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A metaproteomics approach reveals changes in mandibular lymph node microbiota of wild boar naturally exposed to an increasing trend of *Mycobacterium tuberculosis* complex infection

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ABSTRACT

Constraints in the characterization of microbiota community that circulates in the host have limited the extent of co-infection studies in natural populations. In this study, we used a metaproteomics approach to characterize the mandibular lymph nodes microbiota of wild boar (*Sus scrofa*) naturally exposed to an increasing trend of *Mycobacterium tuberculosis* complex (MTC) infection. Our results showed a reduction in microbiota diversity and changes in the composition, structure and functionality of the microbiota community associated with an increase in tuberculosis prevalence, from 45% in 2002/06 to 83% in 2009/12. These temporal changes were accompanied by an increase in the relative abundance of *Babesia*, *Theileria* and Pestivirus genera and a decrease in the *Ascogregarina* and *Chlorella*. A positive association was also evidenced between the prevalence of tuberculosis and the presence of microbial proteins responsible for carbohydrate transport and metabolism. Our findings suggest MTC-host-microbiota interactions at the population level, which may occur in order to ensure sufficient metabolic resources for MTC survival, growth and transmission. We strongly recommend the use of metaproteomics when studying microbiota communities in wildlife populations, for which traditional diagnostic techniques are limited and in which new organisms with a pathogenic potential for domestic animals and humans may appear.

1. Introduction

Tuberculosis (TB), which is caused by *Mycobacterium tuberculosis* complex (MTC), is a chronic infectious disease that affects domestic animals [1], wildlife species [2] and humans [3]. Despite the huge efforts made to control animal TB in the industrialized world [4], this zoonotic disease is still highly prevalent in the cattle of certain countries as a consequence of MTC being maintained in wild reservoirs [5–7] and other domestic animals [8]. In the Iberian Peninsula, the Eurasian wild boar (*Sus crofa*) is considered to be the main wild reservoir of MTC [9], since these Mediterranean populations have the highest prevalence reported worldwide [10]. Studies have shown that the genetic factors of hosts [11–13], pathogen traits [14] and environmental conditions [10,15] are the main drivers of MTC infection in these populations. However, little is known about the impacts of microbiota interactions on a host population that is naturally exposed to MTC infection. The interaction between MTC and other microorganisms has been poorly characterized, and those results that exist were obtained from studies that focused on a limited number of microorganisms, which were principally macroparasites and viruses [16].

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Notwithstanding, increasing evidence suggests that the co-occurrence of multiple microorganisms circulating within a host population may profoundly alter the infection, either by favoring infections through a host via immuno suppression and resource depletion, or by hampering infections through the induction of cross-effective immune responses or the competition for resources within the host [17–19].

Co-infections (i.e., multiple microorganisms circulating within a host) are expected to occur on a widespread basis in natural populations owing to the hosts' exposure to various microorganisms [19]. The co-infection of mycobacteria and parasites has been studied in human [20,21], domestic animal [22] and wildlife hosts [16,23]. In the case of wild boar, positive interactions have been found between MTC infection and porcine circo virus type 2, Aujeszky's disease virus, or *Metastrongylus* spp [16]. However, despite the relevance of these findings as regards a better understanding of TB dynamics, they were derived from studies that assessed a limited number of pathogens from the microbiota community that might co-occur in the host. In addition, co-infection studies have often focused on the known pathogens of a specific host, thus placing less relevance on the role of the commensal and other potential pathogenic microorganisms. Until recently, constraints with which to characterize the host microbiota have limited the capability to understand the complex processes underlying microorganism ecosystems and their interaction within the host populations [24].

Recent advances in “meta-omics” approaches have substantially improved the ability to investigate microbial ecology [25,26]. While metagenomics makes it possible to account for microbial potential, metaproteomics goes further and establishes a direct link between the genetic potential and a functional metabolism [27,28]. Metaproteomics studies have provided phylogenetic and functional insights into complex microbial communities at a given point in time, and namely into the analysis of soil [29], marine and freshwater ecosystems [30,31], the intestinal tract of humans and animals [32,33], natural and bioengineered systems [34,35] and tick microbiota [36,37]. However, few studies have used metaproteomics in order to attain a better understanding of the infection patterns and mechanisms that are responsible for co-infections in the same host system [38]. These molecular mechanisms may provide target proteins or biological pathways with which to develop new intervention protocols for the diagnosis, prevention, and control of infectious diseases [36].

The Eurasian wild boar is the ancestor of the domestic pig, and is one of the most important model species used when researching human infectious diseases owing to the similarities in the physiology and immune response of both species [39,40]. Wild boar are highly susceptible to mycobacteria and reproduce some of the clinical signs observed in humans [9,41]. Infection occurs mostly through oral-nasal routes, with the mandibular lymph nodes being the tissue most frequently affected (i.e., more than 90% of MTC lesions are present in the mandibular lymph nodes [42]), and is possibly the main organ responsible for the progression of the disease after the dissemination of the infection throughout the organism. Communities of viable microorganisms have been described in the lymph nodes of healthy wild ungulates [43] and slaughter pigs [44,45], suggesting that the commensal, endemic and potential pathogenic microorganisms that circulate within a host population may be concentrated in these tissues [46–48]. We, therefore, used the Eurasian wild boar as a model and applied a metaproteomics approach in order to characterize the structure and functionality of the microbiota community co-existing in a host population that is naturally exposed to MTC infection, and for which an increasing trend of TB prevalence was observed.

2. Materials and methods

2.1. Ethical approval and consent to participate

All animal sampling took place post-mortem. The wildlife samples were obtained from hunter-harvested individuals that were shot during control programs implemented on a nature reserve independently and prior to our research. According to EU and National legislation (2010/63/UE Directive and Spanish Royal Decree 53/2013) and to the University of Castilla – La Mancha guidelines, no permission or consent is required to conduct the research reported herein.

