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The effect of a diet with fructan-rich chicory roots on intestinal helminths and microbiota with special focus on *Bifidobacteria* and *Campylobacter* in piglets around weaning

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The restrictions on the use of antibiotic and anthelmintic treatments in organic pig farming necessitate alternative non-medical control strategies. Therefore, the antibiotic and parasite-reducing effect of a fructan-rich (prebiotic) diet of dried chicory was investigated in free-ranging piglets. Approximately half of 67 piglets from nine litters were experimentally infected with Ascaris suum *and* Trichuris suis *in the suckling period (1 to 7 weeks of age) and 58 of the piglets were challenged daily with* Eschericia coli *O138:F8 for 9 days after weaning to induce weaning diarrhoea. The litters were fed either chicory (30% dry matter) or a control diet. The effect of chicory on intestinal helminths, intestinal microbiota, especially* Bifidobacteria *and* Campylobacter *spp. and* E. coli *post-weaning diarrhoea was assessed. The weight gain of the piglets was not impaired significantly by chicory. The intestinal* A. suum *worm burden was reduced by 64% (*P = *0.034) in the chicory-fed piglets, whereas these same piglets had 63% more* T. suis *worms (*P = *0.016). Feeding with chicory elicited no changes among the main bacterial groups in ileum according to terminal restriction fragment length polymorphism analysis. However, the terminal-restriction fragment (T-RF) 208 bp, which may belong to* Lachnospiraceae, *was stimulated by the chicory feed (*P = *0.03), and T-RF 370 bp that matches* Enterobacter *belonging to the* Enterobacteria *was reduced (*P = *0.004). In addition, chicory increased the level of* Bifidobacteria *(*P = *0.001) and the faecal* Campylobacter *excretion level was transitorily reduced in chicory-fed piglets at 7 weeks of age (*P = *0.029). Unfortunately, it was not possible to assess the effect of chicory on post-weaning diarrhoea as it did not develop. In conclusion, feeding piglets chicory around the time of weaning caused complex changes of the microbiota and parasite communities within the intestinal tract, and feeding piglets chicory may therefore serve as an animal-friendly strategy to control pathogens.*

Keywords: *Ascaris suum*, *Campylobacter*, chicory, *Trichuris suis*, *Bifidobacterium*, weaning, diarrhoea

Implications

Feeding piglets with dried chicory roots has been suggested as a promising non-chemical approach to reduce infection levels of gastrointestinal pathogens, such as helminths and bacteria, in pigs. If successful, chicory feeding may reduce or even eliminate the need for medicines. This is of particular relevance for organic pig production.

Introduction

In conventional pig production, piglets experience major stress at weaning, often accompanied by transitory anorexia and diarrhoea, caused by, for example, *Eschericia coli* (Pluske *et al.*, 1997). Animal welfare is supposedly promoted in both free-range and organic pig production systems. However, due to the increased exposure to parasitic and bacterial pathogens in outdoor production, diarrhoea and other diseases still represent a problem (Thamsborg and Roepstorff, 2003; Jensen *et al.*, 2004). For example, *Campylobacter jejuni*, the major source of human campylobacteriosis was found in 29% of 48 organic pigs tested (Jensen *et al.*, 2006) and the presence of this foodborne pathogen and others such as *Salmonella* may impair the food safety associated with pork. Consequently, the restrictions on the use of medical treatments in organic pig farming necessitate alternative strategies to prevent diarrhoea and other diseases

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including a means to reduce the load of human pathogens. Feeding piglets chicory root is a promising non-medical strategy due to the prebiotic effect of fructans (Grizard and Barthomeuf, 1999; Bosscher *et al*., 2006). For example, experimental inulin diets reduced the worm burdens of *Oesophagostomum dentatum* and *Trichuris suis* in pigs by 97% and 71%, respectively (Petkevičius *et al*., 2003 and 2007), and a recent study has demonstrated a marked effect of crude chicory on *Ascaris suum* (Mejer, 2006). Furthermore, a fructan-rich diet was successful in preventing the development of swine dysentery (Thomsen *et al*., 2007), and inulin-supplemented diets reduced the incidence of post-weaning diarrhoea in conventional-weaned pigs (Halas *et al*., 2009).

This study aimed to assess whether dietary supplementation with chicory helps to alleviate the severity of post-weaning diarrhoea in piglets experimentally exposed to enterotoxigenic *E. coli* and intestinal helminths. Furthermore, it aimed to assess whether chicory affects the intestinal microbiota, particularly bifidobacteria, and whether chicory may serve as a means to reduce the load of the important human pathogen *Campylobacter*.

Methods

Pigs and paddocks

Nine helminth-free gestant Danish Landrace/Yorkshire sows all mated with Duroc boars were purchased from a specific pathogen-free indoor herd. Upon arrival at the research farm early July 2006, the sows were placed in a common clover-grass paddock (15×30 m, house 24.8 m^2) to allow for 39 ± 0.6 (s.e.) days to adapt to the outdoor environment before farrowing. The sows were vaccinated twice (5 to 6 and 2 to 3 weeks before farrowing) against colibacillosis (Toxicol®, Orion Pharma, Nivå, Denmark), dewormed with fenbendazole (Panacur®, Intervet, Skovlunde, Denmark) 12 ± 0.6 (s.e.) days before farrowing, and subsequently allocated to individual farrowing paddocks (10×24 m, farrowing hut 4.8 m^2) 1 week before the expected farrowing (Figure 1). After farrowing (week 0), the 4 to 11 piglets in each of the nine litters (in total 67 piglets) were allocated randomly to treatments (see below) within litters and stayed with the sow for 7 weeks before removal of the sow (weaning). After another 2 days in the farrowing paddocks, piglets were relocated into five weaning paddocks (4×15 m, house 24.8 m^2) according to experimental treatments. None of the paddocks had ever been used for pigs before. All pigs had free access to water, an insulated house with straw bedding and a wallowing area. The experiment was performed according to the law on animal experimentation and approved by the Danish Animal Experimental Inspectorate (license: 2005/561-1060).

