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Prokaryotic microbiota in the digestive cavity of the jellyfish *Cotylorhiza tuberculata*

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ABSTRACT

The microbiota associated to the gastric cavity of four exemplars of the jellyfish *Cotylorhiza tuberculata* has been studied by means of cultured-dependent and -independent methods. The pyrosequencing approach rendered a very reduced diversity of *Bacteria* with four major groups shared by the four exemplars that made up to 95% of the total diversity. The culturing approach recovered low abundant organisms and some of them also detected by the pyrosequencing approach. The major key organisms were related to the genera *Spiroplasma*, *Thalassospira*, *Tenacibaculum* (from the pyrosequencing data), and *Vibrio* (from the cultivable fraction). Altogether the results indicate that *C. tuberculata* harbors an associated microbiota of very reduced diversity. On the other hand, some of the major key players may be potential pathogens and the host may serve as dispersal mechanism.

Introduction

Cotylorhiza tuberculata is a scyphozoan of the phylum *Cnidaria*, often occurring in massive blooms in the Mediterranean waters, that follows an annual life-span cycle [36]. The medusae pelagic stage occurs at the late summer after abrupt temperature increases promoting blooms that may generate tons of biomass in relatively small seawater volumes. The life span of this species in the stage of pelagic jellyfish is one year [20,36]. Practically, all the population of adult *C. tuberculata* disappears during late autumn – beginning of winter, a fact that cannot be explained just by mortality due to physical damage related to boats, or other human activities. The cause that triggers this mass mortality is unknown but has further consequences due to the release of organic and inorganic components [35] and the activation of bacterial degradation and planktonic microbial community shifts [10,46,47].

Outbreaks of jellyfishes as *C. tuberculata* among others, have important consequences on tourism and fisheries [32], as well as some of them have been reported as carriers of fish pathogens [8]. Despite its relevance for human health, extensive aquiculture and touristic economies, not much is known on the lifestyle of jellyfishes, nor about the microbiota associated to them and its relevance. Some studies on molecular microbial ecology have been done on ctenophores [7,17], and on specific detection of fish pathogens in cnidarians [8,11,12]. However, exhaustive culture-dependent and -independent studies to reveal the associated microbiome, its diversity, relevance and potential use as indicators are yet to be reported.

The present study represents the first (to our knowledge) study on the microbiome of members of the class *Scyphozoa* by means of molecular microbial ecology tools independent and dependent of culture approaches. We focused the studies in the gastric cavity of the jellyfish *C. tuberculata*. This medusa feeds on microplanctonic organisms, which enter into the stomach through the numerous mouth arm openings, and remains of *Ciliata*, *Crustacea* and *Gastropoda* had been found among the particulate food items [20]. The gastric cavity is tightly connected with the surrounding waters, and thus a source of microorganisms' exchange that may constitute an effective dispersal mechanism, and a source of biological indicators. To this purpose, we combined high quality pyrosequencing (long nucleotide sequences and large amounts of reads; [27]) with culturing of aerobic heterotrophs to reveal the microbial composition of the gastric cavity. The identification approach was based on the recognition of the Operational Phylogenetic Units (OPUs)

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that rely on phylogenetic inferences rather than sequence identity clustering, to better reflect the diversity observed [27,50].

Materials and methods

Sampling and processing

On September 2013 four exemplars of adults *C. tuberculata* (M1, M2, M3 and M4) were caught with a landing net. The sampling site was located in the Alcudia Bay, in the north of the Island of Mallorca, at about 0.5 miles from the shore (39°45′00″N; 13′10″E). The four exemplars were closely swimming in an area of about 10 m^2, and about 0.5 m below the surface. The sampling site is considered as one of the most pristine areas at the shore of Mallorca given the high water circulation rates [41]. The four exemplars M1 (1.75 kg; male), M2 (1.5 kg; male), M3 (2 kg; female) and M4 (1 kg; male) were dissected onboard with sterile scalpel and the material of the digestive cavity was collected with sterile syringes and kept on ice in 50 ml sterile Falcon tubes. Samples were transferred to the laboratory (about 2 h later) and the material used for culturing purposes was mixed with 20% (v/v) glycerol (1:1) and stored at −80 °C.

