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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.

Keywords: Enterobacteria; Intestinal microbiota; Lactobacilli; Pig; Real-time PCR

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

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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% CO_2 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 ($13{,}000 \times g$, 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 ($20{,}000 \times g$, 20 min). The DNA was extracted and purified from the pellet using the same commercial QIAamp DNA Stool Mini Kit and procedures described above.