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

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

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ABSTRACT

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

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

INTRODUCTION

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

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

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

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

T-RFLP profiles were done as described by Leser *et al*. (2000). Essentially, 200-mg of intestinal content was suspended in 600-µl of phosphate-buffered saline [pH 7.4] (0.13 M NaCl, 3 mM Na_2HPO_4 per litre, 7 mM KH_2PO_4), vortexed thoroughly and then centrifuged for 2 min at 200×g. The supernatant was centrifuged (12,000×g, 5 min) and the resulting pellet was resuspended in 570-µl of Tris-EDTA [pH=7]. The suspension was added 30-µl of 10% sodium dodecyl sulfate and homogenized with 400-mg of 100-µm Zirconia-silica beads (BioSpec Products Inc., Bartlesville, USA) using a minibead-beater at high speed for 4 min (Biospec Products Inc). The DNA was extracted from the lysed cells and purified by the CTAB method (Ausubel et al., 1988) and stored at -20°C. The DNA was adjusted by spectrophotometry to a concentration of 5-µg of DNA ml^{-1}. Four replicate 50-µl PCR mixtures were made from each sample using the universal bacterial primers S-D-Bact-0008-a-S-20 (5´-AGAGTTTGATCMTGGCTCAG-3´) and S-D-Bact-0926-a-A-20 (5´-CCGTCAATTCCTTTRAGTTT-3´) (Leser et al. 2000). Primer S-D-Bact-0926-a-A-20 was 5´FAM

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

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

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

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

RESULTS

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

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

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

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

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

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

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

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DISCUSSION

The T-RFLP profiles of ileum.

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

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

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

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

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

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

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

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

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

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

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

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

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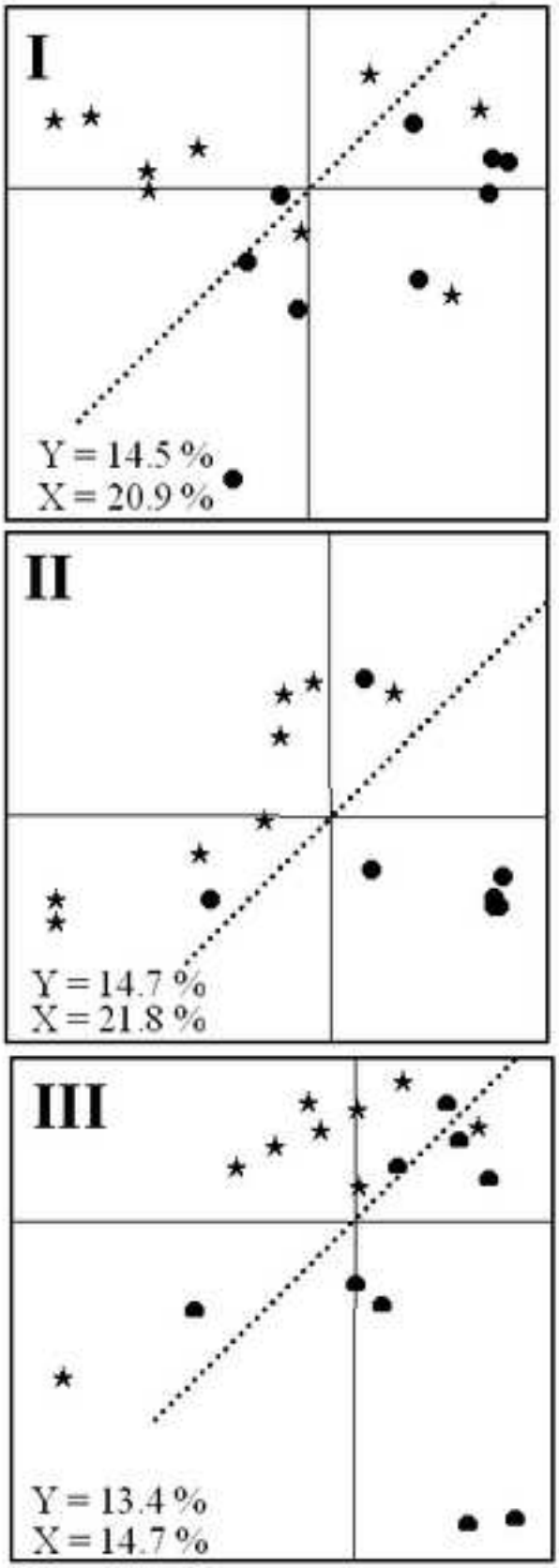