Microbial DNA extraction, PCR amplification and pyrosequencing

The DNA was obtained from either direct extraction of the gastric content (total biomass, BT, generating the samples M1BT, M2BT, M3BT and M4BT) by sizing with scalpel the gastric filaments, or from a previous separation of tissue material (BF, generating the samples M1BF, M2BF, M3BF and M4BF). The latter was prepared by mixing 300 μl of the gastric filaments with 500 μl of PBS 4× (548 mM NaCl, 10.8 mM KCl, 40 mM Na_2HPO_4, 7.2 mM KH_2PO_4). The sample was gently broken up with a sterile mortar in a 2 ml microtube, followed by a centrifugation (10 min, 5400 × *g*, 4 °C). The supernatant was stored in a sterile tube, and the pellet was washed 8× with 500 μl of PBS 4×, collecting the supernatants. Finally the pooled supernatants were centrifuged (10 min, 15,700 × *g*, 4 °C) and the pellet was used for further DNA extraction. Both sorts of raw material were extracted according to previously published protocols [48]. PCR reaction was performed in a volume of 50 μl using the Master Mix (5 PRIME GmbH, Deutschland) following the manufacturer's instructions. 16S rRNA genes were amplified using the specific primer pairs GM3 and S for *Bacteria* and 21F and 1492R *for Archaea* (Supplementary Table S1). PCR amplifications were performed as previously published using 30 cycles at 53 °C (Melting temperature; [21]). A second short PCR (five cycles) was performed in a final volume of 25 μl in triplicate to incorporate tags and linker into the amplicon using 1:25 dilution of the original products as templates, and also using the same protocol that for the first PCR, primers GM3-PS and a variant of 907-PS (Supplementary Table S1). The PCR products were purified and sequenced as previously published [27]. The set of sequences has been deposited at the ENA sequence repository under the study project accession number PRJEB8518.

Sequence trimming, Operational Taxonomic Unit (OTU) clustering and OPU design

Sequences with <300 nucleotides were removed, and low-quality sequences were trimmed with a window size of 25 and average quality score of 25. No ambiguities and mismatches in reads with primer pairs and barcodes were allowed. Chimeras were removed with the application Chimera Uchime implemented in Mothur. The trimming process was performed using Mothur software [40]. The adequate selected sequences were clustered in OTUs at 99% using the UCLUST tool in QIIME [6]. We consider one OTU each unique cluster of sequences with identities ≥99%. The longest read of each OTU was selected as representative, and was introduced to the non-redundant SILVA REF115 database using the ARB software package [25]. Sequences were aligned with the SINA aligner [37], using LTPs115 database [54]. The OPU design by phylogenetic inference was performed as previously described [27,50].

Growth media, plating and isolation

In all cases, a surface-spread plating method was used to isolate aerobic heterotrophic prokaryotes. One milliliter of homogenized gastric material was used to prepare the serial dilutions (until 10^{-4}) in PBS 4×. All samples and their respective dilutions were plated in duplicate in four different media: Seawater (SW; [39]) at 5% and 10% salts, both with 0.05% yeast extract (YE, Cultimed, Scharlab); R2A agar (suplemented with NaCl 3.5% o 0.6 M, Scharlab), and Marine Broth (0.5% peptone, 0.1% yeast extract, seawater and 1.6% agar). Plates were incubated at room temperature (22 °C), and monitored for at least two months. Colonies were selected taking into account different size, form and color to obtain the largest diversity possible making a mean of 48 ± 7 for each sample and culture media. Selected colonies were brought to pure culture by re-streaking them on their respective solid media ensuring the recovery of a pure culture for each. For storage purposes, individual isolates were grown in their respective liquid medium, and the grown suspensions were mixed (1.5:1) with 50% (v/v) glycerol and stored at −80 °C.

