Manipulating the environment in the porcine large intestine to help control swine dysentery

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By

J.R. Pluske, C.F. Hansen[A], D.J. Hampson, A. Hernandez, J. Mansfield, J.-C. Kim[B] and B.P. Mullan[B]

Animal Research Institute, School of Veterinary and Biomedical Sciences, Murdoch University; [A]Current address: Department of Large Animal Sciences, Faculty of Life Sciences, University of Copenhagen, Groennegaardsvej 2, 1870 Frederiksberg C, Denmark; [B]Department of Agriculture and Food, 6983 WA

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Executive Summary

Two experiments were conducted in this project to evaluate the effects of different dietary carbohydrate sources on the incidence of swine dysentery (SD), on aspects of fermentation behaviour in the gastrointestinal tract (GIT), and on the molecular characterisation of the microbiota in the GIT.

Experiment 1 tested the hypothesis that inulin in the chicory root rather than galactans in lupins was responsible for protective effects against SD.

A 2 × 2 factorial experiment was undertaken using pigs fed barley- and triticale-based diets, with the main effects being protein source [185 g/kg canola meal (low in galactans) or 220 g/kg lupins (high in galactans)] and inulin supplementation (0 or 80 g/kg). Forty Large White × Landrace pigs weighing 21 ± 3 kg, with 10 pigs per diet, were allowed to adapt to the diets for 2 wk and then each pig was challenged orally four times with a broth culture containing *B. hyodysenteriae* on consecutive days. Pigs were euthanized when they showed clinical signs of SD or 6 wk post-challenge.

Pigs fed diets without inulin had a 8.3 times greater risk ($P = 0.017$) of developing SD and were 16-times more likely ($P = 0.004$) to have colon contents that were culture-positive for *B. hyodysenteriae*, compared to the pigs fed a diet with 80 g/kg inulin. Diets containing lupins did not prevent pigs from developing clinical SD; however, inclusion of lupins or inulin or both in the diets delayed the onset of disease compared with the diet based mainly on canola meal ($P < 0.05$). Diet did not influence the total concentration of organic acids in the ileum, caecum, or upper and lower colon; however, the molar proportions of the organic acids were influenced ($P < 0.05$). Consequently the pH values in the caecum, and upper and lower colon were not influenced by diet. However the pH values of the ileal digesta were lower in pigs fed the diet with both lupins and inulin compared to the diet containing only lupins ($P < 0.05$). In conclusion, this study showed that diets supplemented with highly fermentable carbohydrates from inulin, protected pigs against developing SD. Furthermore, use of the molecular terminal restriction fragment length polymorphism (T-RFLP) technique showed that T-RFLP analysis was useful for demonstrating changes in the bacterial communities in pigs fed inulin and lupins and infected with *B. hyodysenteriae*.

This experiment showed that the pigs' intestinal microbiota responds to dietary changes with inulin and lupin and infection with *B. hyodysenteriae*, with infection destabilising the bacterial community structure and protection against SD being associated with the presence of particular identifiable bacteria.

In **Experiment 2**, a total of 60 surgically castrated pigs (Large White x Landrace; 41.1 ± 4.47 kg; mean ± SD) were used in a randomized experiment to examine the effects of added dietary inulin (0, 20, 40, 80 g/kg) on the incidence of SD and fermentation characteristics in the large intestine after experimental with *B. hyodysenteriae*, and also the (pre-infection) composition and activity of the faecal microbiota.

The pigs were allowed to adapt to the diets for two weeks before each pig was challenged orally four times with a broth culture containing *B. hyodysenteriae* on consecutive days. Pigs were euthanized when they showed clinical signs of SD or six weeks post-challenge.

Post-infection, increasing the dietary levels of inulin linearly ($P = 0.001$) reduced the risk of pigs developing SD, but nevertheless 8 out of 15 pigs fed the diet with 80 g/kg inulin developed disease. The pH values in the caecum tended ($P = 0.072$), and in the upper colon decreased ($P = 0.047$), linearly with increasing inulin levels in the diets, most probably due to a linear increase in the total VFA concentration that was observed in both the caecum ($P = 0.018$), upper colon ($P = 0.001$) and lower colon ($P = 0.013$). In addition, there was a linear reduction in the proportion of the branched-chain fatty acids (BCFAs) iso-butyric acid and iso-valeric acid in the caecum ($P = 0.015$ and $P = 0.026$) and upper colon ($P = 0.011$ and $P = 0.013$) with increasing levels of dietary inulin. This experiment showed that only a diet supplemented with a high level of inulin (80 g/kg) reduced the

risk of pigs developing SD possible through a modification of the microbial fermentation pattern in the large intestine, with intermediate protection noted at a level of 40 g/kg inulin in the diet. Pre-infection, the DM content of the faeces decreased linearly (P = 0.008) in response to increased supplementation with inulin, whereas the proportion of valeric acid increased (P = 0.081). Furthermore, there was a linear reduction in the proportion of the BCFAs iso-butyric (P = 0.045) and iso-valeric (P = 0.056) and of faecal NH_3-N (P = 0.034) with increasing dietary inulin content. T-RFLP analysis of faeces revealed that pigs fed inulin had a more similar bacterial diversity compared with individuals fed the control diet, but only the pigs fed 80 g/kg inulin had a greater number of identifiable terminal restriction fragments (T-RF; P= 0.008). Terminal restriction fragments (T-RFs) identified tentatively as *Lactobacillus vitulinus* and *Megasphaera elsdenii* were more abundant in the pigs fed the 80g/kg-inulin diet. Contrarily, a T-RF tentatively identified as *Lactobacillus delbruekii* sp. l*actis* was found less frequently in pigs receiving the inulin diet with 80 g/kg.

Collectively, data from these two experiments has demonstrated that:

(i) Including 80 g/kg dietary inulin reduced the relative risk of pigs having SD compared to lupins and canola meal, although this depended on the intensity of infection (challenge) pressure with *B. hyodysenteriae* (Experiment 2). Using 80 g/kg inulin in the diet was associated with a concomitant marked reduction in the population of *B. hyodysenteriae* present in the large intestinal contents. Some degree of protection against SD was observed at an inclusion level of inulin of 40 g/kg of diet in the second study;

(ii) Using lupins rather than canola meal in diets was associated with a reduced risk of SD under the dietary and challenge conditions used;

(iii) Dietary lupins and inulin can change the fermentation behaviour and metabolic characteristics of the microbial community in the large intestine. In association, and particularly with dietary inulin at 80 g/kg, the composition of the microbial community is changed, with inulin in the diet being associated with identifiable bacteria potentially implicated in the pathogenesis of SD.

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1. Introduction

Swine dysentery (SD) is a major endemic disease in a number of pig-rearing areas in the world (Hampson et al., 2006), and is currently present in a number of herds within the key production groups in Australia. SD results from infection of the caecum and colon by *Brachyspira hyodysenteriae*, a pathogenic anaerobic intestinal spirochaete. In association with other anaerobic members of the microbiota, which are present at these intestinal sites, the spirochaete can cause severe mucohaemorrhagic colitis resulting in morbidity and mortality. The disease is seen particularly in grower and finisher pigs, and has a severe impact on production efficiency. SD can be controlled using selected antimicrobials that kill or inhibit the spirochaete. Unfortunately this approach is becoming more problematic due to the occurrence of antimicrobial resistance in *B. hyodysenteriae* strains (Karlsson et al., 2002) and (or) the prohibition on the use of other antimicrobials in pigs. For example, *in vitro* resistance to tylosin and lincomycin is now common amongst *B. hyodysenteriae* isolates in Australia, and strains resistant to tiamulin are emerging. Although dimetronidazole is still an effective treatment for SD, this has been withdrawn from use. Concerns are also in place with regard to olaquindox use; collectively, these matters reinforce the need to investigate alternative means of controlling SD.

The pigs' diet can have a strong influence on colonisation by *B. hyodysenteriae,* and on the occurrence of clinical signs of SD. Two different forms of dietary manipulation have been shown to ameliorate SD. In one method, pigs are fed highly digestible (starch-rich and protein sources based on animal materials) diets typically based on cooked white rice. This method limits the amount of fermentable substrate reaching the large intestine, which in turn seemingly establishes conditions that are unfavourable to *B. hyodysenteriae* (Pluske et al., 1996; Siba et al., 1996; Pluske et al., 1998). These conditions are not clearly defined, but could include specific changes to the microbiota, and (or) to physical conditions in the large intestine (viscosity, pH, hydration etc). In the second approach, specific ingredients are added to the diet, which cause changes in the nature of the fermentation in the large intestine (e.g., Prohaszka and Lukacs, 1984; Thomsen et al., 2007). In this regard, Thomsen et al. (2007) found that a moderately fermentable diet that included sweet lupins and dried chicory root (containing inulin) prevented SD following experimental challenge with *B. hyodysenteriae*. Follow-up work from this particular study showed that the protective effect of the diet containing lupins and inulin was associated with changes in populations of two specific bacteria, a *Bifidobacterium* spp. and a *Megasphaera* spp. (Molbak et al. 2007). However these workers were unable to determine whether the dietary protection was due to the inulin from the dried chicory roots or the galactans supplied by the sweet lupins, or if both carbohydrate sources were needed to provide protection. Nevertheless, this shows that changes in the large intestinal microbiota and (or) other physical changes at the colonisation sites can influence the incidence and severity of SD.

Physiologically, fructo-oligosaccharides, like inulin, are classified as dietary fibre and hence resistant to endogenous enzymatic degradation in the small intestine. Undigested fibre entering the caecum and colon function as substrate for fermentative processes causing a higher luminal concentration of volatile fatty acids (VFA), which in turn can result in a lower luminal pH (Jensen and Jørgensen, 1994). In this context, inulin is mainly fermented in the large intestine by *Bifidobacteria* and *Lactobacilli* spp. to VFAs, lactate and gas (Roberfroid et al., 1998). In addition, dietary inulin supplementation may regulate metabolic activity by decreasing the protein:carbohydrate ratio in the hindgut. As a result, carbohydrate fermentation

may suppress the formation of branched chain fatty acids (BCFAs) and ammonia, produced from protein fermentation (Macfarlane and Macfarlane, 2003).

Two experiments were conducted in this project. In Experiment 1, the aims were to: (i) examine whether diets incorporating inulin and lupins could prevent SD, (ii) determine whether either inulin or lupins independently could prevent SD, (iii) examine if any interaction between the two dietary carbohydrate sources (inulin, galactans) could be demonstrated, and (iv) examine microbiota changes in response to these diets. The hypothesis tested was that a diet supplemented with inulin but not lupins would decrease the incidence of SD. In Experiment 2, the design of which was based on data from Experiment 1, the effects of increasing dietary inulin level on (i) the severity and incidence of SD and (ii) the composition and activity of the faecal microbiota in growing pigs prior to infection, were evaluated. The specific hypotheses tested were first, that increasing levels of inulin supplementation will reduce the incidence of SD, and second, that inulin would enhance the proliferation of faecal lactic acid-producing bacteria, reduce faecal BCFAs, and reduce faecal ammonia nitrogen (NH_3-N) concentration in pigs prior to infection.

2. Methodology

The studies were reviewed and approved by the Animal Ethics Committees of Murdoch University and the Department of Agriculture and Food, Western Australia.

2.1 Experiment 1

Animals and Housing

Forty male pigs (Large White × Landrace) were obtained at weaning from a commercial specific-pathogen-free piggery known to be free of SD (Wandalup Farms Ltd., Mandurah, WA, Australia). The pigs were housed in one group at Murdoch University and all were offered the same commercially formulated diet without any feed additives or antimicrobial compounds until they reached a live weight of 20.9 ± 2.8 kg (mean ± SD). At this time, the pigs were allocated based on live weight to one of the four experimental diets. The pigs were housed in a temperature-controlled animal house in two identical rooms. Each room had four pens in a square arrangement so that each pen was adjacent to two other pens. The pens were raised above the ground and had fully slatted plastic floors and wire-mesh sides that allowed contact between the animal and passage of manure between pens. In each room there was one pen of five pigs per experimental diet. Each pen was equipped with a dry-feed single space feeder without water, and two drinking bowls. Throughout the experiment, the pigs had *ad libitum* access to feed and water. Group housing was chosen to facilitate transmission of the pathogenic bacteria within and between groups (Pluske et al., 1996; Pluske et al., 1997). The pigs were allowed to adapt to the diets for 2 wk before challenge with *B. hyodysenteriae*.

Diets and Experimental Design

The experimental design was a 2 × 2 factorial arrangement with the main effects being protein source [185 g canola meal (low in galactans) or 220 g lupins (high in galactans)/kg] and inulin supplementation (0 or 80 g/kg). Four experimental diets with contrasting proportions of fermentable carbohydrates (galactans and inulin) were formulated as shown in Table 1. The experimental diets were based on barley and triticale and supplemented with canola meal, lupins, and inulin. Pea protein concentrate, canola oil, crystalline amino acids, minerals, and vitamins were used to balance for essential nutrients and ensure that the diets were iso-energetic. The diets

were produced in mash form using the same batches of raw materials and did not contain any antimicrobials.

Table 1. Diet ingredients and chemical composition of the experimental diets (Experiment 1).

Inulin, g/kg:	0		80	
Lupin, g/kg:	0	220	0	220
Ingredient, g/kg (as-fed)				
Triticale	299.1	326.6	244.8	281.9
Barley	448.6	391.8	401.9	338.4
Canola meal	185.0	-	185.0	-
Lupins	-	220.0	-	220.0
Inulin[1]	-	-	80.0	80.0
Pea protein concentrate[2]	34.6	18.9	56.1	40.2
Canola oil	10.0	14.5	10.0	12.9
Limestone	9.9	12.2	9.1	11.4
Dicalcium phosphate	7.1	7.1	8.5	8.4
Salt	3.5	3.7	3.6	3.7
DL-Met	-	0.8	-	0.7
L-Lys	1.2	2.5	-	1.2
L-Thr	-	0.7	-	0.1
L-Trp	-	0.2	-	0.1
Vitamin/mineral premix[3]	1.0	1.0	1.0	1.0
Calculated composition				
Potential physiological energy,[4] MJ/kg	7.9	7.9	7.9	7.9
SID[5] crude protein, g/MJ PPE[6]	17.6	17.6	17.6	17.6
SID lysine, g/MJ PPE	1.00	1.00	1.00	1.00
Crude protein, g/kg	174	164	180	170
Crude fat, g/kg	39.2	39.5	37.7	36.5
Analyzed composition				
Crude protein, g/kg	157	143	163	148
Crude fat, g/kg	49.5	41.7	50.1	40.0
Calculated carbohydrate composition, g/kg (DM)				
Glucose, sucrose and fructose	32	39	32	39
Fructans (inulin)	6	6	85	85
Cellulose	34	50	31	47
Soluble NCP[7]	36	31	33	27
Insoluble NCP[7]	86	151	77	142
NCP[7]	122	181	110	169
NSP[8] (cellulose + NCP[7])	156	232	141	216
Klason lignin	39	20	37	18
Dietary fiber (NSP[8] + lignin)	196	252	178	234

[1]BeneoST, ORAFTI, Tienen, Belgium.
[2]KIRKMAN, Portland, OR.
[3]Supplied per kg of diet: 60.0 mg Fe ($FeSO_4$); 10.0 mg Cu ($CuSO_4$); 40.0 mg Mn (MnO); 100.0 mg Zn (ZnO); 0.30 mg Se (Na_2SeO_3); 0.50 mg I (KI); 0.20 Co ($CoSO_4$); vitamin A, 7,000 IU; vitamin D_3, 1,400 IU; vitamin E, 20.0 mg; vitamin K_3, 1.0 mg; thiamin, 1.0 mg; riboflavin, 3.0 mg; pyridoxine, 1.5 mg; vitamin B_{12}, 0.015 mg; pantothenic acid, 10.0 mg; folic acid, 0.2 mg; niacin, 12.0 mg; and biotin 0.03 mg.
[4]Potential physiological energy (Boisen, 2001).
[5]SID: Standardized ileal digestible.
[6]PPE: Potential physiological energy.
[7]NCP: Non-cellulosic polysaccharides.
[8]NSP: Non-starch polysaccharides.

