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To cite this article: M. Delhoumi , V. Catania , W. Zaabar , M. Tolone , P. Quatrini & M. S. Achouri (2020) The gut microbiota structure of the terrestrial isopod *Porcellionides pruinosus* (Isopoda: Oniscidea), The European Zoological Journal, 87:1, 357-368, DOI: [10.1080/24750263.2020.1781269](https://doi.org/10.1080/24750263.2020.1781269)

To link to this article: <https://doi.org/10.1080/24750263.2020.1781269>



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Published online: 14 Jul 2020.



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The gut microbiota structure of the terrestrial isopod *Porcellionides pruinosus* (Isopoda: Oniscidea)

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(Received 9 January 2020; accepted 1 June 2020)

Abstract

We used a combination of culture-dependent and independent approaches to study in depth the microbial community associated with the digestive tract of the terrestrial isopod *Porcellionides pruinosus* (Brandt, 1833). Specimens from different sampling sites in Tunisia harbored distinct microbiota profiles indicating the impact of both host origin and environmental factors on shaping the microbial flora within *P. pruinosus*. Our results revealed unexpected bacterial diversity especially via metagenomic analysis; a total of 819 operational taxonomic units (OTUs) assigned to two major bacterial phyla; *Proteobacteria* and *Bacteroidetes*. We used Nutrient Agar to isolate the cultivable fraction of bacteria associated with the gut of three geographically distant populations of *P. pruinosus*. The isolated bacteria belong to *Actinobacteria*, *Firmicutes* and *Proteobacteria*. Enrichment cultures on carboxymethylcellulose (CMC) medium gave evidence that the gut of this Oniscidea harbors cellulolytic *Firmicutes* and *Proteobacteria* probably involved in the lignocellulose degradation and then in mediating the functional role of terrestrial isopods as litter decomposers and regulators of nutrient cycling in soil ecosystems.

Keywords: *Oniscidea*, *intestinal flora*, *metagenomic analysis*, *cellulolytic bacteria*, *Hepaticola*

Introduction

Originating from an aquatic environment, terrestrial isopods (Isopoda: Oniscidea) have successfully colonized terrestrial habitats differently to other crustacean taxa. This colonization took place in the light of morphological, behavioral and ecophysiological adaptations (Schmalfuss 1984; Carefoot 1993; Hornung 2011) to the terrestrial environment.

Oniscidea represent a keystone actor for litter degradation in terrestrial ecosystems beyond their contribution to the nutrient cycling and the regulation of the soil microbial activity (Zimmer & Topp 1999; Zimmer 2002a; Crowther et al. 2015). They feed on decaying plant litter that mainly consists of recalcitrant polymers as lignin and cellulose characterized by a low nutrient

content. So, given the lack of the necessary enzymatic machinery for the degradation of these compounds, Oniscidea rely on symbiont-derived enzymes (Kukor & Martin 1986; Zimmer & Topp 1998; Zimmer 2002a). Recent studies showed that terrestrial isopods depend on numerous Carbohydrate Active EnZymes (CAZymes) affiliated to several families to degrade lignocellulose mainly composed by cellulose, lignin and hemicellulose. These enzymes were provided by the host and especially by its associated microbiota (Bredon et al. 2018, 2020).

In fact, the acquisition of bacterial symbionts seems to be a crucial step to facilitate isopods progression on land areas by acquiring new digestive capabilities especially the oxidative breakdown of phenolic compounds like lignin and tannin

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(Zimmer & Topp 1998) and the hydrolysis of cellulose (Zimmer 2002a). In addition, bacterial symbionts provide a high-quality nutritional source that allows isopods to overcome the dietary deficiencies of terrestrial leaf litter (Horváthová et al. 2016).

The digestive system of terrestrial isopods consists of a hindgut and a midgut or hepatopancreas where the secretion of digestive enzymes and the absorption of nutrients take place (Kostanjšek et al. 2002). It has been shown that the hepatopancreas can host two kinds of symbiont “*Candidatus Hepatoplasma crinochetorum*” (Mollicutes, hereafter “*Hepatoplasma*”) and “*Candidatus Hepatincola porcellionum*” (Rickettsiales, hereafter “*Hepatincola*”) (Wang et al. 2004a, 2004b). Despite the mechanism of mutual exclusion, these bacteria are introduced as mutualistic partners that enhance the nutritive utilization of the food sources of isopods (Zimmer 2002a). Fraune and Zimmer (2008) showed that *Hepatoplasma* improves the survival rate of Oniscidea under conditions of poor diet. Thus, the acquisition of the hepatopancreatic bacteria *Hepatoplasma* and *Hepatincola* is considered a potential evolutionary prerequisite for the terrestrial colonization by isopods (Bouchon et al. 2016).