MALDI-TOF MS analyses and identification by 16S rRNA gene sequencing

The initial screening of the isolated strains was carried out with MALDI-TOF MS using whole cell biomass as previously published [50]. Each single similarity cluster in the dendrogram was regarded as an Operational Taxonomic Unit (OTU), and was the basis for further identification. The almost complete 16S rRNA gene sequences were obtained for one or two isolates of each OTU following the protocols previously published [50]. The phylogenetic analysis was performed using the ARB software package [25]. Newly added sequences were aligned using the LTPs115 database as template [54], with SINA aligner [37], but the final alignment was manually improved. Tree reconstructions were performed using the Neighbor-Joining method with the Jukes–Cantor correction with the filter of 30%. For it, 750 high quality-supporting sequences from the LTP [54] were incorporated to stabilize the tree topologies and obtain the final tree. OPUs were circumscribed based on the visual inspection of the tree [50]. The sequences have been deposited at the EMBL repository under the accession numbers (LN812982–LN813005).

Statistical analyses

Presence or absence of isolates detected for each OTU was coded as binary matrix and imported into the statistical program. The statistical significance of the differences among samples (for both culture dependent and independent approaches) was tested using a PERMANOVA analysis (*Permutational Multivariate Analysis of Variance Using Distance Matrices;* [3]) using the Adonis function as implemented in the *Vegan* [31] package of R v 3.1.1 (www.r-project.org). Ecological indexes (Shannon, Dominance and Good's coverage) and rarefaction curves were calculated using the PAST software version 3.01 [16].

Results

The prokaryotic diversity thriving in the samples analyzed was amplified using bacterial and archaeal primers. No amplicon

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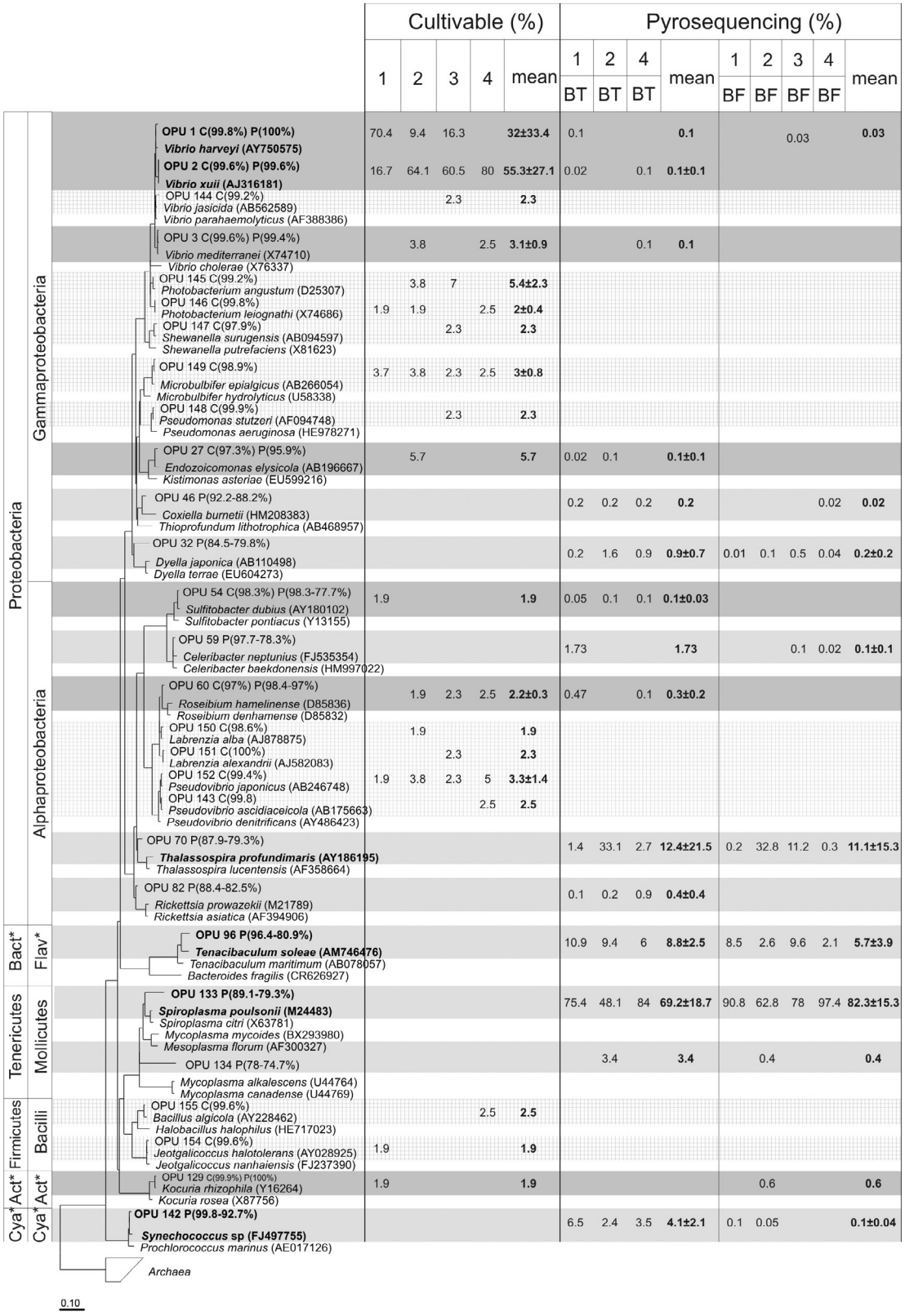