Challenge with B. hyodysenteriae and Assessment of Swine Dysentery

Australian *B. hyodysenteriae* strains WA1 and B/Q02 were obtained as frozen stocks from the culture collection at the Reference Centre for Intestinal Spirochetes, Murdoch University. They were thawed and grown in Kunkle's pre-reduced anaerobic

broth containing 2% (vol/vol) fetal bovine serum and 1% (vol/vol) ethanolic cholesterol solution (Kunkle et al., 1986), and were incubated at 37°C on a rocking platform until early log-phase growth was achieved.

Each morning for four consecutive days, all pigs were challenged via a stomach tube with 80 mL broth culture containing approximately 10^8 cfu per mL of *B. hyodysenteriae*. At this time the pigs had an average live weight of 29.4 ± 3.7 kg (mean ± SD).

The pigs were weighed weekly and rectal swabs were taken from all pigs two times per week for spirochete culture. Visual fecal consistency scoring (1: firm, well formed, 2: soft, 3: loose, 4: watery, 5: watery with mucus/blood) was conducted daily. Watery diarrhea with mucus/blood was considered as clinical signs of SD, with pigs showing these signs removed for post-mortem examination within 48 h. All other pigs were removed for necropsy 42 d after the first day of challenge.

Post-mortem procedures

Euthanasia was by captive bolt stunning followed by exsanguination. The entire gastrointestinal tract (GIT) was removed immediately and divided into seven segments by ligatures. The segments were as follows: stomach, duodenum, jejunum, ileum, caecum, upper colon, and lower colon. The presence, distribution, and nature of gross lesions in the large intestine were recorded (La et al., 2004), and swabs were taken from the wall of the caecum and proximal colon for spirochetal culture. The luminal contents were then removed by gently squeezing the material from the gut segment. The empty segments and collected material were weighed and representative samples were collected in sterile plastic tubes that were snap frozen in liquid nitrogen within 10 min of euthanasia. Samples for dry matter (DM) and VFA examination were stored at -20°C until analysis.

At the time of euthanasia, approximately 5 g of digesta from the ileum, caecum, and upper and lower colon segments were collected for ATP analysis. The samples were placed into sterile 15 mL Falcon tubes kept on ice, extracted with 10 mL of 2 mol/L cold perchloric acid containing 10 mmol/L EDTA, vortexed thoroughly, and then frozen in liquid nitrogen within 10 min of collection from the pig. Samples were stored at -80°C until analysis.

The pH of the digesta was measured by inserting the electrode of a calibrated portable pH meter (Schindengen pH Boy-2; Schindengen Electric MFG, Tokyo, Japan) into the collected sample. The DM content of samples was measured using the AOAC method (930.15; AOAC, 1997).

Histology

A cross-section of the ileum was collected for histological examination and immediately fixed in 10% neutral buffered formalin. After fixation, the samples were dehydrated through an alcohol series, embedded in paraffin wax, sectioned at 3 µm, and stained with haematoxylin and eosin for histopathological examination. Measurements of villous height and crypt depth were taken only from sections where the plane of the section ran vertically from the tip of the villus to the base of an adjacent crypt. A calibrated eyepiece graticule was used to measure 10 of the tallest well-oriented villi from tip to crypt mouth, and 10 associated crypts from crypt mouth to base. For each pig, the average of the 10 measurements were used in statistical analysis.

Bacteriological Analysis

Bacteriology swabs taken from faeces, caecum, or colon were streaked onto selective agar plates (Jenkinson and Wingar, 1981), consisting of Trypticase Soy agar (Becton Dickinson Microbiology Systems, Cockeysville, MD) containing 5% (vol/vol) defibrinated sheep blood, 400-µg/mL spectinomycin, and 25 µg/mL each of colistin and vancomycin (Sigma, St. Louis, MO). The plates were incubated for 5 to 7 d at 37°C in a jar with an anaerobic environment generated using a disposable hydrogen + carbon dioxide generator envelope with palladium catalyst (GasPak Plus; Becton Dickinson Microbiology Systems, Franklin Lakes, NJ). The presence of low flat spreading growth of spirochetes on the plate and any hemolysis around the growth were recorded. Spirochetes were confirmed by selecting areas of suspected growth, resuspending in PBS and examining the suspension under a phase contrast microscope at 400 × magnification. Spirochetes were identified as *B. hyodysenteriae* on the basis of strong ß-hemolysis, microscopic morphology and results of an NADH oxidase gene PCR of cell growth on the plates. The PCR primers and conditions have been described previously (La et al., 2003).

Viscosity

Digesta viscosity was measured within 10 min of euthanasia in fresh contents collected from the ileum by first placing a sample of digesta in an Eppendorf tube, mixing on a vortex, and centrifuging at 12,000 × g for 10 min at room temperature (Sigma Benchtop Centrifuge 1-15, Quantum Scientific Pty Ltd, Milton, QLD, Australia). The supernatant fraction (0.5 mL) was placed in a coneplate rotational viscometer (CP40 Brookfield LVDV-II; Brookfield Engineering Laboratories Inc., Stoughton, MA) where the viscosity of all samples was measured (mPa·s) at 12 and 30 rpm at 37°C.

Feed, Organic Acids, and ATP

The nitrogen content of the feed was determined with a nitrogen analyzer (FP-428; LECO Corp., St Joseph, MI) using a combustion method (990.03; AOAC, 1997). Crude protein was calculated by multiplying the nitrogen content by 6.25. Crude fat was measured using AOAC *Soxhlet* method (960.39; AOAC, 1997).

The concentrations of organic acids (formic acid, VFA, lactic acid, and succinic acid) in the ileal contents were analyzed by the method described by Jensen et al. (1995). The VFA concentrations in caecal and colon contents were determined as described by Heo et al. (2008).

The concentration of ATP in the digesta from the ileum, caecum, and upper and lower colon was measured by the luciferin-luciferase method to provide an indicator of overall microbial activity. Collected samples were thawed, mixed thoroughly, and then centrifuged at 5,500 × g for 30 min at 4°C. One milliliter of the supernatant was added to 200 µL Tris buffer (0.2 mol/L, pH 7.4) before neutralization with 0.5 mol/L KOH (to pH 7.4 to 7.6). After re-centrifugation (5,500 × g for 30 min at 4°C), the supernatant was removed, stored on ice, and then the amount of ATP was determined using an assay kit (Enliten ATP Assay System, Promega, Madison, WI) with a plate luminator (Beckman Coulter DTX 880, Beckman Coulter Australia, Gladesville, NSW, Australia).

Statistical Analysis

All statistical analyses were performed using SAS (version 9.0; SAS Inst. Inc., Cary, NC) with each pig regarded as the experimental unit, given that each pig was challenged. A binary response was recorded for each pig with respect to colonization with *B. hyodysenteriae*. Data were analyzed with a logistic regression model using the GENMOD procedure in SAS:

$$\text{Logit}(P_{ijk}) = \mu + \alpha_i + \beta_j + \gamma_k ,$$

where P_{ijk} is the probability for a pig showing clinical signs of swine dysentery or being culture positive for *B. hyodysenteriae*; μ is the overall mean; α_i is the effect of lupin (galactans); β_j is the effect of inulin inclusion and γ_k is a random effect of room.

The effect of lupin and inulin inclusion on the various quantitative variables measured were analyzed by the GLM procedure of SAS using the following model:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_k + \varepsilon_{ijk},$$

where Y_{ijk} is the observed response; μ is an overall mean; α_i is an effect of lupin; β_j is effect of inulin; $(\alpha\beta)_{ij}$ is effect of interaction between lupin and inulin; γ_k is effect of room and ε_{ijk} is the residual error, which is assumed independent and normal distributed. If the interaction between lupins and inulin was not significant ($P > 0.05$), it was excluded from the model. When the interaction between lupin and inulin was significant, differences between means were compared by Student's *t*-test. Statistical significance was accepted at $P \leq 0.05$ and $P \leq 0.10$ was considered a trend.

T-RFLP (*terminal restriction fragment length polymorphism*) Analysis

The DNA was extracted from digesta using a Qiagen DNA stool mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction and adjusted spectrophotometrically to a concentration of 5 µg of DNA per mL.

Polymerase chain reactions (PCRs) were performed on a Corbett Research / Hewlett Packard Palm-Cycler apparatus. The reaction mixtures (50 µg) contained 0.2 µM of each primer (Sigma-Aldrich, Castle Hill, NSW, Australia), 10 mM deoxynucleoside triphosphate (Promega, Madison,WI, USA), 1.0 U DyNAzyme II DNA polymerase supplied with 10 × PCR buffer (Finnzymes, Espoo, Finland), and approximately 50 ng of template DNA. Amplification was performed under the following conditions: Initial denaturation at 95 °C for 1 min; followed by 30 cycles of melting at 95°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 45 s; and a final extension at 72°C for 10 min. The 16S rRNA genes were amplified by use of the 6-carboxyfluorescenin-labeled forward primer S-D-Bact-0008-a-S-20 (5'-6-FAM-AGAGTTTGATCMTGGCTCAG) and the reverse primer PH1522 (5'-AAGGAGGTGATCCAGCCGCA). Three replicate PCRs in 50-µL reaction mixtures were performed for each sample, pooled and purified with a QIA quick PCR purification kit (Qiagen, Hilden, Germany) before being eluted in a final volume of 50 mL. The PCR amplicons were pooled to obtain enough material, to reduce the sample variance and to avoid PCR artefacts. The purified and combined PCR amplified 16S rDNA products were verified by electrophoresis on 2% agarose gels. Thereafter samples (10 µL) were digested overnight at 37°C with 20 U of the restriction endonuclease HhaI (New England Biolabs, Beverly, *MA, USA*) in 20-µL reaction mixture to produce fluorescently labelled terminal restriction fragments (T-RFs). The labelled T-RFs samples (2 µL) were mixed with 19.5 µL loading buffer and 0.4 µL of a standard size marker before being analysed by electrophoresis in an automatic sequence analyser (Applied Biosystems 3730XL, Melbourne, Vic., Australia) using the GeneMapper mode. Before loading the T-RFs onto a denaturating polyacrylamide gel, the T-RFs mixtures were denatured at 95°C for 2 min, 4°C for 2 min, and 24°C for 2 min. The electrophoresis settings were injector time 1.6kV for 15 s, run voltage 15kV and run time 1600 s with filter G5. After electrophoresis, the length of the T-RFs were determined by using GeneMapper 50_POP7_1 software and determinations of the sizes of the T-RFs in the range of 50 to 600 bp were performed with the size standard GeneScan - 600 Liz (Applied Biosystems,

Melbourne, Vic., Australia). The profiles were alignment and imported into SAS for Windows version 9.13 (SAS Inst. Inc., Cay, NC, USA) for further analysis.

Analysis of T-RFLP Data

Sample T-RFLP data consisted of size (base pair (bp)) and peak height for each T-RF. To standardize the DNA loaded on the capillary, the sum of all T-RFs peak heights were used to normalize the peak detection threshold for each sample. A new threshold value was obtained by multiplying a pattern's relative DNA ratio (the ratio of total peak height in the pattern to the total peak height in the sample with the smallest total peak height) by 40 units (the smallest height detected by the Genescan software in the sample with the lowest total peak height) to create the new threshold value. T-RFs with an area less than the new threshold value for a sample were removed from the data set. New total area was obtained by the sum of all the remaining peak heights in each pattern (Dunbar et al., 2001). In short, and for each T-RF within a pig, semi-quantitative evaluation of the relative abundance was estimated by calculating the ratio of a specific T-RF intensity relative to the total intensity of all T-RFs. The relative peak hight of each T-RF (referred to as relative intensity) was calculated by dividing the intensity of the specific peak by the total intensity of all peaks on the electropherogram. Relative abundance was calculated for all T-RFs that were between 50 and 650 bp in length.

Estimation of variation in bacterial colonization patterns within and between the treatment groups were made based on pairwise comparisons of T-RFs using the Dice similarity coefficients (S_D) as described by Leser et al. (2000). The S_D reflects the similarity between two individual samples by calculating the ratio of two times the number of T-RFs in common between the two samples. The ratio is calculated for all pairwise sample-to-sample combinations, and from this means and their standard errors where calculated within treatment groups. Generally there is a direct relationship between the S_D and the similarity of two compared pigs, with two identical T-RFLP profiles having a similar coefficient of 1.0 (100% similarity) (Leser et al., 2000). Richness was considered as the number of peaks in each sample after standardization.

Tentative identification of specific bacteria characterized by T-RFs was done in silico by inserting primer sequences and restriction enzymes in the MiCA home page (http://mica.ibest.uidaho.edu/digest.php) using the RDP database (Release 10, Update 11, Bacterial SSU 16S rRNA). The reference collection of 16S rRNA gene sequences did not cover all bacterial species present in the faeces of pigs and therefore some T-RF did not have a matching counterpart.

2.2 Experiment 2

Animals and Housing

Sixty surgically castrated commercial pigs (Large White × Landrace) were obtained at weaning from a commercial specific-pathogen-free piggery known to be free of SD. At weaning the pigs were housed in one group at Murdoch University and all were offered the same commercially formulated diets without any feed additives or antimicrobial compounds until they reached a live weight of 31.2 ± 4.28 kg (mean ± SD). At this time, the pigs were allocated based on live weight to one of four experimental diets. The pigs were housed in a temperature-controlled animal house in three identical rooms. Each room had four pens in a square arrangement so that each pen was adjacent to two other pens. The pens were raised above the ground and had fully slatted plastic floors and wire-mesh sides that allowed contact between the animals and passage of manure between pens. In each room there was one pen of five pigs per experimental diet. Each pen was equipped with a dry-feed single space feeder without

water, and two drinking bowls. Throughout the experiment, the pigs had *ad libitum* access to feed and water. Group housing was chosen to facilitate transmission of the pathogenic bacteria within and between groups (Hansen et al., 2010; Pluske et al., 1996). The pigs were allowed to adapt to the diets for two weeks before challenge with *B. hyodysenteriae*.

