Short communication

Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR

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Abstract

Jejunum digesta samples were taken from weaning pigs in order to evaluate real-time PCR (qPCR) as a method for quantifying pig gut bacteria. Total bacteria, lactobacilli and enterobacteria were quantified by qPCR and the results were compared with those obtained with traditional methods: 4\',6-diamidino-2-phenylindole (DAPI staining) for total bacteria, selective culture for lactobacilli and enterobacteria. Real-time PCR showed higher values in terms of 16S rRNA gene copies than DAPI counts or CFU. Despite the differences, the lactobacilli:enterobacteria ratio was similar between methods (2.5 ± 0.58 for qPCR and 3.1 ± 0.71 for selective culture, \( P = 0.39 \)). Possible reasons for the higher PCR counts are discussed considering both an overestimation with PCR by quantification of dead bacteria or free DNA and also an underestimation with conventional methods. Inherent differences in the pre-treatment of the samples could partially explain the discrepancies observed. Regardless of the numerical differences between methods, values obtained by qPCR and traditional methods showed a significant correlation for lactobacilli and total bacteria. In the light of these results, real-time PCR seems a valid method to quantify microbial shifts in the gastrointestinal tract.

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1. Introduction

Recently, molecular methods have shown that the complexity of microbial communities is much greater than previously thought and that the majority of gut bacteria are still unknown (Pryde et al., 1999; Leser et al., 2002). This lack of knowledge is mostly attributed to the failure of many bacteria to grow in a given culture medium (Langendijk et al., 1995; Huijsdens et al., 2003). Quantitative molecular methods could be more sensitive and selective than traditional methods taking into account that they do
not rely on the ability of bacteria to grow. Moreover, DNA-based methods offer the option of storing samples until their analysis, which could be an important advantage in field conditions.

Considering the high complexity of gut microbiota, some authors have tried to find particular microbial groups that could serve as an index of a health-promoting microbiota. Conventionally, the ratio lactobacilli:enterobacteria has been used as a simple index and an increase in this ratio is related with a higher resistance to intestinal disorders (Muralidhara et al., 1977; Reid and Hillman, 1999). Specifically in the weaning pig, lactobacilli could have a predominant role in controlling colibacillosis, which is one of the most common intestinal disorders during the first months of life (Torturero et al., 1995; Nemcova et al., 1999).

The objective of this work was to evaluate the use of real-time PCR to quantify total bacteria, lactobacilli and enterobacteria in pig digesta samples.

2. Material and methods

2.1. Sample preparation

Samples of jejunum digesta were obtained from healthy early weaned (20 ± 2 days) pigs of approximately 40 days old. Animals received commercial diets and were sacrificed with an intravenous injection of sodium pentobarbitone (200 mg/kg body weight). For comparison of qPCR, selective culture and DAPI staining, 32 animals from the same herd were sampled. To study the effect of pre-treatment of samples on microbial counts, 18 animals from a second herd were used. The management, housing, husbandry and slaughtering conditions conformed to the European Union Guidelines.

For microbiological culture procedures and for DAPI staining a fragment of 10 cm from the distal jejunum was tied, cut-off and kept in ice for further dilution. For qPCR counts, 1 g of digesta was kept in tubes that contained 3 ml of ethanol as preservative. Samples were gently mixed with the ethanol and stored at 4 °C until analysis. To assess the effect of pre-treatment of the sample on the total bacteria qPCR counts, approximately 5 g from jejunum digesta were sampled and frozen until analysis.

2.2. Bacteria quantification by traditional methods

For selective culture, digesta samples were serially diluted (w/v) in sterile PBS and plated in selective media. Enterobacteria were enumerated using MacConkey agar at 37 °C (24 h) (CM-115, Oxoid, Madrid, Spain) and lactobacilli in Rogosa agar at 37 °C in a 5% CO₂ atmosphere (48 h) (CM-627, Oxoid).

Direct quantification of total bacteria was carried out by epifluorescent direct count method (Hobbie et al., 1977) using 4',6-diamidino-2-phenylindole (DAPI) staining. One gram of sample was diluted 10 times with sterile PBS and 0.5 ml of this suspension was fixed with 4.5 ml of 2% formaldehyde. Samples were stained with DAPI (10 min, 1 μg/ml) and filtered through polycarbonate membrane filters (0.22 μm, Whatman International, Kent, UK). Bacteria were enumerated using an ocular graticule and 10 random fields per sample were counted. (Olympus NCWHK 10 ×, Olympus, Barcelona, Spain).

2.3. Bacteria quantification by real-time PCR (qPCR)

2.3.1. DNA extraction

The equivalent volume to 400 mg of digesta samples preserved in ethanol was precipitated by centrifugation (13,000 × g, 5 min). The DNA from the precipitate was extracted and purified using the QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK). The lysis temperature was increased to 90 °C and an incubation with lysozyme was added (10 mg/ml, 37 °C, 30 min) to improve the bacterial cell rupture. The DNA obtained was stored at −80 °C.