Among Crustacea, Oniscidea are the most successful colonizers of terrestrial habitats. In contrast to several species of marine isopods which don't show microbiota in their digestive tract (Ray & Julian 1952; Besser et al. 2018), all the studied terrestrial species showed diverse and rich microbial communities in their digestive tract (Zimmer & Topp 1998; Kostanjšek et al. 2002; Zimmer 2002a, 2004; Dittmer et al. 2016; Horváthová et al. 2016; Bredon et al. 2020). A comparative study of the bacterial community associated with the hepatopancreas showed a clear difference between freshwater, semi-terrestrial and terrestrial isopods suggesting that the symbionts acquisition may occur in response to nutritional habits (Wang et al. 2007).

The terrestrial isopod *P. pruinosus* is a synanthropic cosmopolitan species that tolerates a wide range of environmental conditions (Loureiro et al. 2006). This species was used in bioremediation tests like pesticides degradation (Loureiro et al. 2002) and as a bioindicator species of soil contamination (Loureiro et al. 2005, 2009). In addition, many investigations were realized using genetic (Grandjean et al. 2005; Delhoumi et al. 2019a) and biochemical approaches (Achouri et al. 2012) to study the interpopulation polymorphism of *P. pruinosus*. Despite this, little is known about the gut microbiota structure, dynamic and role within this species.

This study aims to (1) investigate the bacterial community associated with the digestive tissues of *P. pruinosus* using a combination of culture-dependent and independent approaches, (2) isolate cellulolytic bacteria from the gut and (3) discuss the functional role of the host-associated microbiota and its contribution to the adaptation of isopods to their environment.

Materials and methods

Sampling

Adult specimens of *P. pruinosus* from Tunisia were manually collected from three geographically distant regions, Metlaoui, Regueb and Sousse in autumn 2016 (Figure 1). Samples from Sousse were collected from a grassland parcel. Animals were sampled from an agricultural area and a phosphate mine in Regueb and Metlaoui respectively (Table I). Before dissection, isopods were kept alive in plastic boxes under laboratory conditions (a temperature between 20 and 25°C and a daily photoperiod of 12 h) and fed on leaf litter from the sampling localities.

Bacterial counts and isolation from the digestive tissues

From each region, five guts of healthy adult isopods were extracted with fine-tipped sterile forceps (Butera et al. 2016), pooled and homogenized to be serially diluted in a sterile physiological solution and plated on Nutrient Agar (NA; Difco, Franklin Lakes, NJ) culture medium. After 72 h of incubation at 37°C, the colonies were counted and the mean number of bacteria per gut was determined. Single colonies of the most diluted NA plates were purified and characterized by colony morphology and microscopy and then subjected to gram staining. The isolates were stored in 20% glycerol at -20°C for further characterization.

Isolation of cellulolytic bacteria from the gut

In order to assess the presence of cellulolytic bacteria in the gut of *P. pruinosus*, enrichment cultures on carboxymethylcellulose substrate were prepared as described in (Butera et al. 2012). 200 µl of each pooled guts homogenate were suspended in enrichment culture flasks containing 20 ml of Medium 1 (Wenzel et al. 2002) enriched with 0.1 g of carboxymethylcellulose (CMC; Sigma).

After 4 weeks of aerobic incubation at 37°C on a rotary shaker, cultures were serially diluted and inoculated onto Petri dishes containing Medium 2 (Wenzel et al. 2002) supplemented with CMC.