Figure 1. Compilation of the results observed regarding the major groups detected by the pyrosequencing and cultivation approaches. The left column shows the phylogenetic reconstruction of the representative sequences of each OPU detected and their affiliation with the closest relative type strains present in the LTP 119 [54]. Each OPU shows in brackets the identity value of the selected sequence and the closest relative reference sequence. Right columns indicate the occurrence (i.e. relative abundances in percentage of the total of each dataset) of each OPU in each of the different exemplars (1–4) studied here. In dark-gray background are indicated the OPUs occurring in both fractions (pyrosequences and cultures); in squared background those only detected by culture; in light-gray background the sequences only detected by the pyrotagging approach. In bold are highlighted the major groups detected by either pyrotagging or culturing.

Table 1
Total number of sequences obtained by pyrosequencing, after trimming and number of OTUs (clustered at 99% identity) and OPUs observed.

	Total	M1BT	M1BF	M2BT	M2BF	M3BF	M4BT	M4BF
Total seq.	59,117	18,533	7866	3241	2421	4035	17,420	5601
Trimmed seq.	50,779 (85.9%)	16,073 (86.7%)	6696 (85.1%)	2668 (82.3%)	1935 (79.9%)	3171 (78.6%)	15,287 (87.7%)	4949 (88.3%)
OTUs (99%)	882	344	83	93	68	27	202	65
OPUs	143	97	20	34	16	10	50	11

was produced with the archaeal primers in any of the samples studied. On the other hand, all samples amplified with the bacterial primers. The pyrosequencing approach generated a total of 59,117 sequences for a total of seven samples, with a size mean of 568 ± 159 nucleotides, and with 12,694 sequences (25%) >700 nucleotides. Unfortunately, we did not get amplification from the M3BT biomass due to unknown reasons. However, as it is reported below, the diversity PERMANOVA analyses did not indicate that the two fractions (BT, total biomass; or BF, bacterial fraction collected by centrifugation and tissue discarding) were significantly different ($p = 0.5$). The sequencing approach of all eight DNA samples of the four exemplars rendered between 18,533 (for M1BT) and 2421 (for M2BF) sequences with a median value of 5601 (for M4BF) sequences. After trimming, the number sequences for the study was reduced to about 78.6% to 88.3% in all samples (Table 1). The clustering approach into OTUs using the identity threshold of 99% sequence identity showed that among all samples we could recognize a total of 882 OTUs, and these ranged between 93 and 344 for the BT samples and between 27 and 83 for the BF samples. Finally, the representative sequence of each OTU was inserted into a reference tree by phylogenetic inference and we could recognize a total of 143 distinct OPUs (Fig. 1 and Supplementary Figure S1 and Table S2) that made the complete diversity observed here. The number of OPUs, or putatively distinct species, observed was always higher for the BT samples (between 34 and 97) than for the BF samples (between 10 and 20). On the other hand, 88 OPUs were detected in single samples including 35 OPUs made of singletons and 18 OPUs made of doubletons.

