

# Freezing at $-80^{\circ}\text{C}$ distorts the DNA composition of bacterial communities in intestinal samples

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## Abstract

Terminal-restriction fragment length polymorphism (T-RFLP) was used to evaluate how to store intestinal specimens for bacterial community analysis. Bacterial communities are increasingly often described by means of DNA-based methods and it is common practice to store intestinal or faecal specimens either at  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$ . In this study, samples of intestines from five different pigs were stored at  $-80^{\circ}\text{C}$  and  $-20^{\circ}\text{C}$ , respectively and a thawing and freezing procedure was carried out three times for each intestinal per pig per temperature. The cumulative sum of the T-RFLP peak heights (T-RF intensities) decreased as the temperature decreased. The composition of the bacterial community changed when stored at  $-80^{\circ}\text{C}$  compared to the samples stored at  $-20^{\circ}\text{C}$ . Thus it is recommended from this study that samples of intestinal content are stored at  $-20^{\circ}\text{C}$  before use for bacterial community analysis, instead of the current practice at  $-80^{\circ}\text{C}$ .

## Introduction

The development of molecular techniques has made it common to study not only unculturable bacteria but also to get a fast and reproducible assessment of bacterial community diversity using the 16S ribosomal RNA gene. Recent studies of the bacterial community in the gastro-intestinal tract of humans, pigs, chickens, dogs, and mice have given information about the bacterial community composition and how different factors such as feeding, or probiotics influence the bacterial community structure (review: Kitts, 2001; Zoetendal *et al.*, 2004). The increasing use of such methods highlights the need for knowledge on sample procession (Ott *et al.*, 2004). The bacterial community most likely changes as the samples are taken, transported to the laboratory and stored. In metabolic studies it is quite normal to excise, section and extrude the intestinal content before freezing at  $-80^{\circ}\text{C}$  (Topping *et al.*, 1993). In microbiological studies intact specimens may be frozen as soon as possible after collection, and they can be thawed and refrozen several

times for retrospective analysis. It is common practice to store intestinal or faecal specimens at  $-20^{\circ}\text{C}$  (Zoetendal *et al.*, 1998; Heilig *et al.*, 2002; Huijsdens *et al.*, 2002; Konstantinov *et al.*, 2003) or at  $-70^{\circ}\text{C}/-80^{\circ}\text{C}$  (Deplancke *et al.*, 2000; Leser *et al.*, 2000; McCracken *et al.*, 2001; Leser *et al.*, 2002; Ott *et al.*, 2004) before their bacterial communities are described by means of DNA-based methods. In order to sub-sample from a frozen intestine it is necessary to cut out intestinal content with a scalpel and this is not possible unless the sample is thawed. When bacteria are subject to freeze and thaw cycles (FT), cell damage will depend on many parameters including the structure and content of the cell itself, the rate of freezing, the rate of thawing, the temperature of storage in the frozen state, and the medium in which the cells are suspended (Mazur, 1970; Morris *et al.*, 1988). It is important that the assessment of the bacterial community is not affected by the FT treatment.