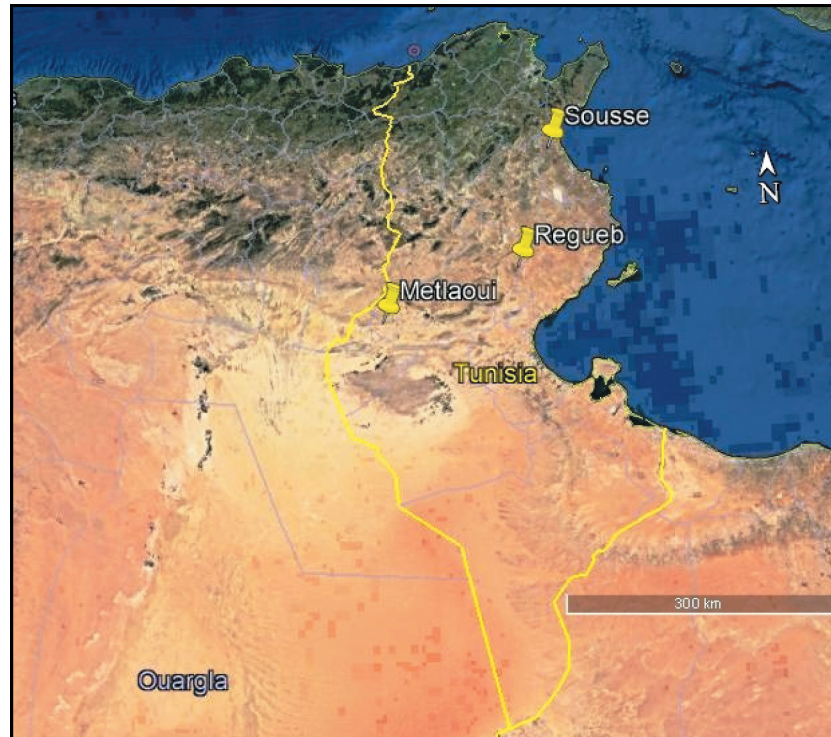


Figure 1. Localization of the sampling sites.

Table I. Characteristics of the sampling sites in Tunisia.

Sampling site	Latitude-Longitude	Type of sampling area	Climate
Sousse	36°02'28.6"N 10°19'31.5"E	Grassland	Semi-arid
Regueb	34°51'13.3"N 9°53'22.6"E	Agricultural parcel	Arid
Metlaoui	34°20'38.7"N 8°13'54.8"E	Phosphate mine	Arid

Plates were incubated at 37°C for 3–5 days, all single colonies of the most diluted plates were streaked to purity on fresh plates of the same medium and tested for cellulose degradation. The isolates were stored as described above for further characterization.

Congo red dye test

To test the ability of gut isolates and of isolates from the enrichment cultures to degrade cellulose, plates with Medium 2 supplemented with CMC were covered with a Congo red dye solution (Cacciari & Quatrini 2002; Delalibera et al. 2005). After dye removal, a clear zone around the colonies indicates the carboxymethylcellulose (CMC) degradation. The hydrolysis activity was determined by the calculation of the ratio of the

clearance zone diameter (mm) to the colony diameter (mm) (Huang et al. 2012).

Identification of the isolated strains

All isolates were grouped into Operational Taxonomic Units based on ITS length polymorphisms. The primers ITSf (5'-TCGTAACAAGGTA GCCGTA-3') and ITSReub (5'-GCCAAGGCA TCCACC-3') (Cardinale et al. 2004) were used to amplify the ribosomal 16 S-23 S rRNA gene intergenic spacer region (ITS) as described in (Catania et al. 2016). The resulting ITS patterns were used to assign the isolates to operational taxonomic units and the ribosomal 16 S rRNA gene of one representative isolate of each OTU was amplified with bacterial universal primers 27 f (5'-AGAGTTTGA TCCTGGCTCAG-3') and 1492 r (5'-TACG YTACCTTGTTACGACTT-3') (Frank et al.

2008) and then sequenced. The 16 S rDNA sequences were analyzed as described in Djahnit et al., (2019). Sequences were deposited in GenBank under accession numbers MH594510 to MH594526.

DNA extraction from digestive tissues

Adult isopods (both males and females) were surface-sterilized with 70% ethanol, washed twice with distilled water and surface dried; their guts were dissected and used freshly for DNA extraction (Butera et al. 2016). From each population, five pooled guts were used for total DNA extraction and purification using the QIAamp® DNA Stool Kit (QIAGEN, Venlo, The Netherlands) according to the manufacturer's instructions. DNA concentration and quality was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The extracted DNA was utilized for the metagenomic analysis of the microbiota.

Metagenomic sequencing of the 16 S rRNA

Procaryotic primers Pro 341 f and Pro 805 r were used to amplify the 16 S rRNA hypervariable region V3-V4 of procaryotes. A final volume of 25 µl of reaction mixture contained 10 ng of genomic DNA and 0.25 µM of each primer. The PCR program was: 98°C for 2 min followed by 35 cycles beginning at 65°C and ending at 55°C for 15 s and a final extension at 68°C for 30 s (Takahashi et al. 2014). Sequencing was performed on Miseq Illumina with 300 bp paired-end reads.

The sequencing reads were processed and analyzed using QIIME version 1.9 (Caporaso et al. 2010a). Reads of quality higher than 20, with a minimum number of consecutive high quality base calls to include a read of 0.95 as a fraction of the input read length were analyzed further. USEARCH61 algorithm (Edgar 2010) was used to detect chimeras using a taxonomy assignment based approach. The remaining sequences were clustered into OTUs using a 97% sequence identity threshold. A representative sequence set was formed by picking the most abundant from each OTU and aligned against the Greengenes core set database (DeSantis et al. 2006) (August 2013 version) by PyNAST (Caporaso et al. 2010b) with a minimum sequence length of 75% of the median input sequence length and a minimum identity of 75%. The Ribosomal Database Project (RDP) classifier program was used to assign taxonomy to the aligned sequences.