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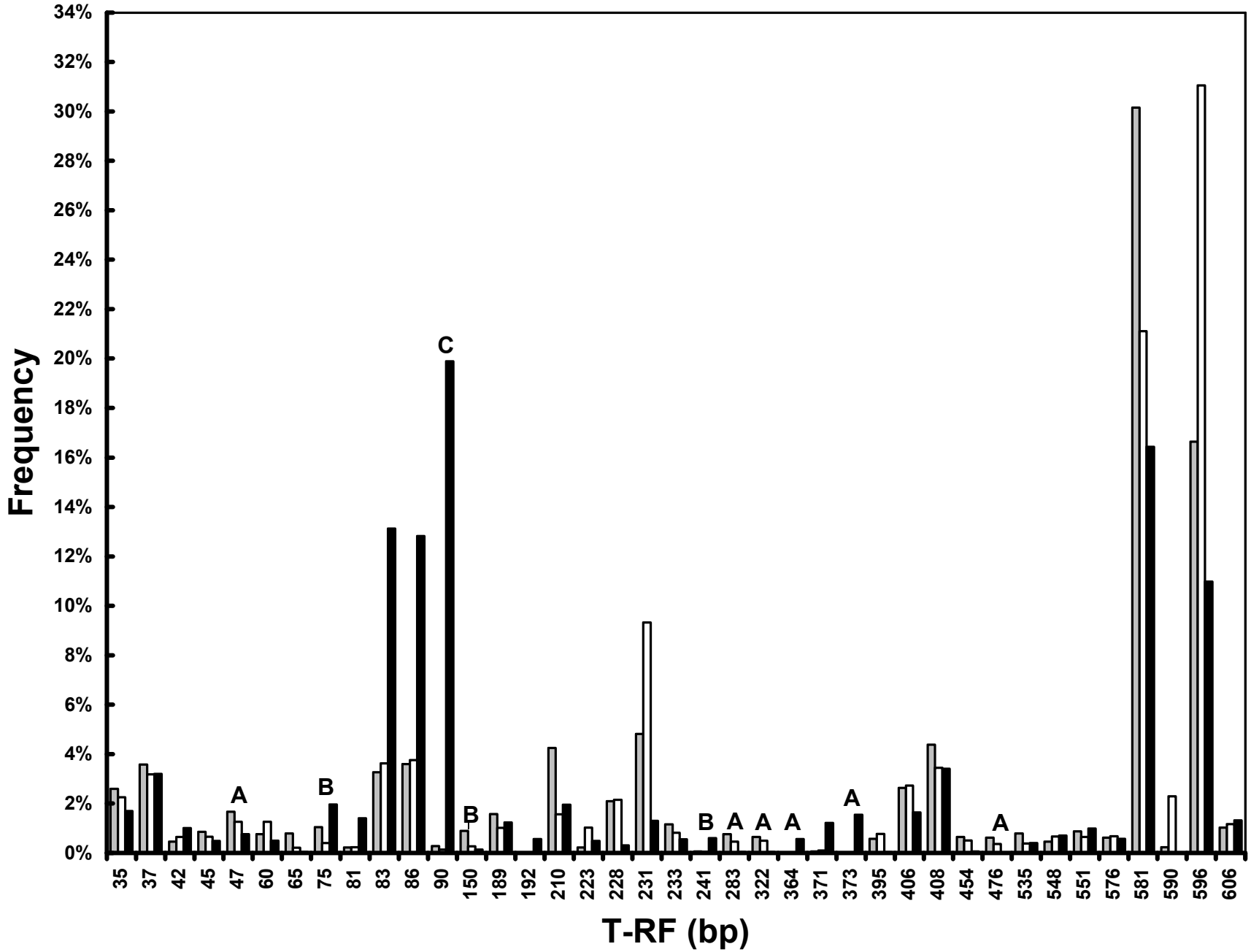