Diets and Experimental Design
The experimental design was a completely randomized block arrangement with four dietary treatments differing in amount of added dietary inulin (0, 20, 40, and 80 g/kg). The four diets were formulated as shown in Table 2, to meet or exceed the nutrient requirements for pig of this genotype and all diets contained the same energy and protein (amino acids) contents. Inulin (BerneoST, ORAFTI, Tienen, Belgium) was added to the diets at the expense of triticale and barley. The diets were produced in mash form using the same batch of raw materials and did not contain any antimicrobials.

Challenge with B. hyodysenteriae and Assessment of Swine Dysentery
Australian *B. hyodysenteriae* strains WA1 and B/Q02 were obtained as frozen stocks from the culture collection at the Reference Centre for Intestinal Spirochetes, Murdoch University. They were thawed and grown in a Kunkle's pre-reduced anaerobic broth containing 2% (vol/vol) fetal bovine serum and 1% (vol/vol) ethanolic cholesterol solution (Kunkle et al., 1986), and were incubated at 37°C on a rocking platform until early log-phase growth was achieved.

Each morning for four consecutive days, all pigs were challenged via a stomach tube with 100 mL broth culture containing approximately 10^9 cfu per mL of *B. hyodysenteriae*. At this time the pigs had an average live weight of 41.1 ± 4.47 kg (mean ± SD).

The pigs were weighed weekly and rectal swabs were taken from all pigs two times per week for spirochete culture. Visual fecal consistency scoring (1: firm, well formed, 2: soft, 3: loose, 4: watery, 5: watery with mucus/blood) was conducted daily. Watery diarrhea with mucus/blood was considered as clinical signs of swine dysentery, with pigs showing these signs removed for post-mortem examination within 48 h. All other pigs were removed for necropsy 42 d after the first day of challenge.

Post-mortem procedures
The same methods were used as in Experiment 1.

Histology
The same methods were used as in Experiment 1.

Bacteriological Analysis
The same methods were used as in Experiment 1.

Analysis of Feed, Organic Acids and Ammonia N
The nitrogen content of the feed was determined with a nitrogen analyzer (LECO FP-428; LECO Corp., St Joseph, MI) using a combustion method (990.03; AOAC, 1997). Crude protein was calculated by multiplying the nitrogen content by 6.25. Crude fat was measured using the *AOAC* Soxhlet *method (960.39; (AOAC, 1997)*.

The concentrations of organic acids (formic acid, VFA, lactic acid, and succinic acid) in the ileal contents were analyzed as per Experiment 1. Concentrations of NH_3-N were measured according to the method by Weatherburn (1967). In short, the supernatant was deproteinized using 10% trichloro-acetic acid. Ammonia and phenol were oxidized

by sodium hypochlorite in the presence of sodium nitroprusside to form a blue complex. The intensity was measured calorimetrically at a wavelength of 623 nm. Intensity of the blue color is proportional to the concentration of ammonia present in the sample.

Statistical Analysis

All statistical analyses were performed using SAS (version 9.2; SAS Inst. Inc., Cary, NC) with each pig regarded as the experimental unit, given that each pig was challenged. A binary response was recorded for each pig with respect to colonization with *B. hyodysenteriae*. Data were analyzed with a logistic regression model using the GENMOD procedure in SAS with the effect of inulin and room as fixed effects. The Pearson Chi-square correction (pscale) was applied to correct for overdispersion.

The effect of inulin inclusion on the various quantitative variables measured were analyzed univariately by the GLM procedure of SAS with room and dietary inulin level included in the model. Polynomial regression was used to determine the presence of linear or quadratic treatments effects as inulin levels were increased. Statistical significance was accepted at $P < 0.05$ and $P < 0.10$ was considered a trend.

Table 2. Diet ingredients and chemical composition of the experimental diets used (Experiment 2).

Inulin, g/kg	0	20	40	80
Ingredient (g/kg as-fed)				
Triticale	400.3	390.2	379.9	359.2
Barley	306.1	288.0	270.0	234.1
Canola meal	185.0	185.0	185.0	185.0
Soy bean meal, 48%	76.5	85.1	93.5	110.4
Inulin[1]	-	20.0	40.0	80.0
Canola oil	10.0	10.0	10.0	10.0
Limestone	10.3	10.1	9.9	9.5
Dicalcium phosphate	5.6	5.8	6.0	6.4
Salt	3.5	3.5	3.5	3.5
L-Lysine	1.6	1.4	1.2	0.8
Vitamin/mineral premix[2]	1.0	1.0	1.0	1.0
Calculated composition				
Potential physiological energy[3] (MJ/kg)	7.9	7.9	7.9	7.9
SID[4] crude protein, (g/MJ PPE[5])	17.6	17.6	17.6	17.6
SID[4] lysine (g/MJ PPE)	1.00	1.00	1.00	1.00
Crude protein (g/kg)	173	175	176	179
Crude fat, (g/kg)	39.3	38.9	38.4	37.6
Analysed composition				
Crude protein (g/kg)	183	185	183	188
Crude fat (g/kg)	48	49	42	47
Calculated carbohydrate composition (g/kg DM)				
Glucose, sucrose and fructose	42	43	44	46
Fructans (inulin)	7	25	44	81
Cellulose	36	35	35	34
Soluble NCP[6]	34	33	33	32
Insoluble NCP[6]	92	90	88	85
NCP[6]	126	123	121	116
NSP[7] (cellulose + NCP[6])	162	159	156	150
Klason lignin	37	36	36	35
Dietary fibre (NSP[7] + lignin)	199	195	192	185

[1]BeneoST, ORAFTI, Tienen, Belgium
[2]Supplied per kg of diet: 60.0 mg Fe ($FeSO_4$); 10.0 mg Cu ($CuSO_4$); 40.0 mg Mn (MnO); 100.0 mg Zn (ZnO); 0.30 mg Se (Na_2SeO_3); 0.50 mg I (KI); 0.20 Co ($CoSO_4$); vitamin A, 7,000 IU; vitamin D_3, 1,400 IU; vitamin E, 20.0 mg; vitamin K_3, 1.0 mg; thiamin, 1.0 mg; riboflavin, 3.0 mg; pyridoxine, 1.5 mg; vitamin B_{12}, 0.015 mg; pantothenic acid, 10.0 mg; Folic acid, 0.2 mg; niacin, 12.0 mg and biotin 0.03 mg.
[3]Potential physiological energy(Boisen, 2001).
[4]SID: Standardized ileal digestible.
[5]PPE: Potential physiological energy.
[6]NCP: Noncellulosic polysaccharides.
[7]NSP: Nonstarch polysaccharides.

Blood and Faecal Sampling (Pre-Infection)

Blood samples (10 mL) were collected into vacutainer tubes coated with lithium heparin via jungular vein puncture two weeks after the experiment commenced. The blood samples were immediately placed on ice and then centrifuged at 2,000 × g for 10 min at 5°C. Plasma was stored at -20°C until analysed for plasma urea nitrogen.

At the same time, a provoked faecal sample was collected from all pigs. For determination of faecal N-NH_3 faecal samples were diluted 1:1 (wt/vol) with trichloro-acetic acid (10%), mixed, snap frozen in liquid nitrogen and stored at -80°C until analysis. Samples for VFA and T-RFLP analysis were collected in separated containers, snap frozen in liquid nitrogen and stored at -20°C for later processing.

T-RFLP Analysis
The same methods were used as in Experiment 1.

3. Outcomes

Experiment 1

One pig fed the diet with lupins and inulin died 17 d after challenge. The exact cause of death was not identified at a post-mortem examination; however, the pig did not have SD and the gross and histological findings indicated that an acute, terminal septic infection was the cause. Generally, the protein content in all the experimental diets was lower than expected (Table 1) due to unanticipated differences in the fat and protein contents of the lupins and canola meal. The crude fat content was higher than anticipated in the diets containing canola meal, again due to the greater than expected fat level in this ingredient.

Diets and Pig Performance, and the Incidence of Swine Dysentery and Re-isolation of B. hyodysenteriae
Pigs fed diets containing lupins tended to grow more slowly than pigs fed diets based on canola meal (P = 0.068) in the 2 wk adaptation period before being challenged with *B. hyodysenteriae* (Table 3). In the first 3 wk post-challenge, pigs fed the diet containing canola meal without inulin supplementation grew more slowly than pigs fed the other experimental diets (P < 0.05). When expressed as number of days from challenge with *B. hyodysenteriae* until the pigs were euthanized, both lupin and inulin inclusion delayed the onset of disease compared with the diet based mainly on canola meal (Table 3).

The incidence of SD and re-isolation of *B. hyodysenteriae* in faeces and colon digesta are shown in Table 4. The pigs fed diets without inulin had a 8.3 times (P = 0.017) greater risk of developing SD and were 16 times (P = 0.004) more likely to have colon contents that were culture-positive for *B. hyodysenteriae* at euthanasia compared with the pigs fed a diet with 80 g/kg inulin. Accordingly, pigs fed diets without inulin had a tendency (P = 0.084) to show a greater risk of having fecal samples culture-positive for *B. hyodysenteriae* during the experiment. The diets containing lupins failed (P = 0.687) to protect the pigs in this experiment from developing SD or reduce the risk of having *B. hyodysenteriae* in faeces (P = 0.278) and the colon contents (P = 0.484).

Table 3. Average number of days until pigs fed diets containing inulin or lupins or both developed clinical SD or were euthanized, and the weight gain of these pigs.

Inulin, g/kg:	0		80			P-value		
Lupin, g/kg:	0	220	0	220	SEM	Inulin	Lupin	Inulin × Lupin
Item								
No. of pigs	10	10	10	10				
No. of days[1]	18.3[a]	34.5[b]	41.1[b]	36.7[b]	1.5	0.001	0.012	0.008
ADG, g/d (2 wk adaptation period)[2]	654	578	626	562	38	0.500	0.068	0.873
ADG, g/d (3 wk post- challenge)[3]	421[a]	826[b]	950[b]	889[b]	109	0.010	0.122	0.039

[a,b]Values not having the same superscripts differ (P < 0.05).

[1]Average number of days until pigs developed clinical SD or were euthanized after challenged with *B. hyodysenteriae*. Pigs were euthanized when they developed clinical SD or at the end of the experiment 42 d post-challenge.

[2]ADG of the pigs in the first 2 wk of the experiment before challenge with *B. hyodysenteriae*.

[3]ADG of the pigs in the first 3 wk after challenge with *B. hyodysenteriae*.

Table 4. Number of positive pigs and relative risk[1] of a pig being culture-positive for *B. hyodysenteriae* or showing clinical signs of SD[2].

Inulin, g/kg:	0		80		Effect of treatment[3]	
Lupin, g/kg:	0	220	0	220	Inulin	Lupin
Item						
Pigs challenged, *n*	10	10	10	10		
Pigs with clinical SD	7	3	0	3		
Relative risk of clinical SD	12.3	1.0	0.0	1.0	Inulin: 1 No inulin: 8.3 (1.7 to 58.0) P = 0.017	Lupin: 1 Canola meal: 1.4 (0.3 to 7.3) P = 0.687
Pigs shedding *B. hyodysenteriae* in faeces (culture)	7	3	2	3		
Relative risk of culture-positive *B. hyodysenteriae* faeces	15.6	1.9	1.0	1.9	Inulin: 1 No inulin: 3.8 (0.9 to 19.4) P = 0.084	Lupin: 1 Canola meal: 2.3 (0.5 to 10.8) P = 0.278
Pigs with culture-positive *B. hyodysenteriae* colon content at euthanasia	8	5	1	2		
Relative risk of culture-positive *B. hyodysenteriae* colon content at euthanasia	79.8	14.0	1.0	3.2	Inulin: 1 No inulin: 16.0 (3.0 to 142.2) P = 0.004	Lupin: 1 Canola meal: 1.8 (0.4 to 10.1) P = 0.484

[1]Relative risk: the risk of an event in the group of interest compared with the reference group.

[2]The relative risk and 95% confidence intervals are given.

[3] These main effects are compared in these columns.

Viscosity, DM, and pH values

The ileal contents of pigs fed diets containing lupins were more viscous (P = 0.010) compared with pigs fed the diets without lupins (Table 5). At the same time, there was a tendency (P = 0.078) for a higher DM content (1.7 percentage units) in the ileum of pigs fed diets containing 80 g/kg inulin. The DM content in the caecum was not

influenced by the diets fed; however, in the upper colon, the DM content was lower ($P < 0.001$) in pigs fed the diet without inulin and lupins. In the lower colon, the DM content was higher ($P = 0.005$) in pigs fed the diet containing inulin compared with pigs fed the diet without lupins and inulin. The pH values of the ileal digesta were lower in pigs fed the diet with both lupins and inulin compared to the diet containing only lupins ($P = 0.017$). In the caecum, and upper and lower colon, the pH values were not influenced by diet (Table 5).

ATP and Organic Acids in Digesta

Elevated concentrations of ATP ($P < 0.003$) were found in the upper colon of pigs fed diets containing inulin compared to the other diets. No dietary effects ($P > 0.05$) on ATP concentration were detected in the ileal, caecal, or lower colon digesta (Table 5).

Diet did not affect ($P > 0.05$) the total concentration of organic acids in the ileum, caecum, or upper or lower colon (Table 6). However, the molar proportions of the organic acids were influenced by diet. In the ileum the proportion of lactic acid was higher ($P = 0.040$) and the proportion of acetic acid lower ($P = 0.033$) in pigs fed diets containing inulin. The percentage of acetic acid was lower ($P = 0.006$) in the caecum, whereas the percentage of butyric ($P = 0.003$) and valeric ($P < 0.001$) acids were higher in pigs fed inulin. The percentage of acetic acid was lower ($P < 0.05$) in the upper colon in pigs fed the diet without inulin and lupins, but the percentage of propionic acid was higher ($P = 0.003$) in the pigs fed lupins. The percentage of butyric and caproic acids were higher ($P < 0.05$) in the upper colon of pigs fed the diet containing only inulin (Table 6).

Table 5. Ileal viscosity, DM, pH, and ATP concentration in digesta[1].

Inulin, g/kg:	0		80			P-value		
Lupin, g/kg:	0	220	0	220	SEM	Inulin	Lupin	Inulin × Lupin
Item								
Viscosity, mPa·s								
Ileum	5.77	8.05	4.66	8.96	1.20	0.917	0.010	0.405
DM, %								
Ileum	13.4	14.6	16.1	15.3	1.0	0.078	0.809	0.311
Caecum	16.0	17.6	15.2	14.8	1.5	0.233	0.650	0.502
Upper colon	13.5[a]	18.9[b]	19.2[b]	19.9[b]	1.0	0.002	0.002	0.024
Lower colon	14.8[a]	19.1[a,b]	23.0[b]	18.1[a,b]	1.9	0.070	0.875	0.024
pH								
Ileum	6.6[a,b]	6.9[a]	6.8[a,b]	6.4[b]	0.2	0.313	0.728	0.015
Caecum	5.6	5.8	5.3	5.8	0.2	0.404	0.131	0.492
Upper colon	5.6	5.7	5.4	5.7	0.2	0.465	0.200	0.630
Lower colon	6.0	6.1	5.9	6.4	0.2	0.767	0.162	0.323
ATP,[2] µg/g digesta								
Ileum	10.6	8.4	11.1	11.6	2.4	0.461	0.739	0.581
Caecum	17.2	16.3	19.0	17.4	2.8	0.606	0.657	0.906
Upper colon	23.7	23.6	39.2	29.7	3.6	0.003	0.196	0.196
Lower colon	23.4	22.0	24.9	28.0	4.4	0.412	0.865	0.604

[a,b]Values not having the same superscripts differ ($P < 0.05$).