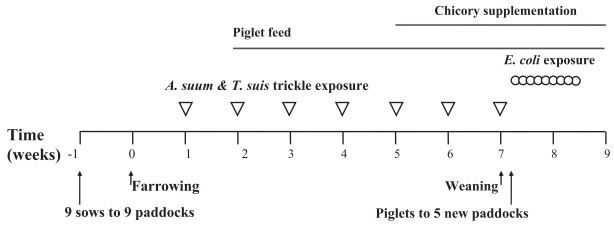

Figure 1 Time scale for the study, which included nine sows and their litters. All piglets were provided piglet feed from 2 weeks of age. From 5 to 7 weeks of age, four out of nine litters had chicory added to the piglet feed, and these piglets continued on a chicory-supplemented feed after reallocation into five new paddocks after weaning. In each litter, 29% to 56% of the piglets were trickle infected with *Ascaris suum* and *Trichuris suis* in weeks 1 to 7. Pigs in all but one paddock were trickle infected with *Eschericia coli* after weaning.

Experimental protocol

The experimental design is outlined in Figure 1. During the suckling period, 29% to 56% of the piglets in each of the nine litters (31 piglets in total) were trickle-infected orally with *A. suum* (25 eggs/kg live weight per day) and *T. suis* (10 eggs/kg live weight per day) once a week when aged 1 to 7 weeks. In total, each piglet was given 7 doses containing a total of 13 000 *A. suum* and 5300 *T. suis* eggs. The *A. suum* eggs were isolated from the uteri of female worms recovered at an abattoir and embryonated in 0.05 M H_2SO_4 (pH = 1). The *T. suis* strain was originally isolated from faeces collected from an organic herd, embryonated in vermiculite and stored in 0.005 M H_2SO_4 (pH = 2). The strain has been passed through pigs several times since the initial strain was obtained.

All 67 piglets were provided with piglet feed (control feed, see details below) from 2.5 weeks of age and onwards. From week 5, this feed was supplemented with chicory (see details below) for four out of the nine litters (30 piglets in total).

Two days after removal of the sow, piglets (7 weeks and 2 days old) were moved to the five weaning paddocks according to their feeding regimens (groups fed chicory *v.* control feed referred to as CHIC and CTRL, respectively) and parasite (groups with parasite exposure *v.* no exposure referred to as +PAR and −PAR, respectively) resulting in the following four main groups: (i) CTRL + PAR, 15 piglets; (ii) CTRL − PAR, 13 piglets; (iii) CHIC + PAR, 16 piglets; (iv) CHIC − PAR, 14 piglets. On the day of reallocation of the piglets 2 days post-weaning, all piglets in groups 1 to 4 were given an infection dose equivalent to 10^8 CFU *E. coli* O138:F18 challenge strain 9910297-2^{STM} (Frydendahl *et al*., 2003) per piglet in the morning meal, which was repeated daily the following 9 days. One additional group (Group 5, CTRL − PAR − *E. coli*) consisted of nine piglets on control feed without exposure to parasites or *E. coli*, thereby serving as a non-*E. coli* control for spontaneous post-weaning diarrheoa. At 9 weeks of age, the piglets were euthanized by stunning with a captive bolt and exsanguination, followed by parasite recovery, *E. coli* detection and general bacterial DNA extraction.

Feeding and feed analyses

All sows were given a feed consisting of a commercial supplement (33%) for sows and ground barley (67%) throughout the study. The sows were fed restrictively up to farrowing, thereafter feeding was gradually increased to *ad libitum*. All nine litters were given *ad libitum* access

Table 1 *Ingredients and chemical composition of two diets fed* ad libitum *to piglets weeks 5 to 9 of age; weaning week 7*

	Diet	
	Control	Chicory
Dry matter (%)	88.9	90.3
Ingredients (g/kg)		
Wheat	315	315
Dried chicory root	–	300
Barley	300	–
Oat	135	135
Fish meal	131	131
Potato protein concentrate	50	50
Casein whey powder	35	35
Rape seed oil	10	10
Vitamins and minerals	24	24
Calculated chemical composition (g/kg dry matter)		
Protein	223	202
Fat	46	36
Ash	69	77
Low molecular weight sugars		
Glucose, sucrose and fructose	14	47
Fructans (inulin)	12	160
Starch	417	261
Dietary fibre	143	160
Net energy (MJ/kg)	9.70	9.65

to feed during weeks 2.5 to 5, which consisted of a commercial pelleted feed for piglets (70%) and ground barley (30%; standard feed; Table 1). However, in weeks 5 to 7, the barley was substituted with dried chicory roots (30%) for four litters. After weaning (weeks 7 to 9), three out of the five experimental groups (Groups 1, 2 and 5) continued on the control feed (37 piglets in total), whereas two groups (Groups 3 and 4) continued on the chicory feed (30 piglets in total). This feed was administered twice daily according to live weight. All feed components were organically produced, including dried chicory roots (Hansen *et al*., 2006). The composition of protein, fat ash, low molecular weight sugars (sucrose, fructose, glucose and fructans), starch and dietary fibres (non-starch polysaccharides and klason lignin) in the control and chicory feed was analysed as described by Thomsen *et al*. (2007) and presented in Table 1. The total energy content was estimated as by Thomsen *et al*. (2005) and also shown in Table 1.

Sampling of faeces and clinical examination

Rectal faecal samples for enumeration of *Campylobacter* spp. were collected when the piglets were 4, 7 and 9 weeks old, whereas samples for parasite faecal egg counts were collected when the piglets were 6, 6.5, 7, 8 and 9 weeks old. Rectal faecal samples for enumeration of *E. coli* were collected daily during the 9 days of *E. coli* challenge.

