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Evaluating protocols for porcine faecal microbiome sampling, storage and extraction: from the farm to the lab

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Running head: **Porcine faecal microbiome sampling**

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Evaluating protocols for porcine faecal microbiome recollection, storage and DNA extraction: from the farm to the lab

Abstract

There is a growing interest in understanding the role of the gut microbiome on productive and meat quality related traits in livestock species in order to develop new useful tools for improving pig production systems and industry. Faecal samples are analyzed as a proxy of gut microbiota and here the selection of suitable protocols for faecal sampling and DNA isolation is a critical first step in order to obtain reliable results, even more to compare results obtained from different studies. The aim of the current study was to establish in a cost-effective way, using automated ribosomal intergenic spacer analysis technique, a protocol for porcine faecal sampling and storage at farm and slaughterhouse and to determine the most efficient microbiota DNA isolation kit among those most widely used. Operational Taxonomic Unit profiles were compared from Iberian pig faecal samples collected from rectum or ground, stored with liquid N_2, room temperature or RNAlater, and processed with QIAmp DNA Stool (Qiagen), PowerFecal DNA Isolation (Mobio) or SpeedTools Tissue DNA extraction (Biotools) commercial kits. The results, focused on prokaryote sampling, based on DNA yield and quality, OTU number and Sørensen similarity indexes, indicate that the recommended protocol for porcine faecal microbiome sampling at farm should include: the collection from porcine rectum to avoid contamination; the storage in liquid N_2 or even at room temperature, but not in RNAlater; and the isolation of microbiota DNA using PowerFecal DNA Isolation kit. These conditions provide more reliable DNA samples for further microbiome analysis.

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Keywords: porcine microbiome, ARISA, DNA isolation, OTU

Introduction

The gut microbiome is now firmly established as a key factor influencing productivity and meat quality related traits in livestock species [25]. Microbiome populations (e.g. Bacteria, virus or fungi) exist in the gastrointestinal tract of mammals, having a great impact on the host biology [23]. Previous studies on humans and mice have evidenced the association between several syndromes and diseases with changes in the microbiota [3]. These sort of changes in livestock species are expected to impact productive and meat quality traits [9, 16, 22]. For instances, some evidences have been linked obesity, metabolic syndromes and diabetes to the microbiota composition [18, 11]. The studies conducted by Bäckhed [1] and Boets [2] reported that the gut microbial community regulates the expression of genes affecting fatty acid oxidation and fat deposition in adipocytes.

Recently, some studies showed that the gut microbiota composition in swine is influenced by the genetics of the host [6, 17], and perturbations to the microbiome occur in response to many factors including stress, antibiotics or diet [9, 15, 16 22]. Ramayo-Caldas study [20] have evidenced the link between the microbial ecosystems and porcine growth-related traits. From here, there are many aspects of the microbiome that need to be analyzed in order to develop new useful tools for improving pig production systems and industry.

Sampling faecal material is considered an effective and non-invasive strategy to determine the gut microbiology. Hence, faecal samples can be utilized as a proxy of gut microbiota [24]. However, the selection of suitable protocols for faecal sampling and DNA isolation that minimise contaminations and DNA degradation is a critical challenge that needs to be overcome in order to obtain reliable results that are comparable and reproducible between different studies [12]. This step is even more critical when analysing large farm animals such as pigs, where sampling uses to take place at commercial farms or remote sites where optimal sampling and storage conditions are unavailable.

The main factors affecting bias from faecal sampling at farms include the sampling procedure (collected from the rectum or from the ground), storage condition (at room temperature, into liquid N_2 or embedded into reagents for stabilisation), and differences in DNA isolation protocols. These factors have been reported to impact DNA yield and sequencing profiles which hamper the ability to detect fine-scale microbiota changes [5].

Currently, the most widely implemented culture-independent approaches employed to analyze the microbiome are based on massive sequencing of the genome or specific target gene (16S rRNA) analysis. However, microbiome fingerprint analysis for the detection of overall patterns in microbial communities is a time and cost effective technique, with enough power for protocol comparison and optimization [13, 14]. One such fingerprint technique is the automated ribosomal intergenic spacer analysis (ARISA), based on the natural inherent length variability of regions within the bacterial ribosomal RNA operon [7]..