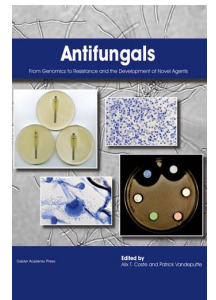
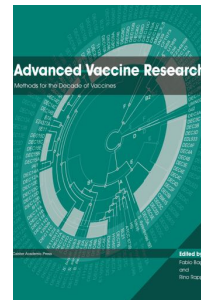
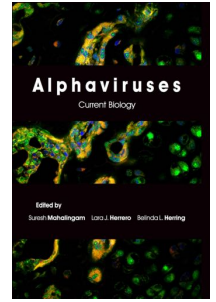
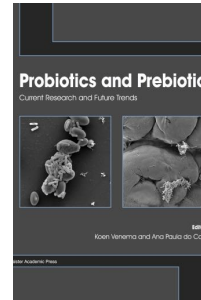
The aim of this study was to investigate the sample sizes influence on the assessment of the bacterial community (Experiment A) and the influence of storage temperature and of repeated FT of samples (Experiment B). The bacterial community structure was described by using the terminal-restriction fragment length polymorphism technique (T-RFLP) (Liu *et al.*, 1997). The T-RFLP technique is fast and simple to perform, which makes it ideal for screening of many samples. T-RFLP analysis provides quantitative data about each T-RF detected, including size in base pairs and intensity of fluorescence (peak height). Since each fragment ideally represents a single group of bacteria, the method furthermore gives the possibility both to identify and to assess certain groups of bacteria in a complex microbial community. In the present study we analysed the microbial community of the pig colon. Colon of humans and other omnivores ferment parts of the diet-derived substrates that the host has been unable to digest and some of the end products of this fermentation are short chain fatty acids. The colonic contents typically contain about  $10^{10}$  microorganisms per gram (dry weight) and it is likely that several hundred different bacterial types may be present (Tannock, 1999). The microbial community was divided up into three groups, based on a bacterial clone library from the pig intestine (Leser *et al.*, 2002). The three groups were: 1) Gram-positive bacteria, 2) Gram-negative bacteria and 3) unidentified bacteria according to Gram-positive and Gram-negative bacteria, the last group representing approximately 25% of all the T-RF in the samples. In the statistical analysis two types of endpoints were used 1) total T-RF intensity, which is the sum of all intensities from all groups of bacteria in a given sample and 2) proportion of Gram-positive bacteria, which are calculated as the sum of all intensities from Gram-positive bacteria, divided by the total T-RF intensity for the given sample.

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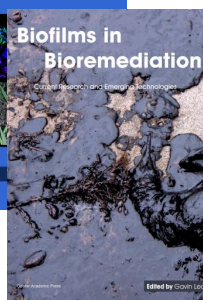
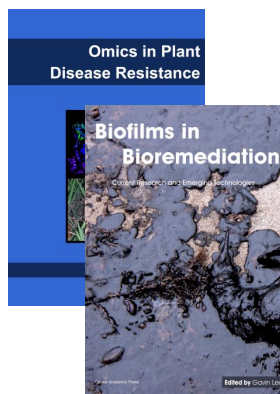
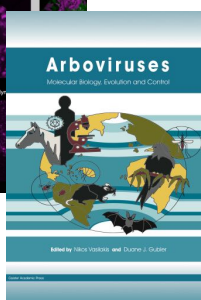
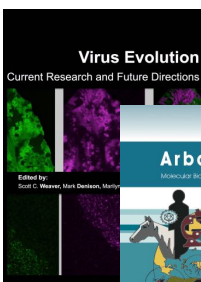
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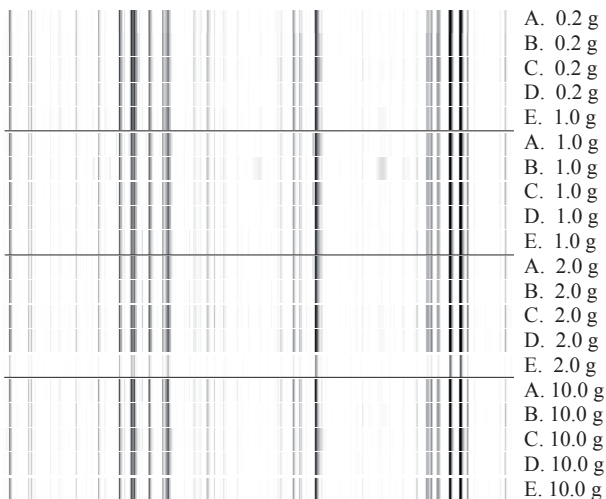


