
Met jejunum	0.04	− 0.10	0.37	− 0.09	0.82 *											
Glu jejunum	− 0.22	− 0.03	0.15	− 0.19	0.51 *	0.73 *										
FITC colon	− 0.45 *	0.52 *	0.22	− 0.34	0.42 *	0.43 *	0.12									
TRITC colon	0.01	0.15	0.21	0.33	0.24	0.08	− 0.24	0.16								
Flow NH_3	0.70 *	− 0.68 *	0.32	0.38 *	0.07	0.02	− 0.09	− 0.41 *	0.03							
Flow IBA	0.51 *	− 0.62 *	0.43 *	0.33	0.21	0.20	0.15	-0.30	0.15	0.79 *						
Flow IVA	0.46 *	− 0.53 *	0.39 *	0.27	0.18	0.18	0.19	− 0.27	0.12	0.70 *	0.96 *					
Flow VA	0.62 *	− 0.59 *	0.11	0.21	− 0.01	− 0.02	− 0.15	− 0.29	0.03	0.76 *	0.45 *	0.35 *				
Flow AA	0.69 *	− 0.65 *	0.24	0.37 *	0.05	− 0.02	− 0.20	− 0.36 *	0.04	0.90 *	0.62 *	0.48 *	0.71 *			
Flow PA	0.70 *	− 0.63 *	0.21	0.34	0.05	− 0.01	− 0.13	− 0.38 *	0.03	0.89 *	0.67 *	0.54 *	0.66 *	0.97 *		
Flow BA	0.78 *	− 0.65 *	0.22	0.29	0.05	0.02	− 0.11	− 0.34	− 0.03	0.80 *	0.65 *	0.55 *	0.61 *	0.90 *	0.94 *	
Flow CA	− 0.05	0.20	− 0.32	− 0.47 *	− 0.04	0.18	0.00	0.39 *	0.22	− 0.22	− 0.17	− 0.12	− 0.05	− 0.21	− 0.15	− 0.04

[1]FITC = Fluorescein isothiocyanate-dextran (4 kDa), TRITC = Tetramethylrhodamine isothiocyanate–dextran (40 kDa), Lys = lysine, Met = methionine, Glu = glucose, IBA = isobutyric acid, IVA = isovaleric acid, VA = valeric acid, AA = acetid acid, PA = propionic acid, BA = butyric acid, CA = caproic acid. * = correlation is significant ($P < 0.05$).

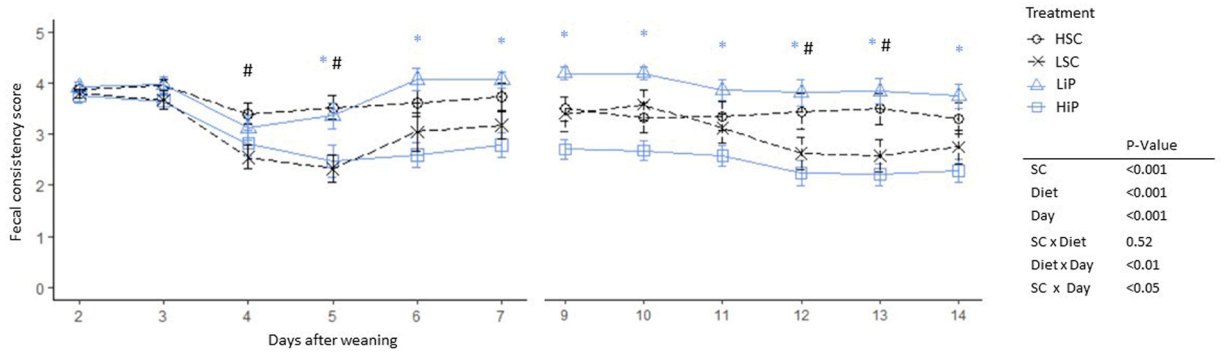

Fig. 1. Average daily fecal consistency score (± SE) over time of weaned piglets (9.1 ± 0.21 kg) kept under high (HSC) or low sanitary conditions (LSC) and fed a diet low (LiP) or high (HiP) in indigestible proteins. 1 =liquid, 5 =solid. * = significant difference on that day between diets ($P < 0.05$), # = significant difference on that day between sanitary conditions ($P < 0.05$).

effect in batch 4 and a lack of SC effect in batch 5 for FITC-dextran and an opposite SC effect in batch 2 for TRITC-dextran.