[1]Digesta samples were collected from different segments of the GIT at the day of euthanasia in pigs fed diets containing inulin or lupins or both and experimentally challenged with *B. hyodysenteriae*. Ten pigs per dietary treatment.

[2]Wet-weight basis.

Table 6. Total VFA and lactic acid concentrations and molar proportion of the organic acids in digesta[1].

Inulin, g/kg:	0		80			P-value		
Lupin, g/kg:	0	220	0	220	SEM	Inulin	Lupin	Inulin × Lupin
Item								
Ileum								
Total VFA + lactic acid,[2,3] mmol/kg digesta	39.1	30.9	29.2	35.1	6.5	0.638	0.839	0.280
Lactic acid, %	62.7	64.1	81.5	82.6	8.9	0.040	0.887	0.985
Acetic acid, %	28.3	27.5	10.5	12.8	7.5	0.033	0.926	0.832
Propionic acid, %	0.4	1.5	0.1	0.7	0.5	0.342	0.114	0.679
Other VFA,[4] %	8.5	6.9	7.9	3.9	2.3	0.439	0.225	0.605
Caecum								
Total VFA,[3] mmol/kg digesta	161.6	159.1	173.9	154.7	13.4	0.754	0.423	0.538
Acetic acid, %	37.4	34.9	31.3	30.4	1.8	0.006	0.345	0.670
Propionic acid, %	36.3	40.3	36.0	37.1	1.8	0.346	0.164	0.433
Butyric acid, %	18.4	17.8	23.1	21.4	1.3	0.003	0.399	0.707
Valeric acid, %	5.3[a,b]	4.8[a]	7.3[b]	9.8[c]	0.8	<0.001	0.204	0.053
Caproic acid, %	0.58	0.38	0.64	0.51	0.11	0.422	0.151	0.759
Iso-butyric acid, %	0.58	0.52	0.15	0.60	0.26	0.496	0.478	0.330
Iso-valeric acid, %	1.5	1.5	1.6	2.4	0.5	0.236	0.393	0.378
Upper colon								
Total VFA,[3] mmol/kg digesta	178.1	189.1	204.0	176.7	15.1	0.637	0.622	0.214
Acetic acid, %	37.1[a]	32.0[b]	30.0[b]	31.8[b]	1.3	0.008	0.199	0.012
Propionic acid, %	36.1	40.9	35.8	40.2	1.5	0.734	0.003	0.879
Butyric acid, %	17.5[a]	17.2[a]	21.6[b]	15.6[a]	1.1	0.288	0.008	0.016
Valeric acid, %	5.9	6.2	9.9	8.4	0.6	<0.001	0.385	0.166
Caproic acid, %	0.62[a]	0.64[a]	1.17[b]	0.62[a]	0.13	0.045	0.042	0.033
Iso-butyric acid, %	0.91	0.94	0.20	0.88	0.32	0.219	0.279	0.310
Iso-valeric acid, %	1.8	2.1	1.4	2.5	0.5	0.974	0.178	0.417
Lower colon								
Total VFA,[3] mmol/kg digesta	151.6	153.5	166.6	142.1	14.0	0.878	0.438	0.351
Acetic acid, %	39.5	35.6	34.7	35.7	1.5	0.118	0.318	0.107
Propionic acid, %	33.5	37.8	39.1	40.7	1.6	0.013	0.070	0.402
Butyric acid, %	16.4	16.4	15.5	12.7	0.9	0.022	0.163	0.144
Valeric acid, %	5.3	5.2	7.3	6.0	0.5	0.004	0.124	0.220
Caproic acid, %	0.83[a]	0.83[a]	1.19[b]	0.75[a]	0.13	0.195	0.047	0.049
Iso-butyric acid, %	1.57	1.39	0.40	1.12	0.30	0.022	0.403	0.140
Iso-valeric acid, %	2.9	2.8	1.9	3.1	0.5	0.473	0.252	0.155

[a,b] Values not having the same superscripts differ (P < 0.05).

[1]Digesta samples collected from different segments of the GIT at the day of euthanasia in pigs fed diets containing inulin or lupins or both and experimentally challenged with *B. hyodysenteriae*. Ten pigs per dietary treatment.

[2]Sum of lactic acid and the measured VFA.

[3]Wet-weight basis.

[4]Sum of butyric, valeric, caproic, iso-butyric, iso-valeric and succinic acid.

Ileal Histology

The villi in the ileum were higher (P = 0.016) in pigs fed the diets containing lupins and tended (P = 0.072) to be higher in the pigs fed diets supplemented with inulin (Table 7). Crypt depth in the ileum was not affected by the dietary treatments, but the villus:crypt ratio was increased (P = 0.042) in pigs fed inulin.

Table 7. Villous height, crypt depth and villous height to crypt ratio in ileal samples[1].

Inulin, g/kg:	0		80			P-value		
Lupin, g/kg:	0	220	0	220	SEM	Inulin	Lupin	Inulin × Lupin
Item								
Villous height, µm	317	354	344	382	15	0.072	0.016	0.961
Crypt depth, µm	259	290	262	252	16	0.277	0.478	0.206
Villus:crypt ratio	1.26	1.23	1.34	1.60	0.10	0.042	0.288	0.152

[1]Ileal samples collected at the day of euthanasia from pigs fed diets containing inulin or lupins or both and experimentally challenged with *B. hyodysenteriae*. Ten pigs per dietary treatment.

T-RFLP Analysis (Post-Infection)

The intestinal microbial diversity in the digesta of the ileum expressed as total number of identified T-RF within a treatment group was markedly greater in the pigs fed lupins ($P = 0.034$). In the caecal ($P < 0.001$), upper colon ($P = 0.050$) and lower colon ($P < 0.001$) contents the diversity was greater in pigs with SD (Table 8).

Table 8. Average number of terminal restriction fragments (T-RFs) and dice coefficients (S_D) for pair wise comparison of T-RFs from digesta contents in various segments of the GIT at the day of slaughter of pigs fed diets containing inulin, lupins and with clinical SD.

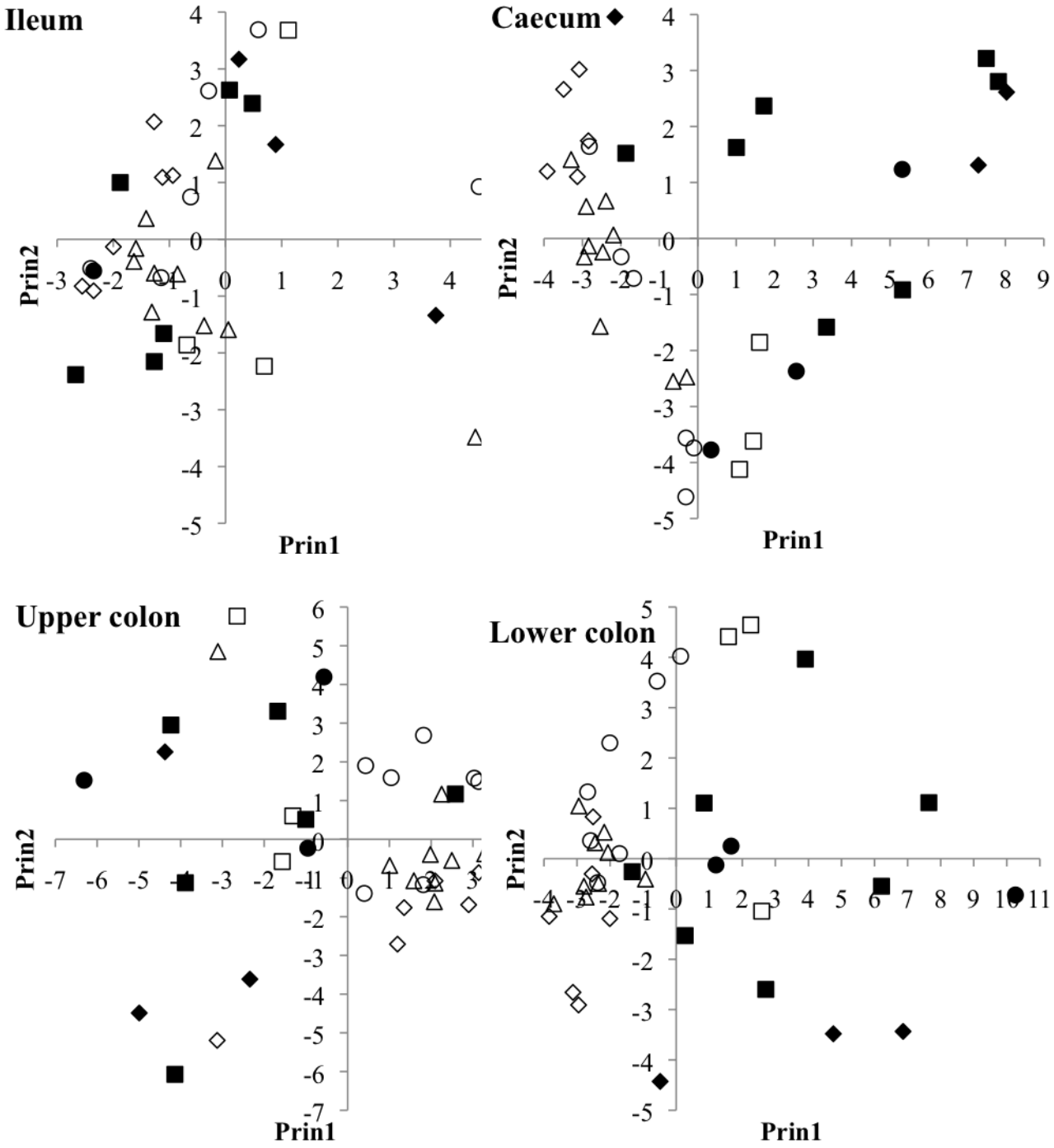
	Inulin, g/kg		Lupin, g/kg		Clinical SD		Pooled	
	0	80	0	220	-	+	SEM	Significance[1]
n	20	19	20	19	26	13		
No of T-RF								
Ileum	11.2	10.7	8.7	13.2	9.7	12.1	1.27	L
Caecum	27.3	26.4	27.3	26.4	22.7	31.0	1.28	SD
Upper colon	21.1	19.5	20.9	19.7	18.1	22.5	1.66	SD
Lower colon	24.9	22.2	22.2	24.9	20.1	26.9	1.20	SD
Dice coefficient, S_D								
Ileum	40.3	47.4	44.8	45.0	48.2	35.6	0.61	I, SD
Caecum	54.3	62.5	56.1	54.6	65.0	49.6	1.37	I, SD
Upper colon	49.0	49.6	49.0	46.4	59.6	33.6	1.55	SD
Lower colon	57.8	63.7	58.8	57.7	66.7	56.8	0.99	I, SD

T-RFs: Terminal restriction fragments; S_D: Dice coefficients

[1]Significance: $P < 0.05$, I = dietary inulin level, L = dietary lupin level, SD = clinical swine dysentery, NS = non-significant:

Dice similarity coefficients were calculated within groups (Table 8). Feeding inulin caused a more similar microbial community in the ileum ($P < 0.001$), caecum ($P < 0.001$) and lower colon ($P < 0.001$). Using lupins as the main dietary protein source caused a more similar microbiota in the ileum ($P = 0.010$), whereas pigs with SD were less alike in the ileum ($P < 0.001$), caecum ($P < 0.001$), upper colon ($P < 0.001$) and lower colon ($P < 0.001$) than healthy pigs. A PCA ordination of the T-RFLP data clearly discriminated between the T-RFLP profiles whether the pigs had SD (Figure 1), but failed to clearly distinguish between the different diet types.

Tentative identification of specific bacteria characterized by T-RFs was done in silico by inserting primer sequences and restriction enzymes in the MiCA home page (http://mica.ibest.uidaho.edu/dizgest.php) using the RDP database (Release 10, Update 11, Bacterial SSU 16S rRNA). The reference collection of 16S rRNA gene sequences did not cover all bacterial species present in the faeces of pigs and therefore some T-RF did not have a matching counterpart. The mean relative intensity of common T-RFs found in the ileal, caecal, upper colon and lower colon contents are given in Figure 2 and Figure 3.



4.

Figure 1. PCA ordination of bacteria terminal restriction fragment length polymorphism (T-RFLP) data for the microbial communities of the ileum, caecum, upper colon and lower colon. Only the T-RF present in at least 4 pigs were included. □: 0 g/kg inulin, 0 g/kg lupin, no SD; ■: 0 g/kg inulin, 0 g/kg lupin, SD; ○: 0 g/kg inulin, 220 g/kg lupin, no SD; ●: 0 g/kg inulin, 220 g/kg lupin, SD; Δ: 80 g/kg inulin, 0 g/kg lupin, no SD; ◊: 80 g/kg inulin, 220 g/kg lupin, no SD; ♦: 80 g/kg inulin, 220 g/kg lupin, SD.