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## Results and discussion

### *The effect of using different sample sizes was examined (Experiment A)*

Bellele *et al.*, 2003 found that the stability of DNA extracts after repeated FT is dependent on the volume in which they were stored. In this study we tested the influence of the sample size on both the proportion of Gram-positive versus Gram-negative bacteria and on the amount of total T-RF intensity. The T-RFLP profiles are shown in Fig. 1. From a visual inspection it seems as though replicate E was weaker than the four others. This sample was considered an outlier, since the total T-RF intensity was only 98 (all other samples ranged from 3,600 to 31,700) and there were no Gram-positive or Gram-negative bacteria identified. It has, in a previous study, been shown that the assessment of bacterial community structure was influenced by the sizes of soil samples (Ellingsøe and Johnsen, 2002). In this study we found from the statistical analysis of variance, that the samples size had an influence on the total T-RF intensity, which decreased somewhat with increasing sample size ( $P = 0.007$ ). Also the levels of the total T-RF intensity for each pig were significant different from each other ( $P = 0.0001$ ). Using a non-parametric analysis we found that the bacterial community was slightly changed as the sample size increased, resulting in a small increase in the proportion of Gram-positive bacteria. In the study of Ellingsøe and Johnsen (2002) with soil samples, the changes in the bacterial community were somewhat larger than for these samples. Intestinal content has both a relative higher amount of bacterial content and a more homogenous structure than soil. This may explain why in this study it was possible to sample from a smaller volume than in the soil study, without having high variation between replicates in the genetic community structure of the intestinal samples. The extraction protocol used in this study is optimised to work on small samples from 0.2 g to 1 g of samples (Leser *et al.*, 2000; Leser *et al.*, 2002) and it is therefore not surprising that we had the highest level of total T-RF intensity at the small sample sizes.

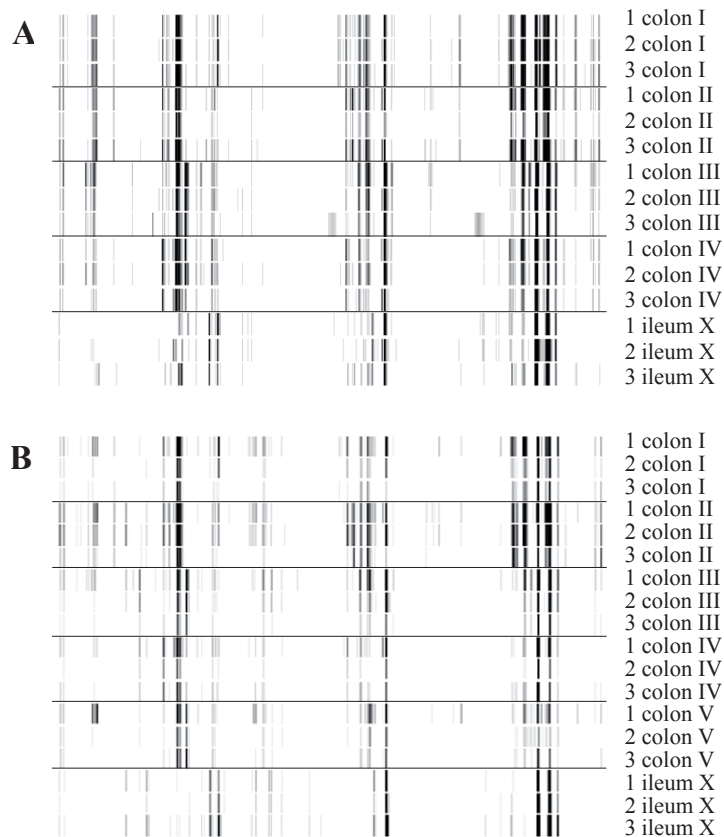


**Fig. 1.** (Exp. A) T-RFLP profiles of 0.2, 1.0, 2.0 and 10 g samples originating from the same pooled sample of colon luminal content from 4 different pigs. The experiment was performed in 5 replicates (A-E), to demonstrate the accuracy and reliability of the T-RFLP profiles.