2.2. Wild boar sampling

The sampling took place on a nature reserve located in the central-southern region of Spain (30S: 408219E, 4363199N). This nature reserve is carrying out a long-term survey program (lasting 20 years) with the objective of monitoring the demographic and health trends of its wild boar population. The prevalence of TB has almost doubled in this population over the last 10 years, without any evident changes in habitat or management practices [12]. We employed retrospective data records as a basis on which to select juvenile (i.e., less than two years of age) and adult (i.e., more than two years of age) animals that were infected and uninfected with MTC in the two sampling periods in which the greatest difference in TB prevalence was recorded: Time 1 (2002/06–45% TB) and Time 2 (2009/12–83% TB). A total of ten groups of animals were sampled, five in each sampling-period: i) juveniles without TB compatible lesions and a negative mycobacteria culture (Juv TB-, n = 5); ii) juveniles with TB compatible lesions and a positive mycobacteria culture (Juv TB + n = 5); iii) adults without TB compatible lesions and a negative mycobacteria culture (Ad TB-, n = 4); iv) adults with TB compatible lesions localized only in the mandibular lymph nodes and a positive mycobacteria culture (Ad TB+, n = 4), and v) adults with disseminated TB compatible lesions (i.e., mandibular lymph nodes and other organs/tissues) and a positive mycobacteria culture (Ad TB++, n = 4). This classification was obtained for each individual after we had performed a detailed necropsy on site [49], which included the dissection of mandibular lymph nodes and the storage of tissue fragments of approximately 2 cm^3 in liquid nitrogen for the subsequent extraction of DNA, RNA and proteins [50]. The remaining portion of the sample was used for the MTC culture [51]. Animals with TB compatible lesions and a negative mycobacterial culture, or vice verse, were not selected.

2.3. Protein extraction and proteomics analysis

Proteins from the mandibular lymph nodes were extracted using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Inc. Valencia, CA, USA) according to the manufacturer's instructions. Precipitated proteins from individual samples were resuspended in 20 mM Tris-HCl pH 7.5 with 4% SDS and the protein concentration was determined by employing the BCA Protein Assay (Thermo Scientific, San Jose, CA, USA), using bovine serum albumin (BSA) as a standard. For both sampling periods, equal amounts of proteins from 4 to 5 individual samples were pooled for each group (Juv TB-, Juv TB+, Ad TB-, Ad TB+ and Ad TB++) and two biological replicates were analyzed individually for each group at the same time. Pooled protein extracts (150 μg per sample) were on-gel concentrated using SDS-PAGE and trypsin digested, as previously described [52]. The desalted protein digests were resuspended in 0.1% of formic acid and analyzed by means of RP-LC-MS/MS using an Easy-nLC II system coupled with an ion trap LTQ mass spectrometer (Thermo Scientific). The peptides were concentrated (on-line) by employing reverse phase chromatography using a 0.1 × 20 mm C18 RP precolumn (Thermo Scientific), and then separated using a 0.075 × 100 mm C18 RP column (Thermo Scientific) operating at 0.3 ml/min. The peptides were eluted using a 180-min gradient from 5 to 40% solvent B (Solvent A: 0.1% of formic acid in water, solvent B: 0.1% of formic acid in acetonitrile). ESI ionization was performed using a Fused-silica PicoTip Emitter ID 10 mm (New Objective, Woburn, MA, USA) interface. The peptides detected in our survey scans were from 400 to 1600 amu (1 mscan), followed by fifteen data-dependent MS/MS scans (Top 15), using an isolation width of 2 mass-to-charge ratio units, a normalized

collision energy of 35%, and dynamic exclusion applied during 30 s periods. The mass spectrometry proteomics data have been placed in the PeptideAtlas repository (www.peptideatlas.org) with the dataset identifier PASS01147.

The raw MS/MS files were searched for in a compiled database containing all the sequences for *Sus scrofa* (34,381 Uniprot entries in March 2015), Actinobacteria (10,508,968 Uniprot entries in March 2015), Alphaproteobacteria (3,703,927 Uniprot entries in March 2015), Viruses (2,226,548 Uniprot entries in March 2015) and Apicomplexa (408,493 entries in February 2015) taxonomies (http://www.uniprot.org) using the SEQUEST algorithm (Proteome Discoverer 1.4, Thermo Scientific). This database was limited to a group of microorganisms, since it was not possible to search all the possible taxa owing to computational limitations. The following constraints were used for the searches: tryptic cleavage after Arg and Lys, up to two missed cleavage sites, and tolerances of 1 Da for precursor ions and 0.8 Da for MS/MS fragment ions, while the searches were performed by allowing optional Met oxidation and Cyscarbamidomethylation. A false discovery rate (FDR) < 0.01 was considered as a condition for successful peptide assignments and at least 2 peptides per protein and a single peptide were the necessary conditions for the identification of protein.

2.4. Data analysis

The composition of host microbiota was determined by assigning all the proteins identified to their phylogenetic categories according to the Uniprot protein database. Microbial proteins were then grouped into three descendent categories, phylum, family and genus, in order to increase phylogenic specificity. When the microorganisms were not categorized in a phylogenetic group, they were maintained as single units. Because large proteins tend to contribute more peptide/spectra than small ones, a normalized spectral abundance factor (NSAF) was used to account for the effect of protein length on the spectral count [53,54]. The NSAF for each protein was further normalized against the total number of NSAFs obtained in each run. An adjusted NSAF makes it possible to compare the abundance of individual proteins in multiple independent samples [55]. The differences between the spectral measurements of the two biological replicates (1 and 2) were assessed using a Student's t-test, and only the identified proteins that were not statistically different regards the spectral counts were considered for further analyses. Microbial community functions were analyzed by grouping proteins into their respective clusters of orthologous group (COG). COG assignments for each microbial protein sequence were obtained by running a rps blast against the COG database from the NCBI using the WebMGA platform [56] and an *E*-value threshold of 0.001, and the top hit was used for the assignment [57].

NSAFs from all the microbial protein groups were added and grouped into their respective COG and phylogenetic categories. The differences in the microbiota community structure and functionality between groups of animals and sampling periods were checked by means of a Student's t-test and assuming a *p-value* of 0.05. The NSAF was transformed into logarithm base ten (Log_{10}) for statistical analyses owing to the heterogeneity of variances observed. A principal component analysis (PCA) was applied in order to identify the key parameters affecting the structure and function of the microbial community associated with the sampling periods and MTC infection. Additionally, although the host proteins were characterized and validated in López et al. [50], a PCA was conducted using the NSAF from the wild boar proteins. All statistical analyses were conducted using R statistical software [58], including the PCA, for which we used the prcomp package.