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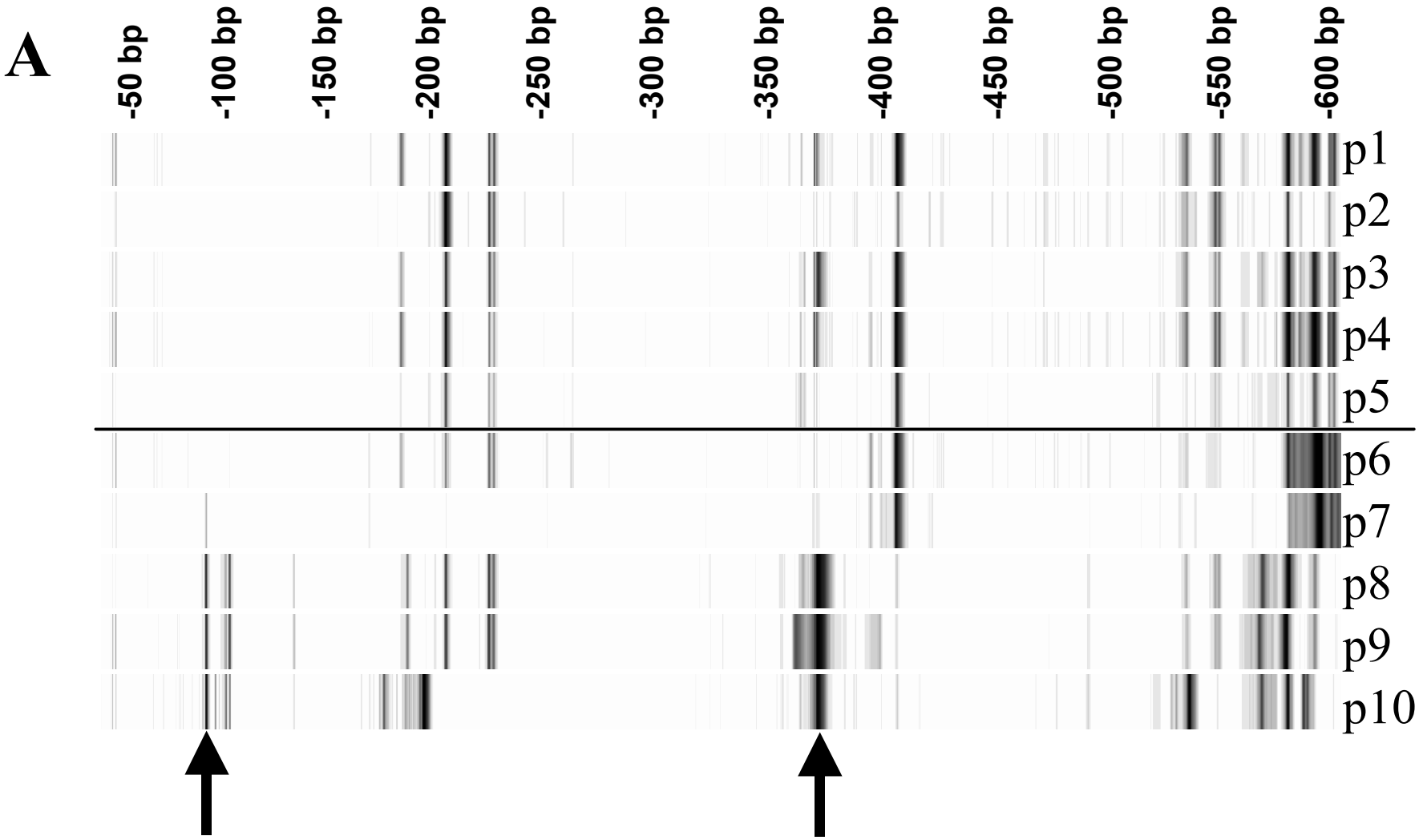