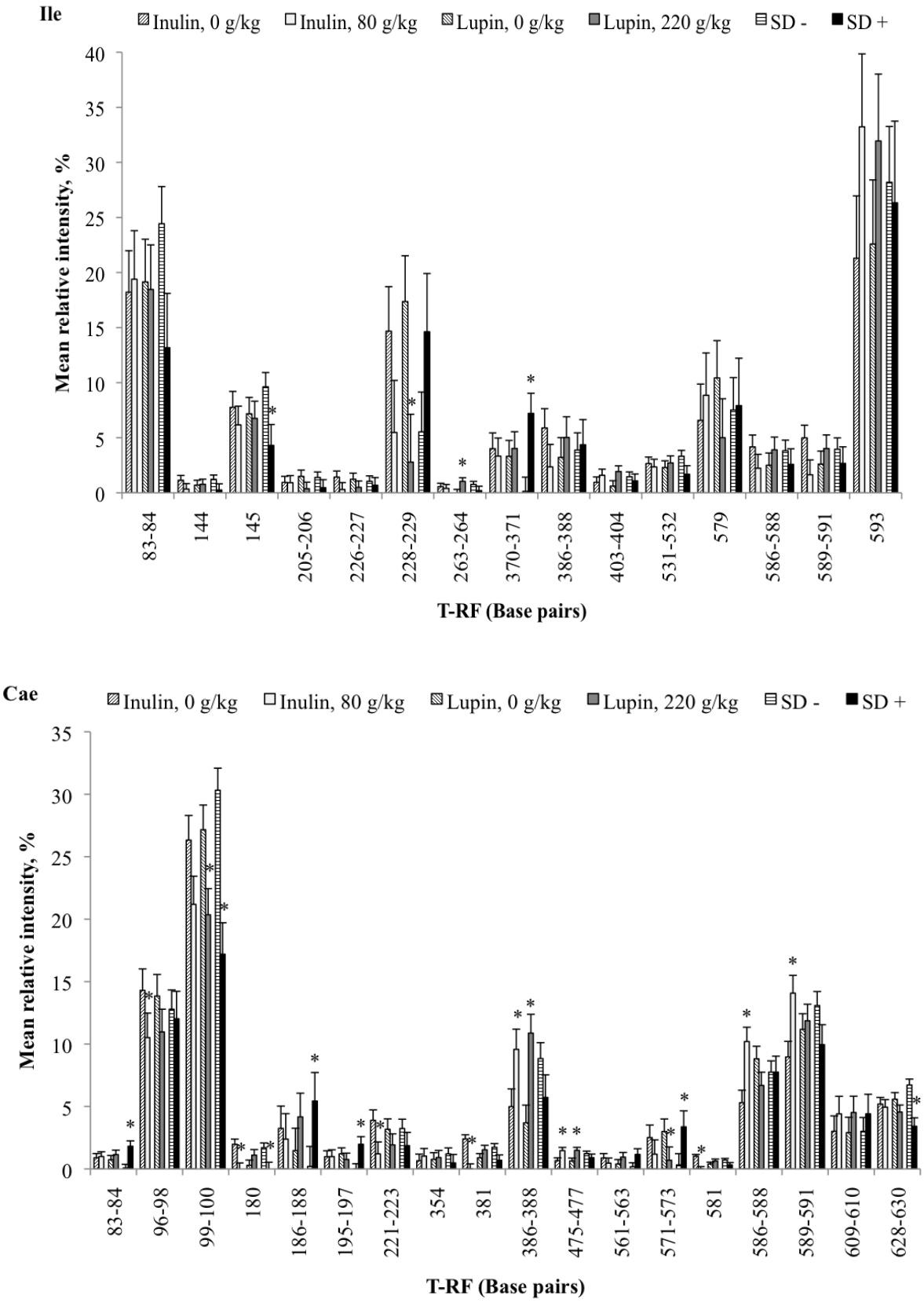


Figure 2. Relative fluorescent intensity of dominant T-RFs determined by T-RFLP analysis of ileal (Ile) and caecal (Cae) digesta the day of slaughter of pigs fed diets containing inulin, lupins and with and with out SD. The figure presents only T-RFs found in minimum 4 pigs and with an average intensity higher than 1% of the total intensity. * Values within inulin, lupin or SD not having the same letter differ ($P < 0.05$).

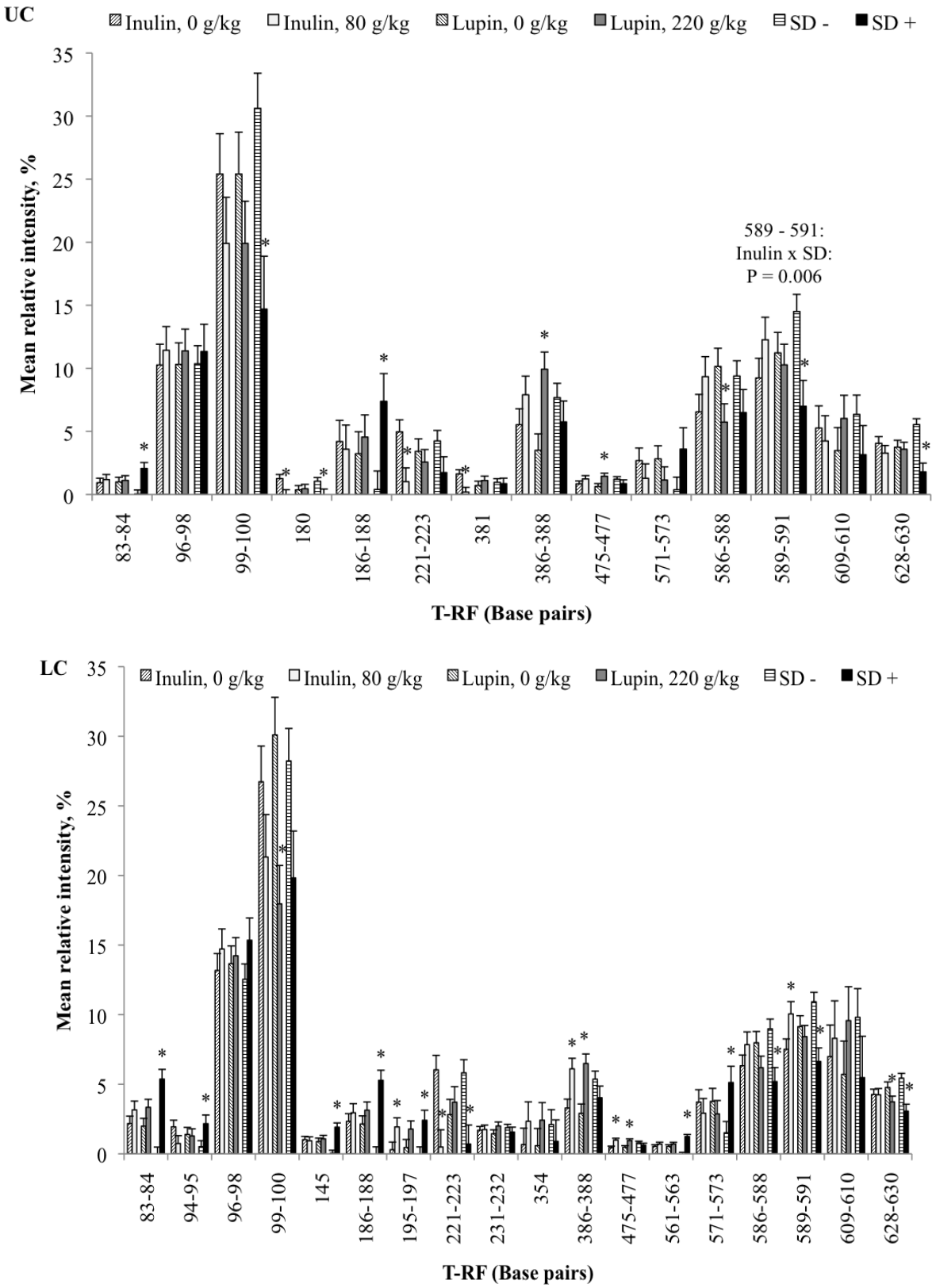


Figure 3. Relative fluorescent intensity of dominant T-RFs determined by T-RFLP analysis of upper (UC) and lower colon (LC) digesta on the day of slaughter of pigs fed diets containing inulin, lupins and with and with out clinical SD. The figure presents only T-RFs found in minimum 4 pigs and with an average intensity higher than 1% of the total intensity. * Values within inulin, lupin or SD not having the same letter differ ($P < 0.05$).

When the pigs had SD the relative abundance of the T-RFs at 145 in the ileum contents, 99-100 and 628-630 in the caecum and upper colon contents, and 221-223, 586-588, 589-591 and 628-630 in the lower colon contents, were reduced. Contrarily in pigs with SD a greater relative abundance of the T-RFs at 370-371 in the ileum contents, 195-197 and 571-573 in the caecal and lower colon contents, 83-84 and 186-188 in the caecal, upper and lower colon contents in addition to 94-95, 145 and 561-563 in the lower colon, was found.

In the upper colon there was a significant interaction between dietary inulin and SD ($P = 0.006$) as the T-RF at 589-591 was not observed in any of the pigs fed inulin that succumbed to SD. Furthermore, the T-RFs at 370-371 in the ileum, 83-84, 195-197 and 571-573 in the caecum, 83-84 in the upper colon and 83-84, 145, 186-188 and 195-197 in the lower colon, were only observed in pigs with SD.

Dietary inulin reduced the abundance of T-RFs at 96-98 in the caecal contents, 180 and 381 in the caecal and upper colon contents along with 221-223 in the caecal, upper and lower colon contents. On the other hand the T-RFs at 586–588 in the caecal contents, 195-197 in the lower colon contents and 386-388, 475-477 and 589-591 in the caecal and lower colon contents, were more abundant in pigs fed inulin.

Lupin inclusion in the diet reduced the relative abundance of the T-RFs at 228-229 in the ileum contents, 571-573 in the caecal contents, 586-588 in the upper colon contents, 628-630 in the lower colon contents and 99-100 in the caecal and lower colon contents. Contrarily the intensity of the T-RFs at 264 in the ileum contents and 386-388 and 475-477 in the caecal, upper and lower colon contents were elevated.

Tentative identification was performed based on the available databases combined with recent pig related publications where the same primers and restriction enzyme were used as in our experiment (Canibe et al., 2005; Højberg et al., 2005; Canibe et al., 2007; Castillo et al., 2007); see Table 9 for tentative identification of the T-RFs.

Table 9. Fragment size of T-RFs found in minimum 4 pigs and with an average intensity higher than 1% of the total intensity in the ileum, caecum upper colon and lower colon of pigs fed inulin, lupins and challenged with *B. hyodysenteriae* (Experiment 1).

Fragment size, bp	Tentative identification of the bacterial species
83 - 84	Unidentified
94 - 95	*Bacteroides* sp.
96 - 98	*Bacteroides* sp.
99 - 100	*Bacteroides* sp.
144	Unidentified
145	Unidentified
180	Unidentified
186 - 188	Unidentified
195 - 197	Unidentified
205 - 206	Unidentified
221 - 223	*Lactobacillus delbruekii* sp. *lactis*
226 - 227	Unidentified
228 - 229	Unidentified
231 - 232	*C. perfringes* / *Sarcina ventriculi*
263 - 264	Unidentified
354	Unidentified
370 - 371	*Escherichia coli*
380 - 381	Unidentified
386 - 388	Unidentified
403 - 404	Unidentified
475 - 477	Unidentified
531 - 532	Unidentified
561 - 563	Unidentified
571 - 573	Unidentified
579	Unidentified
581	*Streptococcus alactolyticus*/*S. hyointestinalis*/*S. suis*/*Lactococcus lactis*
586 - 588	*Megamonas hypermegale*
589 - 591	*Megasphaera elsdenii* / *Selenomonas ruminantium* / *Veionella parvula*
593	*Lactobacillus* sp./*Lactobacillus acidophillus*
609 - 610	*Weisella* sp.
628 - 630	Unidentified

Experiment 2

(i) Post-Infection Data

In the current experiment there was generally a good correspondence between the expected and analyzed contents of nutrients in the experimental diets (Table 2).

***Incidence of Swine Dysentery and Re-isolation of* B. hyodysenteriae**

The incidence of swine dysentery and re-isolation of *B. hyodysenteriae* in faeces and colon digesta are shown in Table 10. Generally the pigs fed 0, 20 and 40 g/kg inulin had a greater risk ($P = 0.002$) of developing clinical SD and were more likely ($P < 0.001$) to have positive colon contents that were culture-positive for *B. hyodysenteriae* at slaughter compared to pigs receiving 80 g/kg inulin. Accordingly, pigs fed 80 g/kg inulin were less likely to have fecal samples culture-positive for *B. hyodysenteriae* during the experimental period. Nevertheless it is

still noteworthy that 8 out of 15 pigs fed the diet with 80 g/kg inulin developed disease.

Table 10. Number of positive pigs and relative risk[1] of a pig being culture positive for *B. hyodysenteriae* or showing clinical signs of SD[2]

					P-value	
Inulin, g/kg:	0	20	40	80	Inulin	Linear
Pigs challenged, *n*	15	15	15	15		
Pigs with clinical SD	15	14	13	8		
Relative risk of clinical SD	1.9	1.8	1.6	1	0.022	0.001
Pigs shedding *B. hyodysenteriae* in faeces (culture)	15	15	14	11		
Relative risk of culture-positive *B. hyodysenteriae* faeces	1.4	1.4	1.3	1	<0.001	<0.001
Pigs with culture-positive *B. hyodysenteriae* colon content at euthanasia	15	15	13	10		
Relative risk of culture-positive *B. hyodysenteriae* colon content at euthanasia	1.5	1.5	1.3	1	<0.001	<0.001

[1]Relative risk: risk of an event in the group of interest compared with the reference group.
[2]Fifteen pigs per dietary treatment.

Dry Matter, pH and Ammonia Nitrogen

The DM content in the caecum decreased linearly (P = 0.007) with increasing dietary inulin levels (Table 11). In the lower colon it was the opposite, with the DM content having increased linearly (P = 0.007) with increasing inulin levels. The DM content in the ileum and upper colon was not influenced by diet.

The pH values in the caecum tended (P = 0.072), and in the upper colon decreased (P = 0.047), linearly with inulin levels in the diets. In the ileum and lower colon the pH values were not influenced by diets (Table 11).

Ammonia nitrogen (NH_3-N) concentrations in the caecum, upper colon and lower colon was unaffected by dietary inulin levels in the current experiment (Table 11).

Organic Acids in the Digesta

Diet did not affect the total concentration of organic acids or the molar proportion of the organic acids in the ileum except for a tendency to a linear decrease in the proportion of propionic acid (P = 0.060; Table 12). On the other hand, a linear increase in the total VFA concentration was observed in the caecum (P = 0.018), upper colon (P = 0.001) and lower colon (P = 0.013). In the caecum, increasing dietary inulin linearly tended to increase the molar proportion of propionic acid (P = 0.067), whereas a linear reduction in the percentage of iso-butyric acid (P = 0.015) and iso-valeric acid (P = 0.026) was observed. In the upper colon there was a linear decrease or tended to be a decrease in the percentage of acetic acid (P = 0.065), iso-butyric acid (P = 0.011) and iso-valeric acid (P = 0.013) with increasing levels of dietary inulin. Contrarily an increase or tendency to a linear increase was found for propionic acid (P = 0.038), butyric acid (P = 0.070) and valeric acid (P = 0.007). In the upper colon, there was a linear increase in the percentage of butyric acid (P = 0.026), valeric acid (P = 0.005) and caproic acid (P = 0.051) but a tendency to a linear decrease in the proportion of iso-butyric acid (P = 0.056; Table 12).

Table 11. The DM, pH and NH_3-N in digesta from different segment of the GIT at the day of euthanasia in pigs fed diets containing inulin and experimentally challenged with *B. hyodysenteriae*[1]

						P-value		
Inulin, g/kg:	0	20	40	80	SEM	Inulin	Linear	Quadratic
DM, %								
Ileum	12.9	11.4	10.2	11.9	0.83	0.160	0.454	0.035
Caecum	18.8[a]	16.1[a,b]	13.9[b]	13.7[b]	1.29	0.024	0.007	0.035
Upper colon	10.4	10.8	9.6	12.2	1.23	0.491	0.307	0.363
Lower colon	6.4	8.2	9.1	11.7	1.34	0.060	0.007	0.885
pH								
Ileum	7.3	7.3	6.9	7.2	0.13	0.112	0.496	0.094
Caecum	6.7	6.6	6.4	6.3	0.18	0.311	0.072	0.755
Upper colon	6.6	6.7	6.6	6.4	0.12	0.174	0.047	0.411
Lower colon	7.0	6.9	7.0	6.9	0.10	0.972	0.735	0.953
NH_3-N, mg/kg								
Caecum	485	385	404	327	69.8	0.432	0.131	0.768
Upper colon	556	522	690	413	76.6	0.085	0.237	0.086
Lower colon	531	502	520	485	48.4	0.911	0.554	0.962

[a,b]Values not having the same superscripts differ ($P < 0.05$).
[1]Fifteen pigs per dietary treatment.