The consistency of faeces from each piglet was visually assigned a score from 0 to 3 as follows: (0) normal/firm, (1) soft, (2) liquid/runny and (3) watery. The piglets were weighed weekly from time of birth until the age of 9 weeks. All piglets were observed daily for clinical symptoms during the whole study.

Parasitological examination

Faecal egg counts were enumerated using the concentration McMaster technique of Roepstorff and Nansen (1998). The flotation fluid (saturated NaCl with 500 g glucose monohydrate/l) had a high specific gravity ($\rho = 1.27$ g/ml) and the lower detection limit was 20 eggs/g faeces.

For recovery of *A. suum*, the small intestine was opened and emptied. The mucus was scraped off the intestinal mucosa, which was washed in 0.9% NaCl solution. The combined contents, mucus and saline were embedded 1 : 1 in 2% agar followed by incubation in 0.9% NaCl at 37°C (Slotved *et al*., 1997) for 3 h. The *A. suum* that migrated out of the agar were collected using a 20 μm sieve and stored in 70% ethanol.

For recovery of *T. suis*, the large intestine was opened and washed in 0.9% NaCl. The contents and saline were mixed and diluted with water to a total volume of 10 l. A 10% subsample was washed on a 212 μm sieve for recovery of lumen-dwelling *T. suis*. The sieved material was stored in iodine (80 g iodine and 400 g potassium iodine in 800 ml distilled water) and decolourised by 30% sodium thiosulphate before examination. Immature *T. suis* were recovered from the intestinal mucosa by incubation of the intestinal tissue in 10 mM EDTA in 0.9% NaCl solution (pH 7) at 37°C overnight (Kringel *et al*., 2002). Released larvae were collected using a 90 μm sieve and stored in 70% ethanol.

Enumeration and isolation of E. coli *and* Campylobacter *spp.*

The faecal samples for enumeration of the *E. coli* O138 challenge strain were cooled during transport to the laboratory and subsequently stored at −18°C until testing according to Frydendahl *et al*. (2003). The faecal material was suspended in phosphate buffered saline (10 ml/g) and homogenised by stomaching. Serial 10-fold dilutions were prepared and 100 μl plated on blood agar with streptomycin (Sigma-Aldrich Denmark A/S, Brøndby, Denmark) 100 μg/ml. After aerobic incubation at 37°C overnight, the number of CFU was determined. From each positive sample, two colonies were confirmed as O138 by slide agglutination.

The faecal samples for enumeration of *Campylobacter* spp. (from 4, 7 and 9-week-old piglets) were stored at 4°C until testing the next day. Tenfold dilution series of faeces (1 g) were cultured by direct plating on modified charcoal-cefoperazone-deoxycholate agar plates (mCCDA; Campylobacter Blood Free Selective Agar Base, Oxoid, Basingstoke, UK) with CCDA Selective Supplement (Oxoid, SE155E, UK). After incubation of mCCDA plates (48 h at 41.5°C; microaerobic conditions), *Campylobacter* spp. colonies (based on colony and cell morphology by microscopy) from direct plating were counted (detection limit 100 CFU/g).

DNA extraction

Total DNA was extracted from the ileal content of the 56 piglets in the four groups trickle-infected with *E. coli* by the

stool DNA extraction kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions except for an added bead beating step. Essentially, 200 mg of intestinal content were suspended in 1.4 ml of Buffer ASL (Qiagen) and homogenised with 400 mg of 100 μm Zirconia-silica beads (BioSpec Products Inc., Bartlesville, USA) using a minibead-beater at high speed for 3 min (Biospec Products Inc.). Subsequently, the suspension was centrifuged (20 000× **g**, 1 min), the supernatant was transferred to a new Eppendorf tube, and the stool kit procedure continued after the lysis step in the protocol where the InhibitEX tablet is added. The DNA was stored at −20°C until further analysis.

Terminal restriction fragment length polymorphism analyses
Terminal restriction fragment length polymorphism (T-RFLP) profiles and analyses were performed as described by Mølbak *et al.* (2007). In short, extracted total DNA was adjusted by spectrophotometry to a concentration of 5 μg of DNA/ml. Four replicate 50-μl PCR mixtures were made from each sample using the universal bacterial primers S-D-Bact-0008-a-S-20 (5′-AGAGTTTGATCMTGGCTCAG-3′; Leser *et al.*, 2002) and S-D-Bact-0926lam-a-A-20 (5′-CC GTCAATTCCTTTRAGTTT-3′; Mølbak *et al.*, 2007). Primer S-D-Bact-0926lam-a-A-20 was 5′FAM (carboxy-fluorescein-N-hydroxysuccinimide-ester-dimethyl-sulfoxide) labelled. Purified PCR products (200 ng) were digested with 20 U of *Hha*I (Boehringer, Mannheim, Germany) restriction enzyme in 20-μl reaction mixtures for 3 h at 37°C. The four PCR replicates were pooled into two samples, which were analysed by electrophoresis on an automatic sequence analyzer (ABI-PRISM-373-DNA-Sequencer; PE Biosystems, Foster City, CA, USA). ABI traces were imported into BioNumerics version 4.5 (Applied Maths, Sint-Martens-Latem, Belgium) and aligned using internal standards. Terminal-restriction fragments (T-RFs) were found by autosearch at a position tolerance of 0.2, and only T-RFs between 35 and 625 bp observed in both PCR duplicates were included in the succeeding analysis. The mean relative intensity of each T-RF within a sample was calculated by dividing the area of that individual peak with the total area of all peaks on the electropherogram. To identify possible bacterial species corresponding to the individual T-RFs in this study, a virtual digest was done on a previously published 16S rRNA gene bacterial clone library from pigs (Leser *et al.*, 2002) by inserting the primer sequences and restriction enzymes used in this study into the online program silico MiCA virtual digest (Shyu *et al.*, 2007). The 16S rRNA gene sequences matching significant T-RFs for either treatment group were classified to bacterial groups by using the classifier function in RDP Release 10 (http://rdp.cme.msu.edu/classifier/classifier.jsp). The Shannon–Weaver diversity index (H′) was calculated for each treatment group as $H' = -\Sigma p_i \ln(p_i)$, where p_i is the number of different T-RFs in each animal.