The aim of the current study was to establish a protocol for in-farm and slaughterhouse porcine faecal sampling and storage conditions using a cost-effective strategy like ARISA, and to determine the most efficient microbiota DNA isolation kit among those most widely used.

Materials and methods

Sampling

Faecal samples were collected from Iberian pigs in two sampling processes:

Case 1. Four replicates from the same homogenised stool portion (≈ 2g each) were obtained *post mortem* from rectum from eight males (14 months old) at slaughterhouse, and collected in cryotubes of 2 ml. For each animal, two out of these four replicates were stored in liquid N_2 and the other two replicates were maintained at room temperature (20-25ºC) for 3 hours until arrival in the laboratory where both sample types were stored at -80ºC. These samples were employed to evaluate DNA isolation kits and liquid N_2 vs room temperature storage conditions

Case 2. Four replicates from the same homogenised stool portion (≈ 2g each) were obtained *in vivo* from the rectum of seven males (3 months old), and preserved in cryotubes. Two of these replicates were stored in liquid N_2 and the other two were embedded into RNAlater stabilisation buffer for 3 hours until arrival in the laboratory where all samples were storage at -80ºC. Simultaneously, two of the same homogenised stool portion were dropped to the ground, concrete open air, collected after 1-2 min, simulating actual approach for faecal sampling from live animals without manipulation, and preserved in cryotubes in order to test potential ground contamination. Then, the

samples were stored in liquid N_2 until arrival in the laboratory where they were stored at -80C. These samples were employed to evaluate collection procedure (rectum vs ground collection) and liquid N_2 vs RNAlater storage conditions.

Animal manipulations were performed according to the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 about the protection of animals used in experimentation.

DNA isolation

All DNA isolations were conducted in duplicates for each sampling replicate from 0.25 grams of homogenized faecal samples. The QIAmp DNA Stool (Qiagen), PowerFecal DNA Isolation (Mobio) and SpeedTools Tissue DNA (Biotools) extraction commercial kits were used following manufacturers' instructions, except lysis incubation step that was modified at 95ºC for all three protocols in order to improve cell lysis. DNA quantification and quality was checked using a NanoDrop Spectrophotometer and electrophoresis in agarose gels.

ARISA analysis

All amplification reactions were performed in duplicates for each isolated DNA in a final volume of 25 µl, containing 30 ng of isolated DNA, 1 unit of Taq polymerase (Biotools), specific buffer, 2.5 mM of dNTPs and 0.5 µM of specific primers [14] (16S-1392Fw:5´-GYACACACCGCCCGT-3´ and 23S-125RRv: 5´-GGTTBCCCCATTCRG -3´, 5´-fluorescently labelled with TET,). Thermocycling was carried out under the following conditions: 94ºC for 3 min, 32 cycles of 94ºC for 1 min, 52ºC for 1 min and 72ºC for 1 min and 30 s, with a final extension of 72ºC for 20 min. The PCR reactions

were carried out in a GeneAmp PCR System 9700 (Applied Biosystems, Warrington, UK). The successful amplification of the PCR products were checked in agarose gels and 1 µl aliquots were run in an ABI PRISM 3100 capillary electrophoresis system, using LIZ1200 as internal size, in the Genomic Unit facility at Complutense University in Madrid.

Raw fragment data were analyzed using Peak Scanner v2 (Applied Biosystems, Warrington, UK) for size calling and results were exported to csv files. The Operational Taxonomic Units (OTUs) determination was carried out following specific criteria as previously reported by Kovacs [14]. Briefly, A 0.1% threshold of total intensity was applied for fragment filtering, and each of the remaining fragments were assigned to length bins of ±3 bp up to 700 bp and ±5 bp for fragments between 700 and 1200 bp length. Amplicon duplicates were compared and the OTUs that appeared in only one of the duplicates were excluded from the analysis (amplicon duplicate correlations ranges between 0.98-1.00, supporting technical reliability). Relative intensities were calculated after filtering and fragment assignment.