### *Repeated freezing of samples at different temperatures was examined (Experiment B)*

T-RFLP profiles from colon of 5 pigs and one sample from ileum stored at either  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$  are shown in Fig. 2A and 2B, respectively. The total T-RF intensities of these samples were compared in the variance analysis and it was found that the total T-RF intensity was significantly ( $P = 0.0001$ ) lower at  $-80^{\circ}\text{C}$  compared to at  $-20^{\circ}\text{C}$ , approximately 1/3 of the values at  $-20^{\circ}\text{C}$  (Fig. 3). This may indicate that a higher proportion of the  $-80^{\circ}\text{C}$  DNA was degraded compared to the  $-20^{\circ}\text{C}$  DNA. The FT procedure had no influence on the total T-RF intensities.

The proportion of Gram-positive bacteria in the community from each sample was compared by the use of the non-parametric Friedman's rank test. The result showed that there was no significant ( $P = 0.39$ ) effect of the freezing/thawing procedure on the proportion of Gram-positive bacteria in the communities (*sample* was insignificant). However there was a significant effect ( $P = 0.013$ ) of the temperature resulting in a decrease in the proportion of the Gram-positive bacteria at temperatures of  $-80^{\circ}\text{C}$  relatively compared to the proportion at  $-20^{\circ}\text{C}$ . When carrying out the same analysis with Friedman's test, as described above, but with Gram-negative data as the response, it resulted in no significant effect of the FT procedure (as for Gram-positive) and no significant effect of the temperature. The conclusion is that the proportion of Gram-positive bacteria T-RFs, decreased as the temperature decreased, the proportion of Gram-negative bacteria was unchanged, but the proportion of unknown bacteria increased as the temperature decreased. The decrease of the proportion of Gram-positive bacteria with decreasing temperature can thus only be an indication, since the group of unknown accounted for about 25% of which we don't know the fraction of Gram-positive and if these also decreases with decreasing temperature. Several studies have shown the stability of DNA after extended storage at temperatures varying from  $-70^{\circ}\text{C}$  to  $4^{\circ}\text{C}$  (Shikama *et al.*, 1965; Ross *et al.*, 1990; Jerome *et al.*, 2002). Various storage method studies on the preservation of bacterial isolates have shown the potential occurrence of single- and double-stranded DNA breaks and membrane damage during several FT cycles (Alur and Grecz, 1975; Calcott and Macleod, 1975). Only few studies have shown the effect of multiple FT on the quantity and quality of DNA in clinical samples (Krajden *et al.*, 1999; Durmaz *et al.*, 2002). They demonstrated that the quantity of viral DNA remains stable after several short-term FT cycles at either  $-20^{\circ}\text{C}$  or  $-70^{\circ}\text{C}$ . In this study we moreover found that the FT cycles of the colon specimens from  $-80^{\circ}\text{C}$  to  $0^{\circ}\text{C}$  did not significantly change the bacterial community regarding the proportion between Gram-positive and Gram-negative bacteria. The storage at different temperatures did have an effect on both the total intensity (decrease in total T-RF intensity with a decrease in temperature) and on the proportion of Gram-positive and Gram-negative DNA. Gram-positive bacteria DNA seems to be most susceptible to the treatment (storage at  $-80^{\circ}\text{C}$ ). It could be emphasized that the reduction of the proportion of Gram-positive bacteria DNA could be explained by the reduction of the total T-RF intensity if many of the Gram-positive bacteria detected at the  $-20^{\circ}\text{C}$  treatment had a peak that were less than the detection



**Fig. 2.** (Exp. B) T-RFLP profiles in the range of 30 to 650 bp from the colon and ileum luminal contents of 6 different pigs (I, II, III, IV, V, X). **A)** T-RFLP profiles from samples that were freeze and thawed (FT) from  $-20^{\circ}\text{C}$  to  $0^{\circ}\text{C}$  for up to three times (1,2,3). **B)** T-RFLP profiles from samples that were FT from  $-80^{\circ}\text{C}$  to  $0^{\circ}\text{C}$  for up to three times (1,2,3).