Results

Bacterial isolation from the gut of P. pruinus

The bacterial communities colonizing isopods guts sampled from three geographically distant regions in Tunisia (Metlaoui, Regueb and Sousse) (Figure 1) was analyzed by cultural methods. A total of 29 isolates were obtained on Nutrient agar medium from Metlaoui and Regueb regions, while no isolates were obtained from Sousse region. Counting of bacteria colonizing isopod gut showed an abundance ranging between 1 and 5×10^5 CFU/gut of *P. pruinus*. Four isolates were obtained from CMC enrichments. Both NA isolates and CMC isolates were grouped in twenty-one operational taxonomic units (OTUs) by ribosomal 16 S-23 S intergenic spacer region analysis. The representative isolates of each OTU were affiliated to the 3 phyla: *Actinobacteria*, *Firmicutes* and *Proteobacteria*. The bacterial community isolated from the gut of specimens from Metlaoui showed the highest bacterial diversity, while that of isopods sampled from Regueb was represented only by *Actinobacteria* (Table II). The NA isolates were affiliated to *Klebsiella*, *Bordetella*, *Microbacterium*, *Staphylococcus*, *Promicromonospora*, *Cellulosimicrobium*, *Streptomyces* and *Brachybacterium* genera.

Cellulolytic isolates in the gut of P. pruinus

The degrading cellulose activity was defined as the ratio between the clearance zone diameter and the colony diameter on CMC plates. 51% of the NA isolates were positive to Congo red test with a ratio fluctuating between 1 and 5.6. This ratio ranged between 1 and 3.3 for the carboxymethylcellulose enrichment culture isolates that were 100% positive to Congo red test. The 16 S rDNA sequencing analysis exhibited 99–100% of nucleotide homology with bacterial strains available in the NCBI database which belong to *Firmicutes* and *Proteobacteria* (Table II).

Metagenomic analysis of the microbiota associated with P. pruinus

A total of 69,233 raw reads (30,170; 15,888 and 23,175 for Metlaoui, Regueb and Sousse samples respectively) were processed using QIIME. A final number of 57,847 reads were grouped into 819 OTUs at 97% of similarity (Table III).

Unsurprisingly, *Proteobacteria* phylum was unique in guts of *P. pruinus* from Sousse and Regueb and predominant from Metlaoui samples (Figure 2(a)). The *Proteobacteria* phylum was dominated by

Table II. Phylogenetic identification of intestinal bacteria isolated from *P. pruinosus* by direct plating on Nutrient Agar (NA) and enrichment on carboxymethylcellulose.

Site	Media	Sequenced isolate	Phylogeny (RDPII) ^a				Blast results			
			Phylum	Class	Family	OTU	No. of isolates in the same OTU	Most closely related sequence	Id. %	Acc. No.
Metlaoui	NA	MET9	Proteobacteria	Gammaproteobacteria	Enterobacteriaceae	1	<i>Klebsiella aerogenes</i> strain AR 0161	99%	CP028951.1	0
	CMC enrichment	MET 1			Xanthomonadaceae	1	<i>Stenotrophomonas rhizophila</i> strain e-p10	100%	NR_121739.1	1
	NA	MET10	Actinobacteria	Betaproteobacteria	Alcaligenaceae	1	<i>Bordetella</i> sp. 2-12	99%	FJ598328.1	1
	NA	MET1	Actinobacteria	Actinobacteria	Microbacteriaceae	5	<i>Microbacterium</i> sp. LHR-08	99%	HE716934.1	3
	NA	MET16				1	<i>Microbacterium</i> sp. strain PYRL23	100%	MF458882.1	0
	NA	MET8				1	<i>Microbacterium</i> MRS84B1 L3 2E	100%	KP789118.1	0
	NA	MET22	Firmicutes	Bacilli	Staphylococcaceae	1	<i>Staphylococcus swarneri</i> strain HBUM06959	100%	MF662231.1	0
	NA	MET17				1	<i>Staphylococcus petrasii</i> subsp. pragensis strain CCM 8529	94%	NR_136463.1	0
	NA	MET18				1	<i>Staphylococcus pasteurii</i> strain ATCC 51129	99%	NR_024669.1	0
	NA	MET23				3	Uncultured bacterium clone MD-178-10-3292_KP6-2 N-P_S150_01B03	99%	QJ816867.1	0
Regueb	NA	REG1	Actinobacteria	Actinobacteria	Microbacteriaceae	1	<i>Microbacterium</i> sp. strain YJYP303	100%	MF084211.1	0
	NA	REG9				1	<i>Microbacterium arthrosphaerae</i> strain CC-VM-Y	100%	NR_117046.1	0
	NA	REG5			Promicromonosporaceae	4	<i>Promicromonospora</i> sp. VP 111	99%	KJ024374.1	5.6
	NA	REG12				3	<i>Cellulosimicrobium cellulans</i> strain PSBB019	100%	CP021383.1	4
	NA	REG8			Streptomycetaceae	1	<i>Streptomyces</i> sp. SC5IO11863	95%	KC904267.1	4
	NA	REG13				1	<i>Streptomyces tacrolimicus</i> strain ATCC 55098	100%	NR_116991.2	3
	NA	REG3			Dermabacteraceae	3	<i>Brachybacterium paraconglomeratum</i> strain IC B5.2	99%	KY775505.1	0
	CMC enrichment	REG 1	Firmicutes	Bacilli	Bacillaceae	1	<i>Bacillus</i> sp. 210_48	100%	GQ199750.1	3.3
	CMC enrichment	REG 3	Proteobacteria	Gammaproteobacteria	Pseudomonadaceae	1	<i>Bacillus cereus</i> strain L-05	100%	KJ534398.1	2.75
	CMC enrichment	SOU 1				1	<i>Pseudomonas</i> sp. strain LDB3	99%	KX245362.1	2.5

