



HAL
open science

The microbiota of pigs influenced by diet texture and severity of infection

Lars Mølbak, Kaare Johnsen, Mette Boye, Tim K. Jensen, Markku Johansen, Kristian Møller, Thomas D. Leser

► To cite this version:

Lars Mølbak, Kaare Johnsen, Mette Boye, Tim K. Jensen, Markku Johansen, et al.. The microbiota of pigs influenced by diet texture and severity of infection. *Veterinary Microbiology*, 2008, 128 (1-2), pp.96. 10.1016/j.vetmic.2007.09.012 . hal-00532325

HAL Id: hal-00532325

<https://hal.science/hal-00532325>

Submitted on 4 Nov 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: The microbiota of pigs influenced by diet texture and severity of *Lawsonia intracellularis* infection

Authors: Lars Mølbak, Kaare Johnsen, Mette Boye, Tim K. Jensen, Markku Johansen, Kristian Møller, Thomas D. Leser



PII: S0378-1135(07)00446-4
DOI: doi:10.1016/j.vetmic.2007.09.012
Reference: VETMIC 3823

To appear in: *VETMIC*

Received date: 4-7-2007
Revised date: 5-9-2007
Accepted date: 11-9-2007

Please cite this article as: Mølbak, L., Johnsen, K., Boye, M., Jensen, T.K., Johansen, M., Møller, K., Leser, T.D., The microbiota of pigs influenced by diet texture and severity of *Lawsonia intracellularis* infection, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2007.09.012

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The microbiota of pigs influenced by diet texture and severity of *Lawsonia intracellularis* infection.

Lars Mølbak^{1*}, Kaare Johnsen¹, Mette Boye¹, Tim K. Jensen¹, Markku Johansen², Kristian Møller¹ and Thomas D. Leser^{1a}

¹National Veterinary Institute, Technical University of Denmark DK-1790 Copenhagen, Denmark.

²The National Committee for Pig Production, DK-8620 Kjellerup, Denmark

1) Running title: The microbiota of pigs infected with *Lawsonia*

* Corresponding author: National Veterinary Institute, Technical University of Denmark ,
Bülowsvej 27, DK-1790 Copenhagen V, Denmark. Phone: 0045 72346361, Fax: 0045
72346001, E-mail: lam@vet.dtu.dk

Present address: ^aChr. Hansen A/S, DK-2970 Hørsholm, Denmark

1 ABSTRACT

2 Pigs with and without naturally occurring *Lawsonia intracellularis* infection were fed diets with
3 different texture. In a previous study from 79 pig herds using a similar feeding on pelleted or non-
4 pelleted form showed that the non-pelleted diet was associated with a reduced prevalence of *L.*
5 *intracellularis*. In this study a mechanistic approach was taken for explaining and testing this
6 observation by studying the microbiota and the occurrence of *L. intracellularis* in the distal ileum of
7 54 pigs by terminal restriction fragment length polymorphism (T-RFLP) analysis, Real-Time PCR
8 and in situ hybridization. The texture of the diet influenced the microbiota, and from a quantitative
9 discriminative analysis of the terminal restriction fragments (T-RFs) of ileum samples it was
10 deduced that *Clostridium* spp. and *Lactobacillus* spp. were associated with the non-pelleted diet and
11 *Streptococcus* spp. with the pelleted diet. In experimentally infected pigs it was verified that 89 bp
12 and 90 bp sized T-RFs (*Hha*I) from ileum represented *L. intracellularis*. The non-pelleted diet
13 seemed to reduce the relative amount of *L. intracellularis* in the total microbiota of the ileum, but
14 the number of pigs detected positive with *L. intracellularis* by Real-Time PCR was not influenced.
15 The five pigs with highest *L. intracellularis* content showed T-RFs that were not present in profiles
16 from less or non-infected pigs, which may indicate that some bacterial species were associated with
17 *L. intracellularis* infection.

18
19 **Keywords:** T-RFLP, microbiota; *Lawsonia intracellularis*, intestinal community, pig, diet texture.

20

21 INTRODUCTION

22 *Lawsonia intracellularis* is an obligate intracellular bacterium and the causative agent of
23 proliferative enteropathy (PE) (McOrist et al, 1993; McOrist et al., 1995). The infection causes
24 diarrhea, retarded growth and/or sudden death in pigs and is one of the economically most

25 important diseases in the swine industry worldwide. The specific PE lesions in the intestinal tract
26 occur in the distal small intestine and less commonly in the large intestine (Rowland & Rowntree,
27 1972). *L. intracellularis* has been detected in tissue sections by immuno-techniques with polyclonal
28 (Lawson et al., 1985; Guedes & Gebhart, 2003) or monoclonal antibodies (McOrist et al., 1987;
29 Guedes & Gebhart, 2003; Boesen et al. 2005), or by *in situ* hybridization with rRNA-targeting
30 probes (Boye et al., 1998). Several PCR studies have been described for detection of *L.*
31 *intracellularis* in intestine and faecal samples (Jones et al., 1993; Møller et al., 1998).

32

33 *L. intracellularis* has only been cultured in cell cultures, and the pathogenesis of *L. intracellularis*
34 infections is not fully understood. Interestingly, it has been shown in a pig model that the germ-free
35 or gnotobiotic piglets with addition of only a simple contaminating microbiota appear to be less
36 disposed to infection by *L. intracellularis*. Nevertheless, the role of a more complex, but incomplete
37 intestinal microbiota allows *L. intracellularis* infection, but the role of bacterial members of the
38 microbiota in facilitating *L. intracellularis* infection is undefined (McOrist et al., 1993; McOrist et
39 al., 1994) and has not yet been studied with cultural independent techniques.

40 In studies of intestinal pathogens in Danish pig herds, the prevalence of herds infected with *L.*
41 *intracellularis* was very high (93.7%). However, it was found that home-mixed and/or non-pelleted
42 diets seemed to be associated with reduced in-herd prevalence of *L. intracellularis* (Stege et al.,
43 2001) and that the animals' diets (Boesen et al., 2004) and/or a changed intestinal microbiota might
44 influence infection with *L. intracellularis*. In pigs, pelleting generally results in an increase in
45 weight gain and an improved feed utilisation (Vanschoubroek et al., 1971; Wondra et al., 1995a;
46 Wondra et al., 1995b; Mikkelsen et al., 2004). These studies have emphasized the need to study the
47 relationship between diet, the microbiota, and the disease in more detail. To address some of the

48 questions related to these interactions in the intestinal environment, this paper describes a survey of
49 the texture of the diet with regard to influence on the microbial community structure.