Quantitative real-time PCR of target groups of organisms
The DNA amounts of *E. coli* O138:F18, *Bifidobacterium* spp. and total bacteria in samples from ileum were measured according to published quantitative real-time PCR (qPCR) assays, using the same primers and similar PCR conditions (Frydendahl *et al.*, 2001; Palmer *et al.*, 2007; Delroisse *et al.*, 2008). For each analysis, 10 ng of total DNA was used as template. The samples were run in triplicates. In each assay, a standard curve with known DNA concentrations was used, and sterile, filtrated water (0.2 μm) was used as negative controls. In the qPCR assays, measuring the *E. coli* O138:F18 and the total amount of bacteria, respectively, 10-fold serial dilutions of DNA from *E. coli* O138:F18 was used for the standard curve. In the bifidobacteria qPCR assay, DNA from *B. thermophilium* was used. The relative amount of bacteria was estimated from the C_t values compared with the standard curves.

Statistical analysis
The effect of chicory supplementation on worm counts was analysed using logistic regression taking litter variation into account using the generalised estimating equation (GEE) approach with the working independence assumption (Liang and Zeger, 1986).

Logistic regression was also used to analyse diarrhoea, taking into account that we had multiple measurements per pig using GEE with the working independence assumption. The included factors were time, chicory (treatment), parasites, sex and baseline. It was not possible to run the model when including interactions with time, and hence only main effects were considered. Time and parasites were the only significant factors and therefore kept in the model, whereas the other factors were removed. For testing the effect of chicory on *Campylobacter* excretion levels, a mixed model of analysis was applied with random piglet and litter effects. For the repeated measurements on growth, we also applied a linear mixed model with ante (1) as the best fitting model (Diggle *et al.*, 1994). These statistical analyses were performed by SAS version 9.1.3 (SAS Institute Inc., Cary, NC, USA). For comparison between the individual T-RFs from the four main groups and between the chicory and control treatments and piglets exposed or not exposed to parasites, we used two-tailed Monte Carlo estimates from the non-parametric analysis (Mann–Whitney U-test and Kruskal–Wallis tests). T-RFs smaller than 35 bp and larger than 625 bp were excluded as these fragments were outside the range of the standards. This statistical analysis was performed by SAS Enterprise Guide 3 (SAS Institute Inc.).

Analysis of variance (ANOVA) was used to test if the DNA concentration measured by real-time qPCR assays of either total bacterial DNA or bifidobacteria DNA were different between treatments. ANOVA was performed in Microsoft Office Excel 2003 (Microsoft Denmark APS, Hellerup, Denmark). $\alpha = 0.05$ was used as the critical level of significance.

Results

Clinical observations and production results
At weaning, the nine litters were composed of 4, 6, 6, 7, 7, 8, 9, 9 and 11 piglets, with a total of 67 piglets, of which three

piglets from group 2, 3 and 5, respectively, were euthanized because they were moribund approximately 1 week before termination of the experiment at 9 weeks of age. The weight gain of the piglets between 5 to 9 weeks of age tended to be negatively affected by parasite exposure ($P = 0.059$) and to some degree by chicory supplementation ($P = 0.1$). The parasite infected pigs on the CTRL and CHIC diet gained 450 g/day and 400 g/day, respectively, while the non-parasite pigs on the CTRL or CHIC diet gained 470 g/day and 430 g/day, respectively.

Post-weaning E. coli *challenge*

Before the first challenge with *E. coli* all piglets were negative for haemolytic *E. coli*. During the 9 days of challenge and at termination of the study, *E. coli* O138 was only reisolated once from each of the five pigs (with 6.5×10^2 CFU/g as a maximum) from the groups CTRL − PAR (two pigs, days 2 and 7), CHIC − PAR (two pigs, day 5) and CTRL − PAR− *E. coli* (one pig, day 5), and was not detected in the small intestine by real-time PCR analysis at the termination of the study. In addition, none of the challenged piglets developed watery diarrhoea (faecal score 3) nor showed other clinical symptoms of post-weaning diarrhoea.

Parasitology

Neither *A. suum* nor *T. suis* eggs were recorded in any of the faecal samples during the study. Worm counts within the four main treatment groups are shown in Figure 2a (*A. suum*) and 2b (*T. suis*). A low level of contamination of the uninfected piglets was noted; 52% and 70% of piglets in groups CTRL − PAR and CHIC − PAR harboured low numbers of *A. suum* (mean abundance: one worm/pig) and *T. suis* (mean abundance: six worms/pig), respectively. In groups CTRL + PAR and CHIC + PAR, 84% to 87% of piglets harboured much higher worm burdens (*A. suum* mean abundance: 29 worms/pig; *T. suis* mean abundance: 148 worms/pig).

Inclusion of dried chicory roots in the piglets' feed reduced the *A. suum* mean abundance by 64% as compared with no chicory ($P = 0.034$) and the odds ratio (OR) indicated an overall 4.3 95% CI = (1.1, 16.8) times lower risk of piglets having *A. suum*, if given chicory. Apart from one female (17 cm), all of the recovered *A. suum* were immature (most worms measured only 0.5 to 1 cm). Similarly, almost all *T. suis* were immature. However, the risk of having *T. suis* was 3.5 95% CI = (1.3, 9.6) times higher for piglets fed chicory (group CTRL + PAR *v.* CHIC + PAR: $P = 0.016$; equal to a 63% higher mean abundance) according to OR.