Ecological indexes (Supplementary Table S3) for the nonculturable data showed high dominance in most samples with values higher than 0.5 with exception of M2BT. The diversity index (H') ranged between 0.14 and 1.36. Richness estimation (Chao-1) and rarefaction curves (Supplementary Figure S3) showed that BT samples exhibited higher coverage reflected in a higher richness (Chao-1 = 51.8–117.2) in comparison with BF (Chao-1 = 14–27.2). On the other hand, culturable data presented similar diversity (0.9–1.4) as pyrosequencing, the dominance was slightly lower (0.4–0.7). In all cases, the jellyfish M4 showed the highest dominance and lower diversity values. Chao-1 in culturable fraction was considerably lower (10.6–31), and the rarefaction curves showed non-saturation.

Altogether the results pointed to a reduced diverse community of microorganisms in where four major groups of sequences made up to at least 95% of the total diversity. The major groups observed in decreasing abundances OPU 133 (affiliating with the genus *Spiroplasma;* summing about 69.2% ± 18.8 for BT or 82.3% ± 15.3 for BF), OPU 70 (affiliating with the genus *Thalassospira;* summing about 12.4% ± 21.5 for BT or 11.1% ± 15.3 for BF), OPU 96 (affiliating with *Tenacibaculum soleae;* summing about 8.8% ± 2.5 for BT or 5.7% ± 3.9 for BF), and OPU 142 mainly detected in the BT samples (affiliating with the genus *Synechococcus;* summing about 4.1% ± 2.1 for BT or 0.1% ± 0.04 for BF). Additionally, OPU 32 (*Dyella japonica*) was also present in all samples, but the abundances of this OPU were much lower (0.9 ± 0.7 for BT or 0.2 ± 0.2 for BF). The remaining OPUs (Supplementary Figure S1 and Table S2) summed a maximum diversity of 2.5% and were very diverse. These low abundant OPUs affiliated mostly with the classes *Gammaproteobacteria* (OPUs 1–35; OPUs 45–53), *Betaproteobacteria* (OPUs 36–44), *Alphaproteobacteria* (OPUs 54–90); and with the phylum *Bacteroidetes* (OPUs 96–119). There were other minor representative taxa of *Planctomycetes, Delta-* and *Epsilonproteobacteria, Firmicutes* and *Actinobacteria*. From the low abundant taxa it is worth to mention a presence of several members of the *Vibrionaceae* (some also obtained in culture as indicated below), *Pseudoalteromonadaceae, Rhodobacteraceae* as well as representatives of the clade SAR116.

Samples were cultured onto four different media. All but SW 10% salt, rendered colony forming units (CFUs) that were in the range between 1.3 and 6×10^5 CFU/ml (Table 2). The highest counts were obtained for M4, but the samples M1–M3 rendered equivalent CFU yields for almost all media. A total of 190 valid profiles clustered in 19 distinct OTUs/OPUs (Table 2 and Supplementary Figure S2). Eight major OPUs were most relevant due to their presence and abundance in almost all samples (Table 3). The major group of isolates belonged to the *Vibrio* species *V. xuii* (101 isolates, OPU 2), *V. harveyi* (50 isolates; OPU 1) and *V. mediterranei* (3 isolates; OPU 3), which made up to 79% of the total set. Other relevant, but smaller groups of isolates belonged to OPU 145 (*Photobacterium angustum*; 5 isolates); OPU 146 (*Photobacterium leiognathi*; 3 isolates); OPU 149 (*Microbulbifer epialgicus*; 6 isolates); OPU 152 (*Pseudovibrio japonicus*; 6 isolates); and OPU 60 (*Roseibium hamelinense*; 3 isolates). The remaining OTUs (Table 3) occurred in very low numbers and just in one of the four samples, thus not treated as relevant.

The cultivable fraction made a very small percentage of the detected sequences by the pyrosequencing approach. However, seven of the cultured representatives (*V. harveyi, V. xuii, V. mediterranei, Endozoicomonas elysicola, Sulfitobacter dubius, R. hamelinense* and *Kocuria rhizophila*; Fig. 1) were detected in the sequencing survey, but in very minor amounts (always <0.3% of the total). The 11 additional cultured OPUs remained undetected by pyrosequencing.