50

51 The intestinal microbiota of pigs is complex and the majority of the bacterial species colonizing the
52 gastrointestinal tract have not been characterized or cultured yet (Pryde et al., 1999; Leser et al.,
53 2002). In this study the bacterial community structure was therefore described by using the well-
54 established method terminal-restriction fragment length polymorphism technique (T-RFLP) (Liu et
55 al., 1997). Terminal restriction fragment (T-RF) patterns are generated and analysed in a series of
56 steps that combine PCR, restriction enzyme digestion and gel electrophoresis. Since each T-RF
57 ideally represents a single species or taxonomic group of bacteria, it is possible to relate the
58 empirically found T-RFs to theoretically digested 16S rRNA genes of bacteria or clones from 16S
59 rRNA clone libraries (Liu et al., 1997, Kaplan et al., 2001; Jernberg et al., 2005). This method
60 provided the possibility both to identify and to assess the presence of *L. intracellularis* in the
61 intestinal microbial community and to describe the total microbiota of pigs with *L. intracellularis*
62 infection.

63

64 MATERIALS AND METHODS

65 **Experimental infection.** 24 pigs (Landrace×Yorkshire×Duroc) were 5-6 weeks of age at the
66 beginning of the experiment (Day 0). The pigs were purchased from a high health herd known to be
67 free of *B. hyodysenteriae* and *L. intracellularis* (Boesen et al., 2004). The pigs were randomly
68 divided into two pens according to weight and sex with 10 pigs in pen A and 14 pigs in pen B. The
69 pens had slatted floors and were separated by wood walls and a corridor. The pigs were fed with a
70 finely ground diet, which was pressed into a pellet form under heat. The absence or presence of *L.*
71 *intracellularis* was confirmed at days 0, 14, 21, 28 and 34 by a specific *L. intracellularis* Real-Time

72 PCR analysis (see description below). The pigs in pen B were after 14 days experimentally infected
73 with 25 ml *L. intracellularis* homogenate inoculum as previously described (Boesen et al., 2004).
74 The pigs from pen A received the same treatment as the pigs from pen B but instead of the bacterial
75 homogenate they received 25 ml of a sucrose-potassium-glutamate [pH 7.0] solution. Five
76 prechosen pigs from each of the two pens were killed 21 days after the experimental infection. The
77 pigs were euthanized by bolt pistol followed by exsanguinations. A 10-cm section from the ileum
78 was tied off not to lose intestinal content and stored at -20°C until the final processing. All
79 procedures of animal handling and experimentation were performed under veterinary supervision
80 and according to recommendations by the Danish Committee of Animal Experimentation.

81

82 **Field study:** Pigs (Landrace×Yorkshire×Duroc) from a Danish herd (Forsøgsstation Grønhøj;
83 Denmark) were included in this study. After weaning, piglets from the herd were mixed (at an
84 average weight of 8.4 kg) and allocated to one of the treatment groups based on average weight
85 (pelleted and non-pelleted diets) and housed in pens with raised mesh floors. Each pen had an
86 average of 24 pigs. Eight weeks after weaning (at an average weight of 32 kg), the pigs were moved
87 from the nurseries to the grower-finisher. For the T-RFLP analyses three to five pigs were randomly
88 picked from four separate pens when the pigs were moved from nurseries to the grower-finisher,
89 and sacrificed. The sampling was done sequential three times and at each time eight weeks after
90 weaning, and a total of 52 pigs were sacrificed for the T-RFLP study (27 fed a pelleted diet and 27
91 fed a non-pelleted diet). The average weight (kg) of a pig from the pellet and non-pelleted feeding
92 groups were, when they were killed, 33.6 ± 4.6 and 31.9 ± 8.3 ($p>0.05$), respectively. None of the T-
93 RFLP-analysed animals were from pens treated with antibiotics. The pig herds use of antibiotics
94 treatments (antibiotics treatments \times pig⁻¹ \times pen⁻¹) in the 32-100 kg weight class of pigs (pen=240)

95 were significantly different ($p < 0.05$) and in average 0.57 and 0.38 for the pelleted and non-pelleted
96 diet, respectively.

97

98 **Feeding and sampling from pigs.** The pigs were either fed with a coarsely ground grain diet (non-
99 pelleted) or with a more finely ground diet, which was pressed into a pellet form under heat. The
100 feed was mixed from the same batch of feed ingredients. No major difference was monitored
101 between the pelleted and the non-pelleted diets (Supplementary table 1a and 1b). The pigs were
102 sacrificed by an intracardial injection of pentobarbital. Each animal was necropsied immediately
103 after euthanasia, and a 10-cm section from the ileum was tied off and stored at -80°C until the final
104 processing. The samples were only thawed once to minimize DNA degradation (Mølbak et al.,
105 2006).

106

107 **T-RFLP profiles** were done as described by Leser *et al.* (2000). Essentially, 200-mg of intestinal
108 content was suspended in 600- μl of phosphate-buffered saline [pH 7.4] (0.13 M NaCl, 3 mM
109 Na_2HPO_4 per litre, 7 mM KH_2PO_4), vortexed thoroughly and then centrifuged for 2 min at $200\times g$.
110 The supernatant was centrifuged ($12,000\times g$, 5 min) and the resulting pellet was resuspended in 570-
111 μl of Tris-EDTA [pH=7]. The suspension was added 30- μl of 10% sodium dodecyl sulfate and
112 homogenized with 400-mg of 100- μm Zirconia-silica beads (BioSpec Products Inc., Bartlesville,
113 USA) using a minibead-beater at high speed for 4 min (Biospec Products Inc). The DNA was
114 extracted from the lysed cells and purified by the CTAB method (Ausubel et al., 1988) and stored at
115 -20°C . The DNA was adjusted by spectrophotometry to a concentration of 5- μg of DNA ml^{-1} . Four
116 replicate 50- μl PCR mixtures were made from each sample using the universal bacterial primers S-
117 D-Bact-0008-a-S-20 (5'-AGAGTTTGATCMTGGCTCAG-3') and S-D-Bact-0926-a-A-20 (5'-
118 CCGTCAATTCCTTTRAGTTT-3') (Leser et al. 2000). Primer S-D-Bact-0926-a-A-20 was 5' FAM

119 (carboxy-fluorescein-N-hydroxysuccinimide-ester-dimethyl-sulfoxide) labelled. Purified PCR
120 products (200-ng) were digested with restriction enzyme for 3 h at 37°C with 20 U of either *Hha*I;
121 *Msp*I; *Rsa*I or *Hae*III (Boehringer, Mannheim, Germany) in 20- μ l reaction mixtures. The
122 fluorescently labeled T-RFs were analyzed by electrophoresis on an automatic sequence analyzer
123 (ABI-PRISM-373-DNA-Sequencer; PE Biosystems, Foster City, Calif.).