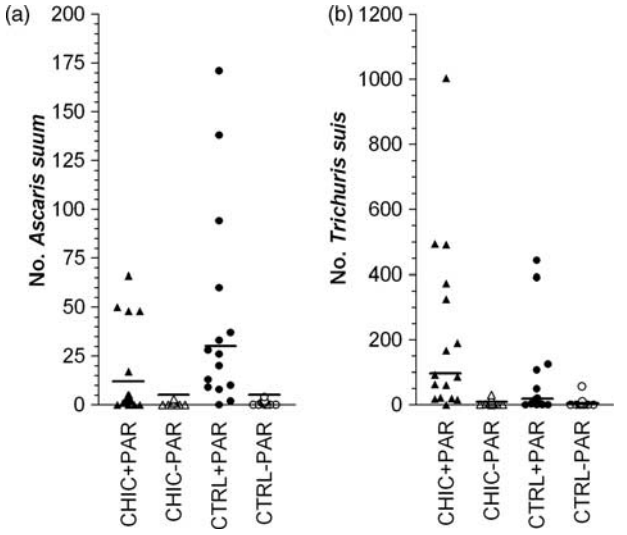

Figure 2 Individual *Ascaris suum* (a, $P = 0.034$) and *Trichuris suis* (b, $P = 0.016$) worm burdens of 9-week-old pigs ($n = 58$) given different treatments. Initially, all four groups were given the same control feed, but from the age of 5 to 9 weeks, two groups were given a chicory-supplemented feed. One group of each feed treatment was trickle infected with parasites from 1 to 7 weeks of age. Horizontal bars show the geometric mean.

Faecal consistency scores

Clinical post-weaning diarrhoea did not develop, but a softening of the faeces was observed and the highest frequency of animals with scores >0 was recorded on days 4 to 7 post-weaning. Overall, 64% of piglets not experimentally infected with parasites (groups CTRL − PAR and CHIC − PAR) had faecal scores >0 at one or more occasions after weaning, whereas the same was the case for 94% of the infected piglets (group CTRL + PAR and CHIC + PAR). The OR correspondingly indicated a 2.5 times higher risk for softer faeces after weaning, if the piglet had been experimentally exposed to *A. suum* and *T. suis* ($P = 0.005$). The mean faecal scores for non-exposed piglets (groups CTRL − PAR and CHIC − PAR) and exposed piglets (groups CTRL + PAR and CHIC + PAR) are depicted in Figure 3.

T-RFLP and real-time PCR analysis of the microbiota

The bacterial microbiota in the ileum of piglets from the four different treatments was assessed by T-RFLP analysis. A total of 59 T-RFs were found in the range of 38 to 612 bp, of which the 18 most abundant T-RFs are shown in Figure 4. The average number of T-RFs in the four different treatment groups were 27 (CTRL + PAR), 25 (CTRL − PAR), 18 (CHIC + PAR) and 20 (CHIC − PAR). Fewer T-RFs were observed in the piglets fed chicory when compared with the piglets on the control diet ($P = 0.02$). Furthermore, the

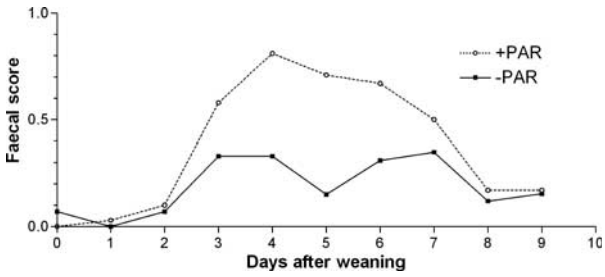

Figure 3 Mean faecal consistency scores after weaning (7 weeks of age) of piglets experimentally infected weekly with *Ascaris suum* and *Trichuris suis* from age 1 to 7 weeks (+PAR, $n = 31$) or uninfected (−PAR, $n = 36$). Scores were visually assigned from 0 to 3 as follows: (0) normal/firm, (1) soft, (2) liquid/runny and (3) watery.

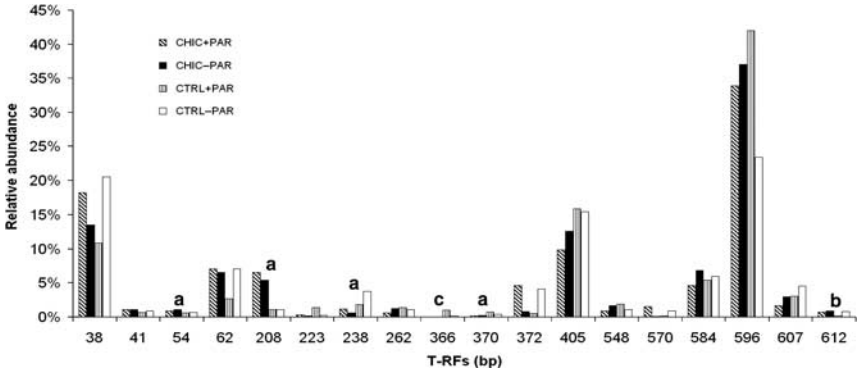

Figure 4 Average relative abundance of specific terminal-restriction fragments (*Hha*I T-RFs) as measured by the T-RFLP analyses of the piglets from four different treatments ($n = 56$). The figure presents only T-RFs found with a frequency higher than 1% of the total intensity. The medians of the T-RFs were different ($P < 0.05$) (a) between the pigs fed chicory or control diets, (b) between the pigs exposed or not exposed to parasites and (c) across the four treatment combinations. See Table 2 for further information about the potential strain matches of the individual T-RFs.

