

SHORT COMMUNICATION

Phylogenetic network analysis applied to pig gut microbiota identifies an ecosystem structure linked with growth traits

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The ecological interactions within the gut microbial communities are complex and far from being fully understood. Here we report the first study that aims at defining the interaction network of the gut microbiota in pigs and comparing it with the enterotype-like clustering analysis. Fecal microbiota of 518 healthy piglets was characterized by 16S ribosomal RNA gene sequencing. Two networks were constructed at the genus and operational taxonomic unit levels. Within-network interactions mirrored the human gut microbiota relationships, with a strong co-exclusion between *Prevotella* and *Ruminococcus* genera, and were consistent with the two enterotype-like clusters identified in the pig microbiota. Remarkably, the cluster classification of the individuals was significantly associated with the body weight at 60 days of age ($P=0.005$) and average daily gain ($P=0.027$). To the best of our knowledge, this is the first study to provide an integrated overview of the porcine gut microbiota that suggests a conservation of the ecological community interactions and functional architecture between humans and pig. Moreover, we show that the microbial ecosystems and porcine growth traits are linked, which allows us to foresee that the enterotype concept may have an important role in the animal production industry.

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Introduction

The intestinal microbiota is a complex ecosystem that is intimately connected with the biology of the host and contributes to its homeostasis and health (Clemente *et al.*, 2014). Recent studies have revealed that the structure of several microbial ecosystems can be effectively studied, with a network analysis approach. Such studies have suggested non-random patterns of community structure and functionalities, and illustrated the role of phylogeny (Faust *et al.*, 2012; Lima-Mendez *et al.*, 2015) and environmental factors (Berry and Widder, 2014) as determinants of co-occurrence patterns. Pig is both a major source of meat for human consumption and a relevant biomedical model. Our aim was to reconstruct the

phylogenetic co-occurrence network structure of the porcine gut microbiota, which to our knowledge has not been reported yet, and to investigate its contribution to the production traits. Thus, we propose a methodology to reveal microbiota interactions, and investigated whether this approach is complementary to the enterotype clustering analyses for the characterization of the microbial communities in the pig gut.

Results

Microbiota composition and functional prediction

A total of 1127 operational taxonomic units (OTUs) and 44 genera were identified in the sequence data from 518 piglets at 60 days of age (Supplementary Table S1). In agreement with the previous reports (Costa *et al.*, 2014; Mach *et al.*, 2015), the dominant bacterial phyla were *Bacteroidetes* and *Firmicutes* (Supplementary Figure S1), and the most abundant genera were *Prevotella* and *Roseburia* (Supplementary Figure S2). By using PICRUSt (Langille *et al.*, 2013), we were able to predict a

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functional map that contains 4245 KEGG orthology groups (KOs), which could be related to microbiota functions (for example, carbohydrate and energy metabolism, amino acid, lipid and vitamins metabolism, and DNA replication and repair).

Network inference

The genera ($n=35$) and OTUs ($n=528$), which were present in $>20\%$ of the animals in the cohort analyzed here, were selected to estimate sparse correlations, using the SparCC method (Friedman and Alm, 2012). Significant interactions were determined by using the partial correlation and information theory (PCIT) algorithm (Reverter and Chan, 2008), in an approach that we have termed Sparse-PCIT (SPCIT). Our results show a high level of connectivity within the pig gut microbiota, which is dominated by phylogenetic assortativity (Figure 1). The SPCIT–genera network led to the identification of 35 nodes and 105 edges (Figure 1a), whereas, after reduction, the SPCIT–OTU network presented 69 nodes and 163 interactions (Figure 1b). The centrality parameters of the SPCIT–genera and SPCIT–OTU networks were consistent with the literature (Faust *et al.*, 2012; Friedman and Alm, 2012; Berry and Widder, 2014). In addition, the power-law model (equivalent to a free scale network) was better fitted to the data when the network density was reduced ($OTU_{non-reduced} = 0.44$ vs $OTU_{reduced} = 0.98$), which suggests that a few OTU nodes act as hubs with a larger number of links to other OTUs. These global trends were verified in independent predictions by using SparCC and PCIT

algorithms, which confirmed the SPCIT results in terms of the network topology and number of overlapping interactions (Supplementary Information).