Statistical analysis

Sørensen index [21] was employed to estimate similarity in all pairwise comparisons among isolated DNA and sampling replicates. Taxonomical classification based on 16S-ITS-23S region lengths for specific OTUs was conducted using ADAPT tool (https://edwards.sdsu.edu/cgi-bin/adapt-test/ADAPTHome.cgi).

Generalized linear models were implemented to determine statistical differences. The model were:

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$$y_i = \mu + x_{ij} loc_j + x_{ik} kit_k(loc_j) + x_{il} Cons_l(loc_j) + e_i$$

Where y_i was the trait analysed (either relative abundance or richness) for sample *i*, loc_j is the effect of sample location (*j*= {*rectum, ground*}), $kit_k(loc_j)$ is the extraction kit (*k*= {*Qiagen, Mobio, Biotools*}) within sample location, $Cons_l(loc_j)$ is the conservation method (*l*={*room temperature, RNAlater, liquid* N_2}) nested to location and e_i is the residual of record *i*.

ANOVA analyses were conducted, and statistically significant differences between effect levels were determined using Tukey comparison test.

Results and discussion

Isolation kit evaluation

Three DNA isolation kits suitable for microbiota DNA extraction, QIAmp DNA Stool (Qiagen), PowerFecal DNA Isolation (MoBio) and SpeedTools Tissue DNA extraction (Biotools) were evaluated from 0.25 g of eight faecal samples.

The yield and quality, measured as the A260/A280 and A260/A230 ratios, of the DNA isolated from the faecal samples collected in liquid N_2 and processed with each one of the kits is shown in Table 1. The highest yield was obtained with QIAmp DNA Stool kit (8-15μg), however abnormally high A260/A230 ratios were observed (>2.6), which indicates some kind of contamination during isolation. Additionally, the DNA samples were loaded in agarose gel in order to confirm the absence of porcine gDNA, the result

are shown in Fig. 1. No contamination with porcine or human gDNA could be detected for any of the samples processed with the three kits.

The OTU numbers, which indicate richness, identified per sample processed with each one of the three isolation kits is represented in Figure 2a. The OTU numbers ranged from 32 to 45, being the PowerFecal DNA Isolation kit the most successful one in terms of detecting richness, because the samples processed with this kit, regardless the sampling cases, showed between 36 and 45 OTUs (median= 39), while the same samples processed with QIAmp DNA Stool kit showed between 34 and 45 OTUs (median= 36) and the ones processed with SpeedTools Tissue DNA extraction kit between 32 and 42 OTUs (median= 37).

The OTU richness between replicates, measured by Sørensen index for the samples processed with each one of the isolation kits is represented in Fig. 2b. The highest similarity was found for the replicates processed with PowerFecal DNA Isolation kit (Sørensen index = 0.98), indicating higher repeatability for the samples processed with this kit. Therefore, the following comparisons were conducted using PowerFecal DNA Isolation kit

Sampling procedure evaluation

Sampling from rectum of a live large animals is a laborious task that requires immobilization, being unfeasible in large population sizes. An alternative could be to sample from stool on the ground immediately after deposition, which could facilitate the sampling process and minimize animal disturbance, but it could imply some contamination. In the current study, the microbiota sampled from stools on the ground (1-

2 min after deposition) was compared to the samples collected directly from rectum in the same live pig and faecal deposition.

The yield and quality of the DNA isolated from faecal samples collected from rectum and from ground and stored in liquid N_2 is shown in Table 1. No relevant differences in DNA yield and absorbance ratios could be observed between collection strategies. However, slight differences in the richness could be detected (Fig. 3a). The OTU numbers ranged from 35 to 51 (median= 42) when the samples were collected from rectum, while the OTU numbers ranged from 40 to 53 (median= 43) when the samples were collected from ground. The OTU similarities between replicates (Fig. 3b) did not show any essential differences between sampling places, although the highest median similarity was found for the replicates collected from the rectum (Sørensen index = 0.97). The larger richness identified from stools collected from the ground may indicate some degree of contamination. In fact, all the OTUs identified in the samples collected from rectum were detected also in the samples collected from ground but some of the OTUs (1040, 1090 and 1181, according to the fragment lengths) were specifically identified in the stools collected from the ground. Furthermore, the taxonomical classification (Table S1) based on 16S-ITS-23S regions (ADPAT tool) reported potential matching fragment lengths for OTU1090 in agreement with *Bradyrhizobium* and *Nitrobacter* genus, playing central roles in the nitrogen-cycle and widely found in soil [19], which supports potential contamination of the samples collected from ground.