To evaluate possible disregard of bacteria attached to particulate material during pre-treatment of the samples for culturing and DAPI staining, DNA extraction was also performed after a previous 1/10 dilution of the samples. One gram of each sample was diluted 10 times with sterile PBS and homogenized 1 min with a vortex mixer. Diluted samples were let to stand on the bench during another minute and 4 ml of the liquid phase were centrifugated (20,000 × g, 20 min). The DNA was extracted and purified from the pellet using the same commercial QIAamp DNA Stool Mini Kit and procedures described above.
The DNA from pure cultures of *Lactobacillus acidophilus* (CECT 903NT) and *Escherichia coli* (CECT 515NT) was harvested from the bacterial pellet obtained by centrifugation of 6 ml of culture using the same Qiagen Kit. Pig genomic DNA was obtained from blood samples that were collected aseptically using the Mammalian Genomic DNA extraction kit (CAMGEN, Cambridge Molecular Technologies Ltd., Cambridge, UK).

2.3.2. Quantitative PCR

To quantify total bacteria, lactobacilli and enterobacteria different primers were used: F-tot (forward) 5’GCAGGCCTAATACATGCAAGTC3’ (adapted from Marchesi et al. (1998)) and R-tot (reverse) 5’CCTACTTCTTTTGCAACCCACTC3’ (adapted from Amann et al. (1995)) for total bacteria. For lactobacilli: F-lac 5’GCAGCAGTAGGAATCT-0-CCTACTTCTTTTGCAACCCACTC3’ (adapted from Walter et al. (2001)) and for enterobacteria F-ent 5’ATGGCTGGTGTCAGTC-0-GCTA3’ (adapted from Leser et al. (2002)) and R-ent 5’GCATTYCACCGCTACACAT-TCCA3’ and R-lac 5’GCATCYACCGCTACACAT-G3’ (adapted from Marchesi et al. 1998 and R-tot (reverse) 5’C-TGCTGCCCTCCGTAGGAGT 3’ (adapted from Leser et al. (2002)) and R-ent

The oligonucleotides were adapted from published specific primers or probes using the Primer Express Software to qPCR recommendations (Applied Biosystems, CA, USA). The different primers were also checked for their specificity using the database similarity search program nucleotide-nucleotide BLAST (Alstchul et al., 1990) and the absence of amplification of porcine DNA was tested empirically by PCR using the DNA extracted from pig blood.

Standard curves were constructed using PCR product of the 16S rRNA gene of *E. coli* and *L. acidophilus*. Primers and PCR conditions were those published by Leser et al. (2002). The PCR product was purified with the commercial kit DNA purification system (Promega Biotech Ibérica, Spain) and the concentration measured at 260 nm (Biophotometer, Eppendorf Ibérica S.L., Spain). Products obtained were also sequenced (ABI 3100 Genetic Analyzer, PE Biosystems, Warrington, UK) to confirm them, and number of copies calculated. Serial dilutions were performed and 10^2, 10^3, 10^4 and 10^5 copies of the gene per reaction were used for calibration. Amplicons from *E. coli* were used for quantification of the total bacteria and enterobacteria and amplicons from *L. acidophilus* for quantification of lactobacilli. The functions describing the relationship between C_t (threshold cycle) and x (log copy number) for the different assays were: C_t = -3.19x + 53.66; R^2 = 0.99 for total bacteria; C_t = -2.60x + 46.82; R^2 = 0.99 for lactobacilli; C_t = -2.32x + 43.88; R^2 = 0.99 for enterobacteria.

Real-time PCR was performed with the ABI 7900 HT Sequence Detection System (PE Biosystems, Warrington, UK) using optical grade 96-well plates. The PCR reaction was performed on a total volume of 25 μl using the SYBR® Green PCR Core Reagents kit (PE Biosystems). Each reaction included 2.5 μl 10× SYBR Green buffer, 3 μl MgCl\_2 (25 mM), 2 μl dNTPs (2.5 mM), 0.25 μl AmpErase UNG\_R™ (1 U/μl), 0.125 μl AmpliTaq Gold® (5 U/μl), 1 μl of each primer (12.5 μM) and 2 μl of DNA samples (diluted 1/10). The reaction conditions for amplification of DNA were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. To determine the specificity of amplification, analysis of product melting curve was performed after the last cycle of each amplification.

3. Results and discussion

Minimum levels of detection for the different PCR reactions ranged from 10^5 to 10^6 gene copies/g fresh matter (FM) and were conditioned by the minimum dilution of sample DNA that did not inhibit the PCR reaction, and by the presence of contaminating *E. coli* DNA in the commercially supplied reagents. Dilution 1/10 was found not to affect the efficiency of amplification, giving equivalent values to 1/100 and 1/1000 dilutions. On the other hand, the degree of contamination of the reagents was variable but ranged between 10 and 200 copies/reaction. Similar contamination has been previously described (Suzuki et al., 2000; Nadkarni et al., 2002).