124

125 **Monitoring of *L. intracellularis*.** The presence of *L. intracellularis* in intestinal samples were
126 detected with a Real-Time PCR assay routinely used for *L. intracellularis* diagnostics at The
127 National Veterinary Institute (Lindecrona et al., 2002). Positive controls (DNA extracted from
128 faeces from pigs diagnosed positive with *L. intracellularis*) and negative controls were included.
129 For histology a cross-section of ileum was fixed in 10% neutral buffered formalin, embedded in
130 paraffin, sectioned 6- μ m thick, and mounted on Super Frost slides (Menzel-Gläser, Braunschweig,
131 Germany). The slides were deparaffinized in xylene and dehydrated in alcohol. One slide from each
132 ileum sample analyzed by T-RFLP was haematoxylin and eosin stained and processed routinely for
133 histology. The fluorescent *in situ* hybridisation (FISH) was done as previously described (Boye et
134 al., 1998) using the Cy3-labelled probe specific for *L. intracellularis* Law1: (5'-
135 AACCGGAGCAGTCTCTCTAG-3'); a fluorescein-labelled probe specific for the *Campylobacter*
136 genus CAMP653: (5'-NCTGCCTCTCCCTYACTCT-3') (Schmid et al., 2005) or/and the general
137 bacterial probe S-D-Bact-0338-a-A-18 (5'-GCTGCCTCCCGTAGGAGT-3') (Amann et al., 1990).
138 The fluorescence cross scans of the intestine were acquired by using an ArrayWoRx white-light
139 charge-coupled-device-based scanner (Applied-Precision, Issaquah, Washington) with a 0.5-s
140 exposure time in both the FITC and the Cy3 channels at a 5- μ m image resolution. An Axioimager
141 M1 epifluorescence microscope equipped for epifluorescence with a 100-W HBO lamp and filter
142 sets 43 and 38 were used to visualize CY3 and fluorescein, respectively. Images were obtained

143 using an AxioCam MRm version 3 FireWiremonochrome camera and the software AxioVision
144 version 4.5 (Carl Zeiss, Oberkochen, Germany). All Images were processed for display by using
145 Photoshop software version 8.5 (Adobe, Mountain View, CA)

146

147 **Statistical methods.** Pig statistics were analysed with SAS (Proc-Logistic, SAS Institute,
148 Heidelberg, Germany). Logic regression was used to analyse the number of days where pigs had
149 diarrhoea. Chi-square tests with a Yates' Correction were used according to the formula to test
150 observed values against expected. The Mann-Whitney U-test and Kruskal-Wallis tests were used to
151 compare the medians of individual T-RFs between the two diet groups and/or the severe infected
152 pig. BioNumerics generated principal component analysis (PCA) on band patterns, where the T-RFs
153 were the variables and the pigs were the objects (n). The first and second principal component was
154 found for each of the three experimental repetitions. Division by the variances over the entries and
155 subtraction of the averages over the characters was included in the PCA. A quantitatively
156 discriminative analysis (Applied-Maths, Kortrijk, Belgium) allowed the determination of characters
157 (T-RFs) that were responsible for the separation of user-delineated groups (pelleted and non-
158 pelleted). We used TORAST, a commercial T-RFLP operation results analysis software tool
159 (<http://www.torast.com/>) to find potential species or genera from specific T-RFs.

160

161 RESULTS

162 **The T-RFLP profiles of ileum.** T-RFLP profiles were retrieved from the ilea of 54 pigs from a
163 Danish herd. At each sampling time, 3-5 pigs from 4 pens fed with a pelleted or non-pelleted diet,
164 respectively, were sacrificed. A comprehensive analysis of the T-RFLP patterns was done for all
165 samples, and a total of 325 *Hha*I Terminal-restriction fragments (T-RFs) were included in the
166 analysis. Two fragments (T-RFs 35- and 37 bp) were found in all the T-RFLP patterns cut with

167 *HhaI*. A non quantitative principal component analysis (PCA) ordination of the 3 independent
168 experiments showed that it was possible to differentiate whether the pigs had been fed with pelleted
169 or non-pelleted diet, indicating that the diet texture had an effect on the bacterial community
170 structure for ileum samples (Fig. 1). Especially, a combination of the first and second principal
171 components ($x=y$ variance line) was able to separate the two diet groups at a variance ranging from
172 13.4% to 21.8%. We found no unique T-RFs (only present in pigs from one treatment group) for
173 either the pelleted or non-pelleted diets, but the relative frequency out of the total population for
174 four or the T-RFs were significantly different between the two diet groups (Fig. 2). One of these
175 was T-RF 90 bp that was recognised as belonging to *L. intracellularis* (see later). The three other T-
176 RFs could not be affiliated to any bacterial group or species. A quantitative discriminative analysis
177 found that T-RFs 231 bp and 596 bp were responsible for the grouping of pigs fed with non-pelleted
178 diet and T-RF 581 bp for pelleted diet, which means that these groups of bacteria changed in
179 relative numbers from one diet form to the other. In a pig clone library (Leser et al., 2002) T-RFs
180 231 and 596 bp were identified as *Clostridium* spp. and *Lactobacillus* spp., respectively. The T-RF
181 581 bp corresponded to *Streptococcus* spp. (Supplementary data 2).

182

183 ***L. intracellularis* detection.** The theoretical *HhaI* T-RF for *L. intracellularis* is under the given T-
184 RFLP conditions 90 bp. An experimental *L. intracellularis* infection on a relative small number of
185 pigs was done to verify that non-infected pigs did not have a T-RF of 90 bp (Fig. 3A). The pigs
186 were killed three weeks after the infection and four of the five pigs infected with *L. intracellularis*
187 had a detectable T-RF at 90 bp. The one challenged pig that did not have the detectable *L.*
188 *intracellularis* T-RF was detected negative with the specific *L. intracellularis* Real-Time PCR as
189 well. The pigs not detected positive for T-RF 90 bp clustered together and the first principal

190 component in a PCA separated the pigs with a high-density *L. intracellularis* T-RF from the rest of
191 the pigs (Fig. 3B).

192 In the analysed T-RFLP profiles of the pigs from the Danish pig herd, two T-RFs (89- and 90 bp)
193 were in 1 bp range from the theoretically *L. intracellularis* T-RF. There was a significant ($p < 0.05$)
194 difference between the pigs fed the non-pelleted diet (48% pigs tested positive for *L. intracellularis*)
195 and the pigs fed the pelleted diet (81% pigs tested positive for *L. intracellularis*). The Real-Time
196 PCR was more sensitive than the T-RFLP in detecting *L. intracellularis* and the total number of
197 pigs detected positive by Real-Time PCR on DNA extracted from the intestinal lumen was 16%
198 higher than the T-RFLP detection. In contrast to the T-RFLP profiles of ileum there was no
199 significant dependency ($p > 0.05$), when the Real-Time assay was used, between the pigs fed the
200 pelleted diet (85% of the pigs were tested positive for *L. intracellularis*) compared to the pigs fed
201 with a diet in a non-pelleted form (78% of the pigs were tested positive for *L. intracellularis*).

