Bacteria-Affecting Cephalopods

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Abstract

Bacterial pathogens contribute to obtain an unsuccessful production of cephalopods. An updated overview of the knowledge of these pathogens must be a valuable tool to improve their aquarium maintenance and aquaculture. The present work provides a description of the main bacterial pathogens associated with larval stages of cultured *Octopus vulgaris,* and juvenile and adults of several cephalopods. *Vibrio* species, reported with ability to cause vibriosis in aquaculture, are the main bacteria associated with skin lesions in adults. Different species of *Pseudomonas* and *Aeromonas,* among others, have also been detected. Furthermore, gram-positive bacteria such as *Bacillus* have been also described. Among them, *V. alginolyticus, V. carchariae, V. parahaemolyticus, V. splendidus* and *V. lentus* have also been isolated from sterile organs or fluids of animals and their potential as invaders proved. However, only *V. alginolyticus* or *V. lentus* has the ability to cause lesions, and, in addition, the last one is proved as the causative agent of death in octopuses. Other organs such as eyes of squids are also colonized by *Vibrio* species or *Micrococcus* sp., and recently *Photobacterium swingsii* and *Lactococcus garvieae* have been reported associated with a retrobulbar lesion in octopus. Rickettsial-like organisms (RLO) are also detected in the gills of the octopus, having a detrimental effect on the respiratory gaseous exchange of the animals. Cultures of octopus paralarvae show a genetically diverse community comparable to those reported previously from other marine hatcheries. Bacteria included in the Splendidus clade is the dominant group in all conditions, except in one of them, where *V. alginolyticus, V. proteolyticus* or *Pseudomonas*

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fluorescens are the main detected groups. Furthermore, *Shewanella* or *Pseudoalteromonas undina* have also been identified. All this shows that pathogenic bacteria are frequent microorganisms associated with aquarium maintenance and culture of cephalopods, and special attention on maintaining a well-balanced community of microorganisms should be applied.

Keywords

Cephalopod diseases • Paralarvae • Microbial community • Pathogenic bacteria • Splendidus clade • Rickettsia-like organisms

8.1 Introduction

Incidence of diseases, several of them caused by bacteria, is one of the most important problems which avoid obtaining a successfully production in the aquaculture of cephalopods or its suitable maintenance in captive conditions (García-Fernández et al. 2016; Sykes and Gestal, 2014).

Knowledge on bacterial species associated with cephalopods culture and its ecological role would be essential for improving industrial culture production. Seawater have a variable microscopic population density (Harder 2009), and it contains a high microbial diversity depending on physico-chemical and geographic factors such as the temperature of the water, latitude and salinity. The ability of bacteria to colonize surfaces (epibiotic bacteria) is very well documented, and they comprise well-organized bacterial communities associated with marine organisms, which are influenced by temporal changes in the environment. However, some bacteria are specifically and persistently associated with particular marine animals, and they are not found in seawater or on other animals (Thakur et al. 2004; Sharp et al. 2007). This can explain the species-specific vulnerability to pathogens previously suggested for *Octopus bimaculoides* and *O. maya* (Hanlon and Forsythe 1990). These 'microepibionts' are multi-functional and are involved in obtaining nutrients, acquiring new genetic traits and providing some measure of chemical defence against pathogens (Wahl et al. 2012). In these epiphytic communities, developing biofilms is the most common, and quorum sensing modulates behaviour of many biofilm-associated bacteria, affecting symbiotic relationships and host interactions (Maximilien et al. 1998; Parsek and Greenberg 2005). Co-infection is another important factor affecting bacterial diseases, in fact in previous studies parasite infection enhanced bacterial invasion in fish (Rodríguez-Quiroga et al. 2016).

Differences in culturable bacteria most frequently isolated from skin of apparently healthy, wild or laboratory-maintained adult squids have been previously found. In fact, a higher diversity is shown in the former ones (Hanlon and Forsythe 1990). Similar data are observed in the gastrointestinal microbiome of wild octopus paralarvae with respect to that of reared ones (Roura et al. 2017). Although aquaculture systems are designed to imitate the natural environment, the maintenance of adequate conditions during all culture production is problematic. Furthermore, diets provided are less diverse than in natural media. Therefore, it can produce alterations in microbial community of seawater and/or surface (external or internal) of cephalopods. This provides an opportunity for the dominance of bacteria, which show the ability to invade the host, and in the end, to cause a fatal infection. On the other hand, this is also facilitated when the surface of the animal is injured.

