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ORIGINAL ARTICLE

Culturable aerobic and facultative bacteria from the gut of the polyphagic dung beetle *Thorectes lusitanicus* Jeckel

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Abstract

Unlike other dung beetles, the Iberian geotrupid *Thorectes lusitanicus* exhibits polyphagous behavior; for example, it is able to eat acorns, fungi, fruits, and carrion in addition to the dung of different mammals. This adaptation to digest a wider diet has physiological and developmental advantages and requires key changes in the composition and diversity of the beetle's gut microbiota. In this study, we isolated aerobic, facultative anaerobic, and aerotolerant microbiota amenable to grow in culture from the gut contents of *T. lusitanicus* and resolved isolate identity to the species level by sequencing 16S rRNA gene fragments. Using BLAST similarity searches and maximum likelihood phylogenetic analyses, we were able to reveal that the analyzed fraction (culturable, aerobic, facultative anaerobic, and aerotolerant) of beetle gut microbiota is dominated by the phyla *Proteobacteria, Firmicutes* and *Actinobacteria*. Among Proteobacteria, members of the order Enterobacteriales (Gammaproteobacteria) were the most abundant. The main functions associated with the bacteria found in the gut of *T. lusitanicus* would likely include nitrogen fixation, denitrification, detoxification, and diverse defensive roles against pathogens.

Key words Actinobacteria, Proteobacteria, Firmicutes, gut microbiota, polyphagy, geotrupidae.

Introduction

Digestion is a basic and key process in heterotrophic animals that provides the necessary nutrients to ensure the continuation of vital activities including development, growth, and reproduction. To achieve maximal digestion efficiency, animals have co-evolved complex symbiotic (mutualistic) interactions with gut bacteria (Ventura *et al*., 2009). The animal gut microbiota develops as an internal ecosystem comprising diverse bacterial species that interact in complex and dynamic ecological networks. This ecosystem includes both persistent residents and transient members introduced from the environment (Lupp & Finlay, 2005). The composition and complexity of gut bacterial communities appear to be species-specific and strongly dependent on environmental (e.g., diet; Turnbaugh *et al*., 2006; De Filippo *et al*., 2010; Wu *et al*., 2011) and host genetic factors (Ley *et al*., 2005; Benson *et al*., 2010). Gut bacterial symbionts provide the host with a broad array of metabolic activities and significantly expand the host digestion spectrum through fermentation of otherwise non-digestible food components (e.g., plant fiber; Dillon & Dillon, 2004; Ley *et al*., 2005). Importantly, these gut bacteria also seem to be actively involved in the development of some health disorders, prevention of infection by invading pathogens via competition for intestinal receptors, the production of antibiotics, and activation of the host immune system (Ley *et al*., 2005; Fava *et al*., 2006; Wen *et al.*, 2008; Kaltenpoth, 2009; Bravo *et al*., 2011; Colman *et al*., 2012; Seipke *et al*., 2012; Waite *et al*., 2012)

Insects, the most diverse group of live organisms, also host highly diverse hindgut microbial communities (Dillon & Dillon, 2004) that await detailed exploration and description. Thus far, gut symbionts have been described in insect groups with different diets such as xylophagous termites (Warnecke *et al*., 2007), leaf-cutter ants (Aylward *et al*., 2012), haematophagous mosquitoes (Gaio *et al*., 2011), pollenivorous bees (Martinson *et al*., 2011)

herbivorous butterflies (Broderick *et al*., 2004), or detritivorous beetles (Andert *et al*., 2010). A recent comparative study using gene sequence data from 58 insect species (Colman *et al*., 2012) showed that both diet and host taxonomy influence the composition and diversity of gut bacterial assemblages (Mrázek *et al*., 2008; Schauer *et al*., 2012).

As bacteria are essential sources of enzymes needed in carbohydrate (e.g., plant xylan and cellulose), lignin, pectin, lipid and protein degradation, polyphagous species normally show the largest diversity in their gut bacterial communities when compared with species that feed on specialized diets (Mrázek *et al*., 2008; Brune & Friedrich, 2000; Suh *et al*., 2003; Schauer *et al*., 2012; Gayatri Priya *et al*., 2012). Among the insects with specialized food habits, xylophagous insects appear to have the richest bacterial gut flora (Colman *et al*., 2012), but to our knowledge no studies have explored the diversity of gut bacteria of coprophagous insect species (but see Frouz *et al*., 2003).

The flightless dung beetle *Thorectes lusitanicus* (Coleoptera, Scarabaeoidea, Geotrupidae) is an endemic species distributed in the south of the Iberian Peninsula. Although dung beetles are normally involved in the recycling and management of herbivore dung and maintain strict coprophagous habits (Nichols *et al*., 2008), *T. lusitanicus* exhibits behavior indicating broad trophic ability; it also feeds on acorns, fresh fungi, carrion, and even plant leaves, as its mouthparts are adapted to the consumption of fibrous material and dry dung (Verdú & Galante, 2004). Furthermore, *T. lusitanicus* is attracted to the acorns of at least two oak species (*Quercus suber* and *Q. canariensis*) and acts as a secondary disperser by collecting viable acorns, partially eating them, and burying them in the soil (Pérez-Ramos *et al*., 2007; Verdú *et al*., 2007). Due to the high polyunsaturated fatty acid content of these acorns, this diet has allowed *T. lusitanicus* to make important improvements in terms of low temperature resistance and ovarian development (Verdú *et al*., 2010).

There are several methods currently available for characterizing gut microbiotas, and each present different advantages. Essentially, these methods can be classified into (i) those that isolate bacteria from the gut to culture them under controlled conditions (Gayatri Priya *et al*., 2012) and (ii) those that use culture-independent molecular methods, such as PCR amplification of bacterial 16S rRNA genes plus direct sequencing or high-throughput metagenomic approaches using next-generation sequencing (NGS) techniques (Kovatcheva-Datchary, 2009; Ventura *et al*., 2009; Qin *et al*., 2010; Shi *et al*., 2010). While the latter methods are able to detect less-frequent species and, in particular, those that cannot be cultured, the former methods, although more labor-intensive, have the advantage that isolated bacteria can be further functionally characterized using traditional microbiology tools and are readily available for potential biotechnological applications. Anticipating that many bacterial species would be reported for the first time in our study, we opted to isolate culturable bacteria because this approach is commonly used for insect microbiomes (e.g., Gayatri Priya *et al*., 2012) and would also enable straightforward characterization of the biological role of isolates of interest (Dillon & Dillon, 2004).

Although, in order to obtain a more real approximation of the "total diversity" of gut microbiota, it could be more appropriated using PCR-based culture independent techniques, in some insects (e.g., bees) it may not be the case. Some studies has demonstrated that the guts of some insects do not present strictly anaerobic conditions, and thus, that a high percentage of insect gut microbiota in these species corresponds to aerobic, facultative aerobic, and aerotolerant bacteria (Mohr & Tebbe, 2006). Therefore, in such cases, culture techniques under aerobic conditions seem to be appropriate and render highly representative results . It is noteworthy that these aerobic, facultative aerobic, or aerotolerant bacteria, in some cases, establish symbiotic associations with insect midgut (Crotti et al., 2010).

In this study, we describe for the first time the culturable aerobic, facultative anaerobic and aerotolerant bacteria present in the gut contents of a coprophagous insect characterized by the ability to efficiently assimilate a wide variety of food resources: the dung beetle *T. lusitanicus*. Taxonomic identification was achieved through 16S rRNA gene sequencing followed by comparative sequence analyses. Bacteria were grouped according to their relative phylogenetic position, and the composition of the obtained fraction of the *T. lusitanicus* gut microbiome was compared with those of other insects. In addition, the putative roles of different bacterial species that might aid the polyphagous diet of this dung beetle were suggested.

Materials and methods

Beetle collection

T. lusitanicus individuals were captured in Los Alcornocales Natural Park in southern Spain in autumn (November 2006). For *T. lusitanicus*, this period coincides with the ingestion of acorns, fungi, madrone fruits and dung (mainly from cattle and red deer). All individuals were maintained in plastic containers at 20ºC until their arrival at the laboratory, where they were maintained at 10ºC in a climate chamber (with a temperature similar to the average experienced in the field during autumn) to minimize stress.

Isolation of the culturable, aerobic, facultative anaerobic, and aerotolerant component of the intestinal microbiota

Culturable, *aerobic, facultative anaerobic,* and *aerotolerant* bacteria were isolated from the intestinal contents of aseptically removed mid- and hindguts. The intestinal contents of 20 *T. lusitanicus* individuals were pooled, homogenized and vortexed vigorously in nine volumes of sterile water. Serial dilutions were made up to 10^{-6}, and 100 μL were spread over

plates with Brain-Heart infusion (BHI) broth (Biomérieux), which were incubated aerobically at 28ºC for 24 hours. Colonies differing in morphology, color, shape and elevation were isolated, streaked on fresh BHI plates, and incubated for 24 hours at 28ºC. Two transfers were needed to get pure cultures, which were stored at –80ºC in skim milk with 10% (v/v) glycerol.

Isolation of DNA, PCR amplification, and sequencing

DNA was extracted directly from bacterial colonies either by lysing them with hot sterile water (50 μL at 100 ºC for 10 min) or by using a commercial purification kit (QIAamp DNA Mini Kit, Qiagen GmbH, Hilden, Germany). One μL of the total volume of each DNA sample was used as template in a polymerase chain reaction (PCR) to amplify the 16S rRNA gene using two overlapping sets of primers (modified from Baker *et al.*, 2003): Ari5 (5′-CTGGCTCAGGACGAACGCTG-3′) and E1541R (5′-AGGAGGTGATCCAACCGCA-3′ that amplify a fragment of about 1500 bp covering the whole molecule; and in those cases that the previous set did not work, E8F (5′-AAGAGTTTGATCATGGCTCA-3′) and U1115R (5′-GTTGCGCTCGTTGCGGGACT-3′) that amplify a shorter fragment of about 1100 bp. PCR reactions (25 μL) contained 10 ng of DNA isolated from bacteria, 2.5 μL (1x) of buffer ($MgCl_2$ added at 2.5 mmol/L final concentration), 0.5μL (0.4 μmol/L) of each primer, 1μL of dNTPs (0.4 mmol/L of each), and 1.5μL *Taq* polymerase (1.5 U). The reaction conditions were: 95ºC for 10 min, 30 cycles of 60 s at 95ºC, 60 s at 52ºC, and 120 s at 72ºC, and a final extension at 72ºC for 10 min. PCR products were purified using the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany). Amplification products were analyzed by electrophoresis in 1.5% (w/v) agarose gels containing 1 ng/mL SYBR SafeTM DNA gel stain (Invitrogen, Oregon, USA). Purified PCR products were sequenced at the Sequencing Service of the Centre for Biological Research (CIB-CSIC; SecuGen, SL) using PCR primers

with the BigDye ® Terminator v. 3.1. kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions.

Sequence similarity searches and phylogenetic analysis.

Obtained sequences were edited with Chromas v. 1.4.3 (Faculty of Science, Griffith University, Australia). Dissimilar sequences were deposited in GenBank under accession numbers HQ326781 to HQ326838 and HQ340154 to HQ340156. Sequences were compared against the NCBI database using the BLAST (http://blast.ncbi.nlm.nih.gov/) tool. Specifically, the program Megablast, which optimizes for highly similar sequences, was selected, and queries were searched against "16S ribosomal RNA sequences (Bacteria and Archaea)." Taxonomic identification was based on the percentage of sequence identity scores, and the first ten hits were retained for further analyses.

For phylogenetic analyses, 16S rRNA sequences obtained from the beetle gut microbiota and best-hit sequences retrieved from GenBank were aligned using MAFFT (Katoh and Toh, 2008) at the EMBL-European Bioinformatics Institute site (www.ebi.ac.uk/Tools/msa/mafft/). Gaps and highly variable portions of the alignment were excluded from phylogenetic analyses taking secondary structure of 16S rRNA into consideration. The trimmed alignment was subjected to maximum likelihood analysis (ML; Felsenstein, 1981) using PhyML (Guindon *et al*., 2010) at the ATGC bioinformatics platform (www.atgc-montpellier.fr/phyml/). GTR+I+G (I = 0.33; α = 0. 63) was selected as the best-fit evolutionary model using jmodeltest v. 2.1.1 (Darriba *et al*., 2012). Support for internal branches was evaluated by performing 100 replicates of non-parametric bootstrapping (Felsenstein, 1985).

Results

Culturing of bacteria from the intestinal contents of *T. lusitanicus* yielded up to 224 phenotypically different colonies. PCR amplification and sequencing of the 16S rRNA genes from these isolates resulted in 56 different DNA sequences (labeled PM; Table 1). Using an arbitrarily defined limit of 99.5% sequence similarity, we could distinguish a minimum of 43 OTUs (operational taxonomic units) that could be ascribed to the corresponding number of species with the BLAST searches and the phylogenetic analysis (see below). On average, the length of the new sequences was 927 bp, with a maximum of 1510 bp (PM37) and a minimum of 236 bp (PM48). BLAST searches were performed using the newly obtained 16S rRNA gene sequences as queries. In Table 1, the best hit of each search is shown. In general, best hits showed 98%–99% identity with their corresponding query sequences, allowing for the direct identification of isolates at the species level (Table 1). In some cases (PM18, PM20, PM43, PM51, PM59, and PM60), the percentage of identity was lower (between 91-94%), which could indicate either new species or higher rates of divergence in a particular group (e.g., *Lactococcus*). According to the BLAST results, the culturable bacteria isolated from the beetle gut were taxonomically highly diverse, comprising 40 Proteobacteria isolates (71%), 10 Firmicutes (18%), and 6 Actinobacteria (11%). Uncorrected *p* distance pairwise comparisons varied between 0.002 and 0.410. Unfortunately, pairwise comparisons involving PM48 and other short sequences, specifically PM1, PM9, PM23, PM31, PM35, PM55, and PM59, could not be estimated because there were no sequence overlaps.

For phylogenetic analyses, a total of 10 best hits per BLAST search were retained. Of the 560 best BLAST hits, a total of 296 were non-redundant. The retrieved non-redundant sequences were aligned with the 56 queries and subjected to ML inference. The initial 16S rRNA alignment was 1814 positions long. After the removal of ambiguous sites (i.e., those of uncertain positional homology), which mostly occurred at the beginning and end of the sequences and around gapped regions, the length of the final alignment was 1480 positions.

In this final alignment, a total of 568 positions were constant, and 760 were parsimony-informative sites. The phylogenetic distribution of the bacteria reported in this work is shown in the reconstructed ML tree (−lnL = 43923.8) in Fig. 1. The intestinal microbiota of *T. lusitanicus* could be separated into six distinct main monophyletic groups: Firmicutes (10 PMs), Actinobacteria (6 PMs), Alphaproteobacteria (2 PMs), Betaproteobacteria (8 PMs), Gammaproteobacteria orders Enterobacteriales and Pseudomonadales (29 PMs), and Gammaproteobacteria order Xanthomonadales (1 PM) (Fig. 1). All six main groups received maximal bootstrap support (Fig. 1). The dung beetle intestinal bacteria (PMs shown in red in Fig. 1) were represented by six recovered monophyletic groups, although these were not evenly distributed (only a few species were recovered for Alphaproteobacteria and the order Xanthomonadales of Gammaproteobacteria). The greatest species richness of the *T. lusitanicus* gut microbiome was located within the order Enterobacteriales.

Discussion

Polyphagous insects are able to eat a wide range of food sources to obtain the most favorable nutrients required for their growth, development and reproduction (Redfearn & Pimm, 1988). Moreover, these insects are able to self-select nutritionally optimal diets from their environment, although the ecological and physiological benefits of this behavior remain unknown. In the case of *T. lusitanicus*, the evolutionary shift from coprophagy to a polyphagous diet may be interpreted as a niche enlargement strategy intended to reduce the risks of consuming a fluctuating and ephemeral resource (Verdú *et al*., 2010). This adaptation provides *T. lusitanicus* with a selective advantage in case of seasonal food shortage, which naturally occurs in the Mediterranean ecosystem (particularly in the southernmost regions such as those inhabited by *T. lusitanicus* in the Iberian Peninsula) and offers additional

physiological and developmental advantages capable of improving fitness (Verdú *et al*., 2010).

Microbial symbioses in the insect gut are known to play a crucial role in facilitating polyphagy in several groups of insects (Cazemier *et al*., 1997), and thus it was expected that the gut of the polyphagous dung beetle *T. lusitanicus* would harbor a high richness of bacteria accompanied by a wide and distinct functional capacity (De Filippo *et al*., 2010; Wu *et al*., 2011). Indeed, our results confirmed this expectation, showing that culturable bacteria of the gut of *T. lusitanicus* are highly rich in terms of sequence divergence, number of species and taxonomic range. Comparable data for taxonomically related species (Coleoptera, Scarabaeidea) are mainly available for some species with larval guts that possess a fermentation sac able to degrade cellulose (Cazemier *et al*., 1997). Despite these studies used culture-independent molecular methods that were also able to detect the anaerobic microbiota, the absolute number of different DNA sequences obtained from *T. lusitanicus* could be considered of the same magnitude to those obtained from the herbivorous larvae of *Melolontha melolontha* (90 clones, Egert *et al*., 2005), *Holotrichia parallela* (21 clones, Huang *et al*., 2012; 205 clones, Huang & Zhang, 2013), and *Costelytra zealandica* (22 clones, Zhang *et al*., 2008), as well as those of the detritivorous larva of *Pachnoda ephippiata* (56 clones, Egert *et al*., 2003) and several xylophagous longicorn beetle species (142 clones, Park *et al*., 2007). The richness of the *T. lusitanicus* gut microbiota also show similar levels to those that occur in other coleopteran and most other insect groups, except in the case of some xylophagous and detritivorous species, mainly termites (Colman *et al*., 2012).

According to our results, aerobic, facultative anaerobic, and aerotolerant bacteria inhabiting the gut community of *T. lusitanicus* coincide with those typically found in soil and plants as well as cattle guts and faeces, a phenomenon that occurs in other polyphagous insects as well (Gayatri Priya *et al*., 2012), whereas microfloras in the anaerobic guts of

specialized insects such as termites are distinct from surrounding free-living bacterial communities because they are dominated by species with fermentative metabolisms (Colman *et al*., 2012). The number of species representing the three phyla was proportional to the diversity of each phylum in nature, with Proteobacteria being the most abundant (Stackebrandt *et al*., 1988). Thus, the gut microbiota composition of *T. lusitanicus* should be similar to those of other non-termite or non-pollenivorous hymenopteran insect species (Colman *et al*., 2012). Clostridiales (Firmicutes) and Bacteroidales associated with a fermentative metabolism have been shown to be prevalent in nearly all termite species as well as in the larval guts of the taxonomically related herbivorous species of Scarabaeidea (Egert *et al*., 2003; Egert *et al*., 2005; Zhang & Jackson, 2008; Huang *et al*., 2012). As in our study, several studies describing the gut microbiomes of xylophagous (Park, 2007; Stackebrandt, 1988) or other herbivorous Scarabaeidea species (Huang *et al*., 2012) also reported a high abundance of Proteobacteria. Within the Proteobacteria of *T. lusitanicus*, most genera belonged to the order Enterobacteriales (*Escherichia*, *Shigella*, *Citrobacter*, *Obesumbacterium*, *Enterobacter*, *Serratia*, *Providencia* and *Yersinia*) of Gammaproteobacteria, which is widespread in animal hindguts in general (Behar *et al*., 2008a; Colman *et al*., 2012) and beetles in particular; for instance, *Rahnella*, *Serratia* and *Yersinia* are abundant in the hindgut microbiome of the Southern Pine beetle *Dendroctonus* (Vasanthakumar *et al*., 2006), and *Enterobacter* is abundant in the bollworm *Helicoverpa* (Gayatri Priya *et al*., 2012).

Unfortunately, we could not isolate any species of the phylum Bacteroidetes, which is well represented in some insect (Schauer *et al*., 2012) and human guts (Turnbaugh *et al*., 2006). This absence could be related to (i) the fact that they are normally found in the foregut (Godoy-Vitorino, 2012), (ii) that they are strictly anaerobic (Gayatri Priya *et al*., 2012), and (iii) low sampling efforts. Additionally, we could not find members of the phylum

Acidobacteria, which have been described to constitute low proportions in the guts of longicorn beetles (Cerambycidae) with woody diets (Stackebrandt *et al*., 1988).

Phylogenetic analyses strongly supported monophyly at the phylum level but failed to recover all Gammaproteobacteria orders together. This is not surprising because the phylogenetic position of the order Xanthomonadales is the subject of a long-standing debate (Comas *et al*., 2006; Gao *et al*., 2009; Cutiño-Jiménez *et al*., 2010). Overall current evidence points to a basal position for Xanthomonadales within Gammaproteobacteria, but more studies are needed to discard the potential presence of shared characters between Xanthomonadales and Betaproteobacteria (Cutiño-Jiménez *et al*., 2010).

The bacterial richness found in the *T. lusitanicus* gut should correlate with a wider functional diversity, which would allow the beetle to improve nutrient supplementation with food sources other than herbivore dung (Shi *et al*., 2010). Although further observations and specific experiments are needed to confirm this hypothesis, several lines of evidence support it. For instance, Enterobacteriales genera such as *Rahnella*, *Serratia*, and *Enterobacter*, which were found in abundance in the gut of *T. lusitanicus*, have been suggested to be involved in nitrogen fixation (diazotrophy) in the guts of bark beetles of the genus *Dendroctonus* (Vasanthakumar *et al*., 2006; Morales-Jiménez *et al*., 2012) and of the Mediterranean fruit fly *Ceratitis capitata* (Behar *et al*., 2008a). In herbivorous ants, bacterial nitrogen fixation is an important source of nitrogen for amino acid synthesis and allows these insects to live on plant-based diets (Eilmus & Heil, 2009). Thus, it is plausible that an enterobacterial diazotrophic community that fixes nitrogen in adults during the reproductive (autumn) and diapause (winter) periods to ensure amino acid synthesis may be required to permit *T. lusitanicus* to feed on acorns (Verdú *et al*., 2007).

It has also been suggested that these Enterobacteriales could be involved in the detoxification of defensive coniferous compounds (e.g., monoterpenes, diterpene acids and

phenolic resins) in *Dendroctonus frontalis* (Vasanthakumar *et al*., 2006). Therefore, it is conceivable that in *T. lusitanicus* these bacteria could also have an important role in the detoxification of defensive acorn substances, which primarily consist of phenolic compounds (Cantos *et al*., 2003). Alternatively, it has been reported that the Mediterranean fruit fly *C. capitata* inoculates Enterobacteriales into fruit, causing fruit decay and accelerating seed maturation (Behar *et al*., 2008b). Similarly, *T. lusitanicus* could inoculate enterobacteria into acorns, triggering germination and contributing to oak seedling recruitment (Pérez-Ramos *et al*., 2007). Other bacteria isolated from *T. lusitanicus* that could potentially be involved in the nitrogen cycle are several Betaproteobacteria genera belonging to the order Burkholderiales including *Achromobacter*, *Alcaligenes*, *Cupriavidus*, and *Comamonas*, which are known to be actively involved in denitrification processes (Ishii *et al*., 2009). Lastly and importantly, bacteria belonging to Enterobacteriales found in the guts of some Scarabaeoidea larvae have also been recently associated with cellulolytic activity (Huang *et al*., 2012; Mabhegedhe, 2012); therefore, we should not discard the possibility that their presence in *T. lusitanicus* may serve to assimilate plant compounds.

The dung beetle *T. lusitanicus* carries in the gut several species belonging to the phylum Actinobacteria. These bacteria are able to produce a great variety of secondary metabolites with antibiotic properties and are mainly considered to be defensive symbionts in insects (Kaltenpoth, 2009; Seipke *et al*., 2012). Moreover, experimental studies in the desert locust (Dillon *et al*., 2005) showed that a diverse gut microbiota is able to provide better resistance against invasion by pathogenic bacteria. In this regard, individuals of *T. lusitanicus* that feed on acorns and harbor a more diverse gut flora would be able to survive longer and remain healthier than individuals eating dung (JRV and JML, personal observations).

In *T. lusitanicus*, we found various *Firmicutes* genera including *Bacillus* and *Lactobacillus*/*Enterococcus* (lactic acid bacteria). It has been reported that lactic acid bacteria

could represent up to 1.8% of the total human gut microbiome (Pessione, 2012), and *Enterococcus* is particularly abundant in the gut of the polyphagous moth *Helicoverpa* (Gayatri Priya *et al*., 2012). Lactic acid bacteria are predominantly saccharolytic (they enhance energy recovery by degrading otherwise indigestible sugars), generate an acid environment, and protect against gas-producing putrefactive and sulphate-reducing bacteria (Pessione, 2012). A cellulose-rich diet increases the presence of this type of bacteria, whereas a protein/fat-rich diet favors the growth of *Clostridium* (Pessione, 2012). Therefore, the presence of Firmicutes (and the relative richness of the genera of this phylum) in *T. lusitanicus* might be directly related to and dependent on the polyphagous behavior of this beetle. However, it is important to note that interpretation of our results regarding Firmicutes is only tentative and highly dependent on future determination of the relative abundance of the anaerobic genus *Clostridium,* which dominates the gut microbiota of e.g., humans and mice (Turnbaugh *et al*., 2006; Ley *et al*., 2005).

In summary, we report the first insight into the composition and richness of a fraction (culturable, aerobic, facultative anaerobic, and aerotolerant) of the microbiota living in the gut of *T. lusitanicus*, an atypical dung beetle with a polyphagous diet. The diversity of the isolated culturable bacteria fraction is of the same level found in other coleopterans and likely provides the required wide functional (saccharolytic, nitrogen-fixing, detoxifying, etc.) capacities associated to polyphagy. This bacterial enrichment could favor the consumption and assimilation of a wider variety of vegetable, animal and fungal tissues. Comparative data on variations in the gut microbiota of individuals subjected to different diets and other control experiments are in progress (and will be published elsewhere) to establish the role played by the gut bacterial community of this species in the physiological and developmental improvements observed with the consumption of acorns.

The design of the present study allowed us to identify culturable aerobic, facultative anaerobic, and aerotolerant bacteria, which represent only a relatively small fraction (that capable of exploiting a protein rich diet) of the total flora of the gut in *T. lusitanicus*. There are several ways in which the present exploratory study could be continued. Isolation media strongly influence the types of bacteria that can be coaxed out of microbial assemblages into culture. Therefore, it is anticipated that additional media types (e.g. 10% tryptic soy agar, M9+cellulose+casamino acids), and especially those mimicking the nutritional profile of the beetle gut (e.g. cattle or red tail deer dung extract solidified with agar or gellan gum) will improve the likelihood of isolating a wider variety of gut residents. In particular, future studies should determine the culturable fraction of strict anaerobic representatives of the beetle gut microflora. In addition, by quantifying colony-forming units of each cultured species, it would be also possible to obtain percentages of relative abundance. This are better approaches for full identification of gut microbiome and accurate quantification of the community members. Moreover, potential functions of the different isolated bacteria, as described here, could be experimentally confirmed through specific functional analyses of the isolates. Furthermore, future studies using genomic tools should also be able to unravel the significant diversity, both aerobic and anaerobic, that is present in the gut of this insect. These studies could be further refined by distinguishing between midgut and hindgut. Moreover, transcriptomic approaches comparing the expression of the gut microbiomes of individuals feeding on acorns or dung diets should also contribute to a better understanding of the evolutionary and ecological changes induced by adaptation to a different dietary selection in the context of the fitness of this dung beetle species.

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Fig. 1 Reconstructed phylogram tree under maximum likelihood based on 16S rRNA sequences (see text) of isolated bacteria living in the gut of the dung beetle *Thorectes lusitanicus*. Labels at the tips indicate Genbank accession number. Branches in red correspond to isolated from the dung beetle (PM). Numbers in the nodes are bootstrap supports. The bar indicates point mutations per nucleotide position.

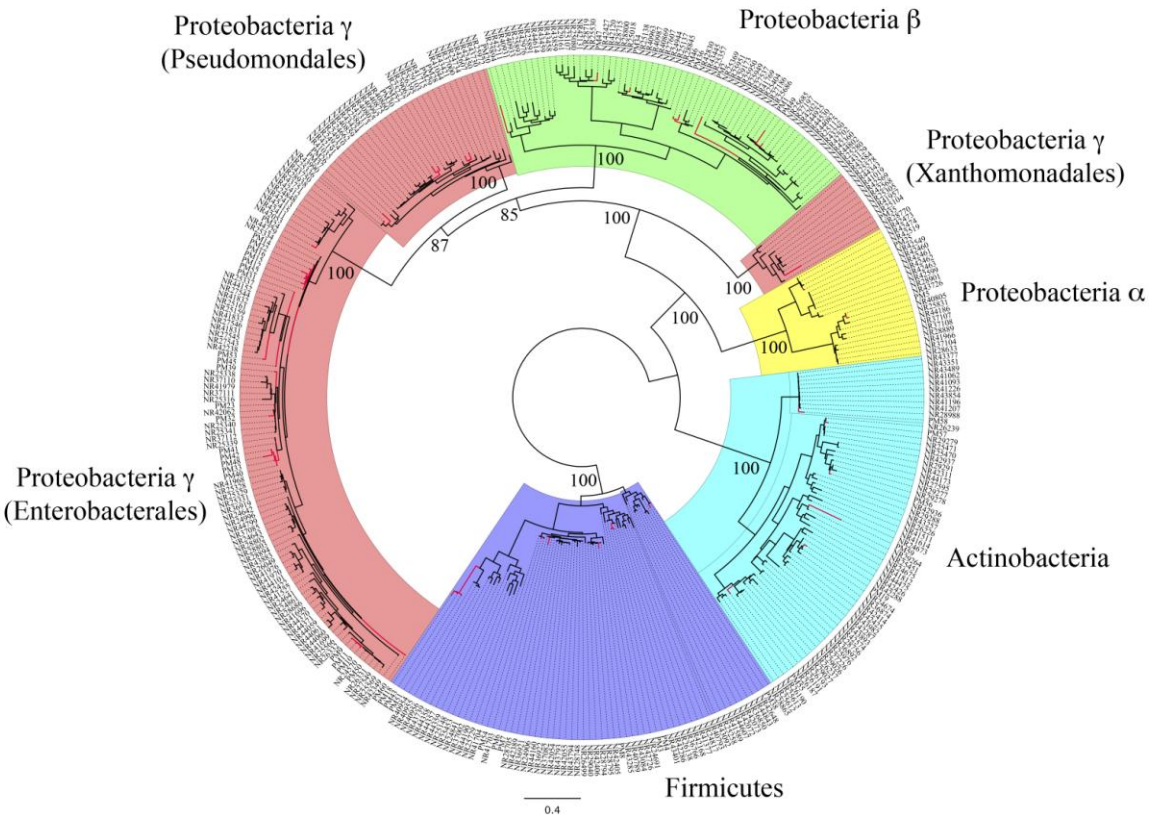

Table 1. Isolated bacteria from the hindgut of *T. Lusitanicus*

Isolate	Length (bp)	Best BLAST hit (Accesion No)	% Identity	% Query coverage	Phylum (tree)	Order
PM1	584	*Achromobacter spanius* strain LMG 5911 (NR 025686.1)	98	99	Beta proteobacteria	Burkholderiales
PM2	987	*Pusillimonas noertemannii* strain BN9 (NR 043129.1)	96	97	Beta proteobacteria	Burkholderiales
PM3	997	*Bacillus megaterium* strain IAM 13418 (NR 043401.1)	99	100	Firmicutes	Bacillales
PM4	1012	*Bacillus megaterium* strain IAM 13418 (NR 043401.1)	99	98	Firmicutes	Bacillales
PM5	963	*Brevundimonas terrae* strain KSL-145 (NR 043726.1)	99	100	Alpha Proteobacteria	Caulobacterales
PM6	847	*Enterococcus devriesei* strain LMG 14595 (NR 042389.1)	99	100	Firmicutes	Lactobacillales
PM7	938	*Enterococcus durans* strain 98D (NR 036922.1)	99	99	Firmicutes	Lactobacillales
PM8	1021	*Enterococcus silesiacus* strain LMG 23085 (NR 042405.1)	99	99	Firmicutes	Lactobacillales
PM9	501	*Escherichia fergusonii* strain ATCC 35469 (NR 027549.1)	99	100	Gamma proteobacteria	Enterobacterales
PM10	1021	*Shigella flexneri* strain ATCC 29903 (NR 026331.1)	99	100	Gamma proteobacteria	Enterobacterales
PM11	1000	*Shigella flexneri* strain ATCC 29903 (NR 026331.1)	99	100	Gamma proteobacteria	Enterobacterales
PM12	937	*Obesumbacterium proteus* strain 42 (NR 025334.1)	99	99	Gamma proteobacteria	Enterobacterales
PM14	1026	*Obesumbacterium proteus* strain 42 (NR 025334.1)	99	99	Gamma proteobacteria	Enterobacterales
PM15	993	*Obesumbacterium proteus* strain 42 (NR 025334.1)	99	99	Gamma proteobacteria	Enterobacterales
PM16	988	*Obesumbacterium proteus* strain 42 (NR 025334.1)	99	98	Gamma proteobacteria	Enterobacterales
PM18	1006	*Lactococcus lactis* strain NCDO 607 (NR 040954.1)	93	96	Firmicutes	Lactobacillales
PM19	905	*Leucobacter albus* strain IAM 14851 (NR 024674.1)	99	100	Actinobacteria	Actinomycetales
PM20	1041	*Vitreoscilla estercoraria* strain Gottingen1488-6 (NR 025894.1)	92	100	Beta proteobacteria	Neisseriales
PM22	1016	*Obesumbacterium proteus* strain 42 (NR 025334.1)	99	100	Gamma proteobacteria	Enterobacterales
PM23	941	*Serratia liquefaciens* strain CIP 103238 (NR 042062.1)	99	99	Gamma proteobacteria	Enterobacterales
PM24	1022	*Providencia rettgeri* strain DSN 4542 (NR 042413.1)	99	99	Gamma proteobacteria	Enterobacterales
PM25	910	*Pseudochrobactrum assacharolyticum* strain CCUG 46016 (NR 042474.1)	99	100	Alpha Proteobacteria	Rhizobiales
PM26	1011	*Pseudomonas tuomuerensis* strain 78-123 (NR 043990.1)	97	99	Gamma proteobacteria	Pseudomonadales
PM27	1467	*Pseudomonas oryzihabitants* strain L-1 (NR 025881.1)	98	98	Gamma proteobacteria	Pseudomonadales
PM28	1000	*Pseudomonas xanthomarina* strain KMM 1447 (NR 041044.1)	98	100	Gamma proteobacteria	Pseudomonadales
PM29	951	*Pseudomonas pseudoalcaligenes* strain Stanier 63 (NR 037000.1)	97	100	Gamma proteobacteria	Pseudomonadales
PM30	1045	*Pseudomonas graminis* strain DSN 11363 (NR 026395.1)	98	99	Gamma proteobacteria	Pseudomonadales
PM31	939	*Serratia grimesii* strain DSM 30063 (NR 025340.1)	99	100	Gamma proteobacteria	Enterobacterales
PM32	1024	*Serratia grimesii* strain DSM 30063 (NR 025340.1)	98	99	Gamma proteobacteria	Enterobacterales
PM33	873	*Serratia fonticola* strain DSM 4576 (NR 025339.1)	99	100	Gamma proteobacteria	Enterobacterales
PM34	1475	*Cupriavidus taiwanensis* strain LMG19424 (NR 028800.1)	98	94	Beta proteobacteria	Burkholderiales
PM35	609	*Stenotrophomanas acidaminiphila* strain AMX 19 (NR 025104.1)	98	99	Gamma proteobacteria	Xanthomonadales
PM36	598	*Alcaligenes faecalis* strain G (NR 025357.1)	98	100	Beta proteobacteria	Burkholderiales
PM37	1500	*Pseudomonas tuomuerensis* strain 78-123 (NR043990.1)	99	91	Gamma proteobacteria	Pseudomonadales
PM38	977	*Solibacillus silvestris* strain HR3-23 (NR 028865.1)	99	100	Firmicutes	Bacillales
PM39	1463	*Serratia proteamaculans* strain DSM 4543 (NR 025341.1)	99	100	Gamma proteobacteria	Enterobacterales
PM40	1013	*Serratia fonticola* strain DSM 4576 (NR 025339.1)	99	99	Gamma proteobacteria	Enterobacterales
PM41	976	*Serratia fonticola* strain DSM 4576 (NR 025339.1)	99	100	Gamma proteobacteria	Enterobacterales
PM42	857	*Serratia fonticola* strain DSM 4576 (NR 025339.1)	99	99	Gamma proteobacteria	Enterobacterales
PM43	987	*Lactococcus lactis* strain NCDO 607 (NR 040954.1)	93	96	Firmicutes	Lactobacillales
PM45	1024	*Serratia grimesii* strain DSM 30063 (NR 025340.1)	98	99	Gamma proteobacteria	Enterobacterales
PM46	1465	*Alcaligenes faecalis* strain IAM 12369 (NR 043445.1)	98	99	Beta proteobacteria	Burkholderiales
PM47	1002	*Comamonas aquatica* strain LMG 2370 (NR 042131.1)	97	100	Beta proteobacteria	Burkholderiales
PM48	236	*Serratia fonticola* strain DSM 4576 (NR 025339.1)	99	93	Gamma proteobacteria	Enterobacterales
PM49	782	*Achromobacter denitrificans* strain DSM 30026 (NR 042021.1)	99	100	Beta proteobacteria	Burkholderiales
PM50	1489	*Shigella flexneri* strain ATCC 29903 (NR 026331.1)	99	99	Gamma proteobacteria	Enterobacterales
PM51	802	*Lactococcus lactis* strain NCDO 607 (NR 040954.1)	91	97	Firmicutes	Lactobacillales
PM52	1013	*Obesumbacterium proteus* strain 42 (NR025334.1)	99	100	Gamma proteobacteria	Enterobacterales
PM53	985	*Yersinia rohdei* strain ATCC 43380 (NR 025161.1)	99	99	Gamma proteobacteria	Enterobacterales
PM54	930	*Enterococcus casseliflavus* (NR 041704.1)	99	100	Firmicutes	Lactobacillales
PM55	908	*Arthrobacter gangotriensis* strain Lz1Y (NR 029026.1)	96	100	Actinobacteria	Actinomycetales
PM56	930	*Sanguibacter inulinus* strain ST50 (NR 029277.1)	99	100	Actinobacteria	Actinomycetales
PM57	922	*Oerskovia enterophila* strain DSM 43852 (NR 026239.1)	99	100	Actinobacteria	Actinomycetales
PM58	917	*Streptomyces microflavus* strain NRRL B-2156 (NR 043854.1)	96	100	Actinobacteria	Actinomycetales
PM59	326	*Pseudoclavibacter helvolus* strain DSM 20419 (NR 029264.1)	93	98	Actinobacteria	Actinomycetales
PM60	918	*Enterobacter amnigenus* strain JCM1237 (NR 024642.1)	94	99	Gamma proteobacteria	Enterobacterales