^aPhylogeny was obtained using the Ribosomal Database Project (RDP) naive Bayesian rRNA classifier, version 2.5, May 2012.

^bCellulolytic activity expressed as the ratio of the zone of clearance diameter to the colony diameter.

Table III. Sequencing information obtained from metagenomic sequencing of the digestive tract of isopod sampled at three out of the four sites of study.

Sample	No. of raw sequences	Filtered for q19 e p095	Chimeras	Post filter
Metlaoui	30170	10554	52	19564
Regueb	15888	359	22	15507
Sousse	23175	385	14	22776
Total				57847

representatives of *Alphaproteobacteria* and *Gammaproteobacteria* in the microbiota profile of three samples. However *Bacteroidetes* phylum was represented by members of *Flavobacteria* (Figure 2(b)).

Apart from classical bacteria widely associated with arthropods like *Wolbachia* (15% and 1% in Metlaoui

and Regueb samples respectively) and *Serratia* (10.4% and 1% in Metlaoui and Sousse samples profiles), the metagenomic analysis revealed the presence of a large spectrum of environmental bacteria (Figure 2(b)). About genera, *Halomonas*, *Microbulbifer* and *Wolbachia* were the most shared between the three samples. *Halomonas* represented 3%, 1.1% and 1.1% of the bacterial community associated with digestive tracts of samples from Metlaoui, Regueb and Sousse respectively. However, *Microbulbifer* represents 13% and 18% of the microbiota of samples from Metlaoui and Sousse respectively.

In order to identify the unassigned Alphaproteo bacteria sequences, a representative sequence was analyzed using BLAST 2.7.1 (Zhang et al. 2000; Morgulis et al. 2008). With a query cover of 99% and identity of 98% a hepatopancreatic symbiont of *Porcellio scaber*