Individual clustering and association with microbial functionalities and host performance

Enterotype analysis on the whole cohort was done using the methodology of Arumugam *et al.* (2011) and showed that piglet samples clustered into two enterotype-like groups (Figure 2a), which were dominated by either *Ruminococcus* and *Treponema* (PEA), or *Prevotella* and *Mitsuokella* genera (PEB; Figure 2b). Cluster assignment was not influenced by pig gender, production batch or nuclear family ($P>0.05$). Diversity analysis revealed a significantly higher level of alpha-diversity and richness for the PEA than for the PEB ($P<0.001$) (Figure 2c). On the basis of the PICRUST analysis to predict KO abundances, 1565 KO that were differentially abundant (DA) between enterotypes were identified (corresponding to 667 different enzymes; Supplementary Table S2). Overall, 9.7% of these DA enzymes matched with the CAZy database (Cantarel *et al.*, 2009), and 96.7% of these were more abundant in the PEB. Moreover, statistical analyses revealed a significant effect of enterotype-like cluster assignment on both body weight (BW; $P=0.005$) and average daily gain (ADG) during the post-weaning period ($P=0.027$). Animals that clustered with the PEB were on average 850 g heavier and had an extra ADG of 17.9 g per day than those that clustered with the PEA (Supplementary Table S3). It should be noted that no