202
203 **Unique T-RFs for *L. intracellularis* infected pigs.** Three of the four pigs detected positive with T-
204 RFLP for having *L. intracellularis* in the experimental infection experiment did also have a unique -
205 T-RF (102 bp) and a significant ($p < 0.01$) higher average proportion of Hha-T-RF 374 bp and 571
206 bp and a lower average proportion of 407 bp, respectively out of the total band intensity when
207 compared to the non-infected pigs. *Campylobacter* spp. have previously been associated with *L.*
208 *intracellularis* infection with conventional culturing methods and the theoretical T-RF values for
209 *Campylobacter* spp. are between 96 bp to 100 bp in length (McCartney et al 1984, Boosinger et al
210 1985). The density of positive in situ targeted *Campylobacter* cells was in general low in the small
211 intestine of both the *L. intracellularis* infected pigs and the control pigs, opposite to a relative high
212 density in the large intestine for both groups (Fig. 4A). We saw no intracellular *Campylobacter*
213 cells in either the small or large intestine at any time. Likewise, using *in situ* hybridization with a

214 *Campylobacter* genus-specific probe we saw no microscopic relation between *L. intracellularis*
215 infected cells and *Campylobacter* spp. (Fig. 4B). In the naturally infected pigs five of the ileum
216 samples (three of pelleted diet and two with non-pelleted diet) had a T-RF of 90 bp with intensity
217 higher than 10% out of each pigs total T-RF intensity (Fig. 2). Examination by hematoxylin and
218 eosin staining (data not shown) and *in situ* hybridization using a probe specific for *L. intracellularis*
219 verified that all five pigs were severely infected with *L. intracellularis* (Fig. 4D). Similar
220 observations were not seen for the ileum samples (N=5) with no T-RFs of 89 or 90 bp (Fig. 4C).
221 Samples from the five pigs with severe *L. intracellularis* infection had 2 unique T-RFs (364- and
222 373 bp) and the T-RFs 83-86 intensity were more than 3 times higher than from the less or not
223 infected pelleted and non-pelleted fed pigs (Fig. 2). The average intensity of a range of T-RFs
224 significant decreased or was not present in the pigs with the severe *L. intracellularis* infections (Fig.
225 2). In order to describe the total microbiota in ilea of severely *L. intracellularis* infected pigs the T-
226 RFLP PCR amplicons were cut independently with 3 other restriction enzymes (*MspI*, *HaeI* and
227 *RsaI*). For all 3 new restriction enzymes, all the new T-RFLP profiles had a high intensity band in
228 the theoretical T-RFs of *L. intracellularis*. The T-RFs present in four of the five pigs with severe *L.*
229 *intracellularis* infections were included in a combination search, which limit the amount of
230 potential bacteria effectively, in the NCBI 16S rRNA gene database (Supplementary data 2).
231 Seventeen percent of the matches in the NCBI database belonged to clones from the pig clone
232 library (Leser et al., 2002). Finally, T-RFs 83 and 86 bp were potentially found to belong to
233 uncultured CFB/Green sulfur group bacterium clone (88% similarity to *Prothecochochloris*
234 *vibrioformis*) and *Alcaligenes* sp. STC1, respectively. T-RF 364 bp corresponded to an uncultured
235 bacterium clone p-1877-s962-3 (92% similarity to *Eubacterium desmolans*) from the pig clone
236 library. T-RF 373-374 bp matched two clones, an uncultured candidate division OP11 bacterium
237 clone and an uncultured clone: pHAuB-N (83% similarity to *Rhodobacter apigmentum*).

238 DISCUSSION

239 **The T-RFLP profiles of ileum.**

240 Using the T-RFLP method, we found a total of 325 *HhaI* T-RF fragments, which is in agreement
241 with a clone library established by Leser et al. (2002), who identified 375 phlotypes in an analysis
242 of the bacterial communities in pigs. We found that *HhaI* T-RFs 35- and 37 bp were present in all
243 the ileum samples. These bands may represent several different species, but are most likely
244 members of the *Clostridium leptum* subgroup (RDP reg. No. 2.30.9.1.3), which is among the most
245 abundant intestinal species including *Ruminococcus spp.*, *Eubacterium siraeum* and
246 *Faecalibacterium prausnitzii* (Leser et al., 2002).

247 With respect to the analysis of T-RFLP profiles, principal component analysis (PCA) is a strong
248 tool for comparison of complex microbial communities (Dollhopf et al., 2001; Kaplan et al., 2001).
249 In this study the combination of the first and second principal components separated the pelleted
250 from non-pelleted fed pigs in all the three independent samplings (Fig. 1). Consequently, the diet
251 structure affected the total microbiota directly or indirectly. With the use of the most comprehensive
252 16S rRNA gene clone library from pigs (Leser et al., 2002) and a quantitative discriminative
253 analysis it was deduced that T-RFs belonging to *Clostridium spp.* and *Lactobacillus spp.* were
254 responsible for the grouping of pigs fed the non-pelleted diet and *Streptococcus spp.* for the pelleted
255 diet. The fact that *Lactobacillus spp.* and *Streptococcus spp.* are predominant in the small intestine
256 is consistent with studies based on cultural experiments (Briggs et al., 1954; Willingale & Briggs,
257 1955). These changes in the microbiota in the small intestine of pigs fed the two different diets may
258 be due to both the pelleting process where physico-chemical changes occur in the ingredients of a
259 diet when they are subjected to physical compression, shear and heat and that coarsely ground grain
260 diet seems to induce physical conditions in the stomach that promote the growth of anaerobic
261 bacteria, accompanied by the production of organic acids, especially lactic acid, and a lowering of

262 the pH, which are favourable conditions for *Lactobacillus* spp. (Vanschoubroek et al., 1971;
263 Thomas et al., 1998; Mikkelsen et al., 2004). Coarsely ground non-pelleted feed has also been
264 reported to affect intestinal morphology in terms of an increase in height and volume of crypts,
265 which may also be interrelated, with the composition of the microbiota (Brunsgaard, 1998). It can
266 of course not be excluded that pigs with a different genetic background or/and other environmental
267 conditions fed the pelleted or non-pelleted diet may have differences in their microbiota compared
268 to what was observed in this study.

269

270 **T-RFLP and Real-Time PCR detection of *L. intracellularis***

271 Earlier study using similar diet types showed that non-pelleted feeding could have an influence on
272 the prevalence and severity of *L. intracellularis* infection (Stege et al., 2001). The T-RFLP
273 technique has in previous studies been used to detect relative abundance values for specific bacteria
274 (Kaplan et al., 2001; Jernberg et al., 2005). In contrast to the Real-Time PCR assay, broad ranged
275 primers targeting bacterial species were used for the T-RFLP profiles and this will give a different
276 measurement. The T-RFLP detection for *L. intracellularis* was based on relative values that are
277 dependent on the ratio of the individual peak heights relative value out of the sum of all peaks. The
278 T-RFLP detection will therefore be influenced by the ratio between total bacterial rRNA genes and
279 the *L. intracellularis* rRNA gene, which also made this technique less sensitive than the specific *L.*
280 *intracellularis* Real-Time PCR. This can explain why the T-RFLP profiles of ileum showed a
281 significant correlation between the T-RFs 89- and 90 bp and the pigs fed with the pelleted diet,
282 which was not seen by the Real-Time PCR. Unfortunately there is still not a publicized Real-Time
283 PCR assay, which can quantify the number of *L. intracellularis* bacteria in a sample, because such
284 an analysis could have supported the T-RFLP data. Our study showed, based on the T-RFLP
285 results, and the apparent difference in the ratio between bacterial rRNA gene and the *L.*

286 *intracellularis* rRNA gene, that it is very likely that at the time the pigs were killed *L.*
287 *intracellularis* was present in a relatively higher percentage of the total number of bacteria in ileum
288 of the pigs fed the pelleted diet compared to the pigs fed the non-pelleted diet. An explanation for a
289 relatively lower amount of *L. intracellularis* in the pigs fed the non-pelleted diet might be because
290 of a changed intestinal environment with ex. a lower pH, different intestinal morphology and/or a
291 different microbiota (as previously described). However it is difficult to discern cause from effect.