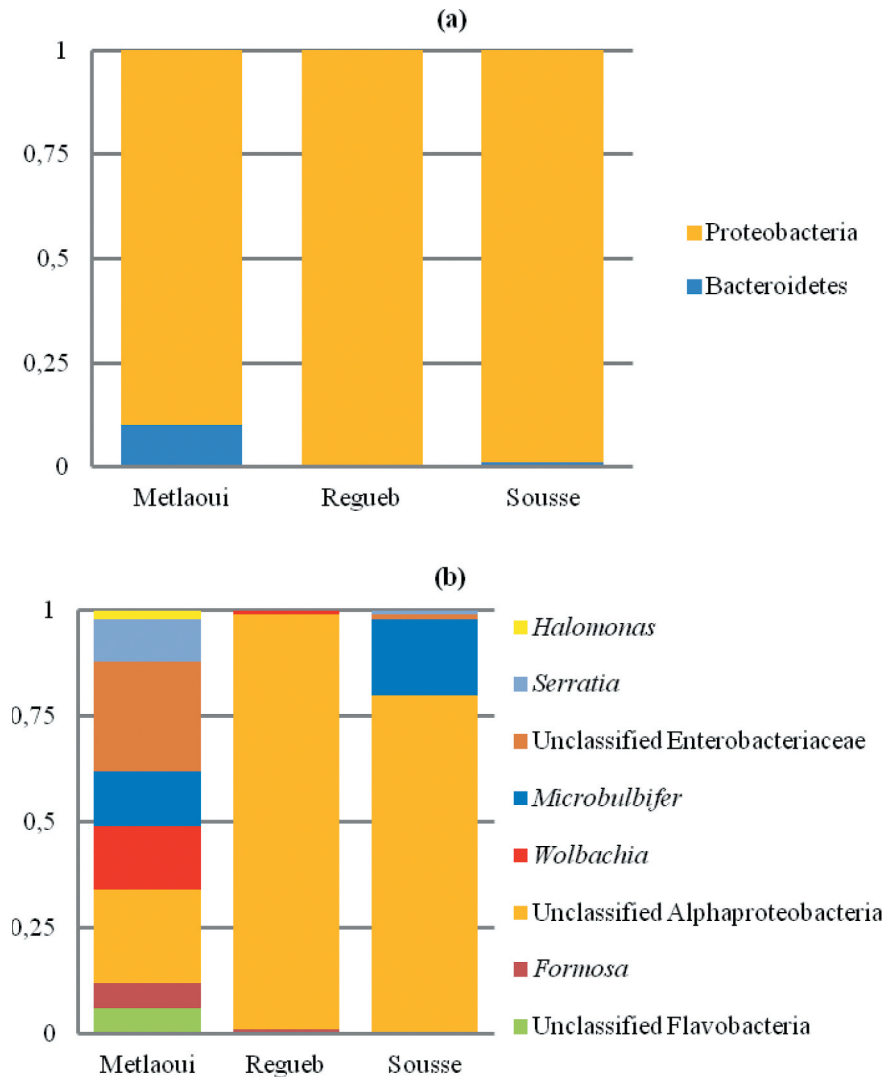


Figure 2. Relative abundance of bacterial groups at the phylum level (a) and most represented genera (b) within the digestive tract of *P. pruinosis*.

clone 1 was identified (*Candidatus* *Hepatincola porcellionum*” (Rickettsiales, Alphaproteobacteria) Accession number AY189806.1) in the sample of Regueb region.

Discussion

In order to deepen our insight into the gut microbiota structure within the terrestrial isopod *P. pruinosus* and its cellulolytic activity, we used a combination of cultivation-dependent and independent approaches. The cultivable fraction of bacteria was targeted by direct isolation on NA and after enrichment culture on CMC.

Herein, the isolated bacteria belong to *Proteobacteria*, *Actinobacteria* and *Firmicutes* phyla which are usually present in the arthropod microbiota such as insects (Huang et al. 2012; Vargas-Asensio et al. 2014; Dantur et al. 2015) (Table II).

The most represented genera among the NA media isolates from *P. pruinosus* are *Microbacterium*, *Streptomyces* and *Staphylococcus*. It has been suggested that, beyond carbohydrate degradation, *Microbacterium* could play other roles in the host intestine (Vargas-Asensio et al. 2014). Members of *Streptomyces* genus, isolated from the gut of Regueb samples, were already isolated from a wide spectrum of arthropods where they contribute efficiently to the degradation of phenolic components like lignin and cellulose in addition to the production of secondary metabolites (Bibb 2005; Vargas-Asensio et al. 2014). *Promicromonospora* sp., isolated also from the same sample, showed the highest cellulolytic activity. It has been mentioned that this cellulolytic bacterium was isolated from the gut of the scarab beetle *Pachmoda marginata* where it was involved in the degradation of plant polymers (Cazemier et al. 2003).

All NA media isolates from the Regueb sample belong to *Actinobacteria* phylum. In fact, representatives of *Actinobacteria* are known to adjust physiological and metabolic equilibrium by production of extracellular enzymes and secondary metabolites (Schrempf 2001). However, representatives of *Actinobacteria*, *Proteobacteria* (*Klebsiella* and *Bordetella*) and *Firmicutes* (*Staphylococcus*) characterize the isolates from the Metlaoui sample. Members of *Staphylococcus* were previously detected in the gut wall of *P. scaber* (Kostanjšek et al. 2002; Lapanje et al. 2010) and other arthropod taxa (Zhang et al. 2014).

The lack of isolated bacteria from Sousse samples on NA media and the low number of isolated cellulolytic bacteria in this study can be ascribed to the

difficulty of isolating and cultivating a wide spectrum of bacteria (Park et al. 2007).

Three of the five strains isolated from the enrichment cultures on CMC shared 99 to 100% of similarity with species from the genus *Bacillus* widely associated with the digestive tract of invertebrates. *Bacillus cereus* group constitute true residents symbionts of the digestive tissues of terrestrial arthropods among which the Oniscidean species *P. scaber* (Swiecicka & Mahillon 2006). Indeed, it has been ensured that *Bacillus* genus produces large quantities of extracellular enzymes involved in the cellulose degradation and then litter breakdown (Gupta & Verma 2015).