Discussion

This is the first report on the associated microbiota in the gastric cavity of the jellyfish *C. tuberculata*, member of the class *Scyphozoa* phylum *Cnidaria*. The very first study on gelatinous zooplankton based on molecular methods was performed studying the microbiota associated to *Mnemiopsis leidyi* and *Beroe ovata* of the phylum *Ctenophora* [7], using clone libraries and molecular fingerprints. However, studies on members of the class *Scyphozoa* have just been studied focusing on the specific detection of the pathogenic bacterium *T. maritimum* [8,11,12], and no report (to our knowledge) on the community structure of the associated microbiome has been published. The study is based on just four exemplars of the jellyfish in just one location and one season. We are aware that the number may be too low to make generalizations, but the very strong coincidence of the results among the exemplars makes the analysis confident.

The high throughput survey revealed that the dominant diversity in this jellyfish cavity was very reduced as four different major OPUs were making at least 95% of the total diversity, and this dominance of few groups was in accordance with the observations made in ctenophores' tissues [7,17]. The composition of the 139 minor OPUs detected, and mainly belonging to the *Gamma-* and *Alphaproteobacteria, Bacteroidetes* and *Planctomycetes* seem to be in accordance with the common microbial composition of seawater [15,26], and specifically west Mediterranean [5,9,30]. Due to

Table 2
Data on the cultivable fraction of the samples.

	SW 5% (CFU·10^5 ml^{-1})	R2A (CFU·10^5 ml^{-1})	Marine broth (CFU·10^5 ml^{-1})	Nr. isolates	Nr. profiles	Nr. OTUs
M1	3.1 ± 0.6	3.8 ± 0.7	1.5 ± 0.7	76	54	8
M2	3.3 ± 0	3.2 ± 0	1.3 ± 0.4	69	53	10
M3	1.3 ± 0.6	3.9 ± 3.8	2.8 ± 1.7	48	43	10
M4	6 ± 0	5.5 ± 3.6	2.1 ± 1.6	46	40	8
Total				239	190	19

the very low abundances of each of them, we cannot discard that these were not part of the *C. tuberculata* microbiome, but accompanied the seawater impregnating the tissues, or part of the ingested microplancton (or picoplancton in this case) through the moutharm openings [20]. On the other side, the four major OPUs detected were present in the four exemplars of jellyfish studied, and may respond to the associated microbiome of this species.

The most abundant sequences, making between 50% and 97% of the complete diversity in all cases, loosely affiliated with the members of the genus *Spiroplasma* (OPU 133), being *S. poulsonii* [52] the closest relative with a maximum identity of 89.1%% in the sequenced stretch (Supplementary Figure S4). Despite the sequence is just partial, and then the observations may be taken with care, this *Spiroplasma* sequence may represent at least a new species given the low identity with the closest relative type strain [53]. *S. poulsonii* belongs to the family *Spiroplasmataceae* of the class *Mollicutes*. The members of this class are wall-less bacteria, generally of very small sized cells (1–2 μM diameter) and very small genomes (530–2220 Kb), and generally host-associated [14]. Specifically *Spiroplasma* has been reported to be a fastidious organism to be cultured, and generally associated to plant and insect diseases [14]. The reports of *Mollicutes* in *Scyphozoa* are very scarce. The first and unique report on the presence of a *Spiroplasma* in a jellyfish was published in the Ph-D thesis of O. Vega-Orellana [49], in four exemplars of the luminescent jellyfish *Pelagia noctiluca* detected with a specific PCR amplification. Given the conspicuous abundance of these sequences in all four exemplars of *C. tuberculata* making >50% of the total diversity (69.2 ± 18.7 for BT and 82.3 ± 15.3 for BF), as well as the healthy aspect of the exemplars (at the sampling date the waters exhibited a bloom of this jellyfishes not showing any morphological anomaly) it is difficult to infer a pathogenic nature of this organism. Actually, despite most of the *Mollicutes* are considered pathogens, some have been considered to establish symbiotic relations with e.g. coral species (i.e. the "*Candidatus Mycoplasma corallicola*" in the species *Lophelia pertusa*; [29]). Finally, also in the ctenophores studied previously [7], some *Mollicutes'* sequences were detected, but due to the short sequence generated, their accurate identification was not possible.