limit at the  $-80^{\circ}\text{C}$  treatment (with a reduction of the total T-RF intensity the detection limit is relatively higher). In our study however this was not the case because the same number of Gram-positive bacteria groups were detected at the  $-80^{\circ}\text{C}$  treatment as at the  $-20^{\circ}\text{C}$  treatment. The diversity of bacteria in general was not reduced as the storage temperature was lowered.

### Conclusions

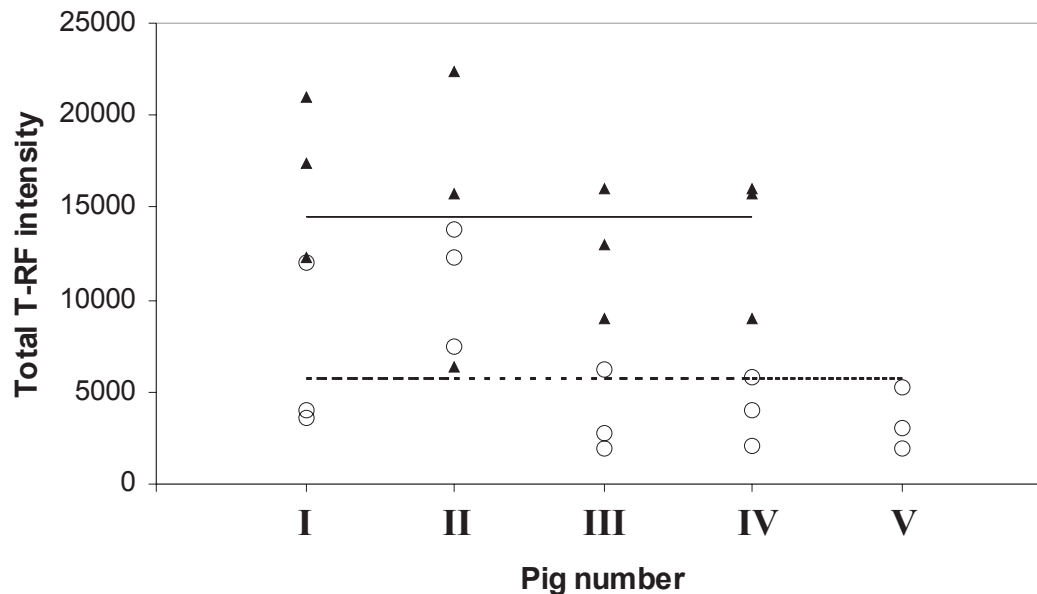
This study shows how important it is to reproduce T-RFLP profiles to detect artefacts and to validate the reliability of the data and it might not only be with intestinal samples that the storage condition of specimens will influence the bacterial DNA composition in a specific way. It is easy to imagine other ecosystems like soil or water that can have the same type of problems. We recommend that samples of intestinal content are stored at  $-20^{\circ}\text{C}$  instead of the current practice at  $-80^{\circ}\text{C}$ . The samples can be quite small, as sample sizes of 0.2, 1.0, 2.0 and 10.0 g all seem to be representative of the bacterial community.