Bacteria of the genus *Pseudomonas*, isolated from the Sousse samples, had long been associated with the hepatopancreas of Oniscidea where they are introduced as a primary specific endosymbiont with a probable role in the degradation of cellulose and phenol (Bouchon et al. 2016). In accordance with our findings, members of *Pseudomonas* and *Bacillus* genera were previously detected in the hindgut of other species of terrestrial isopods like *P. scaber* (Kostanjšek et al. 2002; Lapanje et al. 2010; Horváthová et al. 2016), and *Armadillidium vulgare* (Dittmer et al. 2016). In order to quantify the capacity of isolates to degrade cellulose we utilized the test Congo red. This test established that isolated bacteria belonging to the *Bacillus* (*Bacillus cereus*, *Bacillus wiedmannii* and *Bacillus* sp) and *Pseudomonas* genera exhibited a cellulolytic degrading activity suggesting a close link between these gut colonizers and the digestion process within *P. pruinosus*.

Microbacterium, *Staphylococcus* and *Bacillus*, resistant bacteria to contamination soil, were the most isolated from the gut of Metlaoui sample collected from a phosphate mine. Thus, they are potentially involved in the resistance of *P. pruinosus* to the contamination induced by the production of phosphogypsum. Consistently with our findings, *Bacillus*, *Pseudomonas*, *Klebsiella* *Microbacterium*, *Staphylococcus*, *Cellulosimicrobium* and other members of *Actinobacteria* were the most isolated bacteria from the gut of Oniscidean species *P. scaber* sampled from a polluted area. These bacteria are introduced as resistant microorganisms to mercury pollution and may be involved in the physiological resilience of their host against contaminants (Lapanje et al. 2010). In the same manner, it appears that the isolated bacteria from the gut of *P. pruinosus* from Regueb were closely related to the inhabiting environment marked by agricultural practices. Indeed, *Cellulosimicrobium* and *Streptomyces* genera have been detected in agricultural

soil and plants where they are used as growth promoters and biofertilizers in several crops (Kenzaka et al. 2017; Vurukonda et al. 2018). We can ensure through the culture dependant approach that the intestinal bacteria aid isopods to adapt to their specific environment (contaminant area and agricultural environment for the samples of Metlaoui and Regueb respectively). Furthermore, we found that the digestive tissues of *P. pruinosus* harbor cellulolytic bacteria involved in the breakdown of cellulose and potentially in the degradation of terrestrial leaf litter mainly composed of lignocelluloses. The fact that the microbiota associated with the digestive tissues of *P. pruinosus* includes cellulolytic bacteria producing enzymes involved in the degradation of cellulose makes this species useful in industrial processing of lignocellulose and then biofuel production from biomasses as suggested within plant feeding animals like terrestrial isopods (Bredon et al. 2020) and lepidoptera (Dantur et al. 2015).

The sequencing analysis revealed that intestinal isolated bacteria from *P. pruinosus* belong mainly to *Proteobacteria*, *Actinobacteria* and *Firmicutes* phyla. These phyla dominate the gut microbiota of several invertebrates where they play symbiotic functions like the nitrogen fixation, denitrification, carbohydrate degradation, detoxification, apart from defensive trend against pathogens (Schloss et al. 2006; Shao et al. 2014). Along the same line, Bredon et al. (2018) showed that *Proteobacteria*, *Actinobacteria* phyla characterize the composition of the bacterial community associated with the degradation of lignocelluloses within the terrestrial isopod *A. vulgare*.

The NGS analysis showed the presence of only two bacterial phyla (*Proteobacteria* and *Bacteroidetes*) in comparison to 19 and 20 phyla identified within the Oniscidea species *A. vulgare* (Dittmer et al. 2016) and *P. scaber* (Horváthová et al. 2016) respectively.

On the other hand, the screening of the bacterial community involved in the breakdown of lignocellulose revealed the presence of four phyla within *P. pruinosus*, five phyla in *A. vulgare*, four phyla in *Porcellio dilatatus dilatatus* and *Porcellio dilatatus petiti* respectively and three phyla within the freshwater isopod *Asellus aquaticus* (Bredon et al. 2020). The same study showed that *Proteobacteria* and *Bacteroidetes* phyla dominate the microbiota of the five studied species especially *P. pruinosus* where they encode the most part of lignocellulose degrading CAZymes. In the present study, the three screened samples of *P. pruinosus* exhibited a high bacterial diversity comparable to that found in other litter decomposers like termites (Hongoh

2010; Brune 2014), as demonstrated with the species *A. vulgare* (Dittmer et al. 2016), and in freshwater crustaceans like *Daphnia* (Qi et al. 2009).

As the most of the isolated bacteria were from the sample of Metlaoui, the metagenomic analysis demonstrated that the highest number of raw sequences was identified within samples from the same site. Herein, the high salinity induced particularly by the phosphogypsum dissolution (Yermani et al. 2003) may explain the presence of halotolerant bacteria like *Formosa*, *Microbulbifer* and *Halomonas*. *Formosa*, polysaccharides degrading bacteria, can be found in various marine habitats rich in organic matter in association with invertebrates (Riedel et al. 2013). Likewise, *Microbulbifer* representatives are salt-dependent bacteria involved in the breakdown of cellulose and xylan (Yoon et al. 2007).