292

293 **Unique T-RFs for severely *L. intracellularis* infected pigs.** Observations strongly suggest that the
294 intestinal bacterial microbiota modifies the ability of *L. intracellularis* to colonise the intestinal
295 tract, and that without the presence of some of these bacteria, infection fails (McOrist et al., 1994).
296 This is the first time to our knowledge that a non-cultural dependent study has been used to find
297 bacterial community differences between healthy pigs and *L. intracellularis*-infected pigs. Only the
298 four pigs successfully experimentally infected with *L. intracellularis* had the expected 90 bp HhaI-
299 T-RF compared to the non-infected pigs proving the usefulness of the technique. Three of these pigs
300 had a unique T-RF (102 bp) close to the theoretical value of *Campylobacter* spp. that in previous
301 studies using conventional culturing studies are known to be commonly present in PE infected pigs,
302 though they are not the causal reason for the infection (McCartney et al 1984, Boosinger et al 1985;
303 McOrist and Lawson 1989), but in this study in situ hybridization also did not reveal any obvious
304 relationship between the *L. intracellularis* and *Campylobacter* cells. The most severely
305 experimentally infected pigs had different microbial community structures compared to the rest of
306 the pigs. Following, we used the intensity of T-RFs 90 bp to find the pigs with natural severe *L.*
307 *intracellularis*-infections. Two unique *HhaI* T-RFs characterized the microbiota of ileum of the 5
308 severely infected pigs where *HhaI*-T-RF 373-374 bp also was found in the experimentally infected
309 pigs. Previous results have shown that infections can disturb and reduce the diversity of the normal

310 intestinal microbiota (Leser et al., 2000). The local intestinal environment around the *L.*
311 *intracellularis* infection may have changed both because of the shedding of *L. intracellularis* cells
312 to the lumen or because of the physical changes of the intestine. Interestingly, all the severely
313 infected pigs in this study had unique T-RFs and the frequency of existing T-RFs were present in a
314 higher relative intensity in the intestinal lumen in combination with *L. intracellularis*. Similar for all
315 the potential matches found in the NCBI database for the unique T-RFs were that they were non yet
316 cultured microorganisms, except for *Alcaligenes sp.* STC1, and had low similarity to any culturable
317 strain. In particular, the bacterial species responsible for *HhaI*-T-RF 373-375 bp may be of
318 particular interest because it was found in a significantly higher proportion in all the infected pigs.
319 Future T-RFLP experiments may include time studies with experimentally infected *L.*
320 *intracellularis* pigs to be able to elucidate which role these potential bacterial species have in a *L.*
321 *intracellularis* infection. It will be interesting to determine whether these unique restriction
322 fragments may be due to synergistic bacteria that benefited from the rapid replication of *L.*
323 *intracellularis* and the physical changes of the intestine, or opportunistic pathogens that required
324 prior damage to the host cell. In summary this study showed that the ilea microbiota of pigs were
325 influenced both by the texture of the diet and of infection with *L. intracellularis*.

326

327 ACKNOWLEDGEMENTS

328 We thank Katja Kristensen, Søren Munk Jensen and Annie Ravn Pedersen for excellent technical
329 assistance. This work was supported by The Directorate for Food, Fisheries and Agri Business. Nr.

330 3401-65-03-745

331 **Reference List**

- 332 Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R. and Stahl, D.A. 1990.
333 Combination of 16S ribosomal RNA-targeted oligonucleotide probes with flow cytometry for
334 analyzing mixed microbial populations. *Appl. Environ. Microbiol.* 56: 1919-1925.
- 335 Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Smith, J.A., Seidman, J.G. and Struhl, K.
336 1988. Preparation and analysis of DNA, p. 2.4.1-2.4.5. *In* F. M. Ausubel (ed.), *Current*
337 *protocols in molecular biology*. John Wiley & Sons, New York.
- 338 Boesen, H.T., Jensen, T.K., Schmidt, A.S., Jensen, B.B., Jensen, S.M. and Møller, K. 2004. The
339 influence of diet on *Lawsonia intracellularis* colonization in pigs upon experimental
340 challenge. *Vet. Microbiol.* 5: 35-45.
- 341 Boesen, H.T., Jensen T.K., Jungersen, G., Riber, U., Boye, M. and Møller, K. 2005. Development,
342 characterization and diagnostic application of a monoclonal antibody specific for a proteinase
343 K resistant *Lawsonia intracellularis* antigen. *Vet. Microbiol.* 105: 199-206
- 344 Boosinger, T.R; Thacker, H.L. and Armstrong, C.H. 1985. *Campylobacter-sputorum* subsp.
345 *Mucosalis* and *Campylobacter-hypointestinalis* infections in the intestine of gnotobiotic pigs.
346 *Am. J. Vet. Res.* 46: 2152-2156
- 347 Boye, M., Jensen, T.K., Møller, K., Leser, T.D. and Jorsal, S.E. 1998. Specific detection of
348 *Lawsonia intracellularis* in porcine proliferative enteropathy inferred from fluorescent rRNA
349 in situ hybridization. *Vet. Pathol.* 35: 153-156.
- 350 Briggs, C.A.E., Willingale, J.M., Braude, R. and Mitchell, K.G. 1954. The normal intestinal flora
351 of the pig. I. Bacterial methods for quantitative studies. *Vet. Rec.* 66: 241.