### Experimental procedures

#### Sampling and DNA extraction

Immediately after the 6 pigs were sacrificed, intestinal pieces of approximately 10 cm were excised, the ends were tied off and pieces put on ice and transferred to  $-80^{\circ}\text{C}$  or  $-20^{\circ}\text{C}$ . Sub-samples of intestinal content were taken out by thawing samples from  $-80^{\circ}\text{C}$  or  $-20^{\circ}\text{C}$  to  $4^{\circ}\text{C}$  on wet ice; 0.2 g portions of the intestinal content was cut

out and vortexed. For Experiment A (the effect of sample sizes), a total of 20 samples were taken from the colon of 4 different pigs. The samples from all the pigs were pooled and divided into four groups of 0.2, 1.0, 2.0 or 10 g samples with 5 replicates of each. Essentially, 200 mg of intestinal content was suspended in 600  $\mu\text{l}$  of phosphate-buffered saline, vortexed thoroughly and then centrifuged for 2 min at  $200 \times g$ . The proportion between intestinal content and phosphate-buffered saline [pH 7.4] (8 g of NaCl per litre, 0.2 g of KCl per litre, 1.44 g of  $\text{Na}_2\text{HPO}_4$  per litre, 0.24 g of  $\text{KH}_2\text{PO}_4$  per litre) was the same for all sample sizes in Experiment A. For Experiment B (effect of temperature and repeated freezing and thawing) 27 samples were taken from the colon of 5 pigs. Phosphate-buffered saline was added to the samples in the same proportion as in Experiment A. The following procedure was common for Experiment A and B and the T-RFLP profiles were produced exactly according to Leser *et al.* (2000); 600- $\mu\text{l}$  sub samples of these suspensions were used for the first centrifugation step of the DNA extraction procedure. The supernatant was centrifuged at  $12,000 \times g$  for 5 min and the resulting pellet was resuspended in Tris-EDTA (10 mM Tris [pH 8] and 1 mM EDTA [pH 8]). The suspension was added to 30  $\mu\text{l}$  of 10% sodium dodecyl sulfate and bead-beated. After a brief spin in a microcentrifuge, the samples were transferred to a microcentrifuge tube and the DNA was purified by the CTAB method (Ausubel *et al.*, 1988). DNA was finally dissolved in 50  $\mu\text{l}$  of Tris-EDTA and stored at  $-20^{\circ}\text{C}$ .



**Fig. 3.** (Exp. B) The total T-RF intensity of the  $-80^{\circ}\text{C}$  stored samples (○) were significant lower than for the  $-20^{\circ}\text{C}$  stored samples (▲). The solid line and the dotted line represent the models for the  $-20^{\circ}\text{C}$  and  $-80^{\circ}\text{C}$  samples, respectively.

#### PCR and T-RFLP

The DNA was adjusted spectrophotometrically (GeneQuant II, Pharmacia Biotech, Hørsholm, Denmark) to a concentration of  $5\ \mu\text{g}$  of DNA  $\text{ml}^{-1}$  and this concentration was used for all the PCR analyses. Four replicate  $50\ \mu\text{l}$  PCR mixtures were made from each sample using the 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 (carboxy-fluorescein-N-hydroxysuccinimide ester-dimethyl sulfoxide) labelled and purchased from MWG-Biotech, Ebersberg, Germany. PCR cycling consisted of an initial denaturation at  $94^{\circ}\text{C}$  for 6 min; followed by 30 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 s, annealing at  $57^{\circ}\text{C}$  for 45 s, and extension at  $72^{\circ}\text{C}$  for 2 min; and a final extension at  $72^{\circ}\text{C}$  for 3 min. Amplified DNA was verified by electrophoresis on 1.5% agarose gels. The fluorescently labeled PCR products were purified on QIAquick PCR purification kit columns (Qiagen GmbH, Hilden, Germany) and eluted in a final volume of  $50\ \mu\text{l}$  of double-distilled water. Purified PCR products were digested overnight at  $37^{\circ}\text{C}$  with 20 U of *CfoI* (Boehringer Mannheim, Mannheim, Germany) in  $20\text{-}\mu\text{l}$  reaction mixtures. The fluorescently labeled terminal restriction fragments (T-RFs) were analyzed by electrophoresis on an automatic sequence analyzer (ABI PRISM 373 DNA Sequencer; PE Biosystems, Foster City, Calif.) in GeneScan mode. Aliquots ( $2\ \mu\text{l}$ ) of T-RFs were mixed with  $2\ \mu\text{l}$  of deionized formamide,  $0.4\ \mu\text{l}$  of loading buffer (PE Biosystems), and  $0.6\ \mu\text{l}$  of DNA fragment length standard (New England Biolabs, Beverly, MA, USA). Five-microliter aliquots of the mixture were loaded on a 36 cm, 6% denaturing polyacrylamide gel.