Ileal Histology and Pig Performance

Villous height in the ileum, crypt depth and the villus to crypt depth ratio were unaffected by the dietary treatments (Table 13).

Average daily gain did not differ in the two-week adaptation period before the pigs were challenged with *B. hyodysenteriae*. The pigs fed diets containing 0, 20, 40 and 80 g/kg inulin grew on average 660 g/d (SEM 43.0), 753 g/d (SEM 46.8), 752 g/d (SEM 45.1) and 661 g/d (SEM 43.4), respectively.

Table 12. Total VFA concentration (mmol/kg of digesta), and molar proportion of the organic acids in digesta from different segments of the GIT at the day of euthanasia in pigs fed diets containing inulin and experimentally challenged with *B. hyodysenteriae*[1].

						P-value		
Inulin, g/kg:	0	20	40	80	SEM	Inulin	Linear	Quadratic
Ileum								
Total VFA + lactic acid, mmol/kg	29.0	31.9	33.0	35.4	4.7	0.793	0.318	0.839
Lactic acid, %	50.8	53.0	60.8	53.9	7.1	0.770	0.703	0.401
Acetic acid, %	36.0	39.3	31.3	40.1	5.4	0.655	0.712	0.511
Propionic acid, %	4.3	2.6	2.7	1.2	1.1	0.271	0.060	0.793
Butyric acid, %	4.9	2.5	3.4	3.0	1.2	0.503	0.396	0.395
Other VFA,[2] %	4.0	2.6	1.8	1.8	1.1	0.400	0.142	0.355
Caecum								
Total VFA, mmol/kg	123.4	130.0	131.4	156.8	10.3	0.113	0.018	0.545
Acetic acid, %	52.6	53.4	51.8	51.8	1.7	0.879	0.585	0.964
Propionic acid, %	24.9	26.0	27.1	27.3	1.0	0.268	0.067	0.439
Butyric acid, %	11.6	11.7	11.1	11.8	0.8	0.921	0.857	0.632
Valeric acid, %	4.6	4.0	4.4	5.2	0.5	0.416	0.268	0.248
Caproic acid, %	0.9	0.9	1.0	0.8	0.1	0.814	0.546	0.610
Iso-butyric acid, %	2.2	1.7	1.9	1.2	0.3	0.067	0.015	0.766
Iso-valeric acid, %	3.2	2.3	2.7	1.8	0.4	0.069	0.026	0.959
Upper colon								
Total VFA, mmol/kg	107.0[a]	110.6[a]	113.9[a]	133.1[b]	5.8	0.012	0.001	0.389
Acetic acid, %	53.7	54.0	53.1	50.9	1.2	0.272	0.065	0.493
Propionic acid, %	24.1	24.5	25.1	25.9	0.7	0.236	0.038	0.967
Butyric acid, %	10.2	10.8	10.6	11.9	0.7	0.288	0.070	0.669
Valeric acid, %	4.1[a]	3.9[a]	4.3[a]	5.4[b]	0.4	0.027	0.007	0.173
Caproic acid, %	0.8	0.9	0.9	1.0	0.1	0.416	0.104	0.823
Iso-butyric acid, %	2.9	2.6	2.5	2.0	0.3	0.081	0.011	0.979
Iso-valeric acid, %	4.1	3.4	3.6	2.8	0.4	0.070	0.013	0.959
Lower colon								
Total VFA, mmol/kg	79.9	88.6	89.8	99.9	5.6	0.093	0.013	0.832
Acetic acid, %	55.8	55.2	55.7	54.2	1.0	0.654	0.268	0.707
Propionic acid, %	24.2	23.8	23.8	23.7	0.6	0.927	0.540	0.787
Butyric acid, %	7.9	9.2	9.0	10.3	0.7	0.127	0.026	0.781
Valeric acid, %	3.3[a]	3.4[a]	3.5[a]	4.0[b]	0.2	0.039	0.005	0.510
Caproic acid, %	0.9	1.0	0.9	1.1	0.1	0.119	0.051	0.747
Iso-butyric acid, %	3.5	3.2	3.0	2.8	0.2	0.275	0.056	0.632
Iso-valeric acid, %	4.5	4.1	4.1	3.8	0.3	0.475	0.153	0.655

[a,b] Values not having the same superscripts differ (P < 0.05).

[1]Fifteen pigs per dietary treatment

[2]Sum of butyric, valeric, caproic, iso-butyric, iso-valeric and succinic acid.

T-RFLP Information

Unfortunately and given the large degree of SD observed in this study, compared to Experiment 1 where sickness was milder, it was not possible to present the T-RFLP data in a meaningful physiological/microbiological sense. There was also enormous variation within and between dietary treatments that further rendered the information meaningless.

Table 13. Villous height, crypt depth and villous height to crypt ratio in ileal samples[1] from pigs fed diets with increasing amounts of inulin and experimentally challenged with *B. hyodysenteriae*.

						P-value		
Inulin, g/kg:	0	20	40	80	SEM	Inulin	Linear	Quadratic
Villous height, µm	245	244	232	244	15.0	0.917	0.884	0.582
Crypt depth, µm	202	173	180	186	18.0	0.700	0.658	0.351
Villus:crypt ratio	1.30	1.47	1.33	1.34	0.09	0.551	0.904	0.517

[1] Fifteen pigs per dietary treatment.

(ii) Pre-Infection Data

The animals remained healthy throughout the experimental period and no differences in feed intake were observed between the treatment groups (data not shown).

The DM content of the faeces decreased linearly (P = 0.008) in response to increased dietary supplementation with inulin. There was a linear reduction in faecal NH_3-N (P = 0.034) with increasing dietary inulin content. However, the concentration of PUN was not affected (P > 0.05) by inulin level in the diet (Table 14).

Dietary inulin content had no effect on the absolute amount of total VFA in the faeces and hence faecal pH was not affected by diet (P > 0.05). However, when the individual VFAs were expressed as a molar proportion, increasing dietary inulin linearly tended to increase the concentration of valeric acid (P = 0.081) whereas the concentration of the BCFAs iso-butyric and iso-valeric were decreased (P = 0.040) (Table 15).

Table 14. Effects of feeding increasing levels of inulin on faecal DM, pH, NH_3-N and plasma urea nitrogen (PUN) before infection of pigs with *B. hyodysenteriae*.

						P-value		
Inulin, g/kg	0	20	40	80	SEM	Treatment	Linear	Quadratic
DM, %	28.2^{a}	28.5^{a}	$27.2^{a,b}$	26.7^{b}	0.49	0.034	0.008	0.951
pH	6.0	5.9	5.9	6.0	0.08	0.754	0.766	0.309
NH_3-N, mg/kg	732	638	554	568	55.6	0.096	0.034	0.155
PUN, mmol/L	3.1	3.0	3.4	2.9	0.17	0.279	0.511	0.261

^{a,b} Values not having the same superscripts differ (P < 0.05).

Table 15. Effect of feeding increasing levels of inulin on faecal concentrations of VFA and molar proportions of VFAs before infection of pigs with *B. hyodysenteriae*.

						P-value		
Inulin, g/kg	0	20	40	80	SEM	Treatment	Linear	Quadratic
Total VFA, mmol/kg	186	192	188	180	8.5	0.789	0.487	0.520
Acetic acid, %	48.8	49.0	48.9	50.5	1.07	0.604	0.219	0.577
Propionic acid, %	24.6	24.6	25.5	23.9	0.92	0.651	0.577	0.340
Butyric acid, %	15.6	15.5	15.1	14.5	0.62	0.578	0.158	0.943
Valeric acid, %	4.3	4.7	4.9	5.1	0.32	0.337	0.081	0.536
Caproic acid, %	1.4	1.6	1.6	1.6	0.17	0.626	0.316	0.394
Iso-butyric acid, %	2.0^{a}	$1.8^{a,b}$	1.5^{b}	1.7^{b}	0.13	0.040	0.045	0.048
Iso-valeric acid, %	3.2^{a}	$2.8^{a,b}$	2.4^{b}	2.7^{b}	0.20	0.040	0.056	0.040

^{a,b} Values not having the same superscripts differ (P < 0.05).

The T-RFLP analysis of faeces demonstrated differences in bacterial communities between the dietary groups. The number of identifiable T-RFs was higher ($P < 0.05$) in the pigs fed 80 g/kg inulin compared with the other dietary treatments (Table 16). Inulin influenced ($P = 0.008$) bacterial diversity as indicated by similar S_D for all inulin-fed pigs (Table 16). The S_D revealed that individuals within the groups that received inulin had more similar bacterial diversity than individuals from the control group that did not receive inulin.

Table 16. Effects of feeding increasing levels of inulin on average number of terminal restriction fragments and dice coefficients (S_D) in the faeces of growing pigs before infection of pigs with *B. hyodysenteriae*.

						P-value		
Inulin, g/kg	0	20	40	80	SEM	Treatment	Linear	Quadratic
No. of T-RF	26.8[a]	25.2[a]	26.6[a]	31.5[b]	1.54	0.028	0.012	0.105
Dice coefficient, %	52[a]	58[b]	55[b]	55[b]	1.1	0.008	0.424	0.013

T-RFs: Terminal restriction fragments; S_D: Dice coefficients.
[a,b] Values not having the same superscripts differ ($P < 0.05$).

Despite the similar degree of bacterial diversity among the inulin fed pigs, including 80 g/kg inulin in the diet selectively increased or tended to increase the relative density of bacteria at T-RF 75 ($P = 0.033$), 115 ($P = 0.091$), 386-388 ($P = 0.004$) and 589-591 ($P = 0.071$) while tending to decrease the relative bacterial diversity at T-RF 184-190 ($P = 0.078$) and 221-223 ($P = 0.099$; Figure 4). Tentative identification was performed based on the available databases combined with recent pig related publications where the same primers and restriction enzyme were used as in our experiment (Canibe et al., 2005; Højberg et al., 2005; Canibe et al., 2007; Castillo et al., 2007); see Table 17 for tentative identification of the T-RFs.

Table 17. Fragment size, frequency (number of samples out of 15 per group), and effect of dietary inulin inclusion on T-RFLP profiles in faeces of growing pigs[1] before infection of pigs with *B. hyodysenteriae* (Experiment 2).

	Inulin, g/kg				
Fragment size, bp	0	20	40	80	Tentative identification of the bacterial species
61	3	4	5	6	*Lactococcus lactis*
75	7	10	10	11	*Lactobacillus vitulinus*
76 - 80	13	12	4	8	Unidentified
84	11	11	13	9	Unidentified
96 - 98	15	15	14	15	*Bacteroides* sp.
99 - 105	15	15	15	15	*Bacteroides* sp.
107 - 109	11	8	4	8	Unidentified
115	14	15	14	16	Unidentified
147 - 148	10	11	13	10	*Fibrobacter intestinalis*
184 - 190	15	12	12	12	Unidentified
221 - 223	14	14	12	7	*Lactobacillus delbruekii* sp. *lactis*
386 - 388	10	13	12	15	Unidentified
587 - 588	15	15	14	15	*Megamonas hypermegale*
589 - 591	9	12	12	15	*Megasphaera elsdenii* / *Selenomonas ruminantium* / *Veionella parvula*
609 - 610	7	8	9	11	Unidentified
629 - 630	10	13	12	13	Unidentified

[1]Only T-RFs found with a relative intensity higher than 1% of the total intensity are presented.

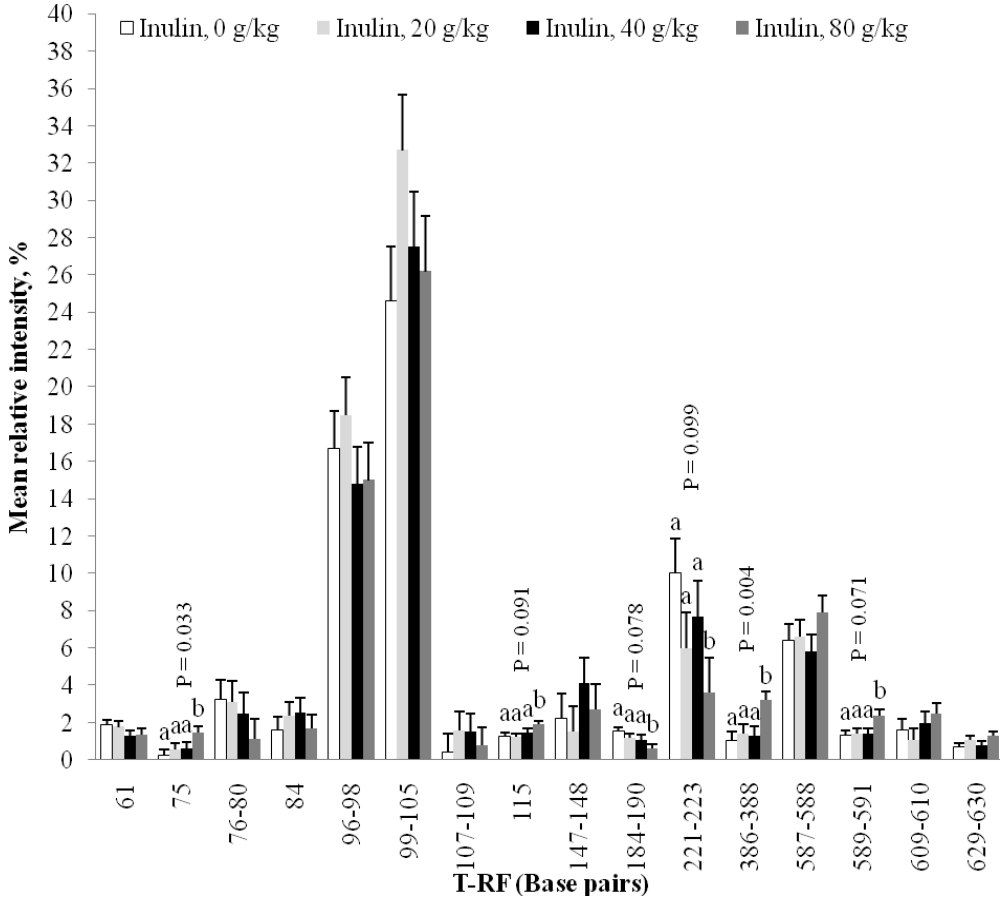


Figure 4. Relative fluorescent intensity of the dominant T-RFs determined by T-RFLP analysis of faeces in growing pigs fed different levels of dietary inulin in Experiment 2. The figure presents only T-RFs found with a frequency higher than 1% of the total intensity. [a,b] Values not having the same superscripts differ ($P < 0.05$).