Storage evaluation

Sample freezing and storage at −80 °C is considered to be the best practice when preserving microbial composition for further sequencing studies [8]. However, it is not

always feasible to have liquid N_2 for fast freeze. Therefore, two alternatives conditions to freezing were evaluated, collection at room temperature for 3 hours and RNA later stabilisation buffer before −80 °C storage at lab facilities.

Liquid N_2 vs room temperature

No differences in DNA yield or quality could be observed between the seven faecal samples collected from rectum and stored in liquid N_2 or at room temperature (20-25 ºC)(Table 1). The richness detected was similar between both storage conditions (Fig. 4a), with a median of 33 when the samples were stored in liquid N_2, and 34 when the samples were stored at room temperature. Furthermore, similarity Sørensen indexes across replicates reported minor differences, with a median of 0.98 in both conditions (Fig. 4b). Therefore, sample collection at room temperature for few hours does not seem to alter richness and repeatability, and it may be necessary longer periods to detect some changes in the microbiota profiles during room temperature storage, as reported by Carroll [4], after 48 hours.

Liquid N_2 vs RNAlater storage

Contrary to the previous evaluation, huge differences in DNA yield and quality could be observed between the seven faecal samples collected from rectum and stored in liquid N_2 or embedded into RNAlater (Table 1) and maintained at room temperature for 3 hours before storage at -80º. Very little yield (0.5-1μg) was obtained from the samples stored into RNAlater, with abnormally low A260/A230 ratios (<0.9), supporting the low yield obtained. Differences in OTU numbers were detected (Fig. 5a). A median of 43

OTUs were detected when the samples were stored in liquid N_2, and median of 37 OTUs when the samples were stored embedded into RNAlater, indicating a loss of richness. However the OTU similarities between replicates (Fig. 5b) did not report essential differences between storage conditions, although the highest median similarity was found for the replicates stored in liquid N_2 (Sørensen index = 0.97), which may point out that the richness loss due to storage condition into RNAlater happens in a similar way in all the replicates. Some OTUs identified when the samples were stored in liquid N_2 did not appear in any of the samples that were stored embedded into RNAlater (OTU667, 959, 210 and 945, according to the fragment lengths). Taxonomical classification with ADPAT tool (Table S1) reports potentially matching fragment lengths for *Yersinia enterocolitica* (OTU959), which lives in domestic animal gut, and *Burkholderia ambifaria* (OTU667) , which belongs to *Proteobacteria* phylum found in the intestinal tracts of humans and animals. These results seem to support richness losses due to the RNAlater use, in agreement with previous studies of faecal microbiota in other species [10].

Based on the obtained results, and although the differences were not statistically significant (p-values>0.10) probably due to the limited number of samples analysed, the recommended protocol for porcine faecal microbiome sampling at farm and slaughterhouse includes collection from porcine rectum, to avoid contamination, and storage in liquid N_2 or even at room temperature, but prevent using RNAlater. For microbiota DNA isolation, the PowerFecal DNA Isolation kit provides more reliable DNA samples for further microbiome analysis.

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Conflict of Interest

There is not conflict of interest.

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Figure captions:

Fig. 1 Electrophoresis of the microbiota DNA isolated from faecal porcine samples. Lane1-2: PowerFecal DNA Isolation kit/ sample collected from rectum in liquid N_2; Lane3-4: QIAmp DNA Stool kit/ sample collected from rectum in liquid N_2; Lane5-6: SpeedTools Tissue DNA extraction kit/ sample collected from rectum in liquid N_2; Lane7: PowerFecal DNA Isolation kit/ sample collected from ground in liquid N_2; Lane8: PowerFecal DNA Isolation kit/ sample collected from rectum at room T^a; Lane9: Porcine gDNA; Lane10: Size ladder (EcoLadderI).