Results for total bacteria, lactobacilli and enterobacteria in jejunum samples using qPCR and traditional methods are shown in Fig. 1. The values obtained, by qPCR and traditional methods, respectively, were 11.1 ± 0.88 log gene copies/g FM and 7.8 ± 0.37 log bacteria/g FM for total bacteria; 10.8 ± 1.66 log gene copies/g FM and 7.9 ± 0.79 log bacteria/g FM for lactobacilli and 8.4 ± 0.56 log gene
copies/g FM and 4.8 ± 1.68 log bacteria/g FM for enterobacteria. It should be noted that regardless of the method used, lactobacilli counts were close to total bacteria counts, confirming Lactobacillus spp. as one of the major groups in upper gastrointestinal tract of pigs (Khaddour et al., 1998; Reid and Hillman, 1999). In all cases, quantification by qPCR gave higher values in terms of 16S rDNA than DAPI counts or CFU (3.4 ± 0.71, 2.9 ± 1.73 and 3.6 ± 1.72 log units higher for total bacteria, lactobacilli and enterobacteria, respectively). However, lactobacilli:enterobacteria ratio (expressed as the difference of logarithms) was similar between methodologies (2.5 ± 0.58 for PCR and 3.1 ± 0.71 for selective culture, P = 0.39). Similar discrepancies between PCR and culturing have been found by other authors (Nadkarni et al., 2002; Huijsdens et al., 2003) and they have been related to the multiplicity of 16S rRNA gene copies (Fogel et al., 1999), to the presence of non-viable, or viable but not culturable bacterial cells, and to free DNA. In that sense, recently, Apajalahti et al. (2003) found that between 17 and 34% of bacteria in fecal samples were dead and thus, permanently beyond any culture method.

The use of real-time PCR with SYBR Green dye could also lead to overestimation due to formation of non-specific amplicons (Hein et al., 2001). However, the dissociation curve obtained at the end of each PCR was checked and always had a similar melting point to the standard samples, without any additional peak, indicating the absence of non-desired PCR products.

Another reason to the overestimation registered, are differences in the pre-treatment of the digesta. The presence of a quantitative important bacterial community attached to the coarse particulate material could have been discharged somehow with culturing and DAPI methods but not with qPCR. In this study, DNA samples were directly extracted from the original material without any previous isolation of the bacterial pellet, whereas for culture or DAPI, a previous 1/10 dilution was performed with a subsequent sub-sampling that generally overlooks most of the coarse digestive material that persists in the bottom of the tubes. To validate this hypothesis we compared qPCR results for total bacteria using DNA extracted directly from digesta samples or from pre-diluted samples. Results confirmed a reduction in numbers when subjecting samples to a previous dilution. Mean values were 11.1 ± 0.60 for directly extracted and 10.3 ± 0.51 log units for diluted samples (n = 18). This would suggest that a high percentage of microbial population remains attached to the coarse particulate material. Previous works have described a high percentage of microbes attached to the solid phase (over 70% in the rumen, Yang et al., 2001). Moreover, for fecal and digesta samples, DNA extraction protocols are diverse (Anderson and Lebepe-Mazur, 2003), some authors extract DNA directly from the samples, while others isolate previously the bacterial pellet. This previous isolation could affect results quantitatively and also compromise the representativity of the species composition taking into account ecological differences between free bacteria and attached populations (Michalet-Doreau et al., 2001). Results obtained indicate the importance of previous treatment of samples whatever the method of microbial quantification we use.

In spite of PCR overestimation of microbial counts, values obtained by qPCR and DAPI for total bacteria showed a significant correlation (r = 0.7; P < 0.001) (Fig. 2). It is interesting to point out that qPCR overestimation was higher with the highest counts than with the lowest counts. It could be due to an increase in the amount of cellular debris and free bacterial DNA with the highest counts or also to an increase in the percentage of bacteria attached to particulate material that had been somehow discarded.
with the DAPI method as we have mentioned before. Another possible reason to consider is a change in the number of 16S rRNA copies related to changes in bacterial species and in metabolic activity of bacteria (Fogel et al., 1999). Similarly to total bacteria, PCR and culture counts for lactobacilli showed a significant correlation ($r = 0.48; P < 0.01$) as did the lactobacilli:enterobacteria ratio ($r = 0.51; P < 0.01$). However, results obtained for enterobacteria did not show significant correlation. It could be due to differences in the bacteria species considered by the two methodologies following a phenotypic (culture) or a genotypic (qPCR) criterion.

4. Conclusion

The results obtained suggest that real-time PCR may well be a practical method for studying quantitative shifts in pig gut bacteria although numerical values are higher than for traditional methods. Differences in absolute values could be related to the amplification of DNA from dead cells with qPCR and to the loss of some particle-attached bacteria with DAPI and selective culture. Relative values between groups such as the lactobacilli:enterobacteria ratio could be used as an index of the gut health status of pigs. The ease and rapidity of qPCR (once implemented) compared with traditional culture, and the possibility of storing samples until analysis, could turn qPCR into the preferred method for quantifying gut bacterial shifts in the near future.

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References