- 352 Brunsgaard, G. 1998. Effects of cereal type and feed particle size on morphological characteristics,
353 epithelial cell proliferation, and lectin binding patterns in the large intestine of pigs. *J. Anim.*
354 *Sci.* 76:2787-2798.
- 355 Dollhopf, S.L., Hashsham, S.A. and Tiedje, J.M. 2001. Interpreting 16S rDNA T-RFLP data:
356 application of self-organizing maps and principal component analysis to describe community
357 dynamics and convergence. *Microb. Ecol.* 42: 495-505.
- 358 Guedes, R.M.C. and Gebhart, C.J. Preparation and characterization of polyclonal and monoclonal
359 antibodies against *Lawsonia intracellularis*. *J. Vet. Diag. Invest.* 15: 438-446
- 360 Jacoby, R.O. and Johnson, E.A. 1981. Transmissible ileal hyperplasia. *Adv. Exp. Med. Biol.*
361 134:267-289
- 362 Jernberg, C., Sullivan, A., Edlund, C. and Jansson, J.K. 2005. Monitoring of antibiotic-induced
363 alterations in the human intestinal microflora and detection of probiotic strains by use of
364 terminal restriction fragment length polymorphism. *Appl. Environ. Microb.* 71:501-506.
- 365 Jones, G.F., Davies, P.R., Rose, R., Ward, G.E. and Murtaugh, M.P. 1993. Comparison of
366 techniques for diagnosis of proliferative enteritis of swine. *Am. J. Vet. Res.* 54: 1980-1985.
- 367 Kaplan, C.W., Astaire, J.C., Sanders, M.E., Reddy, B.S. and Kitts, C.L. 2001. 16S ribosomal DNA
368 terminal restriction fragment pattern analysis of bacterial communities in feces of rats fed
369 *Lactobacillus acidophilus* NCFM. *Appl. Environ. Microb.* 67:1935-1939.
- 370 Lawson, G.H.K., Rowland, A.C. and MacIntyre, N. 1985. Demonstration of a new intracellular
371 antigen in porcine intestinal adenomatosis and hamster proliferative ileitis. *Vet. Microbiol.* 10:
372 303-313.

- 373 Leser, T.D., Amenuvor, J.Z., Jensen, T.K., Lindecrona, R.H., Boye, M. and Møller, K. 2002.
374 Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited.
375 Appl. Environ. Microb. 68: 673-690.
- 376 Leser, T.D., Lindecrona, R.H., Jensen, T.K., Jensen, B.B. and Møller, K. 2000. Changes in
377 bacterial community structure in the colon of pigs fed different experimental diets and after
378 infection with *Brachyspira hyodysenteriae*. Appl. Environ. Microb. 66: 3290-3296.
- 379 Lindecrona, R.H., Jensen, T.K., Andersen, P.H. and Møller, K. 2002. Application of a 5' nuclease
380 assay for detection of *Lawsonia intracellularis* in fecal samples from pigs. J. Clin. Microbiol.
381 40: 984-987.
- 382 Liu, W.T., Marsh, T.L., Cheng, H. and Forney, L.J. 1997. Characterization of microbial diversity
383 by determining terminal restriction fragment length polymorphisms of genes encoding 16S
384 rRNA. Appl. Environ. Microb. 63: 4516-4522.
- 385 McCartney, E., Lawson, G.H.K. and Rowland, A.C. 1984. Behaviour of *Campylobacter sputorum*
386 subsp. Mucosalis in gnotobiotic pigs. Res. Vet. Sci. 36: 290-297
- 387 McOrist, S., Boid, R., Lawson, G.H.K. and McConnel, I. 1987. Monoclonal-antibodies to
388 intracellular campylobacter-like organisms of the porcine proliferative enteropathies. Vet.
389 Rec. 121: 421-422.
- 390 McOrist, S., and Lawson, G.H.K. 1989. Proliferative enteropathies – *Campylobacter* species in the
391 feces of normal and contact pigs. Vet. Rec. 124: 41-41

- 392 McOrist, S., Jasni, S., Mackie, R.A., MacIntyre, N., Neef, N. and Lawson, G.H.K. 1993.
393 Reproduction of porcine proliferative enteropathy with pure cultures of ileal symbiont
394 intracellularis. *Infect. Immun.* 61: 4286-4292.
- 395 McOrist, S., Mackie, R.A., Neef, N., Aitken, I. and Lawson, G.H.K. 1994. Synergism of ileal
396 symbiont intracellularis and gut bacteria in the reproduction of porcine proliferative
397 enteropathy. *Vet. Rec.* 134: 331-332.
- 398 McOrist, S., Gebhart, C.J., Boid, R. and Barns, S.M. 1995. Characterization of *Lawsonia*
399 *intracellularis* gen. nov., sp. nov., the obligately intracellular bacterium of porcine
400 proliferative enteropathy. *Int. J. Syst. Bacteriol.* 45:820-825.
- 401 Mikkelsen, L.L., Naughton, P.J., Hedemann, M.S. and Jensen, B.B. 2004. Effects of physical
402 properties of feed on microbial ecology and survival of *Salmonella enterica* serovar
403 Typhimurium in the pig gastrointestinal tract. *Appl. Environ. Microb.* 70: 3485-3492.
- 404 Mølbak, L., Sommer H.M., Johnsen K., Boye, M., Johansen, M., Møller, K., Leser, T.D. 2006.
405 Freezing at -80° degrees C distorts the DNA composition of bacterial communities in
406 intestinal samples. *Curr. Issues Intest. Microbiol.* 7: 29-34.
- 407 Møller, K., Jensen, T.K., Jorsal, S.E., Leser, T.D. and Carstensen, B. 1998. Detection of *Lawsonia*
408 *intracellularis*, *Serpulina hyodysenteriae*, weakly beta-haemolytic intestinal spirochaetes,
409 *Salmonella enterica*, and haemolytic *Escherichia coli* from swine herds with and without
410 diarrhoea among growing pigs. *Vet. Microbiol.* 62: 59-72.
- 411 Pryde, S.E., Richardson, A.J., Stewart, C.S. and Flint, H.J. 1999. Molecular analysis of the
412 microbial diversity present in the colonic wall, colonic lumen, and cecal lumen of a pig. *Appl.*
413 *Environ. Microb.* 65:5372-5377.

- 414 Rowland, A.C. and Rowntree, P.G. 1972. Hemorrhagic bowel syndrome associated with intestinal
415 adenomatosis in pig. *Vet. Rec.* 91: 235.
- 416 Schmid, M.W., . Lehner, A., Stephan, R., Schleifer, K.H. and Meier, H. 2005.. Development and
417 application of oligonucleotide probes for in situ detection of thermotolerant *Campylobacter* in
418 chicken faecal and liver samples. *Int. J. Food Microbiol.* 105: 245-255.
- 419 Stege, H., Jensen, T.K., Møller, K., Bækbo, P. and Jorsal, S.E. 2001. Risk factors for intestinal
420 pathogens in Danish finishing pig herds. *Prev. Vet. Med.* 50: 153-164.
- 421 Thomas, M., van Vliet, T. and van der Poel, A.F.B. 1998. Physical quality of pelleted animal feed.
422 Contribution of feedstuff components. *Anim. Feed Sci. Tech.* 70: 59-78.
- 423 Vanschoubroek, F., Coucke, L. and van Spaendonck, R. 1971. The quantitative effect of pelleting
424 feed on the performance of piglets and fattening pigs. *Nutr. Res. Rev.* 41: 1-9.
- 425 Willingale, J.M. and Briggs, C.A.E. 1955. The normal intestinal flora of the pig II. Quantitative
426 bacteriological studies. *J. Appl. Bacteriol.* 18: 284.
- 427 Wondra, K.J., Hancock, J.D., Behnke, K.C., Hines, R.H. and Stark, C.R. 1995a. Effects of particle-
428 size and pelleting on growth-performance, nutrient digestibility, and stomach morphology in
429 finishing pigs. *J. Anim. Sci.* 73: 757-763.
- 430 Wondra, K.J., Hancock, J.D., Behnke, K.C. and Stark C.R. 1995b. Effects of mill type and particle-
431 size uniformity on growth-performance, nutrient digestibility, and stomach morphology in
432 finishing pigs. *J. Anim. Sci.* 73: 2564-2573.