4. Discussion

The main objectives of this experiment were to evaluate the effect of sanitary housing conditions on protein digestibility and fermentation and subsequent effects on intestinal health in piglets fed diets varying in indigestible protein content. As anticipated, AID of N was lower for piglets fed the HiP diet containing sunflower seed meal as the main protein source (AID of N HiP =57%), than for the LiP diet based on casein (AID of N LiP =74%), resulting in a 61% increased protein flow ($P < 0.001$) into the hindgut.

4.1. Digesta mean retention time

Solid fractions of digesta remained on average for 2.1–3.8 h in the stomach and 1.6–1.8 h in the small intestine, which is in line with previous observations (Martens et al., 2019; Schop et al., 2020) when taking differences in feed intake level between studies into account (Schop et al., 2019). The mean digesta retention time in the total tract was 18.2–25.3 h, which is shorter than the average digesta transit time of 32.1–34.4 h in growing pigs reported by Le Goff et al. (2002). This difference may well be related to differences in age between studies, and relevant MRT values in weaned piglets are largely lacking in literature.

In piglets kept under HSC, the solid fractions of digesta remained on average 98 min longer in the stomach for piglets fed the LiP diet than the HiP diet. For the LiP diet, gastric mean retention time might be prolonged due to casein coagulation, in combination with increased gastric sieving caused by the coarse particles from sunflower hulls, thereby decreasing the digesta MRT of liquids and increasing the digesta MRT of solids (Bornhorst et al., 2014; Huppertz and Chia, 2021; Martens et al., 2019; Potkins et al., 1991). In the cecum and colon, the digesta MRT in HiP piglets was shorter than in LiP piglets, consistent with the lower fecal consistency score in HiP piglets. Undigested proteins and fibers may have stimulated peristalsis in piglets fed the HiP diet and favored the proliferation of pathogenic bacteria (Choi and Kim, 2019; Le Goff et al., 2002).

Assuming that the feed ingested in the last six hours did not reach the large intestine, marker intake rate from 30 till 6 h before was used to calculate the digesta MRT in the large intestine. Since a small portion of the feed ingested in the last six hours has reached the large intestine, this may have led to a small overestimation of the cecal and colonic MRT.

4.2. Intestinal absorption

Amino acids and peptides are mainly absorbed in the jejunum by active transport, with different transporters for basic, neutral, and acidic amino acids (Bröer and Fairweather, 2018). As acidic amino acids are mainly used as energy source by the intestine, a neutral (methionine) and basic (lysine) amino acid were selected to measure absorption capacity (Blanco and Blanco, 2017), in combination with glucose, which is also transported by active transport, but uses different transporters (Chen et al., 2018). The lysine and methionine flows (nmol/cm^2/min) were considered as the amount of lysine and methionine transported over the basolateral membrane. This hypothesis was supported by the moderate to strong positive correlations between the flow of lysine, methionine, and glucose (lysine and methionine $r = 0.82$, $P < 0.001$, methionine and glucose $r = 0.73$, $P < 0.001$, lysine and glucose $r = 0.51$, $P < 0.01$). The intestinal passage of lysine, methionine and glucose may be influenced by differences in their metabolism in the enterocytes.