5. Discussion and Application of Research

Experiment 1

Dietary Inulin

The results of the current experiment confirm the results of Thomsen et al. (2007), and demonstrate that the carbohydrate fraction of the diet plays an important role in the pathogenesis of SD. The current study identified inulin as being the main protective component of the diet, such that diets containing 80 g/kg of inulin reduced the risk of pigs developing clinical SD and reduced the number of challenged pigs that contained *B. hyodysenteriae* in the colon digesta. In contrast, almost all pigs fed the diet containing canola meal showed SD and had *B. hyodysenteriae* in their colon contents. Other researchers have also found that fermentable carbohydrates influence the development of SD. In a field study, Bilic and Bilkie (2003) observed that a diet supplemented with wheat shorts and cornstarch reduced the incidence of SD compared with a diet containing less fermentable carbohydrates, even though the diet did not protect the pigs from developing SD. In contrast, a North American study using fermentable

carbohydrates from sugar beet pulp, wheat shorts, and potato starch failed to prevent the development of disease caused by *B. hyodysenteriae* (Kirkwood et al., 2000). In a study by Pluske et al. (1996), feeding diets that contained wheat, barley, or oat groats were associated with an almost 100% incidence of SD. The level of total nonstarch polysaccharides (NSP) in these diets was 89, 137, and 77 g/kg DM, respectively. In the present experiment, the total NSP content in the diets varied from 141 to 232 g/kg DM, so clearly the properties of the dietary carbohydrate fraction are important factors when formulating diets to control swine dysentery infections in pigs.

Nevertheless, our data seemingly contradict previous findings where diets supplemented with soluble NSP and resistant starch were found to facilitate the development of SD in comparison with diets lower in fermentable carbohydrates and resistant starch, i.e., diets based on cooked white rice and animal protein were found to be completely protective against SD (Pluske et al., 1996; Siba et al., 1996; Pluske et al., 1998). In these reports, it was suggested that an increased amount of fermentable carbohydrates entering the large intestine was associated with an increased prevalence of SD as diets based on cooked white rice provided little substrate for fermentation in the hindgut. On the other hand, attempts to reproduce these results by Lindecrona et al. (2003) and Kirkwood et al. (2000) failed. In response, Pluske and Hampson (2009) suggested that this could be due to differences in the virulence of the different strains of *B. hyodysenteriae* used in the different studies, different feeding strategies (diet composition, intake) causing a different microbial community before oral challenge, and (or) differences in rice processing that could be of importance.

Fermentation of inulin by the indigenous microbiota results in the production of VFA, gases, and organic acids (Gibson and Roberfroid, 1995), however, the luminal pH values and concentration of VFA in the ileum, caecum, and upper and lower colon were unaffected by dietary treatment in the current experiment. Accordingly, Lynch et al. (2007) failed to detect any differences in caecal or colonic pH or VFA concentration when feeding inulin to finisher pigs. In contrast, Halas et al. (2009) using weaner pigs and Loh et al. (2006) using grower pigs, observed lower total VFA concentrations in the large intestine when supplementing the diet with inulin.

Nevertheless, in the present experiment, feeding inulin influenced the proportion of organic acids in the luminal contents. Inulin-fed pigs had a decreased proportion of acetic acid in the ileum and caecal contents, which is in agreement with observations by Halas et al. (2009) and Loh et al. (2006). Interestingly, feeding inulin increased the concentration of butyric acid in the caecum and upper colon but decreased it in the lower colon. However, according to Cummings and Macfarlane (1991), dietary inulin mainly stimulates lactobacilli that produce lactic and acetic acid, and hence butyrate and valerate production should not be stimulated. In humans, it has been shown that butyrate-producing bacteria can be net users of acetate, and Bindelle et al. (2008) reported that the proportion of acetate is reciprocal to the concentration of butyrate due to bacterial cross-feeding. Therefore, it is probable that the lower proportion of acetic acid in the inulin-fed pigs may be linked with an increased proportion of butyric and valeric acid, and consequently increased numbers of microbes producing those acids. Feeding inulin might have caused an increase in lactate-producing bacteria that in turn could stimulate lactate-utilizing butyrate producers such as *Megasphaera elsdenii* (Mølbak et al., 2007).

Collectively these data indicate that dietary supplementation with inulin most likely influenced bacterial populations in pigs fed these diets, resulting in the observed changes in VFA levels and proportions. Changes in bacterial populations also might have affected the pathogenesis of SD. Generally it is possible to modify either microbial balance or metabolic activity alone (Fonty et al., 1993). Examination of the concentration of ATP in digesta in the current experiment reveals that bacterial activity seemed identical among dietary treatments in the ileum, caecum, and lower colon. On the other hand, an increased ATP concentration in the upper colon of the pigs receiving inulin most likely reflects an increased number of bacteria in these pigs.

A change in the proportion of organic acids in the ileum indicates that the ileum harbors sufficient numbers of bacteria to ferment inulin, which to some extent could explain the absence of an inulin effect on pH values and VFA content in the large intestine. However, it is possible that inulin-induced changes to the ileal microbiota and contents will be disseminated to the large intestine potentially affecting fermentation in the caecum and colon. The different dietary effects on SD expression are most likely linked to diet-related changes in the intestinal microbial community (Leser et al., 2000). Such changes might inhibit the colonization of *B. hyodysenteriae* or any of the synergistic bacteria, which have been reported to facilitate colonization by *B. hyodysenteriae* (Whipp et al., 1979).

Lupin Inclusion

Lupin inclusion at 220 g/kg in the diets did not prevent disease even though the onset of disease was delayed. These observations are in accordance with findings by Siba et al. (1996). In that study, feeding dehulled lupins at 150 g/kg in a cooked white rice-based diet and a whole-wheat diet resulted in 83.3 and 62.5% of the challenged pigs, respectively, developing SD. In the current experiment, lupin inclusion did not affect the concentration or proportion of lactic acid or VFA in the ileum or the caecum. In the upper and lower colon, lupins increased the proportion of propionic acid. In contrast, a Spanish study found a higher concentration of lactate, acetate, and iso-butyrate in the ileal contents of Iberian pigs fed a lupin-based diet compared with a soybean-based diet (Rubio et al., 2005).

Ileal Histology and Performance

The length of the villi and crypts in this experiment were similar to observations in grower pigs made by Hedemann et al. (2005). Similarly, increased villous height in rats fed inulin has been reported (Kim, 2002). The longer villi in the ileum of pigs fed lupins and inulin might be related to a higher absorption of nutrients and energy in these pigs. The villus:crypt ratio expresses the balance between cell loss from the villi and cell production in the crypt. In this study, the inulin-fed pigs had a higher ratio indicating less cell loss, which in turn could have positive implication for the ability of these animals to digest and absorb nutrients (Pluske et al., 1997), thus the potential for better performance of those animals.

In the present experiment, care should be taken when interpreting the slower growth of the pigs fed lupins in the adaptation period before challenge, as the experiment was not designed to detect differences in performance. However, Nørgaard and Fernandez (2009) recently observed reduced performance in organically-reared pigs fed diets containing 250 g/kg lupins. In the present experiment, the pigs fed lupins showed an increase in ileal digesta viscosity, which has been associated with reduced interaction between nutrient and

digestive enzymes and decreased nutrient digestibility (O'Connell et al., 2005), and possibly explains the reduced growth rate. In accordance with the present finding, Pluske et al. (2007) observed an increased ileal viscosity in pigs fed a diet based on wheat, barley, and lupin compared with feeding rice-based diets. The slower growth rate of the pigs fed the diet with canola meal without inulin post-challenge was due to these pigs developing SD sooner than pigs on the other dietary treatments. As the crude protein content was lower than predicted in all the experimental diets, this likely had no influence on the performance observed.

T-RFLP Data

Infection with B. hyodysenteriae
We used T-RFLP analysis in an attempt to characterize bacterial communities in the different segments of the GIT of pigs under infection pressure with SD. The T-RFLP analyses showed that the most abundant T-RF (593 bp) in the ileum was tentatively identified as a *Lactobacillus* sp. In the caecum and colon the most abundant microbes irrespective of dietary treatment and occurrence of disease were *Bacteroides* spp., which have also been the most abundant bacterial groups detected by molecular screening of the human colonic microbiota (Eckburg et al., 2005; Suau et al., 2001). Nonetheless, development of SD had a much larger impact on the microbiota in the large intestine than the dietary treatment as expressed by the PCA-analyses of the T-RFLP data.

In Experiment 1, infection with *B. hyodysenteriae* influenced the intestinal environment and microbial community in pigs irrespective of the diet fed, especially in the caecum and upper and lower colons. A peak characterizing *B. hyodysenteriae* was not identified in this study as we only had access to a 600 bp standard and consequently only worked with T-RFs that were between 50 and 650 bp long. Further work at our laboratory using the same restriction enzyme but a longer standard has reveal that *B. hyodysenteriae* gives a unique T-RF at 662 bp (*unpublished data*).

The different dietary effects on SD are most likely related to diet-related changes in the intestinal microbial community (Hansen et al., 2010; Leser et al., 2000). Such changes might inhibit the colonization of *B. hyodysenteriae* or any of the synergistic bacteria which have been reported to facilitate colonization by *B. hyodysenteriae* (Whipp et al., 1979). Whether the synergism observed between *B. hyodysenteriae* and other anaerobes facilitates colonization or expression of pathogenicity or both has not clearly been demonstrated, however it is noteworthy that there are several observations indicating the disease produced in gnotobiotic pigs colonized with *B. hyodysenteriae* was less severe than in conventional pigs, or even absent (Brandenburg et al., 1977; Meyer et al., 1974; Neef et al., 1994). Several unidentified bacteria, e.g., T-RFs 83-84 and 186-188, were up-regulated and almost exclusively present in the large intestine of pigs with SD potentially indicating a synergism between *B. hyodysenteriae* and these bacteria. Contrarily other bacteria, especially a T-RF at 589-591 tentatively identified as *Megasphaera elsdenii* that is potentially antagonistic to *B. hyodysenteriae*, was less abundant in the large intestine of pigs with SD.

Overall, infection with *B. hyodysenteriae* tended to destabilise the bacterial communities in the caecum and upper and lower colons, which was also expressed by decreased mean Dice coefficients as a consequence of clinical SD. In accordance with the present study, previous investigations have also shown that

the colonic microbiota in pigs changed dramatically when they were infected with *B. hyodysenteriae* (Leser et al., 2000; Leser et al., 1997). A shift in the colonic epithelial microbiota from predominating non-motile Gram-positive bacteria to motile Gram-negative bacteria after infection has previously been reported (Robinson et al., 1984).

An interesting observation, even though the importance is unclear at the present time, was that a T-RF of 370-371 tentatively identified as *Escherichia coli*, that is normally associated with post-weaning diarrhoea (Hampson, 1994), was only observed in the ileal contents of pigs with SD.

Dietary inulin supplementation
One way to manipulate the microbiota is to add selective substrates (e.g., inulin) to the diet. A T-RF tentative identified as *Lactobacillus delbruekii* sp. *lactis* was detected to a lesser extent in pigs fed the highest amount of inulin. *L. delbruekii* sp. *lactis* is an obligately homo-fermentative lactobacilli that ferments glucose exclusively to lactic acid (Stiles and Holzapfel, 1997). *L. delbruekii* sp. *lactis* has previously been isolated in the pig GIT (Castillo et al., 2007), although typically inhabiting dairy products especially Swiss-type cheeses (Delley and Germond, 2002).

In the caecum of pigs receiving inulin, a T-RF indentified tentatively as *Megamonas hypermegale* was more abundant. *M. hypermegale* has previously been found in pigs (Canibe et al., 2007) and poultry, and production of acetic and propionic acids by this species has been thought to inhibit colonization of the poultry intestine with *Salmonella* (Scupham et al., 2008).

Furthermore, in pigs fed 80 g/kg inulin a relatively higher frequency of a T-RF tentatively identified as *Megasphaera elsdenii*, *Selenomonas ruminantium* or *Veionella parvula* was identified. The same observation was made by Mølbak et al. (2007) in the colon of grower pigs fed an inulin-rich diet, however Mølbak et al. (2007) were able to use *in situ* hybridization to show that *M. elsdenii* was the most likely bacteria identified. *M. elsdenii* is a ruminal, non-motile cocci that usually growths on glucose, fructose and/or lactate even though it sometimes can use other substrates too (Stewart et al., 1997). In the rumen, *M. elsdendii* is the main lactate-utilizing bacteria and it has been shown that *M. elsdenii* utilizes lactate in preference to glucose in media where both carbon sources are present (Soto-Cruz et al., 2002). In pigs this means that *M. elsdenii* potentially cross-feeds on lactate produced by other members of the microbial community. Different strains of *M. elsdenii* have been shown to ferment lactate to different VFAs, however acetate, propionate, butyrate and valerate are the main products of lactate metabolism (Soto-Cruz et al., 2002).

Dietary lupin inclusion
Lupins increased the number of identifiable T-RFs in the ileum indicating a more diverse microbiota in the small intestine of these pigs. In the pigs fed lupins that developed SD, a much lower proportion of lactic acid but higher proportion of acetic acid was observed in the ileal contents potentially indicating that manipulation of the microbiota in the small intestine might play a role in the susceptibility of pigs to SD. The observed changes in the proportion of VFAs in the ileal contents in the present study might have been influenced by the lower abundance of the unknown bacteria T-RF 228-229 that was less abundant in the pigs fed lupins. It can be speculated that the bacteria with a T-RF of 386-388 that

were more abundant in the caecum, upper and lower colon of pigs receiving lupins might be involved in the degradation of galactans from this legume.

Experiment 2

The aim of this experiment was to evaluate the effect of increasing dietary levels of inulin on the incidence of SD, and fermentation characteristics and the microbial community in the faeces of growing pigs before infection.

Post-infection
The results confirm findings in Experiment 1 demonstrating that dietary inulin can play an important role in the development and pathogenesis of SD (Hansen et al., 2010). However, it was mainly the diet containing 80 g/kg inulin that was able to reduce the risk of pigs developing SD and reduce the number of challenged pigs that harbored *B. hyodysenteriae* in the colon digesta at slaughter. These findings strongly indicate that a high concentration of dietary inulin is necessary to obtain a protective effect against SD if inulin is used as the sole intervention. In this study, a large proportion of pigs fed 80 g/kg inulin developed the disease compared to Experiment 1, which can be explained by differences in the amount and concentration of the broth used to challenge the pigs with *B. hyodysenteriae*. In Experiment 2, pigs were challenged on four consecutive days with 100 mL of a broth containing approximately 10^9 cfu per mL of *B. hyodysenteriae* whereas in Experiment 1, pigs received 80 mL of a broth culture with around 10^8 cfu per mL viable cells in the previous study (Hansen et al., 2010). The difference in challenge intensity was done to fully "test" the efficacy of inulin in ameliorating SD.