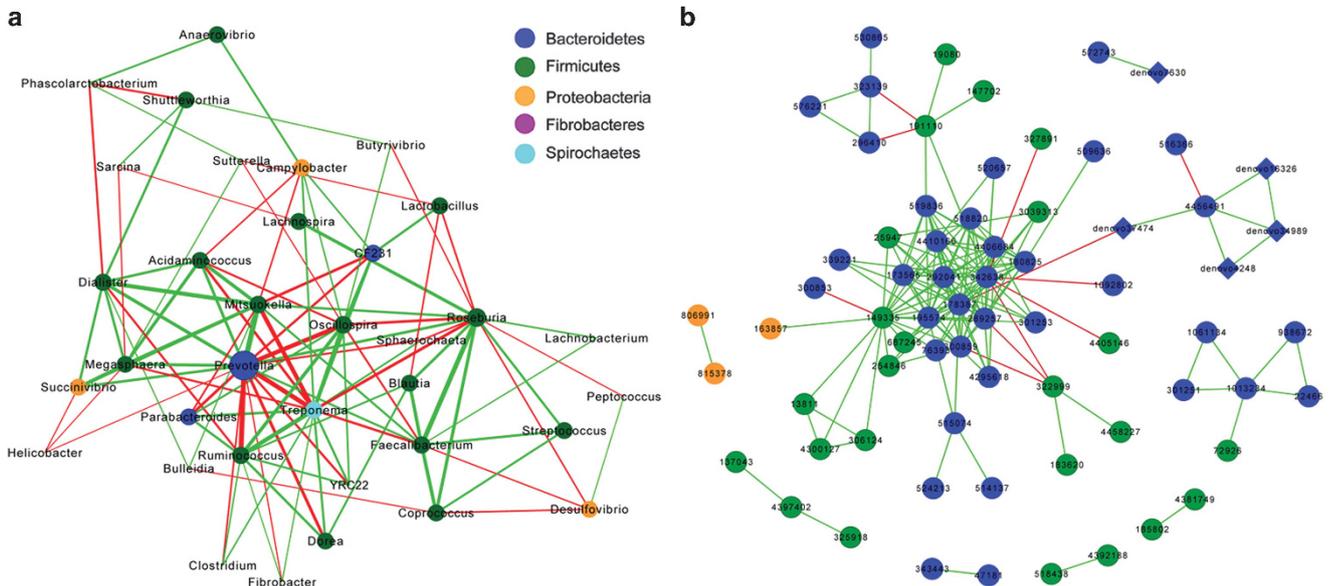


Figure 1 Network analysis applied to the porcine gut microbiota on a cohort of 518 60-days old pigs (a) SPCIT–genera network analysis of the porcine gut microbiota reveals the significant interactions. The size of the node is proportional to the genera abundance. Node color corresponds to phylum taxonomic classification. Edge color represents positive (green) and negative (red) correlations, and the edge thickness is equivalent to the correlation values. (b) Subset of the OTU network representing only the high-confidence interactions with absolute sparse correlations ≥ 0.35 . All networks are displayed graphically as nodes (genera or OTUs) and edges (significant interactions among nodes).