Lysine and methionine concentrations in the buffer at the luminal side of the everted sac were high, to allow quantification by amino acid analysis in the intestinal sacs, exceeding physiological concentrations in digesta. Similar concentrations were used in previous studies and although no evidence of cell damage was described, to our knowledge, dose-effect response of amino acid concentrations on cell damage was not specifically investigated (Adibi et al., 1967; Nolles et al., 2008). Therefore, we tested the effect of adding 8 MM lysine and 8 MM methionine to the buffer on intestinal permeability in eight piglets of the first batch (HSC; LiP+HiP)

by using two jejunal segments in which a buffer with or without lysine and methionine was used. No significant differences in intestinal permeability were observed between segments incubated with or without amino acids (2.1 pmol/cm^2/min for FITC-dextran with lysine and methionine vs. 2.6 pmol/cm^2/min for FITC-dextran without lysine and methionine, $P = 0.28$, and 0.39 pmol/cm^2/min for TRITC-dextran with lysine and methionine vs. 0.46 pmol/cm^2/min for TRITC-dextran without lysine and methionine, $P = 0.40$). Hence, the addition of 8 MM lysine and 8 MM methionine to the buffer did not influence intestinal transport of FITC- and TRITC-dextran.

4.3. Digestion and fermentation

The AID of N of the casein-based diet (LiP) was in line with data on casein digestibility in just weaned piglets (Engelsmann et al., 2022), taking into account the difference in initial weight (7.0 kg in the study by Engelsmann et al. vs. 9.1 kg in the current study). The digestibility coefficients in the current study were lower than those expected for grower-finishing pigs (CVB, 2018) because the digestive system is still developing, and reduced villus height and enzyme activities caused by weaning result in reduced digestion and absorption of nutrients (Engelsmann et al., 2022; Heo et al., 2013; Lindemann et al., 1986; Xiong et al., 2019).

In line with previous studies, ATTD of N for piglets kept under LSC was 9% units lower than for HSC piglets (Kampman-van de Hoek et al., 2016; Le Floc'h et al., 2014; Van der Meer et al., 2016; Van der Meer et al., 2020). This coincided with a 7% units lower apparent ileal N digestibility for LSC piglets than for HSC piglets, indicating that the decrease in N digestibility was already present in the small intestine. Ileal N digestibility may be affected by intestinal transit time, absorption capacity of end-products of protein hydrolysis, the quantity of endogenous N losses, or secretion and activity of gastric and pancreatic enzymes (Heo et al., 2010; Jayaraman et al., 2017; Johnson, 1997; Khan and Collins, 2005; Parra-Suescún et al., 2015). Gastric and ileal digesta MRT were similar between both sanitary conditions and digesta MRT in the duodenum/jejunum tended to be shorter for piglets kept under HSC. Moreover, only weak positive correlations were observed between digesta MRT in the stomach or small intestine and AID of N and between digesta MRT in the cecum or colon and ATTD of N, implying that digesta MRT is not the causative factor for differences in AID of N between sanitary conditions. Also, the absorption capacity of lysine, methionine, and glucose was not affected by SC and was not correlated with AID of N, indicating that absorption capacity does not explain the differences in N digestibility. The unlikely role of digesta MRT and absorption capacity as underlying mechanisms for the differences in AID of N between sanitary conditions in the current study, suggests that the quantity of endogenous losses (Adedokun et al., 2012; Schweer et al., 2018; Schweer et al., 2019; Teng et al., 2021) or secretion and activity of gastric and pancreatic enzymes (Zhang et al., 2016) play a role. Contradictory results are reported on the ileal flow of endogenous N in response to immune challenges (Adedokun et al., 2012., Schweer et al., 2018; Schweer et al., 2019; Teng et al., 2021).