Nonetheless, our findings are in accordance with other researchers who also found that fermentable dietary carbohydrates reduced the incidence of SD (Bilic and Bilkei, 2003; Thomsen et al., 2007). Contrarily, Thomsen et al. (2007) found that an organic diet based on dried chicory roots and lupins offered complete (100%) protection against SD after experimental challenge with *B. hyodysenteriae*. In Experiment 1 we demonstrated that pigs fed inulin had a reduced risk of developing SD, while the onset of disease was delayed in pigs fed lupins, however and unlike Thomsen et al. (2007), a small number of pigs fed the protective diets developed disease. These discrepancies are most likely due to differences in virulence of the different strains of *B. hyodysenteriae* employed or dissimilarity in the experimental diets used. Nevertheless and as discussed for Experiment 1, our findings seemingly contradict previous findings where diets supplemented with soluble NSP and resistant starch were associated with the development of SD compared with diets based on cooked white rice and animal proteins (Pluske et al., 1998; Pluske et al., 1996; Siba et al., 1996).

Fermentation of inulin by the indigenous microbiota results in the production of VFA and gases (Roberfroid et al., 1998), so consequently the luminal pH values in the caecum and upper colon fell with increasing levels of dietary inulin. Likewise the concentration of VFA in the caecum, upper colon and lower colon increased when the pigs were fed higher concentrations of inulin. Generally dietary inclusion of inulin has shown contradictory results with respect to luminal pH and VFA concentrations. We observed in Experiment 1 that feeding 80 g/kg inulin to pigs experimentally challenged with *B. hyodysenteriae* had no influence on large intestinal pH and total VFA concentrations, whereas Halas et al. (2009) using

weaner pigs, and Loh et al. (2006) in grower pigs, observed lower total VFA concentrations in the large intestine when diets were supplemented with inulin.

All the same, in Experiment 2, feeding increasing levels of inulin influenced the proportion of VFAs in the luminal contents, which concurs with previous findings (Halas et al., 2009; Hansen et al., 2010; Loh et al., 2006). Mølbak et al. (2007) suggested that feeding inulin can cause an increase in lactate-producing bacteria that in turn can stimulate lactate-utilizing butyrate producers such as *Megasphaera elsdenii*. In addition, fermentation of protein is normally more evident in the large intestine as carbohydrates can become a limiting factor for fermentation by the microbes (Jensen, 2001). Collectively, this could explain the decline in BCFA in the caecum and colon with increasing levels of dietary inulin as the preferential metabolism of inulin by carbohydrate fermenting bacteria may have reduced the activity of the proteolytic bacteria. In agreement, Jensen et al. (1995) showed that the production of protein metabolites from microbial fermentation might be reduced by inclusion of non-starch polysaccharides.

Protein fermentation is thought to be accompanied by an increased production of ammonia, indoles, phenols amines and sulfuric-containing compounds (Heo et al., 2009). In the current experiment, NH_3-N was measured as a marker of protein fermentation in the large intestine that nonetheless was unaltered by dietary inulin levels. However, this lack of increased luminal NH_3-N concentrations in the large intestine could be a result of nitrogen fixation by bacteria and/or growth of the caecal and colonic biomass possibly coupled with acidification of the large intestinal contents resulting in conversion of ammonia to the less diffusible NH_4^+ ion (Gibson and Roberfroid, 1995).

Collectively, data from Experiment 2 suggests that dietary supplementation with inulin most likely influenced bacterial populations in the gut (see next section), resulting in the observed changes in VFA levels and proportions. These changes have most likely affected the pathogenesis of SD. Different dietary effects on the expression of SD are most probable linked to diet-related changes in the intestinal microbiota (Leser et al., 2000). Such changes could either inhibit the colonization of *B. hyodysenteriae* directly or inhibit any of the synergistic bacteria that have been reported to be facilitate colonization with *B. hyodysenteriae* (Whipp et al., 1979).

Experiment 2, therefore, demonstrated that pigs fed 80g/kg inulin have reduced risk of developing clinical SD after experimental challenge with *B. hyodysenteriae*, thereby confirming the findings in Experiment 1. In addition, this experiment underlines that relative high dietary inclusion levels are necessary to induce changes in large intestinal fermentation characteristics. Diets supplemented with 80 g/kg inulin possibly protect pigs against developing SD by modifying the regular microbiota in the gastrointestinal tract, although intermediate protection against SD was observed at 40 g of inulin/kg of diet.

Pre-infection

Fermentation Characteristics

The linear decrease in the faecal DM content with increasing levels of dietary inulin could possibly be explained by the presence of non-absorbable sugars in the diet, which will increase the load of water and electrolytes in the large intestine (Wiggins, 1984). In addition, the production of VFAs may further increase osmotic

pressure, resulting in accelerated peristaltic movement and decreased transit time (Ruckebusch, 1981). A decreased transit time likely reduced reabsorption of water and electrolytes in the colon, resulting in the lower faecal DM content.

Total faecal VFA concentration was not influenced by inulin level so consequently faecal pH also remained unchanged. In accordance, total faecal VFA concentration in grower pigs was not influenced by 50 g/kg dietary inulin in a study by Rideout et al. (2004), and other reports also found no response in terms of intestinal pH and VFA concentration due to inulin supplementation (Houdijk et al., 1998; Hansen et al., 2010). Furthermore, fermentation of protein is more pronounced in the large intestine where carbohydrates can become a limiting factor for microbial fermentation (Jensen, 2001). Together this could explain the decrease in the concentration of BCFA in the current experiment with increasing levels of inulin. The preferential metabolism of inulin by carbohydrate fermenting bacteria may have decreased the activity of the proteolytic bacteria. In agreement, previous work has shown that the production of protein metabolites from microbial fermentation may be reduced by inclusion of non-starch polysaccharides (Jensen et al., 1995).

As discussed previously, protein fermentation is accompanied by increased production of ammonia, indoles, phenols, amines and sulfuric-containing compounds (Heo et al., 2009). The PUN and faecal NH_3-N levels were measured as "biomarkers" of microbial fermentation of protein and/or protein utilization efficiency (Chen et al., 1995; Coma et al., 1995). The concentration of PUN can increase due to increased microbial production of NH_3-N and its subsequent diffusion into the portal blood stream and conversion to urinary N via the urea cycle in the liver, or to ineffectiveness associated with imbalance or excess of essential amino acids available for protein synthesis, or to a combination of both factors (Heo et al., 2009). In the present experiment PUN probably was not influenced by NH_3-N origination from microbial fermentation of protein as faecal NH_3-N decreased linearly with increasing levels of dietary inulin whereas PUN was unaffected. In man, dietary fibres and non-absorbable sugars are known to reduce blood ammonia and urea levels (Gibson and Roberfroid, 1995). These effects have been associated with the growth of colonic biomass and nitrogen fixation by bacteria in the large intestine possibly coupled with acidification of the colonic content and conversion of ammonia to the less diffusible NH_4^+ ion (Gibson and Roberfroid, 1995). However PUN levels in the current experiment remained unchanged when dietary inulin levels were increased.

Nevertheless and whilst measurement in the faeces of pigs does not necessarily reflect conditions more cranial in the large intestine, our results suggest a reduction in the extent of protein fermentation in the hindgut of pigs fed increasing levels of inulin. This could be a contributing factor to the observed beneficial effects of inulin with respect to reduced boar taint (Hansen et al., 2006) and occurrence of SD after experimental infection with *B. hyodysenteriae* (Hansen et al., 2010).

T-RFLP Analysis of the Microbial Community Prior to Infection

According to the available T-RFLP libraries (RDP database), many different bacteria of different taxonomical groups (genus and species) can share these T-RF. Thus, definitive identification of bacteria responsible for these T-RFs would require further isolation, cloning and genetic sequences.

To evaluate global changes in the faecal microbial ecosystem the T-RFLP method was employed. The most abundant microbes irrespective of dietary treatment were tentatively identified as *Bacteroides* sp., which was also the most abundant bacterial group detected by molecular screening of the human colonic and faecal microbiota (Suau et al., 2001; Eckburg et al., 2005). It is noteworthy that changes in the faecal microbial community using the T-RFLP technique were only detectable in the pigs fed 80 g/kg inulin. The pigs fed this particular diet had a richer microbial community, expressed as more identified T-RFs, compared with the other dietary treatments. In addition, the T-RFLP data showed that a T-RF tentatively identified as *Lactobacillus vitulinus* was more abundant in the pigs fed 80 g/kg inulin. *L. vitulinus* is a non-motile, gram-positive rod that is able to ferment a wide range of sugars including cellobiose, fructose, galactose, glucose, mannose and lactose. The cells are homo-fermentative producing lactic acid (Stewart et al., 1997). Consistent with Experiment 1, a T-RF tentatively identified as *Lactobacillus delbruekii* sp. *lactis* was detected to a lesser extent in pigs fed the highest amount of inulin. *L. delbruekii* sp. *lactis* is an obligately homo-fermentative lactobacilli that ferments glucose exclusively to lactic acid and does not ferment pentoses or gluconate (Stiles and Holzapfel, 1997). *L. delbruekii* sp. *lactis* has previously been isolated in the pig GIT (Castillo et al., 2007).

Furthermore and again consistent with Experiment 1, pigs fed 80 g/kg inulin showed a relative higher frequency of a T-RF tentatively identified as *Megasphaera elsdenii*, *Selenomonas ruminantium*, or *Veionella parvula*. The same observation was made by Mølbak et al. (2007) in the colon of grower pigs fed an inulin-rich diet, however Mølbak et al. (2007) were able to use *in situ* hybridization to show that *M. elsdenii* was the most likely bacteria identified. *M. elsdenii* is a ruminal, non-motile cocci that usually growths on glucose, fructose and/or lactate even though it sometimes can utilise other substrates as well (Stewart et al., 1997). *M. elsdendii* is the main lactate utilizing bacteria in the rumen and has been shown to utilize lactate in preference to glucose in media where both carbon sources are present (Soto-Cruz et al., 2002).

6. Conclusions

Previously, and in reference to Experiment 1, it was not possible to determine whether dried chicory roots supplying inulin or galactans from sweet lupins in the diet were responsible for the observed protection against *B. hyodysenteriae* in pigs that was observed by Thomsen et al. (2007). Experiment 1 clearly demonstrated that pigs fed inulin at 80 g/kg of diet had a reduced risk of developing SD, while the onset of disease was delayed in pigs fed lupins; feeding canola meal alone was associated with the highest incidence of SD. Diets supplemented with highly fermentable carbohydrates from inulin may protect pigs against developing SD by modifying the normal microbiota in the GIT. Furthermore and in conjunction with the T-RFLP analyses, our data indicate that a proposed mechanism by which inulin protects pigs against SD is linked to induced changes in the large intestinal microbiota that are most likely antagonistic with *B. hyodysenteriae*. Tentative bacteria were identified in this regard.

In Experiment 2, it was demonstrated again albeit under conditions of a greater intensity of infection challenge, that feeding 80 g/kg inulin in the diet was required to reduce the relative risk of pigs developing SD, although pigs fed 40

g/kg of inulin showed an intermediate level of protection. Moreover, this experiment showed that before infection with *B. hyodysenteriae*, pigs fed diets with 80 g/kg inulin had T-RFs tentatively identified as *Lactobacillus vitulinus* and *Megasphaera elsdenii* that were more abundant, whereas *Lactobacillus delbruekii* sp. *lactis* was found less frequently. Such changes could be associated with the establishment of an environment that assists with protection against infection with *B. hyodysenteriae*, but we were unable to definitively test this proposition in this study. The experiment indicates that relatively high dietary inclusion levels of inulin (80 g/kg) are necessary to induce changes in the faecal fermentation characteristics and microbial community in grower pigs.

7. Limitations/Risks

Data generated in these two experiments were conducted in a research environment using limited, although statistically sufficient, numbers of animals owing to both the infrastructure confines of the facility and the logistics/cost involved with conducting studies of this nature. Evaluation under field conditions is therefore warranted to confirm or deny these experimental findings. Furthermore, it is unknown at this time whether feeding a diet containing inulin once pigs have been diagnosed clinically with SD would be beneficial; the current project did not allow for this study to be conducted.

The major impediment to uptake of these findings presently is the cost of inulin supplementation. We used a pure product used for human nutrition purposes, however we are aware of cheaper and non food-grade forms of inulin in the marketplace such as FibroFos 60 (60% inulin), manufactured by SOCODE (a division of Cosucra Groupe Warcoing S.A.) in Belgium (http://www.socode-warcoing.be/en/inulin_Fibrofos.htm). In the UK, this currently sells for $1.10-1.30 per kg (Mick Hazzeldine, *personal communication*). Interestingly, this product is being included in the diets of male finishing pigs at 90 g/kg of diet to control boar taint, at a cost of AU $4/pig (Mick Hazzeldine, *personal communication; May 2010*).

Dr Peter McKenzie was consulted for his view on current medications and costs for SD. Dr McKenzie said that the ‘base case’ for the medicated control of SD is currently 100-ppm monensin/salinomycin or 100-ppm Enterodox. Allowing 200 kg of medication @ $5-8, the cost works out to be $1.00-1.60 per pig. Dr McKenzie points out though that these numbers are ‘ballpark’ only, because some of the respiratory (weaner) drugs (tiamulin, lincomycin) are effective against SD, and the cost can be decreased if pigs are cleaned up with Enterodox by end of Grower 1 phase and enhanced hygiene into the grow-finish area. Furthermore, the ‘big issue’ is that ionophores and lincomycin have a modest life at modest use, with tiamulin usually lasting longer but is on the way out due to years of use (resistance). There is also a considerable gap between performance on drugs that stop clinical signs (monensin) and drugs that stop production losses (Econor).

8. Recommendations

As a result of the outcomes in this study the following recommendations have been made:

1. Inulin can be used in the diet of growing pigs at 80 g/kg of diet to reduce clinical SD under conditions of intense infection pressure, with intermediate protection offered at an inclusion level of 40 g/kg diet. Lupins, relative to canola meal, reduced the incidence and severity of SD under the dietary and infection challenge conditions used;
2. Other dietary strategies such as (i) the use of lactic acid (high lactic acid levels were found in the digesta of pigs fed 80 g/kg inulin in Experiment 1, as a consequence of microbial production), (ii) the use of fructose/sucrose (fructans, which is the carbohydrate in inulin, are cleaved to fructose by the microbiota in the large bowel and hence supplying fructose directly could be beneficial), and (or) (iii) probiotic administration (e.g., *Megaspheara elsdenii* was found as a dominant species in pigs fed the protective 80 g/kg inulin diet), used in conjunction with a lower level of inulin being supplied directly (e.g., 40 g/kg), could be investigated further;
3. In this regard, any further studies (experimentally or commercially) should also consider feeding an inulin-enriched diet once the pigs have been diagnosed with SD, since studies conducted herein were with naïve pigs made sick using the challenge model;
4. A cost-benefit analysis be conducted in association with veterinarians and nutritionists to evaluate the merits, or otherwise, of dietary inulin supplementation compared to current medication strategies.

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