2.5. Real-time PCR

The metaproteomics results were validated by selecting two of the most differentially represented microbial genera (*Babesia* and *Theileria*) in order to conduct a semi-quantitative real-time PCR. Individual DNA samples of wild boar mandibular lymph nodes were used together with specific primers for the *Babesia* (BT1-F5′-GGTTGATCCTGCCAGTAGT-3'; BT1-R5′-GCCTGCTGCCTTCCTTA-3′) and *Theileria* genera (8SF 5′-GGTAATTCCAGCTCCAATAG-3'; 18SR 5′-ACCAACAAAATAGAACCA AAGTC-3′) [59–61]. A real-time PCR was performed using the QuantiTec SYBR Green RT-PCR kit and a Rotor Gene Qthermocycler (Qiagen, Inc. Valencia, CA, USA), following the manufacturer's recommendations. Amplification efficiencies were normalized against *S. scrofa* H3E3A-histone and beta-actin [59,60]. We used linear regression models to determine the trend as regards the association between microbial DNA levels and the adjusted NSAF of microbial proteins.

3. Results

3.1. Metaproteomics

A proteome profile of the mandibular lymph nodes of juvenile and adult wild boars was characterized for groups of animals that were infected and uninfected with MTC in the two sampling periods: Time 1 (2002/06–45% TB) and Time 2 (2009/12–83% TB). The average number of microbial proteins (two biological replicates) varied between 81 (Ad TB-) and 88 (Juv TB+) for Time 1, and between 63 (Juv TB+) and 74 (Ad TB+) for Time 2. The host proteins ranged between 154 (Juv TB-) and 173 (Ad TB+) for Time 1, and between 196 (Juv TB-) and 232 (AD T-) for Time 2 (see details in Appendix 1 and Fig. S1). The proportion of wild boar and microbial proteins of the various groups of animals was compared both within and between sampling periods (Fig. 1). A significant decrease in the microbial protein composition was observed during Time 2 in comparison with Time 1 for all groups, with the exception of Juv TB- (see details in Table S1 and Fig. S1).

3.2. Microbiota composition and structure

In total, the microbial proteins were classified in eight phyla, 81 families and 123 distinct genera. The number of phylogenetic groups was similar within each sampling period, although there were differences in the composition of the microbiota between Time 1 and Time 2 at both family and genus levels (Fig. 2 and see details in Table S1). Almost 50% of the families and genera were shared between sampling periods. Moreover, a higher number of families and genera were observed in Time 1 when compared to Time 2 (Fig. 2).

The relative proportion of the normalized spectral abundance factor (NSAF), which was calculated for each phylogenetic group, revealed significant differences among the groups of animals as regards phyla, both within and between the sampling periods (Fig. 3 and see details in Table S2). The major differences between the sampling periods were registered for the Apicomplexa, Clorophyta and Viruses phyla. Although the Apicomplexa and Viruses had higher relative proportions of the NSAF in the case of all the groups during Time 2 when compared with Time 1, with the exception of Viruses for Juv TB-, the opposite pattern was observed for the Clorophyta group, which had a lower proportion in Time 2 when compared with Time 1.

Detailed research into the relative proportion of the NSAF was also performed for each family, focusing particularly on the families belonging to the Apicomplexa, Clorophyta and Viruses phyla (Fig. 4 and see details in Table S3). Chlorellaceae was the only family identified in the Chlorophyta phylum, whereas various families were identified for the Apicomplexa and Viruses. Of the families within the Apicomplexa phylum, those that were significantly different between the sampling periods and for all the groups of animals were Babesiidae, Lecudinidae and Theileriidae. While the relative proportion of NSAFs obtained from Babesiidae and Theileriidae was higher during Time 2 when compared to Time 1, Lecudinidae had the reverse pattern. In addition to the differences between the sampling periods, the Theileriidae and Lecudinidae families were different in the various groups of animals within