The T-RFLP profiles were analyzed using BioNumerics (Applied Maths, Kortrijk, Belgium). ABI traces were imported into the program and aligned using

internal size standards run with the samples. Bands were found by autosearch at position tolerance 0.5%. T-RFs were included in the analysis in the range of 30 to 650 bp, which was the range of the size marker that could be determined reliably under the applied electrophoresis conditions. The T-RF intensities from the different T-RFLP profiles were exported to SAS (Heidelberg, Germany) for statistical analysis. A T-RFLP operation results analysis software tool (TORAST) was used for analyzing the bacterial groups of the T-RFs. The program was developed by Frank Ludwig, Dresden University of Technology, Germany. In the program a database was installed with all T-RFs of a previous established 16S rDNA clone library from pig intestine (Leser *et al.*, 2002). From the Clone library 75 T-RFs were in total found to belong to Gram-positive bacteria and 11 T-RFs were in total found to belong to Gram-negative bacteria (mainly Bacteroides and Proteobacteria).

#### Statistical methods

Two types of statistical analysis, (1) and (2), were carried out using the software program SAS (version 8.1, SAS Institute Inc., Cary, NC, USA) for both Experiment A and for B. (1) a parametric variance analysis with the purpose of examining for possible changes in the total T-RF intensities. The analysis *proc mixed* in SAS were used, starting with a full variance model and reducing as insignificant factors were taken out of the model. (2) a non-parametric rank test with the purpose of examining for possible changes in the proportion of Gram-positive bacteria in the community. The rank test used was the Friedman's rank test (Lehmann, 1975) applied in SAS by *proc rank* followed by *proc anova* for Exp. A (allowing for more than one replicate per cell) and for Exp. B by applying *proc freq* plus the *cmh2* option. A more correct way of analyzing these data would have been by applying a Dirichlet distribution describing the three correlated

proportions for Gram-positive, Gram-negative and unclassified bacteria. However, such an approach would be very time-consuming to implement, since SAS did not have features that could meet our specifications and it therefore would involve the developing of a program to estimate and test the model. For the present study we found the use of Friedman's test to be satisfactory.

The design of Experiment A consists of two factors: *sizes* (of the sample) at 4 levels (0.2, 1, 2, 10 g) and *pig* at 4 levels (1, 2, 3, 4) each combination with 5 replicates. The model is a two-way mixed model with stochastic and deterministic factors, where the pigs are randomly chosen from a population and hence was a stochastic factor. The design of Experiment B consisted of 3 factors: *temp* ( $-20^{\circ}\text{C}$ ,  $-80^{\circ}\text{C}$ ), *sample* (1, 2, 3) and *pig* (5, 6, 7, 8, 9), where *sample* represent the number of times a sample had been frozen and thawed. The model was a three-way mixed model. The statistical analysis of the total T-RF intensity (the variance analysis (2) described above) was carried out in order to determine the possible effect of *temp*, *sample* plus their interactions, on the total T-RF intensity and furthermore if a potential decrease of total T-RF intensity affects the diversity of the bacteria community. The statistical analysis of the proportion of Gram-positive bacteria (the rank test (1) described above) was conducted in order to clarify which type of bacteria (Gram-positive/Gram-negative) that best survives the storage when decreasing the temperature and when repeating the freezing/thawing procedure. Since Friedman's test only accounts for one variable plus a random factor, the analysis were carried out in two steps. First analyzing the effect of *sample* and then analyzing the effect of *temp*.

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