433 **Figure 1.** PCA ordination of bacteria T-RFLP data from 3 experiments (**I, II, III**) for the microbial
434 communities of ileum from a pig herd fed a pelleted diet (●) or a non-pelleted diet (★). The three
435 plots found an overall repetitive trend in grouping the pelleted fed pig and the non-pelleted fed pigs
436 separately by the first and second principal component (group of T-RFs). The amount of variability
437 accounted for by the first and second component is on the left corner in the panel and the line (----)
438 is the X=Y variance line.

439

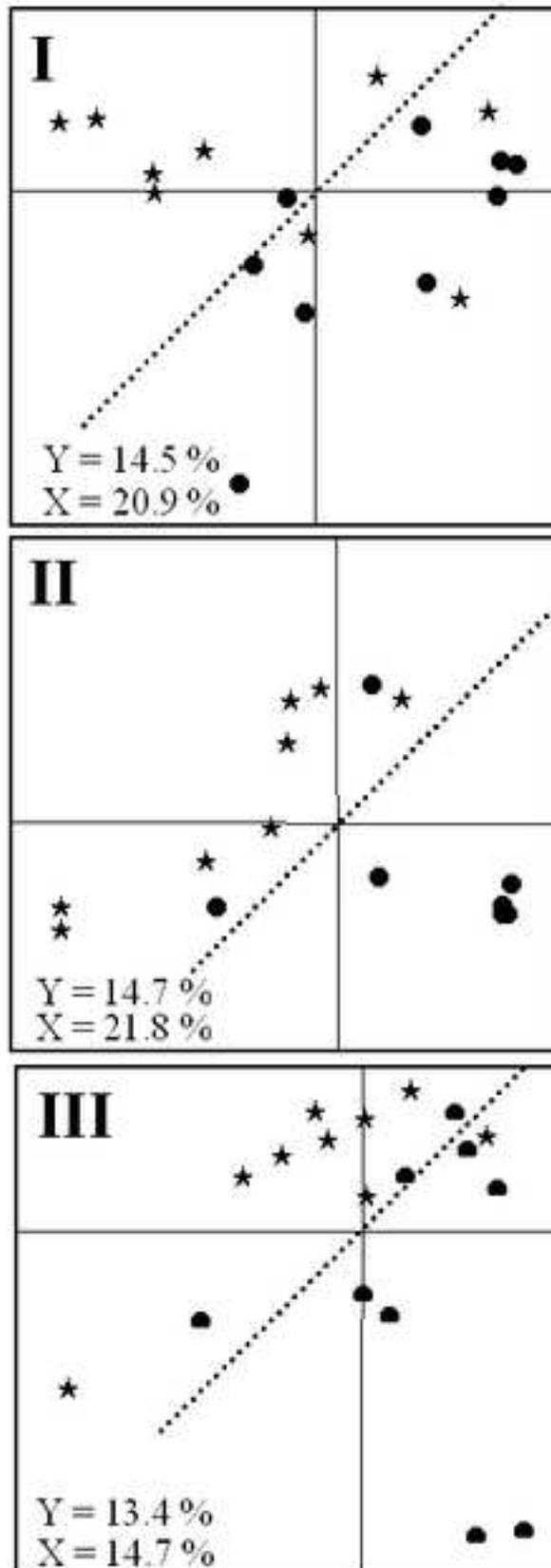
440 **Figure 2.** Average relative abundance of specific terminal restriction fragments (*HhaI* T-RFs) as
441 measured by the T-RFLP analyses of pigs fed with a pelleted diet (n=24; grey) and a non-pelleted
442 diet (n=25; white) and pigs with severe *L. intracellularis* infection (n=5; black). The figure present
443 only T-RFs found with a frequency higher than 1% of the total intensity. The medians of the T-RFs
444 were significantly different (p<0.01) in A) between the pigs having severe *L. intracellularis*
445 infection and the pigs fed either of the two other diets; in B) between the pigs fed pelleted or non-
446 pelleted diets or in C) where all three groups were significantly different.