The second most abundant group of sequences affiliated with the genus *Thalassospira* [24], with identity values below 87.9% with the closest relative species that may be considered as a different species or even genus [53]. *Thalassospira* was first isolated from Mediterranean seawater and under heterotrophic aerobic conditions [24]. There are not many reports on the occurrence of this organism in natural samples, and had been found in consortia degrading aromatic hydrocarbons [13] as well as associated microbiota of *Annelida* in crude oil enrichments with potential production of biosurfactants [38]. In addition, this organism was one of the most prominent in the ctenophores *M. leidyi* and *Beroe* sp. [7,17]. Due to the scarce information on its physiology, we cannot hypothesize any relevant role in *C. tuberculata*.

The third most relevant sequence type due to its abundance affiliated with the fish pathogen genus *Tenacibaculum*, with the closest relative *T. soleae* [33] with identity 96.4% that may indicate that this represents a different species [53]. All 20 species of *Tenacibaculum* hitherto classified (http://www.bacterio.net/tenacibaculum.html) have been isolated from marine environments. Most of them from water (five species) or sediment samples (four species), but others (*T. adriaticum and T. crassostreae*) associated to apparently healthy oysters [22], bryozoan [18] or sponges and green algae [42]. Only the three species *T. dicentrarchi, T. discolor* and *T. soleae* have been isolated from diseased marine fauna [34,51]. Especially *T. maritimum* (which actually belongs to a different phylogenetic clade; Supplementary Figure S5) is a known fish pathogen, for which a detection method based on specific PCR has been developed [12]. With this method, the presence of this organism has been reported in jellyfishes as *P. noctiluca* [8] and *P. quadrata* [11]. However, we did not detect any similar sequence to this species in our survey. As

Table 3
OTUs detected in the MALDI-TOF MS dendrogram and their identification.

	M1	M2	M3	M4	SUM	OPU	Identification
OTU 1	1	0	0	0	1		*Sulfitobacter dubius* (AY180102)
OTU 2	1	0	0	0	1		*Kocuria rhizophila* (Y16264)
OTU 3	0	0	0	1	1		*Bacillus algicola* (AY228462)
OTU 4	0	0	1	0	1		*Shewanella surugensis* (AB094597)
OTU 5	0	0	1	0	1		*Vibrio jasicida* (AB562589)
OTU 6	38	5	7	0	50	1	*Vibrio harveyi* (AY750575)
OTU 7	0	2	3	0	5	145	*Photobacterium angustum* (D25307)
OTU 8	1	1	0	1	3	146	*Photobacterium leiognathi* (X74686)
OTU 9	0	2	0	1	3	3	*Vibrio mediterranei* (X74710)
OTU 10	9	34	26	32	101	2	*Vibrio xuii* (AJ316181)
OTU 11	0	3	0	0	3		*Endozoicomonas elysicola* (AB196667)
OTU 12	0	0	1	0	1		*Pseudomonas stutzeri* (AF094748)
OTU 13	2	2	1	1	6	149	*Microbulbifer epialgicus* (AB266054)
OTU 14	1	0	0	0	1		*Jeotgalicoccus nanhaiensis* (FJ237390)
OTU 15	0	0	1	0	1		*Labrenzia alexandrii* (AJ582083)
OTU 16	0	1	0	0	1		*Labrenzia alba* (AJ878875)
OTU 17	0	0	0	1	1		*Pseudovibrio ascidiaceicola* (AB175663)
OTU 18	1	2	1	2	6	152	*Pseudovibrio japonicas* (AB246748)
OTU 19	0	1	1	1	3	60	*Roseibium hamelinense* (D85836)
	54	53	43	40	190		

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most organisms have not been reported to be associated with disease, but naturally thriving in sediments, seawaters or saprophytes animals or plants, we cannot rule out that in *C. tuberculata* this organism may also be a naturally occurring as commensal or symbiotic microbiota. Some of the isolates had been shown to exhibit properties as synthesis of algaecides [23] that could be relevant for the host.