The lower AID of N in HiP piglets resulted in a higher N-flow in the hindgut compared with LiP piglets, leading to more substrate available for protein fermentation. Indeed, the colonic flow of NH_3 and BCFA and the colonic N disappearance (ATTD - AID) were higher for HiP piglets than for LiP piglets. No diet x SC interaction was observed for the colonic flow of NH_3, but for BCFA the effect of feeding HiP on colonic flow was greater for piglets kept under LSC. Differences in microbial activity in LSC piglets compared with HSC piglets may have changed the production of BCFA from leucine, isoleucine, and valine (Dai et al., 2011). In addition, BCFA absorption may have been reduced (Gilbert et al., 2018), but in this study we found no differences in absorption for other nutrients.

In contrast, the lower AID of N in LSC piglets did not result in a higher N-flow in the hindgut compared with HSC piglets, due to the lower feed intake in LSC piglets. Nevertheless, the colonic flow of NH_3 and BCFA was higher for LSC piglets, caused by a combination of the numerically shorter colonic digesta MRT in LSC piglets and the numerically higher N flow. No differences in colonic N disappearance were observed between LSC and HSC. In addition, ATTD of N was lower in LSC than HSC, implying that not all additional proteins that entered the hindgut were fermented. This could be related to a limited fermentation capacity of N, or more endogenous and bacterial N losses for LSC piglets than for HSC piglets.

Except for crude protein level, we attempted to keep macronutrient concentrations equal between HiP and LiP. To correct for the added dietary fibers with sunflower seed meal in the HiP diet, sunflower hulls were added to the LiP diet. However, based on the ATTD of NSP and the increased colonic flow of SCFA in piglets fed the HiP diet, the dietary fiber fraction from the HiP diet appeared better fermentable, following the higher contents of lignin and cellulose in the hulls, rather than the more fermentable pectic substances and hemicellulose in the sunflower seed meal (Bach Knudsen, 1997; Canibe et al., 1999; Drochner et al., 2004; Lannuzel et al., 2022). This greater NSP fermentability in the HiP diet may have resulted in more N incorporation into bacterial proteins rather than being used as energy source (Pieper et al., 2014), which may have reduced the difference in colonic flow of protein-derived metabolites between piglets fed the LiP and HiP diet, and could lead to a slight overestimate of the difference between HiP and LiP in ATTD of N.

4.4. Intestinal health

In line with previous studies, from day 5 after weaning fecal consistency scores were reduced for piglets fed the HiP-diet compared with the LiP-diet (Heo et al., 2008; Heo et al., 2009; Heo et al., 2010; Wellock et al., 2006). In addition, fecal dry matter and colonic flow of NH_3 and BCFA were negatively correlated. These results indicate that protein fermentation and fecal consistency score are associated, however, this correlation might not be causal (Gilbert et al., 2019).

In addition, low sanitary conditions reduced the fecal consistency score. On days 4,5,12 and 13 fecal scores were lower for LSC piglets than for HSC piglets, on the other days except for day 10, fecal scores were numerically lower for LSC piglets. Therefore, the combination of feeding high protein diet and housing under low sanitary conditions resulted in the lowest fecal scores. Although LSC were expected to aggravate effects of HiP on fecal consistency score, based on a study by Heo et al. (2010), in which the effect of a high protein diet on diarrhea were aggravated in piglets with E. coli compared with non-infected pigs, no significant interaction between

diet x SC was observed.

Under healthy conditions, molecules less than 600 Da are able to pass the intestinal wall through paracellular transport (Watson et al., 2001). In the current study, only 0.6% of the FITC-dextrans and 0.7% of the TRITC-dextrans passed through the jejunal wall into the intestinal sac (concentration in the intestinal sac was 3% of that in the Erlenmeyer flask for FITC- and 3.4% for TRITC-dextran), which is comparable to other studies (Gao et al., 2001; Lambert et al., 2002). Only 0.8% of the FITC-dextrans and 0.9% of the TRITC-dextrans passed through the colonic wall into the intestinal sac (concentration in the intestinal sac was 4.2% of that in the Erlenmeyer flask for FITC- and 4.5% for TRITC-dextran). As the markers used in this study were relatively large (FITC 4 kDa and TRITC 40 kDa), they only passed the intestinal wall if the barrier function was compromised (Watson et al., 2001).