The difference of microbiota structure between Metlaoui, Regueb and Sousse samples can be ascribed to both host diet and environmental factors. In accordance, it has been argued that the host origin is the most important factor shaping the microbiota composition in Oniscidea (Dittmer et al. 2016; Delhoumi et al. 2018). Indeed, the microbiome reacts more rapidly than its host to environmental variability which aids it to evolve and conquer new niches (Bredon et al. 2019, 2020). Moreover, several studies showed that environmental bacteria are involved in Oniscidea performances like reproductive success, growth rate, survival and fertility (Zimmer 2002b; Horváthová et al. 2015; Delhoumi et al. 2019b) given their high nutritive value (Zimmer & Topp 1998). With the same manner, Horváthová et al. (2016) demonstrated the key role the external microbes forming a biofilm on the growth of the isopod *P. scaber*; individuals fed on the a diet source supplemented with a large amount of biofilm gained more mass in comparison with those feeding on a diet source with a marginal biofilm. In line with this interaction (isopod-symbiont), Des Marteaux et al. (2020) found that *P. scaber* and *O. Asellus* modify the microbial abundance and bacterial community structure in the leaf litter which alter the decomposition process.

Interestingly, we evidenced for the first time the presence of *Hepatincola* in the microbiota of *P. pruinosus*. Effectively, the hepatopancreatic symbiont in *P. scaber* *Candidatus* *Hepatincola* porcellionum (Rickettsiales) was identified in the gut of Regueb sample. *Hepatincola* has been detected only in species of the Crinocheta group (*Oniscus asellus*, *Philoscia muscorum*, *P. scaber* and *A. vulgare*) (Fraune & Zimmer 2008; Dittmer et al. 2016). Despite its negative effect on host longevity, it has been suggested

that *Hepatincola* as a hepatopancreatic symbiont is mainly involved in the provisioning of cellulolytic enzymes necessary for the breakdown of lignin and tannins (Zimmer & Topp 1998) and then the adaptation to plant material the typical nutritional source in the terrestrial environment. Hence, despite its importance in the evolutionary progression of terrestrial isopods on land, this bacterium showed a low prevalence in some populations of Oniscidea and this may refer to its environmental transmission (Wang et al. 2007). In the context of terrestrialization process and the importance of intestinal flora, it has been showed that the major part of lignocellulose degrading CAZymes in terrestrial isopods are provided by the microbiota which enhances the food utilization and then the adaptation to their environment (Bredon et al. 2020). So, the complementarity and the diversity of the host's enzymes repertoire and that of their microbiota allow Oniscidea to utilize several kind of plant litter and subsequently to occupy new ecological niches on land. However in marine species, data ensured the endogenous origin of enzymes involved in the breakdown of lignocellulose (King et al. 2010; Besser et al. 2018).

The metagenomic analysis of the microbiota associated with *P. pruinosus* revealed an important fraction of environmental bacteria acquired usually via ingested nutrients and feces. In fact, it's well known that terrestrial isopods ingest bacteria colonizing the plant material and their own feces in order to provide microbial enzymes necessary for digestion (Zimmer & Topp 1998; Zimmer 2002a) and to meet their feeding requirements on the other side (Delhoumi et al. 2019b) given the low nutritive quality of terrestrial leaf litter. The combined use of cultivation-dependent and independent approaches established that environmental bacteria may constitute a source of variability between populations of *P. pruinosus* since the structure of the associated microbiota changes depending on the environment and the origin of the isopod.

Conclusion

The culture-dependent approach allowed the identification of cellulolytic bacteria involved in the breakdown of cellulose within *P. pruinosus* and potentially in the degradation of terrestrial leaf litter. Metagenomic analysis revealed a high bacterial diversity with a variable structure depending on the host origin. Hence, apart from reproductive manipulators (*Wolbachia*, *Serratia* ...) and cellulolytic bacteria involved in the digestive process, the microbial

flora associated with the digestive tissues of *P. pruinosus* includes a wide spectrum of environmentally transmitted bacteria with unknown function. A major finding of this study is the detection of the hepatopancreatic symbiont *Hepatincola* that may mediate the role of Oniscidea in the ecosystem functioning. Further researches on other isopod species are required in order to get a complete image on the microbiota and to advance our knowledge on its role on the land colonization by Oniscidea.

Acknowledgements

The authors express their sincere thanks to the Editor and anonymous referees for their valuable comments, corrections and language assistance.





Funding

This work was supported by the Université de Tunis El Manar (Tunis) and partially by the Università degli Studi di Palermo (Italy).

Disclosure statement

No potential conflict of interest was reported by the authors.

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