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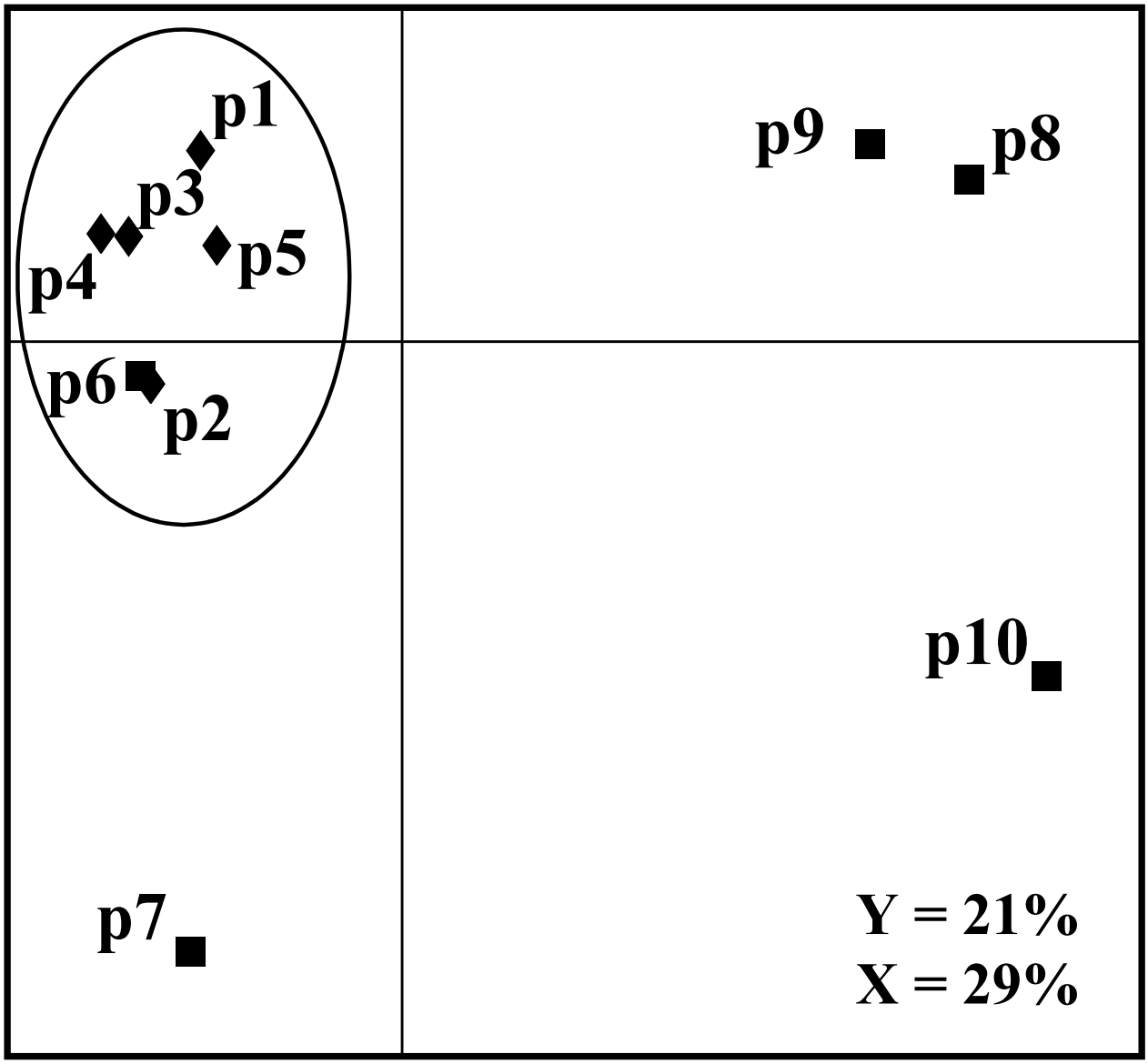