Jejunal permeability to FITC was increased by 8% for piglets fed the HiP diet. Although the predominant site of fermentation is the large intestine, protein fermentation also occurs in the small intestine (Miner-Williams et al., 2009; Pieper et al., 2014). Besides, it is unknown if the negative effects of protein-derived metabolites occur via direct exposure to the intestinal wall, or via the blood circulation. The increase in jejunal permeability in piglets fed the HiP diet supported our hypothesis that indigestible proteins have a negative effect on barrier function. However, numerically the dietary impact is low when compared with the differences among batches. In addition, opposite diet effects for FITC-dextrans were observed in batch 5. Hence, these dietary effects on jejunal permeability should be interpreted with care.

Remarkably, the opposite effect was observed for the large intestine, where colonic permeability was reduced for piglets fed the HiP diet. As mentioned previously, the NSP fraction from the HiP diet was found to be more fermentable compared with that from the LiP, leading to a higher colonic flow of SCFA. Since SCFA may have a positive effect on gut permeability (Khoshbin and Camilleri, 2020), we speculate that potentially negative effects of protein fermentation were compensated for the positive effects of SCFA originating from fiber fermentation.

4.5. Batch effects

Several batch effects and interactions between batch x diet and batch x SC were observed, indicating that treatment effects varied among batches. This is in line with the study of van der Meer et al. (2020), who hypothesized that the batch differences were dominated by differences related to batches of pigs and batches of feces distributed in the pens of LSC. In this study, similar to the study of van der Meer et al. (2020), housing conditions were well standardized. In addition, in the current study, the effect of different batches of feces was excluded by using one batch of homogenized feces throughout the entire study. Hence, different batches of pigs, with concurrent differences in feed intake, may have dominated the batch differences. Applying paired feeding of treatment groups standardized to the group with the lowest intake within batch, resulted in differences in average feed intake between batches ranging from 123 g/day to 159 g/day on days 4–7.

It is important to note that the direction of most treatment effects was not affected by batch; only for intestinal permeability and transport of methionine and glucose, some opposing treatment effects were observed between batches. The observed variation among batches highlights the importance of taking these batch effects into account. For relevance in translating results of trials like these into practice, the use of multiple batches is preferable over the use of a single batch in studies using models to create a contrast in sanitary conditions.

5. Conclusions

In conclusion, LSC reduce apparent ileal and fecal N digestibility substantially to a similar extent. In this study, variation in digesta mean retention time and jejunal absorption capacity were found unlikely as underlying causes, implying that endogenous losses and the secretion and activity of gastric and pancreatic enzymes deserve further study. The increased colonic flow of protein-derived metabolites indicates that more protein fermentation occurred in piglets fed HiP compared with LiP and in LSC compared with HSC piglets. However, colonic N disappearance was similar under LSC and HSC conditions. Results for some, but not all indicators of protein fermentation confirm that protein fermentation induced by HiP diets is aggravated by LSC conditions. The reduced fecal consistency scores for HiP piglets, together with the association between the colonic flow of protein-derived metabolites and fecal scores, indicate that protein fermentation and post-weaning diarrhea are associated. Intestinal permeability in the jejunum was somewhat reduced by feeding HiP; no negative effects of feeding HiP on colonic permeability were observed. The absence of diet x SC interaction for fecal consistency scores and jejunal permeability indicate that the effects of feeding HiP on fecal consistency scores and jejunal permeability were not aggravated by low sanitary housing conditions.

CRediT authorship contribution statement

Lonneke Noorman: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing.**Myrthe Gilbert:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Writing – review & editing.**Bart van der Hee:** Data curation, Methodology, Writing – review & editing. **Sonja de Vries:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing. **Walter Gerrits:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2023.115669.

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