Table 2 *T-RFLP identification of differences in potential intestinal bacterial groups in 9-week-old pigs depending on their feed and parasite exposure (treatment)*[a]

Treatment	Significance level	*Hha*I[b] (bp)	No. hits in clone library[c]	Accession no.	Classification of matching clones[c]
Feed	$P = 0.004$	54	0	–	–
Feed	$P = 0.03$	208	1	AF371594	*Lachnospiraceae*
Feed	$P = 0.003$	238	0	–	–
Feed	$P = 0.004$	370	4	AF371851, AF371852	*Enterobacter*
Parasites	$P = 0.001$	612	1	AF371480	*Weissella*
Across treatments	$P < 0.0001$	366	2	AF371805	*Ruminococcaceae*
				AF371857	*Pasteurella*

T-RFLP = terminal restriction fragment length polymorphism.
[a]Pigs were fed two different diets from weeks 5 to 9 (chicory *v.* control), and were either exposed (+PAR) or non-exposed (−PAR) to parasites (*Ascaris suum* and *Trichuris suis*; see Figure 4 for further details).
[b]*Hha*I base pairs detected through T-RFLP analysis that vary significantly (week 9) between treatments[a] depending on either feed type, exposure to parasites or across the four treatment combinations.
[c]The T-RFs potential matches were found from a search in a previous published 16S rRNA gene pig clone library (Leser *et al.*, 2002).

two groups of piglets fed chicory had a lower Shannon–Weaver diversity index compared with the control groups (CTRL + PAR $H' = 3.95$; CTRL − PAR $H' = 3.87$; CHIC + PAR $H' = 3.74$; CHIC − PAR $H' = 3.75$).

The T-RFLP is a semi-quantitative analysis in which the peak of each T-RF reflects the abundance of the current bacteria group represented by that T-RF. For the most abundant bacteria, there were no differences between the four treatments (Figure 4). In contrast, the composition of six of the less-abundant bacterial groups depended on the treatment (Figure 4). Five of these T-RF bacterial groups (54, 208, 238, 366 and 370 bp) were affected by the chicory diet, while two bacterial groups (T-RF: 366 and 612 bp) were affected by the parasites (Table 2). The chicory feeding led to a reduction of T-RF 370 bp, which according to the clone library of pigs' intestinal bacteria (Leser *et al.*, 2002) matches *Enterobacter* belonging to the enterobacteria (Table 2). T-RF 208 was more abundant in piglets fed chicory compared with control piglets ($P = 0.03$) and Table 2 shows that the T-RF 208 bp matches the *Lachnospiraceae* family, which includes the typically fermenting genera: *Butyvibrio*, *Reseburia* and *Ruminococcus.*

Table 3 *Real-time PCR quantification of bacterial DNA and bifidobacterial DNA in ileum of pigs fed with CHIC or CTRL feed and either exposed or not exposed to parasites (+/−PAR)*

	Threshold cycle, (C_t[a])[b]	
Diet	Bacterial DNA	Bifidobacterial DNA
CHIC + PAR	13.00 (2.78)	10.23 (3.52)[c]
CHIC − PAR	13.62 (1.99)	8.54 (2.24)[c]
CTRL + PAR	13.81 (2.32)	7.27 (2.68)[d]
CTRL − PAR	13.87 (2.18)	6.39 (2.30)[d]

[a]C_t threshold cycle reflects the cycle number at which the fluorescence generated within the PCR reaction crosses the threshold. C_t is inversely correlated to the logarithm of the initial copy number.
[b]The number in parentheses is s.e. of the mean.
[c,d]C_t means with different superscripts differ ($P < 0.001$).

The total number of bacteria, as quantified by qPCR, was not significantly different among the four treatment groups, whereas quantification of *Bifidobacterium* spp. in the ileum by qPCR showed a higher level of these bacteria in the chicory-fed piglets ($P = 0.001$; Table 3).

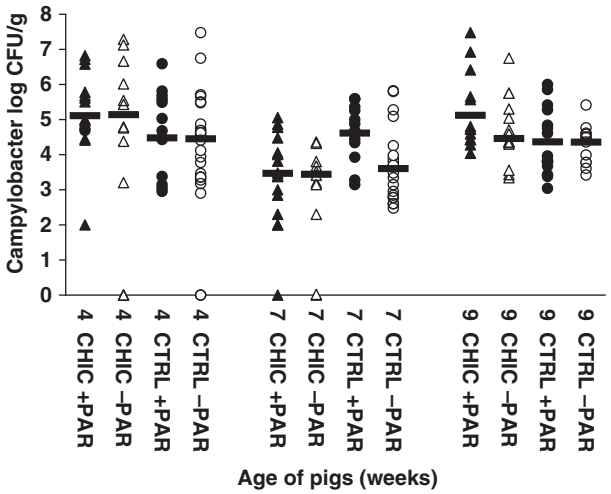

Figure 5 *Campylobacter* spp. excretion levels in piglets at 4, 7 and 9 weeks of age grouped according to feeding (chicory or control feed from 5 to 9 weeks of age) and parasite exposure (*Ascaris suum* and *Trichuris suis* infection from 1 to 7 weeks of age (+PAR) or no infection). There was an effect of chicory, time and parasites ($P < 0.05$), with an interaction between time and chicory ($P = 0.058$). Horizontal bars show the geometric mean.

Campylobacter *spp. excretion level*

Campylobacter spp. was found in 161 of the 172 samples (93.6%) examined in total from the piglets at 4, 7 (58 piglets) and 9 weeks (56 piglets) of age. By the end of the experiment, all piglets except one (which was euthanized at 8 weeks of age as it was moribund) had been *Campylobacter* positive at least once; the excretion levels are shown in Figure 5. The effect of chicory on the *Campylobacter* excretion level interacted with time, though not significant at the 5% level ($P = 0.058$). However, this interaction showed that the *Campylobacter* excretion at 7 weeks of age had decreased by 1 log in the piglets that have been fed with chicory for 2 weeks as compared with the control diet ($P = 0.029$). After another 2 weeks of feeding with chicory, at 9 weeks of age, the *Campylobacter* excretion in the chicory-fed piglets was similar to that found in the pigs fed the control feed ($P = 0.93$).