The fourth major group observed was OPU 142 affiliating with the genus *Synechococcus*. Nearly all the sequences of this OPU were detected in the BT fraction, and nearly insignificant amounts were detected in the BF samples. These results indicated that the cells of *Synechcococcus* should have been pelleted with the tissue material after being disaggregated and centrifuged. *Synechococcus*, together with *Prochlorococcus* are the major microbial primary producers in the oceans [45] and globally distributed. Moreover, it has been hypothesized that this organism can be actively ingested by the dinoflagellate *Symbiodinium*, a widely spread symbiotic organism associated to *Cnidaria*, serving as primary source of nitrogen in oligotrophic waters [19]. *Symbiodinium* in jellyfishes seems to be generally associated to the tentacles and oral endodermic tissue [28], and this would be in accordance with the *Synechococcus* sequences to be just detected in the non-fractionated biomass. Contrarily, the other three major groups (*Spiroplasma, Thalassospira* and *Tenacibaculum*), appear with similar amounts in both fractions may be extracellular or not strongly bound to the tissues, thus probably part of the digestive microbiome.

The culturable fraction made a very small proportion <0.6% of the total diversity detected by the pyrosequencing approach in accordance with the low cultivability in natural ecosystems [2]. This fraction showed also very a low diversity trend, and was strongly dominated by few groups. The most relevant were members of the family *Vibrionaceae*, and in special *V. xuii* and *V. harveyi* that made nearly 80% of the total culturable fraction and were present in all samples. *V. xuii* was initially isolated from shrimp culture waters as well as associated to invertebrates [44], and yet a pathogenic nature of this organism has not been reported. On the other hand, *V. harveyi* is considered a serious pathogen for marine fish and invertebrates [4], promoting diseases as vasculitis, gastro-enteritis and eye lesions.

In summary, *C. tuberculata* seems to have an associated microbiome in its gastric cavity that is of very low diversity with at least three major prokaryotic taxa (i.e. *Spiroplasma, Thalassospira* and *Tenacibaculum*) representing over 95% of the total sequence retrieval in our survey, and specially *Spiroplasma* that summed over 69–82%. But none of the three major taxa had been reported to be abundant free living in plankton, nor in among the emerging taxa associated to jellyfish biomass degradation after blooms [10,46,47]. Contrarily, the low abundant and very diverse taxa detected were more reminiscent of the planktonic marine communities [5,9,15,26,30]. It is remarkable that the three major taxa had been already detected in association with the two ctenophores *M. leidyi* (M.L) and *B. ovata* (B.O) (Supplementary Figure S6; [7]), and thus as hypothesized for corals these should be rather specifically associated to the medusa than the occupation by opportunistic bacteria ingested during the feeding process [1]. These organisms may be just saprophytic colonizers of the digestive organ of this jellyfish. However, due to the fact that at least spiroplasmas may establish symbiotic relations with their hosts [29], it seems plausible that *C. tuberculata* could benefit from the interaction with the hosted microorganisms. Moreover, we cannot discard that such abundant microorganisms may interact with the host releasing substances that may be of very much relevance to induce changes in their life-cycle as occurs in other cnidarian [43], or may perhaps be responsible for the annual life cycle that ends with the mortality of the pelagic forms [36]. On the other hand, some members of *Spiroplasma, Tenacibaculum* and *Vibrio* are potential pathogenic colonizers, and in this case, *C. tuberculata* could operate as dispersive mechanism in a similar way to what it has been reported for *P. noctiluca* carrying *T. maritimum* [8]. In any case, the pathogenic nature of the organisms detected, excepting *V. harveyi*, needs still to be proven. Further investigation on the microbiomes of such pelagic organisms, thriving in all coasts and experiencing seasonal blooms, is necessary to overcome the lack of knowledge about the relevance of the associated microbiota and understand the threatens of disseminating potential pathogens.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2015.07.001

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