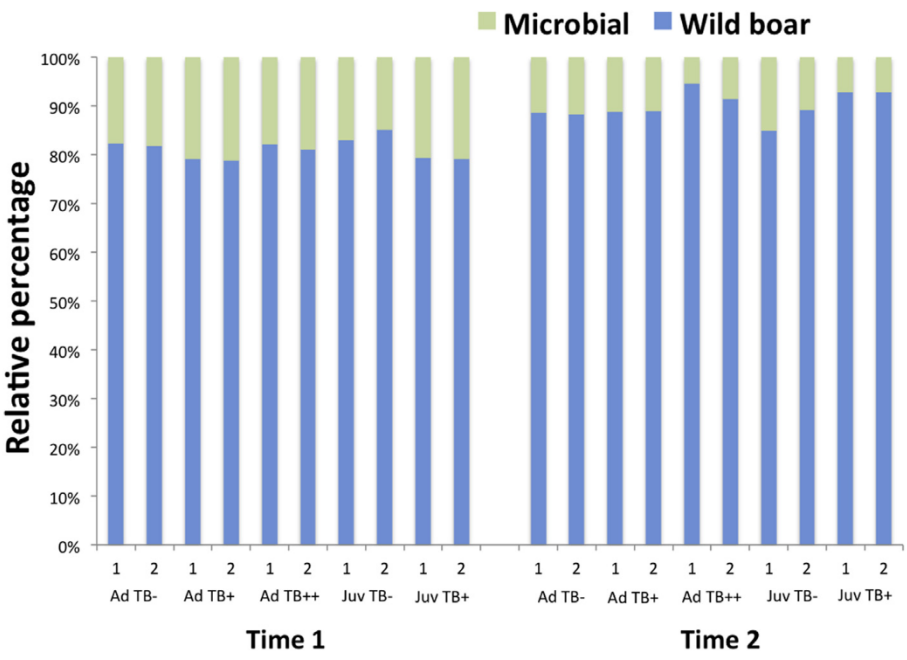


Fig. 1. Distribution of wild boar and microbial proteins for the two biological replicates. The adjusted NSAFs obtained from wild boar (blue) and microbial (green) protein groups were added up for each group of animals (x-axis) and plotted as a percent of the total proteins detected for each age class (adults - Ad; juveniles – Juv), TB category [no tuberculosis– TB-; with tuberculosis – Tb+; and in the case of adults, animals with tuberculosis restricted to mandibular lymph nodes (TB+) and animals with disseminated tuberculosis (TB++)] and sampling period (Time 1–2002/06; and Time 2–2009/12). The variability between the two biological replicates was low, as evidenced by the standard deviation values (shown below as percentages) obtained for both the microbial and wild boar protein groups: Time 1 - Ad TB- = 0.46%, Ad TB+ = 0.29%, Ad TB+ = 0.65%, Juv TB- = 1.54%, Juv TB+ = 0.13%; Time 2 - Ad TB- = 0.24%, Ad TB+ = 0.18%, Ad TB+ = 2.34%, Juv TB- = 2.99%, Juv TB+ = 0.04%. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the first sampling period (Table S3). In the case of Theileriidae, Ad TB++ had higher relative proportions of NSAFs than did Ad TB- (t-test = −5.08, df = 2, p < 0.05) and Ad TB+ (t-test = −12.10, df = 2, p < 0.05). In the case of Lecudinidae, Juv TB- had a higher relative proportion of NSAFs when compared to Juv TB+ (t-test = 4.51, df = 2, p < 0.05). With regard to the Viruses, 19 families were characterized for all the groups of animals and sampling periods, and the major differences were found for the Flaviviridae family between sampling periods and across all the groups of animals (a higher value in Time 2), with the exception of the Ad TB++ animals (see details in Table S3).

In order to further increase the specificity of the phylogenetic analyses, the microbial proteins were grouped according to microorganism genera. In the cases in which a single genus was identified within a family, the same statistical outputs were obtained (Table S4). This occurred for the Babesiidae, Lecudinidae and Theileriidae families, for which only the *Babesia*, *Ascogregarina* and *Theileria* genera were identified, respectively. *Chlorella* was also the only genus identified in the Chlorellaceae family. However, three genera from the Flaviviridae family were identified, although only the *Pestivirus* genus was significantly different as regards the relative proportions of NSAFs between sampling periods for all the groups of animals, with the exception of Ad TB++. Moreover, various genera had different proportions of NSAFs according to the group of animals, both within and between the sampling periods, although no evident pattern was evidenced when comparing MTC infected and uninfected groups and Time 1 and Time 2 (see details in Table S4).

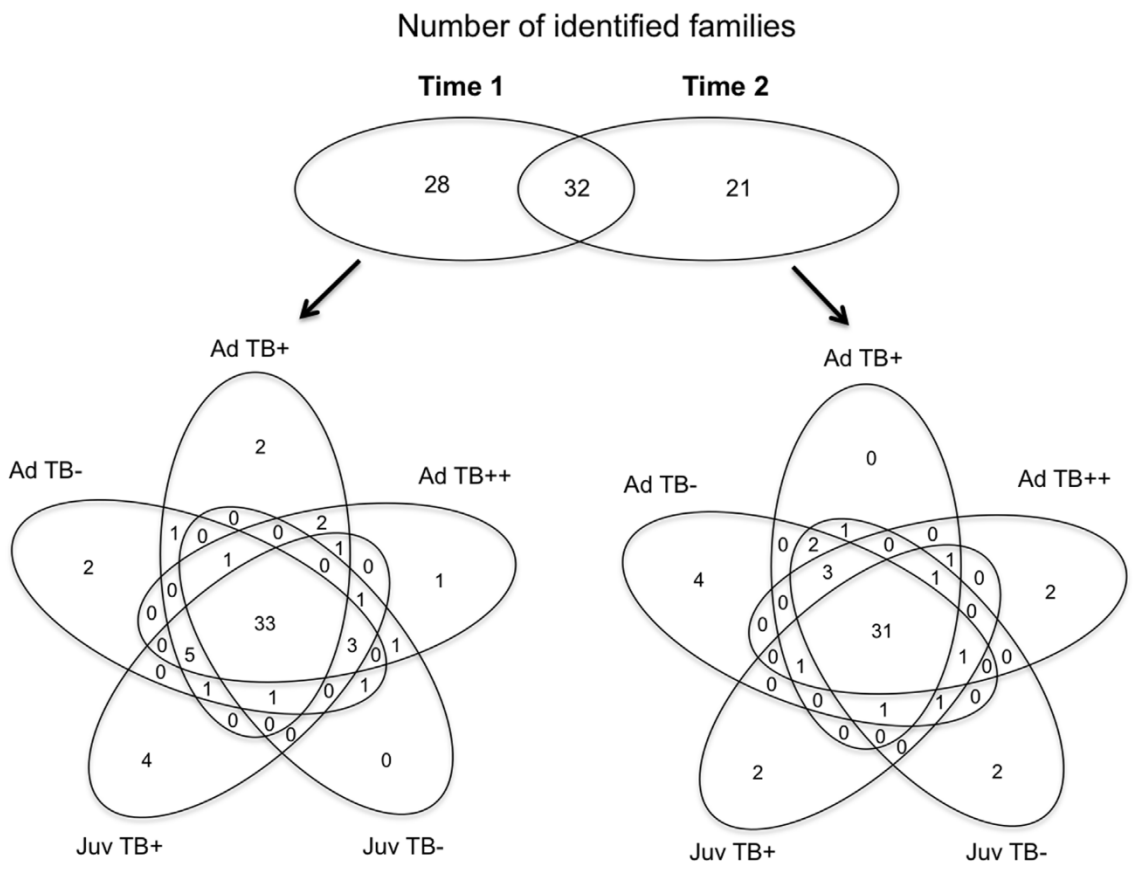
We then used the NSAF adjusted by genus to carry out a principal component analysis (PCA), which clearly segregated the groups of animals sampled at Time 1 from those sampled at Time 2 (Fig. 5). In Time 1, the adult groups (Ad TB-, Ad TB+ and Ad TB++) had a more homogeneous distribution across the axes, while the Juv TB- and Juv TB + animals had a more split divergent structuring. However, the differences between the groups infected and uninfected with MTC or as regards adult and juvenile animals were more evident in Time 2 than in Time 1 (Fig. 5). The relative contribution of each genus to the two principal components was subsequently assessed, and this process revealed that the *Theileria*, *Babesia*, *Plamodium*, *Ascogregarina* and *Chlorella* genera were mostly weighted toward principal component 1, which represented 93.7% of the total variance (see details in Table S5).

3.3. Microbiota functionality

The microbial proteins were characterized according to their function in the respective clusters of their orthologous group (COG), and the adjusted NSAF from each COG was compared as regards animal groups and sampling periods. Differences were particularly observed in most of the animal groups between sampling periods for the RNA processing and modification (COG -A) (Juv TB-, t-test = 7.90, df = 2, p < 0.05; Juv TB+, t-test = 4.11, df = 2, p = 0.054; Ad TB-, t-test = 43.43, df = 2, p < 0.05; Ad TB+, t-test = 11.43, df = 2, p < 0.05; Ad TB++, t-test = 52.10, df = 2, p < 0.05), and carbohydrate transport and metabolism (COG -G) (Juv TB-, t-test = −8.13, df = 2, p < 0.05; Juv TB+, t-test = −6.19, df = 2, p < 0.05; Ad TB-, t-test = −2.70, df = 2, p = 0.114; Ad TB+, t-test = −9.15, df = 2, p < 0.05; Ad TB++, t-test = −7.22, df = 2, p < 0.05). The relative proportion of the NSAF from the COG - A was higher for all the groups (with the exception of Juv TB+) in Time 1 when compared with Time 2, whereas there were higher proportions of COG -G in all the animal groups (with the exception of Ad TB-) in Time 2 when compared with Time 1 (Fig. 6 and see details in Table S6). A PCA was additionally conducted using the relative proportion of the NSAF grouped by COG, which revealed similar results to those observed in the microbial structure analysis (Fig. S2 and Table S7).

3.4. Host protein composition and structure

Although the study was focused on microbial proteins, the global distribution pattern of wild boar proteins was also assessed using a PCA (Fig. 7). This analysis clearly segregated the animal groups into the two different sampling periods. However, in contrast to microbial proteins, the differences among the groups of animals were more evident in Time 1 than in Time 2. In Time 2, a more homogenous distribution across axes was observed among groups, with the exception of Ad TB+, while a divergent split among groups was more evident in Time 1. In the latter, Juv TB+ and Ad TB+ were placed together and far away from Juv TB- and Ad TB-, whereas Ad TB++ was positioned closer to Ad TB- than to Ad TB+. The relative contributions made by each protein to the two principal components are shown in Table S8.



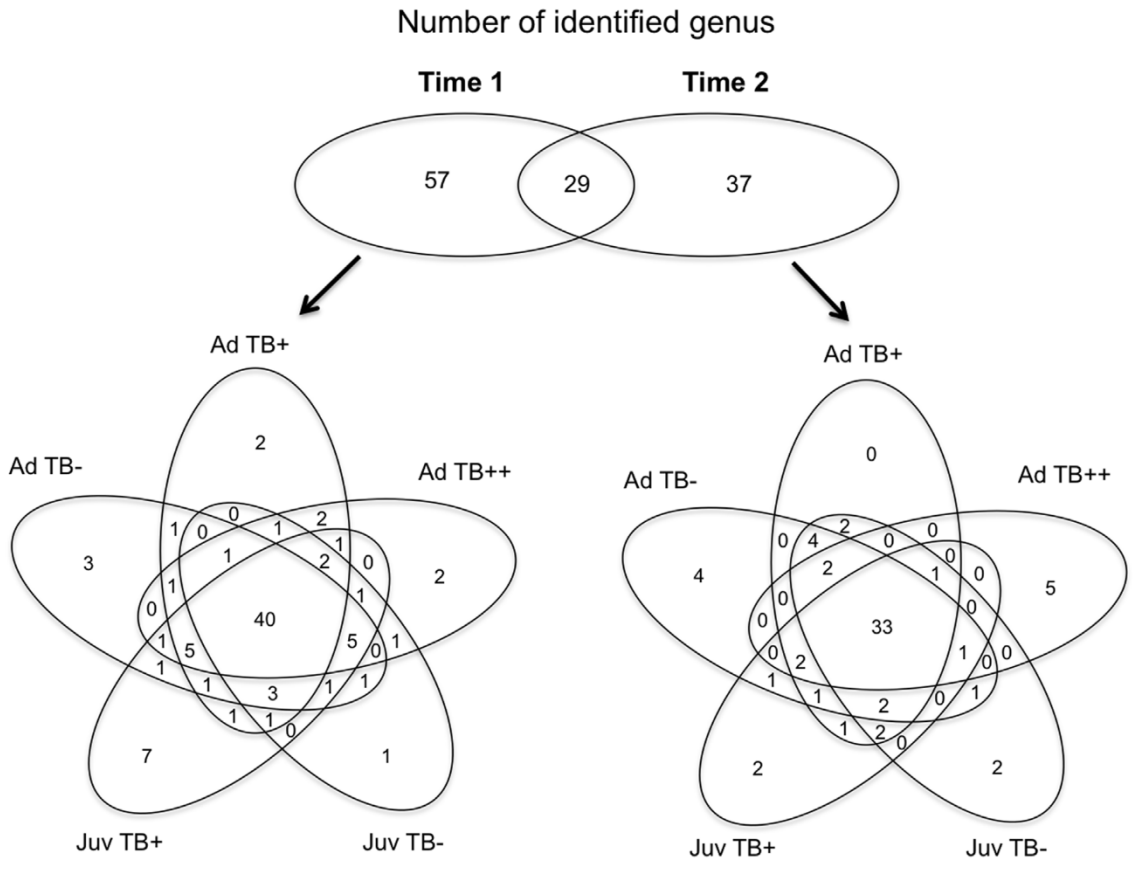


Fig. 2. Number of families and genera identified for each group of animals (adults with no tuberculosis; Ad TB-; adults with restricted tuberculosis, Ad TB+; adults with disseminated tuberculosis, Ad TB++; juveniles with no tuberculosis, Juv TB-; juveniles with tuberculosis, Juv TB+) both within and between sampling periods (Time 1–2002/06 and Time 2–2009/12).

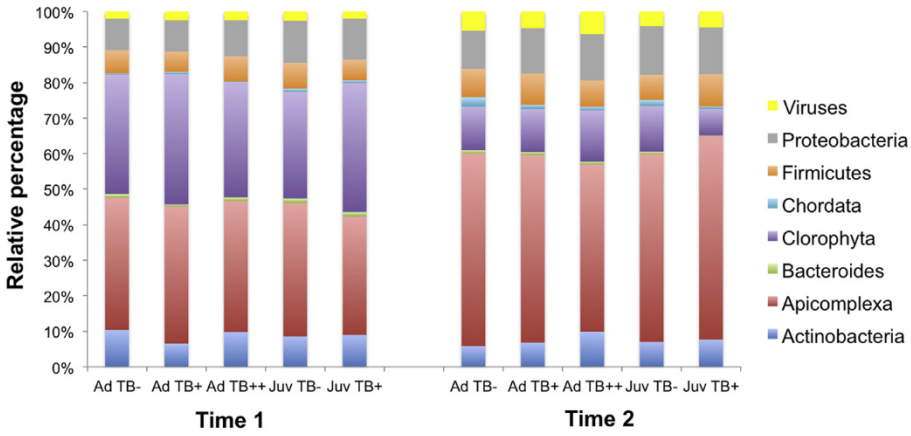


Fig. 3. Microbial proteins assigned by phylum and grouped for each group of animals (adults with no tuberculosis; Ad TB-; adults with restricted tuberculosis, Ad TB+; adults with disseminated tuberculosis, Ad TB++; juveniles with no tuberculosis, Juv TB-; juveniles with tuberculosis, Juv TB+). The relative abundances were calculated from the sum of the NSAFs found for each group in the respective sampling period (Time 1–2002/06and Time 2–2009/12).

3.5. Validation of metaproteomics results

In order to validate the metaproteomics results, the *Babesia* and *Theileria* genera were selected for a semi-quantitative real-time PCR amplification. A significant positive correlation between microbial DNA levels and the adjusted NSAF proteins was obtained for both the *Babesia* (ANOVA: $F = 6.49$, $df = 1$, $p < 0.05$) and *Theileria* (ANOVA: $F = 5.69$, $df = 1$, $p < 0.05$) genera. The microbial DNA levels quantified by a real-time PCR tended to increase as the adjusted NSAF increased in the metaproteomics analysis (Fig. S3). These results provided a validation for the metaproteomics results obtained using a different methodological approach, and provided additional support for the results presented in this study.

4. Discussion

We used a metaproteomics approach to characterize the microbiota community circulating in a wild boar population that is naturally exposed to MTC infection, thus overcoming previous methodological constraints related to the identification of multiple microorganisms in co-infection studies [24–26]. Our main findings revealed a reduction in microbiota diversity (i.e., the number of families/genera) associated with an increase in TB prevalence. The temporal changes in the composition and structure of the microbiota community were accompanied by an increase in the relative abundance of the *Babesia*, *Theileria* and *Pestivirus* genera and a decrease in the *Ascogregarina* and *Chlorella* genera, which had a higher relative abundance during the period of lower TB prevalence.

During the first years of our study (2002/06), TB was present in around 45% of the wild boar studied, which was almost half the prevalence found only a few years later (83% in 2009/12). This rising trend has been associated with an increase in both the hosts' genetic susceptibility to MTC [12] and the hosts' population abundance [10], two recognized TB risk factors for wild boar populations inhabiting Mediterranean Iberia [11,15]. Nevertheless, in this study our results suggested that the hosts' microbial community also plays a role in this temporal change in TB prevalence, particularly the microorganisms assigned to the *Babesia*, *Theileria* and Pestivirus genera. In 2009/12, 83% of the animals were infected with MTC, which might have prompted an increased susceptibility to these pathogens and have favored their dissemination throughout the population. Indeed, it has been shown that TB reduces the health status of wild boar, inducing nearly 30% of the adult mortality in this region [64]. Moreover, microorganisms/strains belonging to the *Babesia*, *Theileria* and *Pestivirus* genera can also debilitate the hosts' health system and, therefore, facilitate infection with MTC and the progression of the disease [65–67]. Furthermore, in the case of *Babesia* and *Theileria* genera, differences between the relative abundance of the NSAF in MTC infected and uninfected groups were also evidenced during the period of low TB prevalence, which reinforce their putative effect on the health status of wild boar. In summary, although a facilitative interaction between mycobacteria and *Babesia*, *Theileria* and *Pestivirus* genera would appear to be evident at the population level, the causes and consequences of these interactions, along with the molecular mechanisms responsible for them, should be investigated in greater depth.

The reduction in microbiota composition and diversity (i.e., measured as the number of families and genera), observed in most of the groups of animals between the sampling periods, might be explained by the increase in the abundance of the wild boar population over time [12], the activation of the hosts' immune system against the pathogenic agents [50], or both. A previous study in pika (*Ochotona curzoniae*) populations has shown an association between the hosts' population

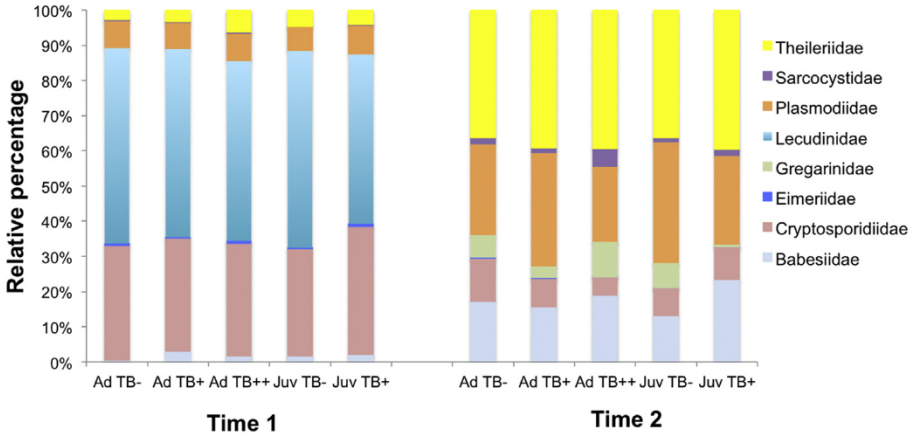


Fig. 4. Microbial proteins from the Apicomplexa phylum grouped into their families and displayed for each group of animals (adults with no tuberculosis; Ad TB-; adults with restricted tuberculosis, Ad TB+; adults with disseminated tuberculosis, Ad TB++; juveniles with no tuberculosis, Juv TB-; juveniles with tuberculosis, Juv TB+). Relative abundances were calculated from the sum of the NSAF found for each group in the respective sampling period (Time 1–2002/06and Time 2–2009/12).

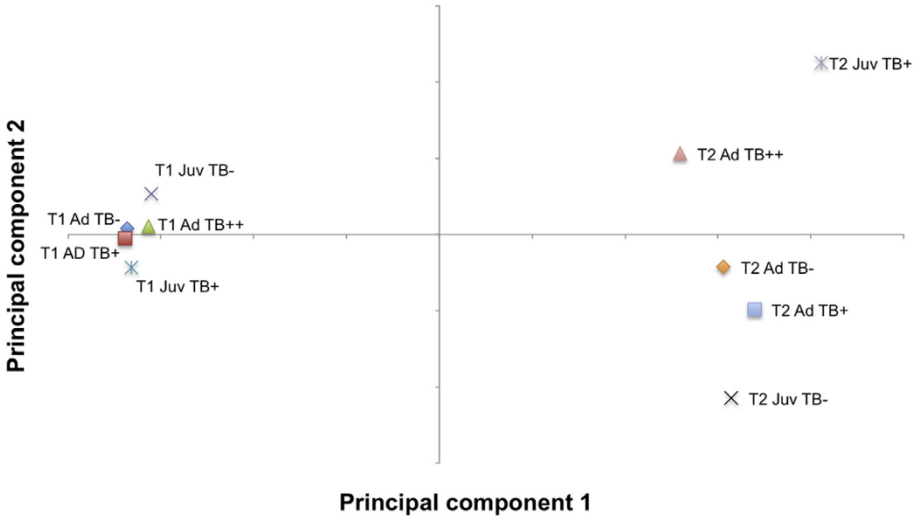


Fig. 5. Plot showing the results of the principal component analysis performed using the relative proportion of NSAFs from the microbial proteins grouped by genus for each group of animals (adults with no tuberculosis; Ad TB-; adults with restricted tuberculosis, Ad TB + ; adults with disseminated tuberculosis, Ad TB + + ; juveniles with no tuberculosis, Juv TB-; juveniles with tuberculosis, Juv TB +) and sampling periods (T1, Time 1–2002/06and T2, Time 2–2009/12). The two principal components that account for 96.3% of total explained variance are shown.

abundance and microbial diversity in the gut [68]. High population abundance was correlated with a lower inter-individual variation in the pika's microbial diversity. This density-dependent effect could be particularly relevant in Mediterranean habits, in which wild boar tend to concentrate at the few waterholes that remain during the dry periods, thus boosting the widespread transmission of microorganisms and their standardization throughout the population. A large amount of MTC was recently found in the mud of these waterholes, thus demonstrating the role of the indirect transmission of MTC in wild populations [69]. Furthermore, a previous study in humans has shown that TB modulates the microbial diversity of the gut, and that MTC infection leads to a loss of microbial diversity as a result of activating the host's immune system [70].

In addition to temporal changes in the host's microbiota community, our findings evidenced that the levels of TB in the wild boar population also influenced the proteome profile of the groups of animals that were infected and uninfected with MTC. During the period of high TB prevalence, there was a high divergence in the composition and structure of microbial proteins and a more homogenous distribution of host proteins between the groups that were infected and uninfected with MTC. However, during the period of low TB prevalence there was a high divergence in the composition of host proteins and a more homogenous composition and structure of microbiota proteins among the groups. The hypotheses of host immune suppression largely explored in co-infection studies, and the implication of several host immune system proteins in determining MTC infection and disease progression in wild boar, might explain these results [50,71,72]. Nevertheless, other factors at the population level might interfere with a host's ability to respond to MTC infection over time. For instance, we observed an increase in the hosts' genetic susceptibility to TB over time, which might have contributed to the similar protein profile of the host population in this sampling period as a result of intense genetic drift