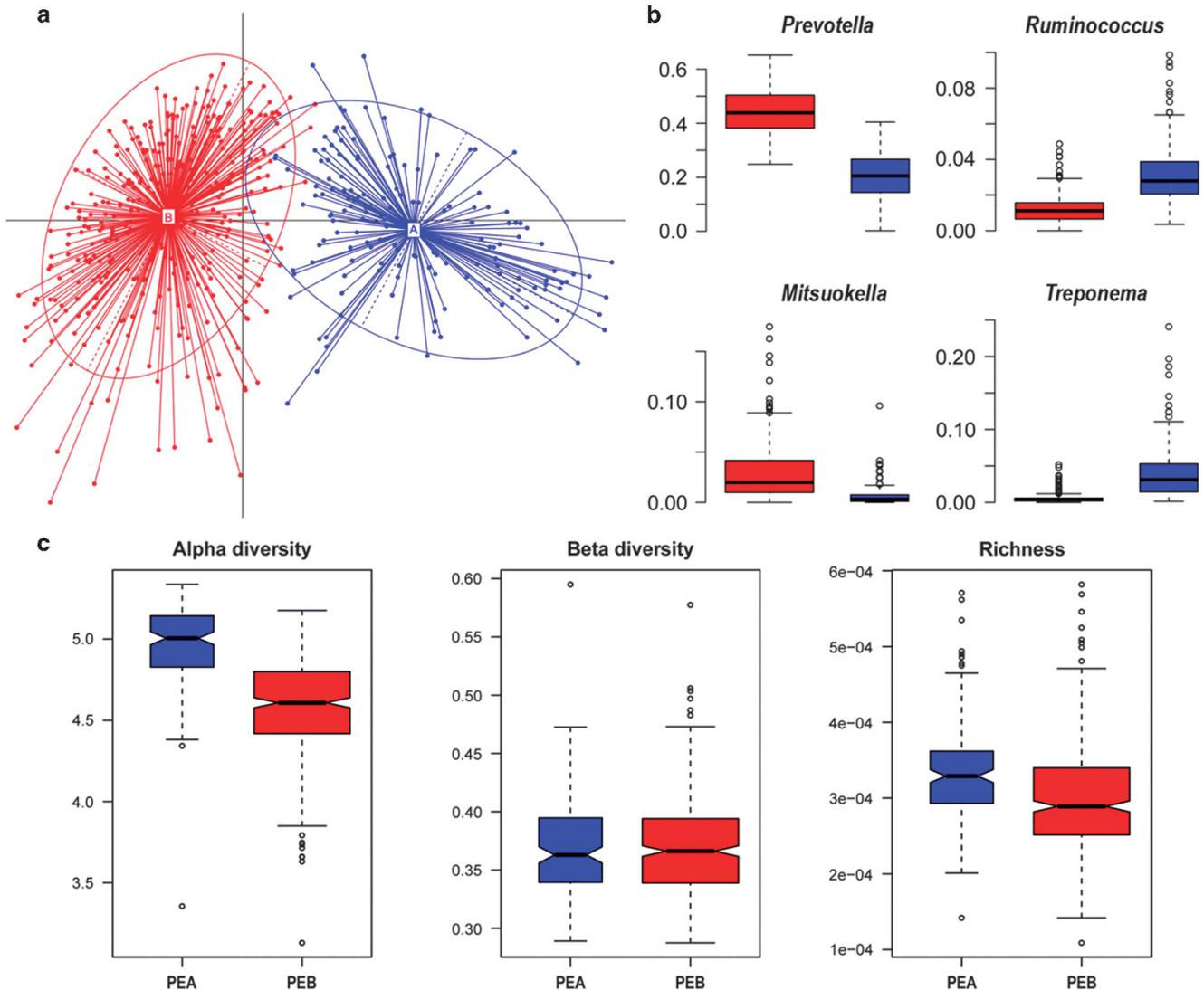


Figure 2 Pig enterotype-like cluster distribution in a cohort of 518 60-days old pigs. (a) Enterotype-like group assignment of samples. (b) Abundances of the two main contributors of each enterotype. Blue color corresponds to the PEA cluster and red color to the PEB cluster. (c) Mean values corresponding to alpha, beta diversity and richness between the pig enterotype-like groups.

significant differences in BW at weaning were observed, which indicates that the growth rate during the post-weaning period is driven in part by differences in the gut microbiota ecosystem.

Discussion

This is the first report on a study that aims at jointly defining the enterotype-like clustering structure of the gut microbiota in pigs and the microbial interaction network that drives the pig gut microbiota, by applying a new approach (SPCIT) that combines sparse correlations in SparCC with the PCIT algorithm. In agreement with our previous report on a subset of 31 animals (Mach *et al.*, 2015), two enterotype-like groups were identified in this new cohort that includes 518 60-day old pigs (Figure 2a).

However, the stability of these two enterotype-like clusters beyond the age of 60 days remains to be determined, as at younger ages, we observed a dynamic enterotype-like clustering assignment that primarily shifted the relative abundance of the genus *Prevotella* (Mach *et al.*, 2015). It should be noted that the occurrence of the two enterotype-like clusters within this larger pig cohort cannot be attributed to variations in the environment, treatments, diets or age, although all these factors have been suggested as possible biases for the definition or even the existence of human enterotype clusters (Knights *et al.*, 2014). Thus, our results demonstrate that enterotypes can be effectively identified in the gut microbiota ecosystem of a species other than humans or mice. Regarding the possible biological explanations for the existence of enterotypes in gut, they may reflect multiple exclusion mechanisms,

including secretory products and competition for nutrients (Mukherjee *et al.*, 2015).

The study of the topological properties of networks provides valuable and complementary information to the classical enterotype clustering analysis. In accordance with the findings of Arumugam *et al.* (2011) on human gut microbiota, a co-exclusion between *Prevotella* and *Ruminococcus* (sparse $r = -0.64$) was found in pig gut microbiota. Moreover, a significant over-abundance of genera such as *Prevotella* and *Streptococcus* was observed within the PEB enterotype-like group, whereas the *Ruminococcus* genus was significantly more abundant within the PEA enterotype-like group (Supplementary Table S4). Accordingly, the co-exclusions between *Bacteroidetes* and *Firmicutes* phyla (Figure 1a), and between *Bacteroidia* and *Clostridia* classes found in the porcine gut microbiota were consistent with human gut microbial networks (Qin *et al.*, 2010; Faust *et al.*, 2012; Friedman and Alm, 2012). In agreement with its proposed discriminatory role in our previous report (Mach *et al.*, 2015), the *Prevotella* genus was the most central node in the network (Figure 1a). Thus, based on the network centralities and the differential abundance analysis between enterotype-like clusters, we suggest that *Prevotella* represents a keystone genus for the PEB group, whereas *Treponema* instead of *Ruminococcus* is the main driver of the PEA. For both *Treponema* and *Ruminococcus*, the strongest positive correlations were found in the PEA (Supplementary Information), which could be explained by the fact that these bacterial genera are involved in cellulose and lignin degradation (Niu *et al.*, 2015). Nevertheless, the contribution of other co-occurrent genera as revealed in our study could also be of fundamental importance. For instance, a positive association of both *Prevotella* and *Mitsuokella* with BW was previously suggested by Mach *et al.* (2015), and these two genera have the strongest co-occurrence patterns (sparse $r = 0.67$) in the PEB.