Piglets experimentally infected with *A. suum* and *T. suis* (+PAR) showed a higher *Campylobacter* excretion level (+0.31 log CFU/g) than non-infected piglets (−PAR; $P = 0.017$).

Discussion

This study found that the fructan-rich (dried chicory roots) diet lowered the small intestinal *A. suum* worm burden and increased the large intestinal *T. suis* worm burden in 9-week-old weaners, reduced the faecal *Campylobacter* excretion at 7 weeks of age and had a positive bifidogenic effect in the ileum. Furthermore, inoculations according to an otherwise well-established *E. coli* O138:F8 infection model (Frydendahl *et al*., 2003) did not give rise to infections in piglets weaned at 7 weeks of age and there were no signs of weaning diarrhoea.

The overall energy content was identical in the two feed types, and the current dietary level of 30% dried chicory did not impair the growth significantly, which is in agreement with previous observations (Hansen *et al*., 2006). The protein level was slightly lower (20.2%) in the chicory diet when compared with the control diet (22.3%). This difference in the protein level was unintended and may theoretically have influenced the results by reducing the immune responses of the chicory groups, as previously demonstrated for induced long-term severe protein deficiency (51% reduction in the diets protein level), on *T. suis* and *A. suum* infections (Pedersen *et al*., 2002). However, because the chicory-fed piglets still reached an almost optimal protein level, this difference is considered of minor importance.

A. suum *and* T. suis *infections*

The growth of the piglets appeared to be affected by the parasite infections, though this was not significant. In contrast, other studies have demonstrated marked effects by both *T. suis* and *A. suum* on feed conversion and growth (Hale and Stewart, 1979; Hale *et al*., 1985), which may be due to a heavier parasite exposure and more large worms than in this study, in which the large majority of worms were small, immature worms.

Inclusion of 30% dried chicory root in the diet (≈16% fructans) reduced the number of immature *A. suum* recovered from the small intestine by 64% in the current trickle-exposure study. Similarly, immature *A. suum* worm burdens have previously been reduced by 55% to 73% by crude chicory root (≈14% fructans), dried chicory root (≈16% fructans) or inulin (commercial purified fructans, ≈17% fructans) in pigs slaughtered 13 to 15 days after a single *A. suum* dose (Mejer, 2006). As suggested for the large intestinal parasites *O. dentatum* and *T. suis* (Petkevičius *et al*., 2004; Thomsen *et al*., 2005), *A. suum* may have been negatively affected by changes in the physico-chemical conditions (e.g. higher production of short-chain fatty acids (SCFA) and lower pH) in the caecum and proximal colon, as these sites are where the newly hatched larvae invade the mucosa before migrating through the liver and lungs and returning to the small intestine (Murrell *et al*., 1997). These changes of living conditions are likely to occur as the caecum and proximal colon are also the main sites for bacterial degradation of carbohydrates, yielding substances such as SCFA and lactic acids (Jensen and Jørgensen, 1994). *O. dentatum* has thus been shown to be almost eliminated from the intestinal tract after infusion of purified SCFA and LA directly into the caecum (Petkevičius *et al*., 2004).

The effect of easily fermentable carbohydrates on *T. suis* infections has in previous studies varied from insignificant (H. Mejer, unpublished data) to different degrees of adverse effects resulting in reduced parasite establishment, persistence, growth and/or fecundity (Thomsen *et al*., 2005 and 2007; Petkevičius *et al*., 2007). However, in this study, the *T. suis* infection levels increased in piglets fed chicory. This overall variable effect on *T. suis* may partly reflect that levels and sources of fermentable carbohydrates as well as the

other feed components varied between studies. Fermentation patterns may therefore have varied accordingly, thus comparisons between studies are complicated. In addition, it should be noted that the regulation of *T. suis* as well as *A. suum* infections is very complex. Both parasites are highly immunogenic so that a high initial establishment of even a single infection is followed by a host-mediated expulsion of the large majority of *A. suum* worms (Roepstorff *et al.*, 1997) or all *T. suis* worms (Kringel and Roepstorff, 2006).

In this study, the infection levels and the population composition in the control groups indicated that the onset of acquired resistance (i.e. expulsion of worms) had begun by the time of slaughter. The current higher recovery of *T. suis* in chicory-fed piglets could thus potentially be the result of a low initial establishment (because of the chicory feeding) causing a delayed onset of those immune mechanisms, which are required to expel the worms. In addition, it has been demonstrated that both *T. suis* and *O. dentatum* worms can be severely stunted by a diet supplemented with easily fermentable carbohydrates (Thomsen *et al.*, 2005; Mejer, 2006). Therefore, the apparent higher *T. suis* worm burdens in this study may possibly result from a delay in the gradual development and emergence of larvae from the intestinal mucosa before establishment in the intestinal lumen.

Infecious *E. coli* has mainly been found adhered to the small intestinal mucosa in the gastrointestinal tract (Kenworthy and Crabb, 1963), and in a study by Halas *et al.* (2009), they found a prebiotic effect of an 8% inulin diet given to pigs infected with *E. coli* O149:K91;K88 by improving the faecal consistency and reducing the incidence of post-weaning diarrhoea. In this study, faecal bacterial excretion and weaning diarrhoea did not develop after repeated inoculations with *E. coli* O138:F18, an infection model established by Frydendahl *et al.* (2003), even though the piglets were expected to be susceptible to the infection and they received daily infection doses of 10^8 CFU *E. coli* O138:F18 for nine consecutive days post-weaning. It is not the aim of this study to experimentally identify the reasons for this lack in establishment of the *E. coli* challenge infections among the piglets. However, it may be speculated to be a combination of microbial colonisation resistance against *E. coli* O138:F18 and a more developed immune system of free-range piglets weaned at 7 weeks of age compared with conventional piglets weaned at a younger age. The lack of establishment and thus lack of induction of post-weaning diarrhoea made it impossible to measure any potential effect of chicory and intestinal helminth infections on *E. coli* O138:F18 excretion and clinical course.