Fig. 2 Graphical representation of the OTU number (richness) (**a**) and Sørensen similarity index (**b**) among microbiota DNA isolation protocols from faecal porcine samples

Fig. 3 Graphical representation of the OTU number (richness) (**a**) and Sørensen similarity index (**b**) between porcine faecal microbiota sampled from rectum and ground.

Fig. 4 Graphical representation of the OTU number (richness) (**a**) and Sørensen similarity index (**b**) between porcine faecal microbiota sampled in liquid N_2 and room temperature.

Fig. 5 Graphical representation of the OTU number (richness) (a) and Sørensen similarity index (b) between porcine faecal microbiota sampled in liquid N_2 and RNAlater

Supplementary material:

Supplementary Table S1: Potentially matching Genus according to ADAPT tool for the OTU length summary

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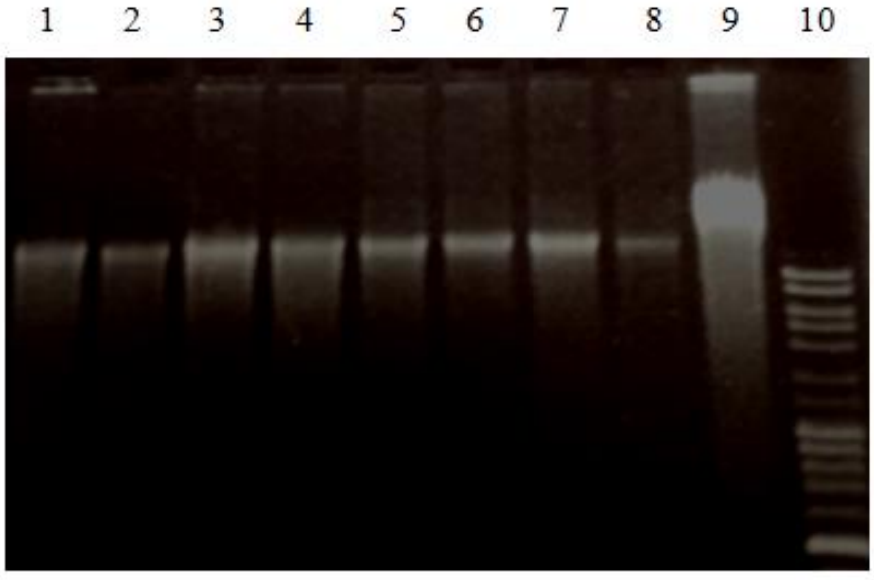
Figure 1:



Lane1-2: PowerFecal DNA Isolation kit/ sample collected from rectum in liquid $N_{2;}$ Lane3-4: QIAmp DNA Stool kit/ sample collected from rectum in liquid $N_{2;}$ Lane5-6: SpeedTools Tissue DNA extraction kit/ sample collected from rectum in liquid $N_{2;}$ Lane7: PowerFecal DNA Isolation kit/ sample collected from ground in liquid $N_{2;}$ Lane8: PowerFecal DNA Isolation kit/ sample collected from rectum at ambient T^{a}; Lane9: Porcine gDNA; Lane10: Size ladder (EcoLadderI)

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Figure 2:

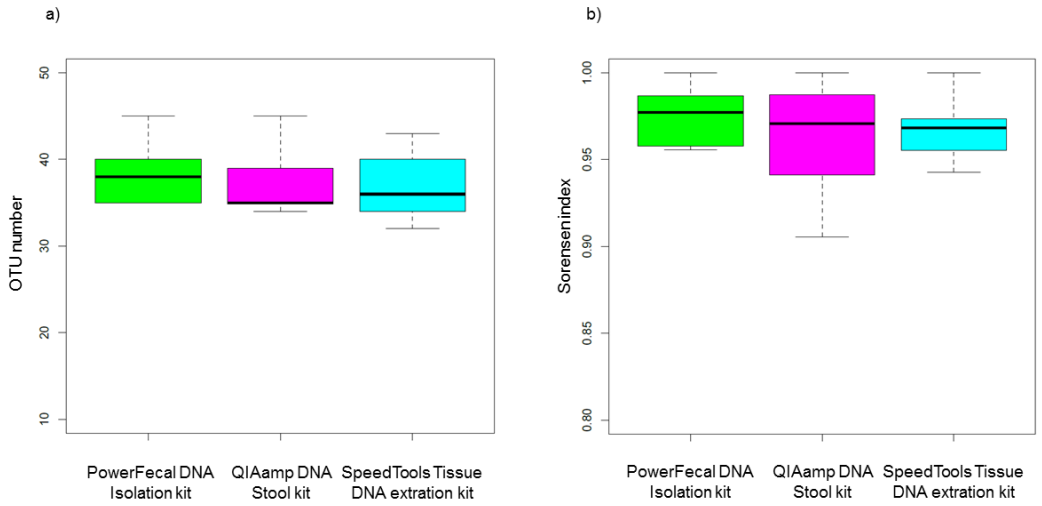

Figure 3:

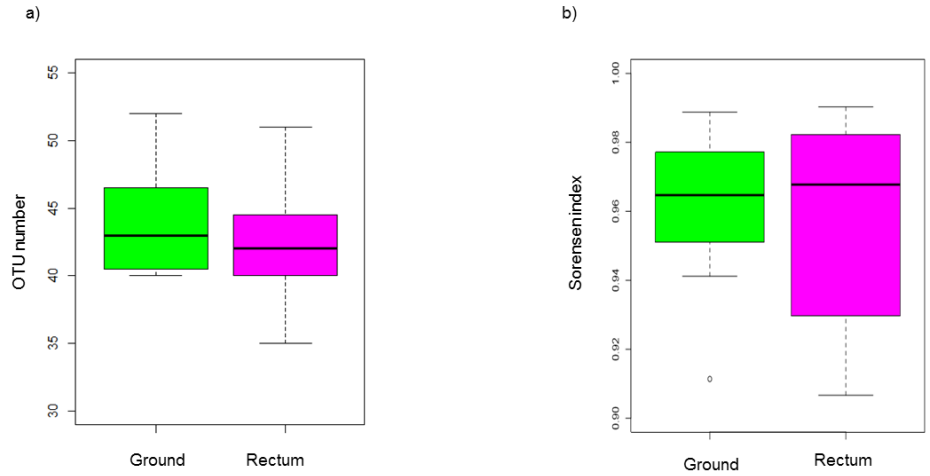

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Figure 4:

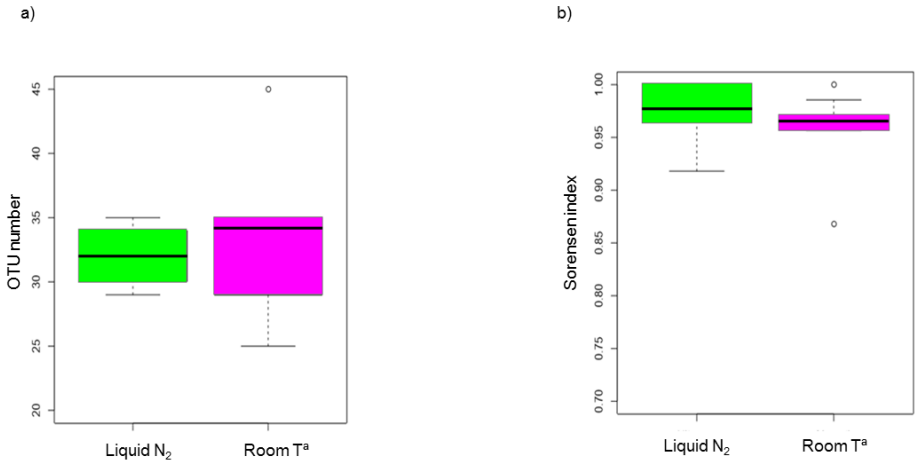

Figure 5: