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Gut and faecal bacterial community of the terrestrial isopod *Porcellionides pruinosus:* potential use for monitoring exposure scenarios

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Short title: *P. pruinosus* bacterial community

Highlights

1. *P. pruinosus* bacterial community (BC) was explored by high-throughput sequencing.
2. Gut and faecal BC were dominated by Proteobacteria, namely *Coxiella.*
3. Faecal BC revealed higher richness and diversity compared to gut BC.
4. Soil, ecological/metabolic-related bacteria, endosymbionts, pathogens were found.
5. Isopods BC signature can be used as endpoint in multilevel approaches.

Abstract

This work aimed to characterize the gut and faeces bacterial communities (BC) of *Porcellionides pruinosus* using high-throughput sequencing. Isopods were collected from the field and kept in laboratory conditions similar to those normally applied in ecotoxicology tests. Faeces and purged guts of isopods (n= 3 x 30) were analysed by pyrosequencing the V3-V4 region of 16S rRNA encoding gene. Results showed that gut and faecal BCs were dominated by Proteobacteria, particularly by an OTU (Operational Taxonomic Unit) affiliated to genus *Coxiella*. Diversity and richness values were statistically higher for faecal BC, mainly due to the occurrence of several low-abundance phylotypes. These results may reflect faecal carriage of bacterial groups that cannot settle in the gut. BCs of *P. pruinosus* comprised: (1) common members of the soil microbiota, (2) bacterial symbionts, (3) bacteria related to host metabolic/ecological features, and (4) bacterial etiological agents. Comparison of BC of this isopod species with the BC from other invertebrates revealed common bacterial groups across taxa. The baseline information provided by this work will assist the design and data interpretation of future ecotoxicological or biomonitoring assays where the analysis of *P. pruinosus* BC should be included as an additional indicator.

Capsule

Terrestrial isopods bacterial communities might support ecotoxicological assays and biomonitoring processes as a valuable tool.

Keywords

Porcellionides pruinosus; Bacterial community; Faeces; Guts; Pyrosequencing; Ecotoxicological indicator.

1. Introduction

Within terrestrial isopods, *Porcellionides pruinosus*, Brandt 1833 (Crustacea: Isopoda) is a synanthropic species with a key role on litter fragmentation, decomposition and nutrient recycling processes (Loureiro et al. 2005). It is also considered a good test-species for ecotoxicological tests, other stress ecology applications, such as soil contamination (Loureiro et al. 2005) or abiotic changes (Morgado et al. 2015). Understanding the bacterial community (BC) of *P. pruinosus* is of significant interest as it may open new insights to unveil the effects of host-BC relationships, particularly the interactions, reciprocal feedbacks and multi-scale effects on host, their BC and the surrounding environment (Borer et al. 2013). This information can hence be used to anticipate stress-related imbalances in host-BC dynamic interaction (i.e. pollution, environmental stressors) further comprising the processes they are involved in, namely in soil function and services, like decomposition, nutrient cycling or biomonitorization (van Gestel et al. 2018). For instance, an analogous species, *Porcellio scaber*, was used to understand the impact of temperature on host symbiont community (Horváthová et al. 2019).

Previous investigations support the idea that isopod-associated BC can be beneficial, neutral or pathogenic, including (1) a well-established resident gut BC associated to the hepatopancreas and, (2) a transient hindgut BC (eliminated via faeces and due to frequent moulting) (Kostanjšek et al. 2004; Ihnen and Zimmer 2008; Horváthová et al. 2016; Bredon et al. 2018). Patterns of dominance by host-symbionts have been extensively reviewed (Bouchon et al. 2016) as well as their importance for ecology and evolution of species, host nutrition, reproduction, immunity, speciation, growth rate and survival, and mode of symbionts' transfer to the host (vertical, horizontal or environmentally) (Horváthová et al. 2015; Horváthová and Bauchinger 2019). Acquired via food, coprophagy or ingestion of old cuticles (Kostanjšek et al. 2005; Horváthová et al. 2015), isopod gut BC has been shown to be relevant for gut homeostasis (Zimmer and

Topp 1997; Zimmer and Brune 2005) and nutrition, either by contributing to the processing of the ingested detritus (Zimmer and Topp 1998; Zimmer 1999; Bredon et al. 2018, 2019; Delhoumi et al. 2020) or actually becoming a food item and source of nutrients (Drobne 1995; Ihnen and Zimmer 2008). By stimulating bacterial growth within their gut compartments (Eisenbeis 2005) and afterwards releasing a considerable proportion through faeces (Gunnarsson and Tunlid 1986), isopods create multiple hotspots of enhanced and differentiated bacterial activity, likely to interact with the neighbouring soil microbiota [microbial community coalescence (see (Rillig et al. 2016))]. Altogether, gut BC, in a concerted action with isopod digestive enzymes, and BC from faeces assist in the rapid degradation of organic matter promoted by isopods (Zimmer and Topp 1998). Moreover, bacterial input and distribution in the terrestrial environment via isopod faeces may have impact on ecological processes such as decomposition and biogeochemical cycling of soil nutrients (Kautz and Topp, 2000; Rillig et al., 2016). The effectiveness in providing these benefits to isopod health and to soil functioning and quality is likely to be dependent on the composition of the isopod BC.

Current knowledge on terrestrial isopods BC has previously been reviewed (Bouchon et al. 2016) along with the essential morphological and physiological aspects of the isopods digestive tract (Zimmer 2002; Kostanjšek et al. 2005). Several authors addressed the BC diversity of *P. scaber* (Kostanjsek et al. 2002; Horváthová et al. 2015). The hepatopancreas BC diversity of aquatic and terrestrial isopod species (*Idotea balthica*, *Ligia oceanica*, *Oniscus asellus*, *P. scaber* and *Asellus aquaticus*) (Wang et al. 2007; Mattila et al. 2014) was also described. Recent works used 16S rRNA gene pyrosequencing to characterize (1) the BC of various tissues (haemolymph, gonads, nerve cord, midgut caeca and hindgut) of the terrestrial isopod crustacean *Armadillidium vulgare* originated from laboratory lineages and field populations (Dittmer et al. 2016) as well as (2) the *Jaera albifrons* species complex and analyzed seasonal, spatial and sex-ratio distorting patterns affecting BC composition (Wenzel et al. 2018). While

the contribution of these and other several studies to expanding our knowledge of the terrestrial isopod gut and faeces BC is undeniable, to our knowledge, the BC of *P. pruinosus* has not been yet characterized using high-throughput sequencing, despite its ecological, ecotoxicological and biomonitoring relevance as well as wide distribution throughout the world (Lefebvre and Marcadé 2005). Only recently, the gut bacteria of *P. pruinosus* was addressed aiming to understand their role on the land colonization by Oniscidea (Delhoumi et al. 2020). Using a metagenomic approach, this study found that the gut BC had variable structure depending on host geographic origin (three locations in Tunisia). Also, cellulolytic bacteria was retrieved from the gut by means of culture-dependent techniques.

Given the relevance of the BC associated with *P. pruinosus*, the lack of baseline information, and the focusing interest of using this excellent model as sentinel, it is of importance to deepening our knowledge concerning their BC (gut and faeces) using similar laboratory-controlled conditions to those used in the ecotoxicological/biomonitoring assays. Thus, this study aimed to (1) characterize both gut and faecal BC of the isopod *P. pruinosus* by high-throughput pyrosequencing of the 16S rRNA gene, (2) compare our results to previous documented BC for other isopods or invertebrate species, and (3) discuss the use of isopods' BC as an additional indicator/tool for several exposure scenarios.

2. Materials and methods

2.1. Sample collection and acclimatization

Isopods (*P. pruinosus*) were collected from horse and cow compost manure of an equestrian centre (Centro Hípico de Coimbra, Portugal), which has been for years the source of isopods to to maintain the laboratory culture at the University of Aveiro. Isopods were brought to the laboratory of the Department of Biology, University of Aveiro, where they were hand-sorted (15-25 mg wet weight) and no gender differentiation was done, although pregnant females were

excluded. External moulting coincides generally with gut cuticular moulting, and consequently cuticular microorganisms were also released/excreted (Drobne et al. 2002). Therefore, only non-molting adults were included in this investigation. A preliminary analysis included a Denaturing Gradient Gel Eletrophoresis (DGGE)-based comparison of the BC of isopods after long-term maintenance in laboratory (>4 years; for maintenance conditions see Loureiro et al. 2006) to those freshly collected from the field. BC of field isopods was clearly distinct from the BC of those maintained at laboratory (S2 Fig), directing our choice towards isopods freshly collected from the field to include a more realistic scenario. Isopods to be used for BC sequencing analysis were brought to the laboratory and left for acclimatization for 2 weeks under culture conditions described as optimal to reduce stress (related to collection, transport and sorting), and to restore/preserve isopod's performance. Isopods were held in LUFA 2.2 soil, moisture at 60% of maximum water holding capacity (WHC), 20ºC and 16h/8h light/dark photoperiod (Løkke and van Gestel 1998; Loureiro et al. 2006), fed *ad libitum* with alder leaves [collected from a riparian vegetation at São Pedro de Alva, Coimbra (40°16'38.8"N, 8°11'52.8"W) since they did not exist at the Centro Hípico de Coimbra as a good nutritional food source (Sousa et al. 1998)].

2.2. Sample preparation

Isopods were then left for 14 days in LUFA 2.2 soil as the only food item. LUFA 2.2 is a non-contaminated natural soil, widely used as reference in ecotoxicology studies (Caetano et al. 2012). To minimize bacterial conditioning: (1) LUFA 2.2 soil was sterilized and (2) the soil adjustment of WHC was made using sterilized water. The remaining conditions were maintained. Thirty isopods were pooled (to obtain per replicate the needed biomass close to the minimum of 250 mg required by the extraction kit) and used as a replicate (n=30) in a triplicate design thus, 90 animals were used in total. The number of isopods was verified at the beginning

and at the end of this 14-days period to ensure that transference of bacteria among isopods as a result of cannibalism (Le Clec'h et al. 2013) did not occur; also, no evidence of predatory behaviour was identified (i.e. lack of antenna).

Isopods were carefully transferred into chambers (plastic boxes) containing moist Plaster of Paris (to keep chamber humidity) and a 2 mm nylon screen suspended 5 mm above, for 48 hours to induce purging. All material involved in faeces collection was sterilized. The use of these purging chambers allowed faecal pellets to fall through the nylon screen and into filter paper (adapted from (Loureiro et al. 2006)), helped in the selection/collection of the faeces (which otherwise would be rapidly decomposed in soil or misidentified as soil particles) and prevented the isopods from ingesting their faeces. Because this behaviour (coprophagy) can occur in isopods probably as a survival strategy or as a nutritional need when foods are of poor nutritional quality (David 2014), it needed to be anticipated after the 14-days period of sterilized soil-feeding imposed in this study. Depurated specimens were immobilized using anaesthetic chloroform (in a soaked cotton within a closed petri dish). Organisms were briefly washed with 70% ethanol followed by sterile distilled water for a few seconds (to remove BC from isopods' outer surface and avoid bacterial transference to other tissues during handling). The hepatopancreas was aseptically extracted by holding the body and pulling out the head. The digestive tract was pulled out as a whole attached to the uropod. Head and uropod were removed immediately after with sterile tweezers and scalpel and the entire guts (hepatopancreas and digestive tract) were used. Only fully purged guts were handled further. Faeces were collected with a sterile spatula. A total of 6 samples (3 of guts and 3 of faeces) were analysed covering 30 isopods. Gut samples (n= 3 x 30 animal guts) and faecal samples (n=3 x total faeces purged by 30 isopods) were conserved separately in 0.5 mL of sterile Phosphate Buffered Saline buffer (0.12 M, pH 8.0) at -20°C until DNA extraction.

2.3. DNA extraction

After slow thawing in ice, samples were crushed with sterilized pestle homogenizers. The total sample amount was transferred into the UltraClean® bead tubes (MoBio Laboratories, Inc., Carlsbad, CA). DNA was then extracted using the commercial UltraClean™ Soil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) following the manufacturers' protocol.

2.4. Pyrosequencing analysis

DNA extracts were prepared for 454 pyrosequencing by nested PCR amplification as described previously (Alves et al. 2016): for the amplification of the 16SrRNA gene were used the universal primers 27F 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R 5'-ACGGCTACCTTGTTACGACTT-3' and, for the amplification of the V3V4 hypervariable region were used the forward primer 5'- ACTCCTACGGGAGGCAG-3' and the reverse primer 5'-TACNVRRGTHTCTAATYC-3' (Wang and Qian 2009). The PCR amplicons were quantified as previously described (Silva et al. 2016; Alves et al. 2016; Mahmoudi et al. 2019) and according to manufacturer's instructions (Roche, 454 Life Sciences, Brandford, CT, USA) at GenoInSeq, the Next Generation Sequencing Unit of the CNC/BIOCANT - Centre for Neuroscience and Cell Biology/Portugal Science & Technology Park for Biotech and Life Science (Cantanhede, Portugal).

The fasta files, with the raw pyrosequencing reads, were processed using Metabiodiverse at GenoInSeq (Cantanhede, Portugal) as described previously (Pinto et al. 2014; Ribeiro et al. 2018; Mahmoudi et al. 2019). Briefly, reads were quality filtered e.g. by eliminating sequence reads with (1) <100 bp, (2) >2 undetermined nucleotides, (3) > 50% of low complexity regions [DustMasker (Welch and Huse 2011)] and, (4) chimera sequences [UCHIME (Edgar et al. 2011)]. Then, the Operational Taxonomic Units (OTU) were created using a phylogenetic distance of 3% [USEARCH (Edgar 2010)]. Rarefaction curves (plotting the number of observed

OTUs as a function of the number of sequences, shown in S1 Fig) and Chao1 estimator were calculated [mothur package (Oakley et al. 2009)].

Identification of the taxonomy of each OTU was made using a BLAST search against the Ribosomal Database Project II (RDP) database (Cole et al. 2009). Quality control included rejection of sequences with an alignment of less than 40%, with an E-value greater than 1^{-50} and a bootstrap test [PHYLIP package (Felsenstein 1989)]. For each identified taxon, the sum of the total number of sequences provided the abundance of all identified organisms. Obtained data (taxonomy of each OTU, taxonomic ID, number of OTUs, number of sequences and bootstrap value for each entry and each sample/replicate) is summarized in S1 Table. The Shannon index, *H'*, was calculated for guts and faeces and plotted to further evaluate the variance within samples from the two origins (Fig 1). PERMANOVA (1000 permutations with "bray" method, R-vegan function adonis) (Oksanen et al. 2013), was used to test if there were differences in the composition of the BC (OTUs relative abundance) in samples from different origins (guts or faeces) (S2 Table).

3. Results

3.1. General analysis of the pyrosequencing-derived dataset

The pyrosequencing-derived dataset (Table 1 and 2) comprised 38055 high quality sequences that were assigned to the domain Bacteria and, from these, 38018 (99.90%) were classified below the domain level corresponding to a total of 273 bacterial OTUs. The number of classified sequences in all samples ranged from 4263 to 8358 with an average of 5106.00 ± 1231.00 in gut samples and of 7579.00 ± 702.06 in faecal samples (Table 1). Only one sequence from trimmed dataset was not closely related to bacterial 16S rRNA genes (belonged to Chlorophyta) and was eliminated from subsequent analysis (S1 Table).

3.2. Bacterial richness and diversity

Faeces comprised 247 OTUs while guts included only 26 OTUs corresponding to 22701 and 15317 sequences, respectively (Tables 1 and 2). Hence, the highest mean bacterial richness according to Chao1 estimator was predicted for faeces (166.87 ± 135.50) while gut estimated richness was 11.94 ± 8.02 (Table 2). Comparison of the rarefaction analysis (S1 Fig) with the number of obtained OTUs (Table 2) and the Chao1 richness estimator (Table 2) revealed that with such bacterial richness (Table 2), the sampling effort (herein measured as N, the total number of individuals in the sample) was not sufficient to completely describe the faecal community (S1 Fig) with only 53.14% ± 6.16% (Table 2) of the estimated taxonomic richness being revealed. For guts, the generated rarefaction curves (S1 Fig) for each gut sample nearly reached saturation, indicating that the study described most of the phylogenetic diversity at 3% 16S rRNA gene sequence divergence. Indeed, coverage was of 85.72% ± 22.77% (Table 2).

Faeces revealed a higher diversity index than guts (Fig 1). An Adonis test showed that 46% (R^2=0.46232) of the variance was explained by the origin of the BC (guts or faeces), and that there were significant differences in the BC composition in samples from different origins ($F_{crit\ (1,4;\ 0.1)}$= 4.545 > F_{model}=3.439, P=0.083; α=0.1) (S2 Table).

3.3. Bacterial composition in *P. pruinosus*

Bacterial OTUs classified below the domain level were assigned to 7 phyla, 12 classes, 25 orders, 48 families, 59 genera (S1 Table). Few OTUs with low relative abundance (0.01% in guts and 0.16% in faeces) could not be affiliated into any known group and were assigned as “unclassified bacteria” (Tables 1 and 2, Fig 2 and 3).

Sequences obtained from faeces were affiliated to 7 bacterial phyla (Gemmatimonadetes, Verrucomicrobia, Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Acidobacteria) while gut sequences were attributed to only 1 phylum (Proteobacteria) (Fig 2). Proteobacteria

was the most abundant phylum in both gut and faeces samples, representing 99.99% (96.15% OTUs) and 99.04% (50.61% OTUs) of the retrieved reads, respectively (Fig 2). The dominant class was Gammaproteobacteria (99.69% reads in guts and 98.32% reads in faeces comprising 65.38% and 28.34% OTUs, respectively) (Fig 2). The remaining classified sequences (0.30% in guts and 0.71% of the faeces) were assigned to Alphaproteobacteria (0.29% reads in guts and 0.55% reads in faeces corresponding to 23.08% and 11.74% OTUs, respectively) followed by Betaproteobacteria (0.01% reads in guts and 0.10% reads in faeces corresponding to 7.69% and 5.67% OTUs, respectively) (Fig 2). Deltaproteobacteria was only detected in faeces samples with an occurrence of 0.06% of the reads (4.45% OTUs) (Fig 2).

The order Legionellales (Fig 2) was almost completely represented by *Coxiella*, with only 1 OTU in faeces, corresponding to 1 sequence, being affiliated to *Aquicella*. Indeed, *Coxiella* was the most abundant genus across all samples, representing 99.46% (30.77% OTUs) and 85.29% (7.69% OTUs) of the whole sequences in gut and faeces, respectively (Fig 2). Within faeces, and though with a smaller number of reads, the second most abundant taxon was the order *Vibrionales* (11.25% reads and 5.26% OTUs) (Fig 2).

The remaining classified sequences (0.54% in guts and 3.30% in faeces) affiliated to other bacterial groups, each bacterial group represented less than 1% of all classified sequences (Fig 2). In guts, these rare bacterial groups were affiliated to 5 genera: *Anaplasma* (Rickettsiales, 0.19% reads; 19.23% OTUs), *Vibrio* (Vibrionales, 0.08% reads; 7.69% OTUs), *Pseudomonas* (Pseudomonadales, 0.03% reads; 3.85% OTUs), *Burkholderia* (Burkholderiales, 0.01% reads; 7.69% OTUs) and *Shewanella* (Alteromonadales, 0.01% reads; 3.85% OTUs) (Fig 3). Rare bacterial groups of guts also included unidentified genera of the following phylogenetic groups (totalizing 0.22% of reads; 23.08% OTUs): Brucellaceae (Rhizobiales, 0.10% reads; 3.85% OTUs), Enterobacteriaceae (Enterobacteriales, 0.06% reads; 3.85% OTUs), Gammaproteobacteria (0.03% reads; 11.54% OTUs), Xanthomonadaceae (Xanthomonadales,

0.02% reads; 3.85% OTUs), and Coxiellaceae (Legionellales, 0.01% reads; 3.85% OTUs) (Fig 3). In faeces, 55 genera were identified at relative abundances that ranged from 0.004% to 0.38% sequences (*Pseudomonas*, Pseudomonadales, corresponding to 2.02% of OTUs) (Fig 3). From these, 28 orders were represented at relative abundances above 0.009% (e.g. *Devosia*, Rhizobiales, corresponding to 0.40% of OTUs) and the remaining 27 orders were identified at relative abundances lower than 0.005% sequences (Fig 3).

3.4. Comparison of bacterial communities between gut and faeces

Besides *Coxiella*, shared OTUs also comprised those affiliated with *Vibrio* (abundant genus in faeces but rare in guts), *Pseudomonas* and *Burkholderia*, along with other rare phylotypes identified in gut samples above genus level (Figs 2 and 3). A comparison of the isopods' gut and faeces BC, using Venn diagrams (Fig 4), showed 79 shared OTUs of a total of 273 OTUs and that shared sequences comprised 99.79% and 94.19% of all gut and faeces sequences, respectively (Fig 4).

Only 7 OTUs (0.20% of all sequences) were unique to guts while faeces comprised a higher number of specific OTUs (173, corresponding to 5.66% of all sequences) (Fig 4). Unique members of gut or faeces were rare bacterial groups. Analysis at genus level revealed that OTUs occurring uniquely in isopods' gut were affiliated to genera *Anaplasma* and *Shewanella* (Fig 3) and to Coxiellaceae. On the other hand, bacterial groups exclusively found in faeces included 53 genera.

4. Discussion

4.1. Common bacteria in the gut and faeces of *P. pruinosus*

Coxiella dominated the BC of both gut and faeces of the isopods (Fig 2). Although this pattern might be referred to as infection along this manuscript, it may result in both positive and negative impacts to the organism (Fraune and Zimmer 2008; Bansal et al. 2014).

The abundance of *Coxiella* might be viewed as a specific symbiotic relationship bacterium-isopod (Klyachko et al. 2007). Bacterial symbionts were found associated with isopods and responsible for obtaining nutrients under conditions of poor diet (Wang et al. 2004, 2007; Bouchon et al. 2016; Delhoumi et al. 2020), or as drivers of the reproductive processes (Dittmer and Bouchon 2018; Wenzel et al. 2018), including in *P. pruinosus* (Michel-Salzat et al. 2001; Cordaux et al. 2012). *Coxiella* has high infectivity rate for several tick species (Almeida et al. 2012; Klyachko et al. 2007), and was shown to be prevalent in cattle tick eggs (Andreotti et al. 2011). However, to our knowledge, *Coxiella* dominance was not reported for terrestrial isopods. Nonetheless, genera closely related to *Coxiella*, namely *Rickettsiella* (Dittmer et al. 2016) among other members of the order Legionellales (Drobne et al. 1999; Kleespies et al. 2014) were found to be predominant members in the BC of other isopods species. *Rickettsiella* is known to cause a lethal disease in isopods (Bouchon et al. 2016), which symptomatology includes opaque white masses in ventral surface, a phenotype not observed in this study. Additionally, *Rickettsiella* OTUs were not found in our samples.

The environmental origin of bacterial symbionts associated to extensive infection was described for other isopods (Wang et al. 2007; Fraune and Zimmer 2008; Bouchon et al. 2016). Considering that *Coxiella burnetii* causes coxiellosis, a worldwide zoonosis occurring in several animal species (Marenzoni et al. 2013), *Coxiella* might have been acquired by isopods while feeding on manure produced by infected animals. This hypothesis is also supported by previous works addressing the isopods' role as reservoirs of disease vectors (Kostanjsek et al. 2002; Kostanjšek et al. 2005; Fraune and Zimmer 2008). Elimination of *Coxiella* via isopod's

faeces corroborates isopods' role in disseminating diseases [similarly to what happens with other known vectors (Rodriguez et al. 2009)].

Similarly to other detritivores (Aira et al. 2015), the isopod may act as a biological filter by favouring the proliferation within the gut and/or elimination via faeces of specific ingested bacterial groups. Here, conditions in the isopod gut appear to have favoured *Coxiella* proliferation and although its elimination through faeces occurred, it was only partial. This abundance may result in both advantageous and adverse effects (Fraune and Zimmer 2008); it may either: 1) hamper the gut colonization by other detrimental organisms (e.g. bacteria, parasites, viruses), providing an additional protection to the isopod (Klyachko et al. 2007; Wang et al. 2007; Koch and Schmid-Hempel 2011; Bansal et al. 2014); or 2) result in gut dysbiosis which, in turn, may result in diminished ability to respond to natural perturbations and environmental stress (Sharma et al. 2011). In the latter case, the loss of an abundant symbiont may result in the dominance of other normally resident or commensal bacterial groups (Stein et al. 2013). Detection of such shifts could serve as a biomarker of exposure to *e.g.*, cattle disease, as this species is synanthropic and it presents the advantage of thriving in environments where human activities take place. In summary, the diversity of negative and positive interactions that may be established between *Coxiella* and the isopods, make it difficult to anticipate the environmental impact of its dominance. Future comparative studies between colonised and non-colonised isopods may provide more insight into this impact.

For 14 days isopods were fed only with sterilized soil. These suboptimal food conditions might impact the isopod BC since leaves' BC are an important source of nutrients for isopods (Horváthová et al. 2016). Also, a large fraction of transient bacteria normally present in the gut might have been eliminated through faeces (decreasing diversity inside the guts and increasing in faeces) allowing dominance of *Coxiella*. Besides *Coxiella*, a limited number of rare phylotypes were detected as common to gut and faeces. Although *Vibrio* was a common

phylotype, it occurred at substantial abundance (>11%) in faeces but not in the gut (<1%). As explained above, this might be explained because while most bacterial groups were fully expelled from the gut via faeces (unique phylotypes of faeces), few still remained in the gut though partially released (common phylotypes), and only a small fraction was not expelled via faeces (unique phylotypes in guts). *Burkholderia* was also a rare phylotype common to gut and faeces. Both genera (*Vibrio* and *Burkholderia*) include pathogens responsible for diseases in horses and cattle. *Burkholderia* and unclassified members of the Xanthomonadaceae family were found in both gut and faeces in our survey but were never previously associated with isopods. Yet, these phylotypes dominate the gut of other terrestrial organisms such as the ant species *Cephalotes varians* (Kautz et al. 2013). Other rare bacterial groups common to gut and faeces included *Pseudomonas* and members of Enterobacteriaceae; these bacterial groups were previously detected in the gut and faeces of the isopods species *O. asellus* and *P. scaber* (Kostanjšek et al. 2005) and in the gut of *P. pruinosus* (Delhoumi et al. 2020). These groups include nitrogen fixers and effective degraders of plant polymers, especially cellulose and hemicellulolytic polymers (Tagliavia et al. 2014) and, consequently may provide benefits for terrestrial isopods. Lastly, Brucellaceae was also found in both gut and faeces but relatively little is known about their associations with isopods; however, since it comprises both pathogenic and typical soil bacteria, their physiological and ecological role might be wide-ranging. By spreading bacteria within and across habitats, isopods play a significant part in the enrichment of the soil providing an important ecological contribution (Kautz and Topp 2000; Rillig et al. 2016). Thus, attention must be given to these bacterial groups, regardless of their abundance, particularly when predicting the effects of environmental stress on soil BC and/or even in the isopod.

4.2. Isopod gut bacterial community

Only a small fraction of all OTUs (0.2%) were exclusive to gut BC and were represented by just two phylotypes: *Anaplasma* and *Shewanella,* both affiliated to Proteobacteria. Despite their low abundance, the presence of these bacterial groups is worth mentioning and explored for different reasons. *Anaplasma* genus includes etiologic agents of cattle anaplasmosis (Rodriguez et al. 2009) and thus its presence supports the idea that *P. pruinosus* BC is sensitive to and constrained by the surrounding environment. *Shewanella* members have previously been detected in the gut of the isopods *P. scaber* (Kostanjšek et al. 2005) and *A. Vulgare* (Dittmer et al. 2016) and due to the diverse metabolic capabilities are known to play a major role in carbon cycling (Fredrickson et al. 2008).

All bacterial groups found in the isopod gut were affiliated to Proteobacteria, similarly to other organisms guts, e.g. California black (*Haliotis cracherodii*), white abalone (*H. sorenseni*) (Gruenthal 2007), soil-feeding termites (*Cubitermes niokoloensis*) (Fall et al. 2007), arthropods (Esposti and Romero 2017) and insects (Jones et al. 2013; Yun et al. 2014). Distinct organisms, and particularly invertebrates detritivores, might conserve some functionally similar bacterial groups, related to the host digestive needs or to their ecological role (Mouchet et al. 2012). Similarities might also partially reflect the BC of the sampling site, as in the case of the earthworm *Eisenia andrei* fed with horse manure (Aira et al. 2015).

4.3. Isopod faeces bacterial community

The isopods' digestive capabilities result from the joint action of the distinct BC in the hepatopancreas and digestive tract (Zimmer and Topp 1998; Zimmer 2002; Fraune and Zimmer 2008; Horváthová et al. 2019). Ultimately, the contribution of the isopods (stressed or not) to the decomposition processes results from what happens in the whole gut, and from what is expelled via faeces. Faeces enable bacterial analysis without sacrificing the isopods which represents an additional advantage as a potential bioindicator.

All phylotypes exclusively detected in faeces were at relative abundance levels below 1%. In contrast to gut BC (where only Proteobacteria were present), faeces harboured bacteria affiliated to seven phyla.

Some phylotypes have been already associated with faeces of different terrestrial isopod species (e.g. members of the phylum Bacteroidetes and order Bacillalles, and genera *Paracoccus*, *Paenibacillus* (Kostanjšek et al. 2005), and *Sphingomonas* (Dittmer et al. 2016)) being linked to the digestion of polysaccharides and aromatic compounds, nitrogen fixation and degradation of environmental pollutants. This confirms the importance and interest of the present study both in an ecological and an ecotoxicological perspective (König 2006). Other phylotypes found in our survey, to our knowledge, were never reported in isopod faeces but may play a significant yet unknown or less understood ecological role. Among these are bacteria related to plants and soil [Xanthomonadales (*Lysobacter*, *Stenotrophomonas*, *Rhodanobacter*), *Geobacter*, *Novosphingobium*, *Methylobacterium* (Rogers and Backus 2014)], soil bacteria related to nitrogen cycling (*Rhizobiales*, *Rhodanobacter* and *Stenotrophomonas*), chitinolytic, cellulolytic and hemicellulolytic bacteria (*Enterobacter* and *Microbacterium*) probably essential for the degradation of the diet compounds of *P. pruinosus* (Tagliavia et al. 2014) or pathogenic bacteria (*Serratia*, the etiologic agent of horses conjunctivitis, also found to be a dominant phylotype in the BC of another detritivore, *L. rubellus* (Aira et al. 2015)). *Microbacterium* was also linked with potential resistance of *P. pruinosus* to soil contamination (Delhoumi et al. 2020).

Overall, the rare phylotypes herein found exclusively in faeces of *P. pruinosus* either reflect bacterial groups inherent to the isopod gut that were fully expelled and therefore had just left the gut via feaces, or possibly reflect transient bacteria that were ingested , not digested, and expelled via faeces. It could be speculated that some bacterial transference from isopod's outer surfaces to our samples could have also occurred, but precautions to avoid bacterial

conditioning were ensured. Despite their low abundance, faeces phylotypes cover a wider range of possible ecological or physiological functions which cannot be underestimated.

4.4. Relevance to ecotoxicological studies

There is increasing evidence that ecotoxicological assessments will benefit from the inclusion of the microbiome as an additional endpoint, as this community is a fundamental interface that interacts with the organism and the environment. One of the main knowledge gaps that is hampering progress in this area is the absence of baseline knowledge regarding the microbiomes of species used as model organisms in ecotoxicology studies, such as *P. pruinosus*. Having this in mind, this study was performed using experimental conditions that mirror those used in ecotoxicology assays (e.g., temperature, photoperiod, soil type). As such, our study provides baseline data that will assist in the design and data interpretation of future multi-level investigations where isopod-BC should be included as an additional indicator complementing the information of the ecotoxicological standard endpoints. The experimental design herein employed allowed to get a broad picture of the BC of the isopods and to identify the most abundant bacterial groups in the isopod BC (probably the ones that were common to most of the analyzed individuals). In a future perspective of using BC of isopods as an additional indicator in ecotoxicological studies, this experimental design will also enable to retain the population response rather than an individual response. The inclusion of more replicates with fewer individuals or even replicates with only one individual as well as individuals obtained from different origins together with collection of samples from the surrounding environment will provide a more complete picture of the BC of this species. The reduction of number of individuals per replicate should be made with caution since variability among replicates will probably increase (more than what it was herein obtained, Figure S1) due to higher variability inter-individuals. Higher inter-individual variability could mask the

impacts of the disturbance that will be highlighted by comparison towards a non-exposed population (control). Future studies should contribute to determine this BC variability inter-individuals and the factors that affect this variability, also including samples of the food sources, geographic origin and type of soil.

In summary, to consider the microbiome in ecotoxicological studies, experimental design should carefully consider microbiome intra- and inter-individual variation and other confounding factors, such as the numerous sources of microorganisms within the experimental setup, and the effect of sex, diet, age and other parameters in the microbiome structure and diversity. A close cooperation between ecotoxicology and microbiology experts is fundamental to the success of such approach.

5. Conclusion

We found prominently important bacterial taxa associated with the gut and faeces of the terrestrial isopod *P. pruinosus* that comprised: (1) common members of the soil BC with significance for the biogeochemical cycles, (2) bacterial symbionts, (3) bacteria possibly related to host metabolic/ecological features and, (4) bacterial etiological agents. The gut included fewer bacterial groups while faeces sustained more phylogenetically and presumably functionally divergent groups (that were not present inside the organism gut probably because they were all expelled via faeces or represent ingested transient bacteria). Both BCs were dominated by Proteobacteria. Similarities found between *P. pruinosus* BC composition and previous reports for other species, particularly those sharing ecological features (e.g., invertebrate detritivores), suggest that some bacterial groups may be conserved among taxa. These similarities support the use of *P. pruinosus* as organism model also when addressing the BC assembly as an additional ecotoxicological endpoint.

A surprising result of this work was the dominance of *Coxiella*. Despite previous reports of *Coxiella* infectivity in other terrestrial organisms, absence of such previous observation for isopods sustain that *Coxiella* presence in such high abundances possibly represent a link between the isopod-associated BC and the BC present in the surrounding environment (in this case, manure of infected cows and horses). This result also highlights the use of this isopod species, or other synanthropic isopod species, to be used in monitoring processes, providing insights on their previous exposure scenarios. Notwithstanding, future work is needed to further explore this possibility. Isopod BC must be viewed as a complex system capturing pressures and anticipating behavioural, reproductive, and/or phenotypic responses of the organism. Thus, the bacterial signature of terrestrial isopods might be of value as an early indicator of exposure effects, providing information on the "historical" exposure of organisms (i.e. soil contamination, anthropogenic stressors, infections, habitat climate change or other factors causing departures from bacterial dynamic equilibrium). So, more than just an enumeration of the bacteria present in the gut and faeces of *P. pruinosus* by a novel expensive and accurate method and comparison with other terrestrial species, the approach herein presented is extremely promising due to the possibility to capture the isopod BC overall response, to analyze the diversity of bacteria that might be involved in perturbance responses and to establish its ecological connections with the environmental conditions/stressors affecting both isopod species and its BC.

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7. Declarations

7.1. Funding

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7.2. Conflicts of interests

The authors declare that they have no conflict of interest. All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

7.3. Ethics approval

This paper does not contain any studies with human participants performed by any of the authors. All procedures performed in our studies involving the invertebrate animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

7.4. Clinical Trials Registration

Not applicable.

7.5. Consent to Participate (Ethics)

Not applicable.

7.6. Plant Reproducibility

Not applicable.

7.7. Data availability

Data supporting the results reported in this article can be found as supplementary data: Table S1.

7.8. Author Contributions

Susana Loureiro, Isabel Henriques and Jacinta M.M. Oliveira contributed to the study conception and design. Material preparation, data collection and analysis were performed by Jacinta M.M Oliveira. Rui G. Morgado and Sara Peixoto collaborated in sample collection and acclimatization. Daniel S. Read and Hyun S. Gweon helped with the generated sequencing data by performing statistical analyses. The first draft of the manuscript was written by Jacinta M.M. Oliveira and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. Isabel Henriques, Susana Loureiro, António Correia and Amadeu M.V.M. Soares provided scientific advice and were responsible for funding acquisition. No other person is entitled to authorship.

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Graphical Abstract