We provide evidence on the existence of links between the microbial ecosystem structure and growth traits in pig, which allows us to foresee that the concept of enterotype may be relevant for the farm animal production industry. Further studies, including experimental validation and longitudinal experiments, meta-transcriptomics or metabolomics data, are needed to better understand the biological relevance of the predicted enterotype-like groups and the different microbial functions in the porcine gut microbiota. Nevertheless, our aim was to analyze the microbiota functions of each enterotype-like group, by using the PICRUSt predictions on KO enzymes, which are based only on known OTUs (~80%) assigned to bacterial species. Even if our results should be treated with caution because they are not based on whole-metagenome sequencing data, they show that 54% of the overrepresented KOs that were previously reported in human gut enterotypes (Arumugam *et al.*, 2011) were DA in the

pig gut (Supplementary Table 8). In the PEA, most of the differentially over-abundant KOs are involved in butyrate, nitrogen and aminoacids (for example, alanine, aspartate or glutamate) metabolism. These associations can be explained either by the co-occurrence within this enterotype-like group of butyrate-producing bacteria belonging to genera such as *Clostridium*, *Blautia* and *Dorea*, or by the suggested role of *Ruminococcus* as a candidate genus associated with butyrate production (Claesson *et al.*, 2012). For the PEB, we observed a significant enrichment of KOs that belonged to the pathways related to carbohydrate metabolism. In fact, 96.9% (63/65) of the DA enzymes mapped against the CAZy database (Cantarel *et al.*, 2009) were significantly more abundant in the PEB than the PEA. This observation is consistent with the fact that glycoside hydrolases and polysaccharide lyase enzymes are known to be produced by *Bacteroidetes* (El Kaoutari *et al.*, 2013), including *Prevotella*. Dominance of *Prevotella* and butyrate-producing bacteria in the pig colonic community was previously reported by Liu *et al.* (2012), who also observed changes in *Prevotella spp.* in response to diets with different fiber fractions. *Prevotella*, which has an essential role in the process of complex dietary polysaccharides (Ellekilde *et al.*, 2014), may promote an increased uptake of monosaccharides in the host and confer performance advantage (McBurney and Sauer, 1993; Anguita *et al.*, 2006). In fact, *Prevotella* is capable of metabolizing plant cell wall dietary fiber and thus producing significant amounts of short chain fatty acids (SCFAs) that are later absorbed by the host. *Prevotella* was also enriched in the gut microbiota from Burkina Faso children that were fed with a diet containing a large amount of vegetal fiber (De Filippo *et al.*, 2010). Therefore, piglets that cluster in the PEB group may be better adapted to the growing pig diet that contains plant polysaccharides because of the significantly higher abundance of SCFAs produced by *Prevotella*, which, in turn, may explain the extra BW and ADG compared with piglets from the PEA cluster. These observations, together with the significant abundance of CAZy enzymes associated to the PEB cluster, suggest a better digestive capacity of the animals that cluster in the PEB for cereal-based diets, which are routinely included in piglets' diet. However, at this stage, we cannot conclude that the presence of PEB in the gut microbiota may improve feed efficiency in post-weaned pigs, but further intervention studies on this issue could provide results of high relevance to the porcine industry.

Overall, our results illustrate the usefulness of SPCIT for microbial network inference and suggest a conservation of the ecological community interactions and functional architecture between human and pig gut microbiota. In addition, we provide evidence that the pig gut ecosystem is linked to growth traits, which suggests that the concept of enterotype might be relevant for translational research applied to the porcine industry.

Conflict of Interest

The authors declare no conflict of interest.

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