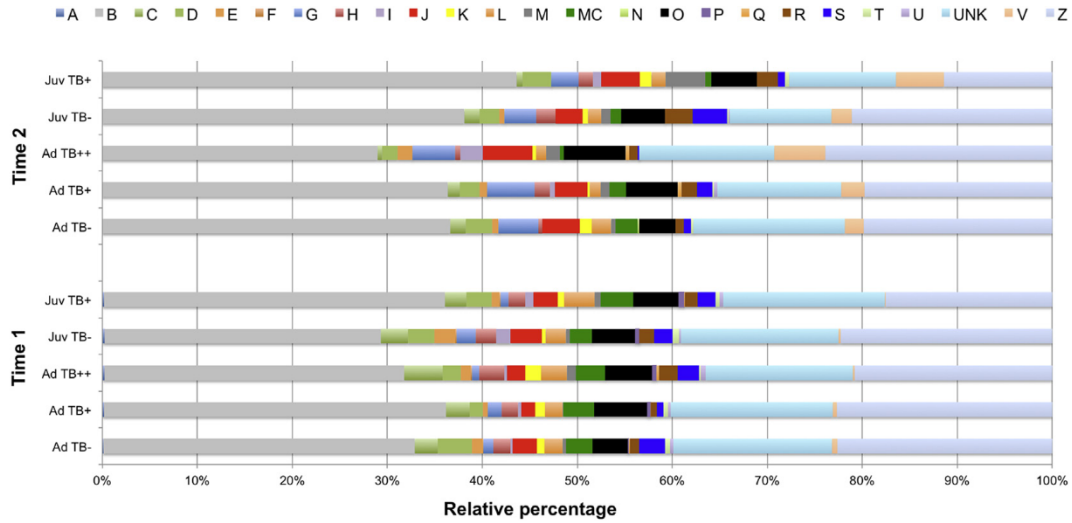


Fig. 6. Microbial proteins assigned to each Cluster of Orthologs group (COG) and displayed for each group of animals (adults with no tuberculosis; Ad TB-; adults with restricted tuberculosis, Ad TB + ; adults with disseminated tuberculosis, Ad TB + + ; juveniles with no tuberculosis, Juv TB-; juveniles with tuberculosis, Juv TB +) and sampling periods (Time 1 and Time 2). Relative abundances were calculated from the sum of the NSAFs found for each group in the respective sampling period (Time 1–2002/06 and Time 2–2009/12). The COGs were grouped as: RNA processing and modification – A; Chromatin structure and dynamics – B; Energy production and conversion – C; Cell cycle control, cell division, chromosome partitioning – D; Amino acid transport and metabolism – E; Nucleotide transport and metabolism – F; Carbohydrate transport and metabolism – G; Coenzyme transport and metabolism – H; Lipid transport and metabolism – I; Translation, ribosomal structure and biogenesis - J; Transcription – K; Replication, recombination and repair - L; Cell wall/membrane/envelope biogenesis – M; Multiple function class – MC; Cell motility – N; Posttranslational modification, protein turnover, chaperones – O; Inorganic ion transport and metabolism – P; Secondary metabolites biosynthesis, transport and catabolism – Q; General function prediction only – R; Function unknown – S; Signal transduction mechanisms – T; Intracellular trafficking, secretion, and vesicular transport – U; Undetermined COG – UNK; Defense mechanisms – V; Cytoskeleton – Z.

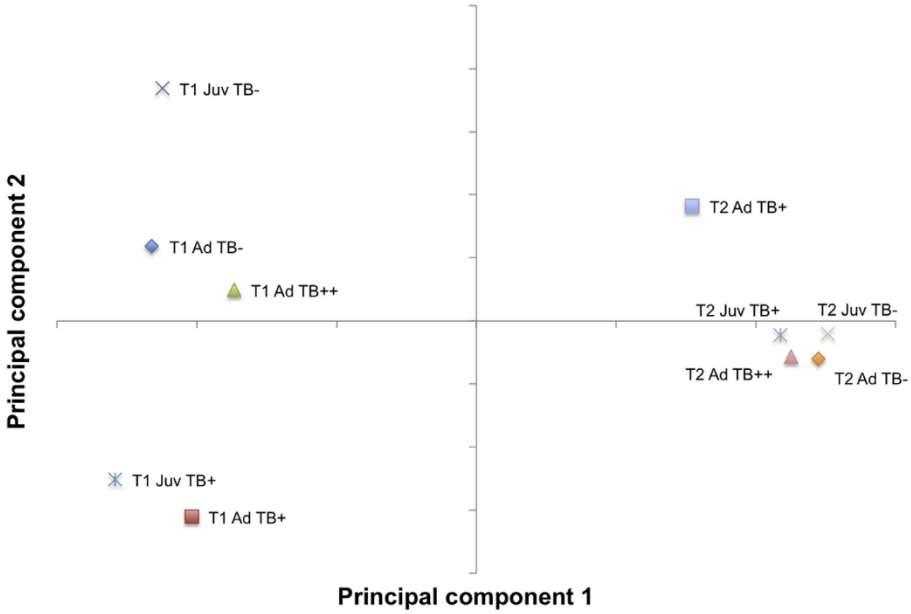


Fig. 7. Plot showing the results of the principal component analysis performed using the relative proportion of NSAFs from the wild boar proteins for each group of animals (adults with no tuberculosis; Ad TB-; adults with restricted tuberculosis, Ad TB+; adults with disseminated tuberculosis, Ad TB++; juveniles with no tuberculosis, Juv TB-; juveniles with tuberculosis, Juv TB+) and sampling periods (T1, Time 1–2002/06 and T2, Time 2–2009/12). The two principal components that account for 77% of total explained variance are shown.

and TB-mediated selection [12].

The evident divergence in the composition of the microbial community between sampling periods and the higher divergence in microbial protein structure between the animals that were infected and those that were uninfected with MTC during the period of high TB prevalence led to the hypothesis that mycobacteria might also modulate the composition and functionality of microbiota in order to ensure favorable conditions for their survival, growth and dissemination. The energy required by MTC to survive and grow within the host [73] may explain the functional differences observed in carbohydrate transport and metabolism in the two sampling periods. The competition/availability of the hosts' resources may also explain these changes in the composition of the microbial community and the positive association between MTC and the microorganisms of the *Babesia* and *Theileria* genera. Like most microorganisms, MTC is absolutely dependent on exogenous iron for its survival within the host [74], and members of both the *Babesia* and *Theileria* genera can infect erythrocytes throughout their life cycle, causing their destruction and thereby an increase in the circulation of free iron within the host's system. Increasing the relative abundance of these two potential pathogenic genera in a wild boar population could, therefore, have beneficial effects on MTC infection in the long term [75]. Hypoferraemia has been shown to be a host defense mechanism during infection and inflammation [76], and MTC has been suggested to manipulate the host immune response mechanisms in order to increase the availability of iron circulation for bacterial growth and transmission [50]. The mechanisms underlying these possible interactions should be explored in greater depth.

Although metaproteomics outputs may introduce a bias toward better-known microorganisms, because it is based on the genomic and protein reference databases available, metaproteomics provides a new perspective with which to study co-infections in natural populations and indicate the functional role of interactions among microorganisms. The epidemiological dynamics of TB in natural populations is very complex owing to the interactions of its multi-host-multi-pathogens. The strategies employed to control TB in natural populations should, therefore, take into account the possible interactions among microorganisms that are circulating within the host population in order to take adequate measures that will increase the efficacy of control programs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2018.12.003.

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