The bacterial community structure was described by using the well-established method, T-RFLP. Since each terminal-restriction fragment (T-RF) ideally represents a single species or taxonomic group of bacteria, it is possible to relate the empirically found T-RFs to theoretically digested 16S rRNA genes of bacteria. The T-RFLP analysis only showed a moderate effect of the fructan-rich diet on the microbiota in the ileum. However, several of the less abundant T-RFs (54, 208, 238, 366 and 370 bp) showed significant differences in peak intensities among the groups. In a previous study, it has been shown that pigs fed a fructan-rich diet had a higher proportion of *Bifidobacterium thermacidophilum* and *Megasphaera elsdenii* in the colon (Mølbak *et al.*, 2007). In this study, we found 10 times or more bifidobacteria in the ileum of the piglets fed the fructan-rich diet (16.0% fructan) compared with the other diet (1.2% fructan). This is to the best of our knowledge the first time a bifidogenic effect is seen in the ileum of pigs. Bach Knudsen and Hessov (1995) found that the recovery of inulin from the small intestine in seven human subjects with ileostomy was 87%. They argued that the 13% loss of inulin during passage through the small intestine was due to hydrolysis by either acids or enzymes and to microbial degradation by the microbiota permanently colonizing the ileum. The suggestion of a microbial degradation was supported by our findings in the chicory-fed piglets, which had an increase in both *Bifidobacteria* and bacteria belonging to *Lachnospirace*, which contains *Butyrivibrio* spp. and *Roseburia* spp. that previously have been reported to degrade fructans (Duncan *et al.*, 2006, Falony *et al.*, 2006). It is difficult to say how efficiently these bacteria fermented the chicory in the ileum, what the end metabolites were, and to what extent they provided energy to the epithelial cells. However, it is interesting that *Megasphera* was not involved in the fermentation as we previously have seen in the colon. Therefore, this study indicated that the fermentation of chicory taking place in the ileum is different from the fermentation known from colon. Future experiments could elucidate whether this difference in fermentation is due to the intestinal environments, diets or experimental set up. Halas *et al.* (2009) showed that the inulin diet considerably increased the total concentration of LA in the caecum and colon of the pigs. Although the concentration of LA was not measured in this study, an increase in LA concentration in the ileum is plausible, which would cause a drop in pH. Such a restricted intestinal environment may explain why the chicory-fed piglets in this study had a lower microbial diversity as compared with the non-chicory-fed piglets. This reduced bacterial diversity may also partly be due to a higher ileal colonisation resistance against potential pathogenic organisms such as enterobacteria, which according to the T-RFLP analysis were reduced in the chicory-fed piglets (T-RF 370 bp in Table 2).

Campylobacter *spp.*

All but one piglet in this study was *Campylobacter* spp. positive at least once. This is in concordance with the general finding of high *Campylobacter* spp. prevalence in pigs and that colonisation may occur within the first day of life (Young *et al.*, 2000; Jensen *et al.*, 2006). However, in the 7-week-old piglets fed chicory for 2 weeks, the *Campylobacter* spp. excretion had decreased by 10-fold as compared with the control piglets. To our knowledge, the effect of chicory on *Campylobacter* spp. excretion has not previously been tested in pigs, but chickens fed with fructooligosaccharides have been shown to have a lower *C. jejuni* colonisation (Schoeni and Wong, 1994). However, the potential

Campylobacter-reducing effect of chicory, observed after 2 weeks of chicory feeding, seemed to disappear over time; after 4 weeks of feeding, the *Campylobacter* spp. excretion level had returned to the level found in the control piglets. *Campylobacter* only constituted a minor part of intestinal microbiota as it was not among the 59 T-FRs derived from the ileum at the time of slaughter, although *Campylobacter* has been shown to reside there (Weijtens *et al*., 1993). The specific mechanism behind the transitory *Campylobacter* reduction is unknown. *Campylobacter* numbers were probably affected by the changing microbiota and fermentation patterns, but adaptations to these changes may have occurred. It has been suggested that bifidobacteria excrete an anti-microbial substance that affects *Campylobacter* and other bacteria (Gibson and Wang, 1994). Furthermore, administration of *Bifidobacteria longum* to poultry as a probiotic for 2 weeks was shown to reduce their *C. jejuni* concentration within that period (Santini *et al*., 2010).

The experimental parasite infection lead to an increased *Campylobacter* spp. excretion level, which supports previous suggestions that parasites can facilitate pathogen invasion. Germ-free piglets given co-infections of *C. jejuni* and *T. suis* thus had more severe diarrhoea and pathology than pigs with single infections (Mansfield *et al*., 2003). Although diarrhoea was absent in this study, the helminth-inoculated piglets had softer faeces than non-inoculated piglets. These findings may indicate the importance of controlling single pathogen infections, although seemingly insignificant, because interactions of multiple infections may aggravate the conditions.

Conclusions

In this study, inclusion of fructan-rich dried chicory root in the diet of weaning piglets altered their intestinal bacterial and helminth communities as demonstrated by a strong bifidogenic effect, a reduced *A. suum* worm burden and the increased number of *T. suis* worms at necropsy. However, interpretations of these results should probably be cautious due to the high variation observed between individual pigs and the generally accepted complexity of whipworm regulation. The expected assessment of the effect of chicory on weaning diarrhoea was not feasible because the previously proven *E. coli* O138:F8 infection model did not establish in the piglets. It is speculated that the late weaning (7 weeks) may have promoted resistance against the challenge strain. The *Campylobacter* spp. excretion level seemed to be affected by chicory. Accordingly, feeding of piglets with chicory may also serve as an animal-friendly strategy to reduce the risk of carcass contamination at slaughter and thereby improve the food safety of pork.

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