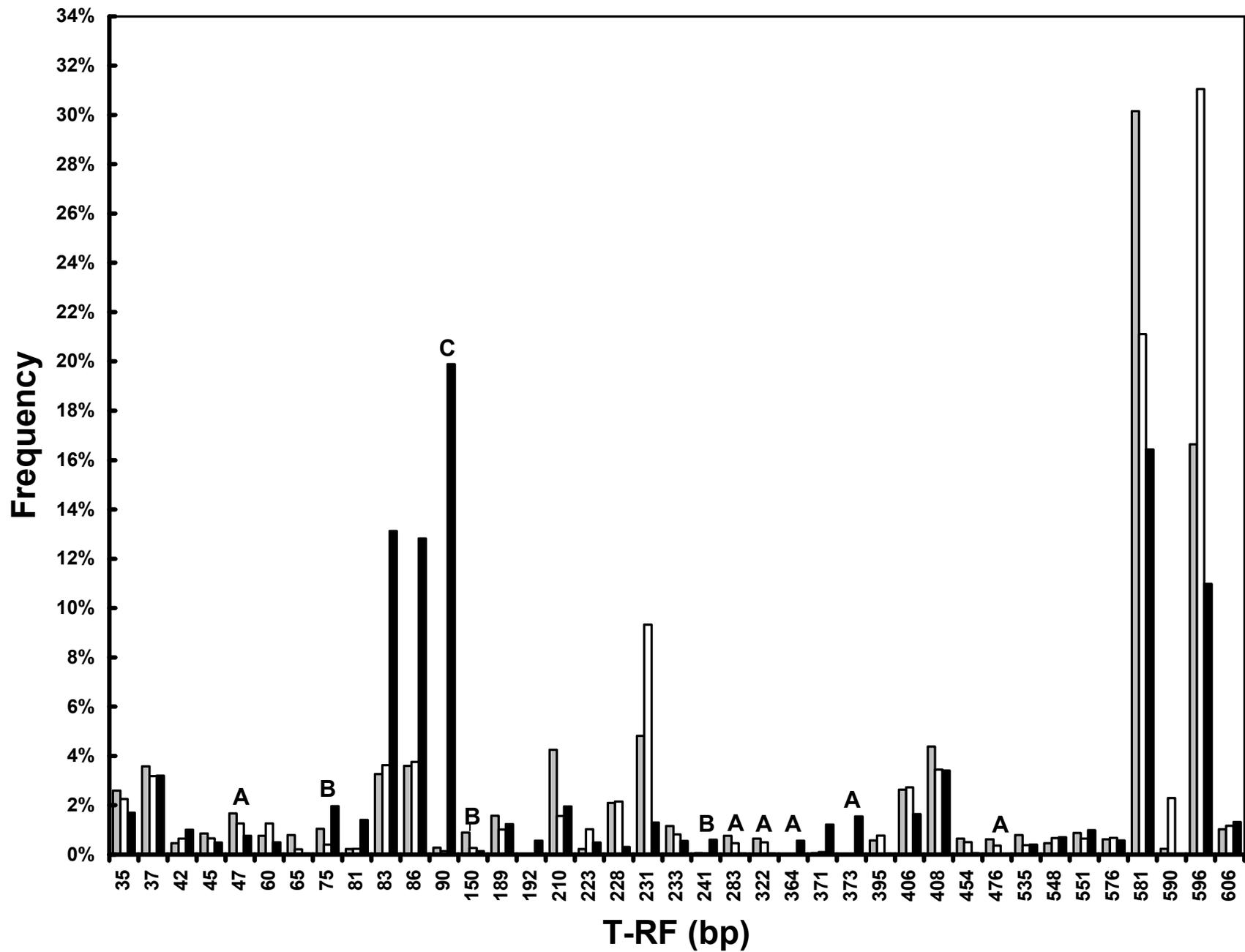
447

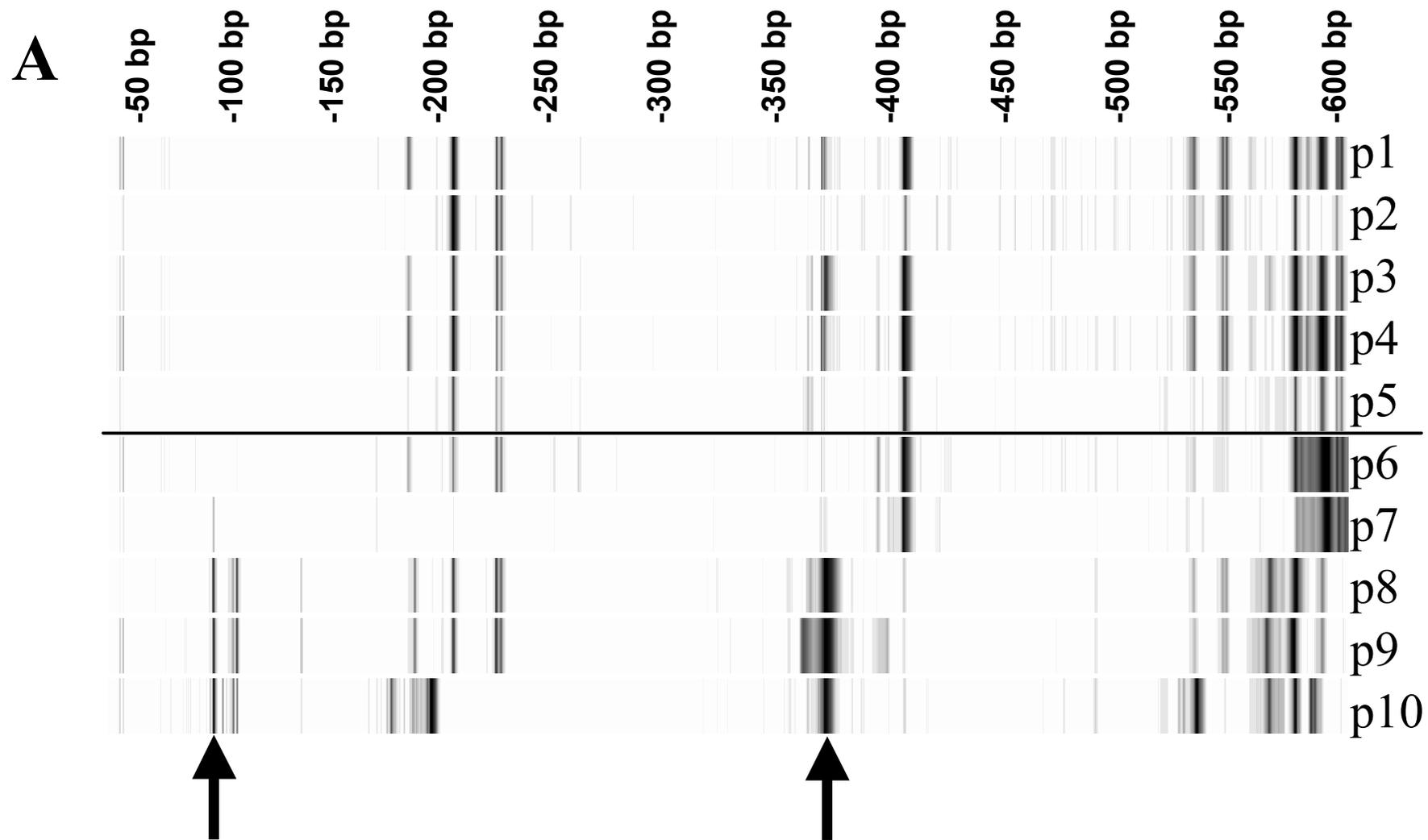
448 **Figure 3. A)** T-RFLP profiles of ileum samples cut with *HhaI*. Pigs p1-5 were not infected with *L.*
449 *intracellularis* and pigs p6-10 were experimentally infected with *L. intracellularis*. All pigs were
450 killed 3 weeks after infection. The arrows indicate T-RF 90 bp that is the T-RF length of *L.*
451 *intracellularis* and T-RF 373 bp found to be related to *L. intracellularis* infected pigs. **B)** The PCA
452 ordination of the T-RFLP profiles of the experimentally infected pigs (■) and the control pigs (◆).
453 The pigs without the *L. intracellularis* T-RF 90 bp clustered together, whereas the first principal
454 component separated p8-10 from the rest of the pigs.

455

456 **Figure 4. A)** Epifluorescence micrographs of hybridised colon tissue samples taken from a pig that
457 has not been experimentally infected with *Lawsonia intracellularis*. The yellow target cells are the
458 fraction of *Campylobacter* cells (green fluorescent 16S rRNA- oligonucleotide probe) out of the
459 total red bacterial cell fraction (red fluorescent 16S rRNA- oligonucleotide probe). **B)**
460 Epifluorescence micrographs of hybridised colon tissue samples taken from an experimental *L.*
461 *intracellularis* infected pig. The green *Campylobacter* target cells found were always extracellular
462 and the red target *L. intracellularis* cells were found independently on the presence of the
463 *Campylobacter* cells. **C) and D)** Epifluorescence micrographs of hybridised cross sections of ileum
464 from both a non-infected and a severely infected pig, respectively. The green auto fluorescence
465 exhibited by the intestinal cell material was used to visualize the cross section surface of the
466 intestine and the specific red fluorescent probe visualized the *L. intracellularis* microcolonies.







B