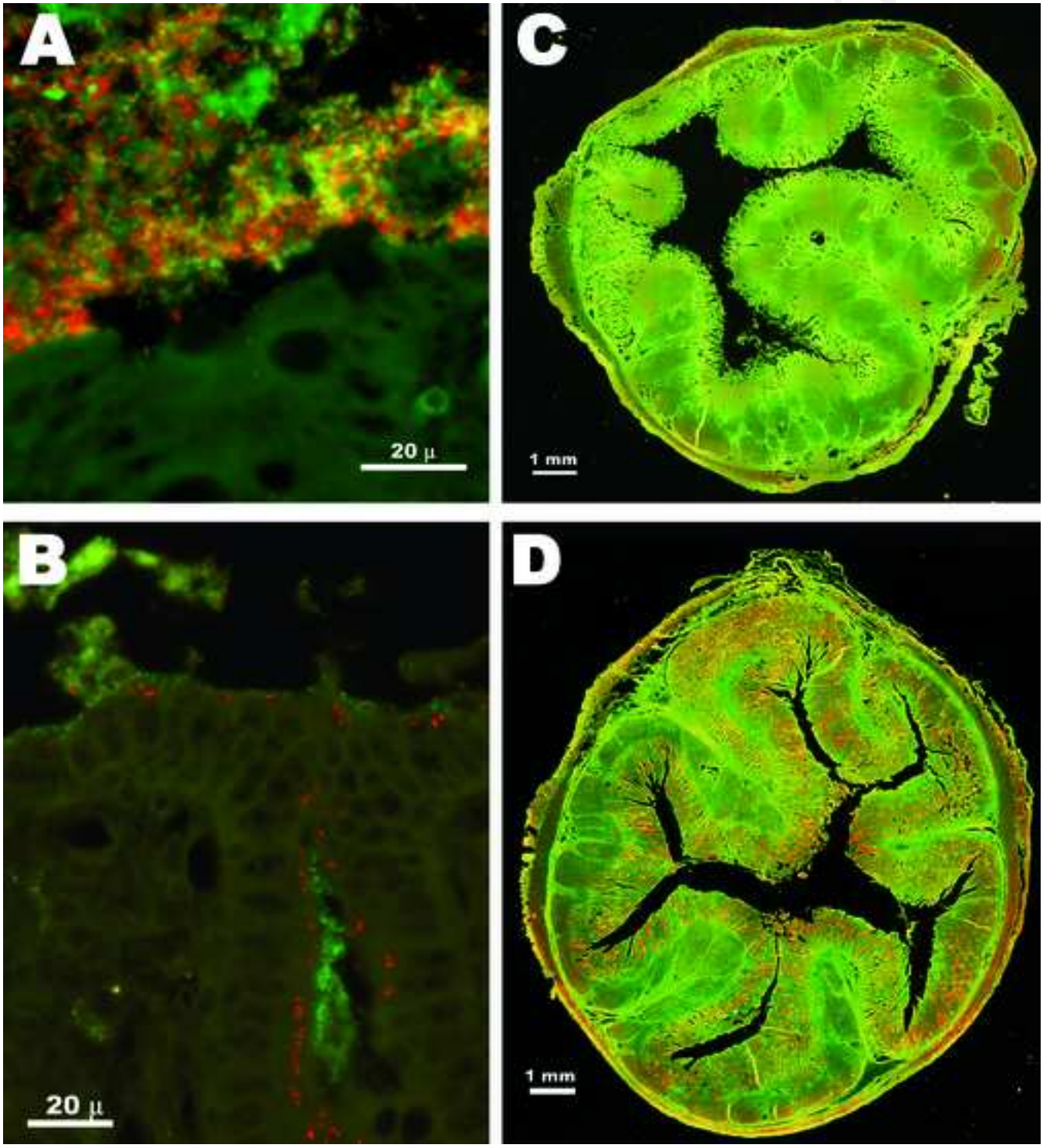
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