In 1990, Hanlon and Forsythe described the main bacterial pathogens associated with juvenile and adults. In fact, they included data from published literature, and their own data collected from analyses resultant of 14 species of different cephalopods for 13 years. Species of *Vibrio*, mainly, but also members of the genera *Pseudomonas, Aeromonas, Staphylococcus* or *Streptococcus*, among others, have been associated with healthy octopuses or squids in nature. Some of these genera have also been associated with the ulcerated skin of octopuses and squids from their natural environment or laboratory-maintained, being this, their route of entrance in animals, and progressing later until a fatal infection. Furthermore, a possible food-borne was the suggested route of entrance for *V. carchariae*, which was associated with sudden death of laboratory-maintained octopuses, which showed lack of any external of behavioural symptoms. Similarly, an unknown route of entrance, not associated with ulcerated skin, was proposed for cuttlefish which suffered a highly virulent systemic infection. Squids with eye damage associated with bacteria were also reported, but no further information on the route of infection was given. However, it is unknown if bacteria are the initial cause of infection, with the exception of one study, where *V. alginolyticus* was proved as causation of lesions in octopus. Similarly, there is a lack of information on culturable bacteria associated to different stages of cephalopods in reared conditions.

In attempting to be comprehensive, we reviewed the knowledge about bacterial disease in the most representative European cephalopod species to date, detailing the main bacterial pathogens associated with juvenile or adults.

Furthermore, data from bacterial associated with larval stages of cultured octopuses (unpubl.), recently qualitatively sampled 'in situ' by us were also included.

The common signs caused by bacteria on cephalopods, and, when possible, images, are shown. In addition, it is included a brief description of characteristics of bacteria, diagnostic and treatments used against bacteria.

8.2 Potential Pathogenic Bacteria for Larval Development Stages

Bacterial septicaemia was occasionally observed in paralarvae (13–15 days old) of *O. vulgaris* reared in aquaculture tanks, which could be causative of the dead of the paralarvae (Fig. 8.1a, b). The identification of the aetiological agent was not established by the authors. Seawater quality, and maintenance and cleaning precautory measures were suggested as factors that could facilitate the growth of pathogenic bacteria that as a result caused the infection. Previous studies have revealed that uncontrolled factors in the culture have an important impact on the establishment and evolution of the microbial community in the culture tanks of *Artemia* (Verschuere et al. 1997). Similar results were also found for culture of clams (Kwan and Bolch 2015). It was also shown that an effective balance of microbial populations included in the same community ensures the final rearing success (Verschuere et al. 1997; Kwan and Bolch 2015; Shi et al. 2017).

Recently, it has been proved that the intestinal microbiome, highly diverse, of octopus paralarvae reared in captivity and feed with *Artemia* is rapidly become less diverse and is mainly suggested as a consequence of culture conditions (Roura et al. 2017). However, to date, there is an absence of published data available on diversity of bacteria from cephalopod hatcheries. Recent qualitative preliminary analysis of seawater associated with different stages of *O. vulgaris* hatchery culture has focused to characterize and identify the culturable bacteria.

8.2.1 Microbial Community Counts

8.2.1.1 Water Samples

Culturable bacteria was isolated on three different dates corresponding to Experiment 1, 2 and 3 from seawater associated with different stages of an *O. vulgaris* culture in Galicia (Table 8.1). After 13–15 days of paralarvae culture, samples were taken on the same day in all stages of each Experiment. Seawater inside the hatchery was filtered until 1 µm for all experiments, but in addition, a high wattage UV treatment was also applied for *Artemia* (*Artemia salina*) culture. Moreover, seawater filtered until 20 µm and treated with low wattage UV was analysed for comparative purposes (Experiment 4). Standard conditions of natural photoperiod, seawater temperature (19–23 °C) and salinity of 33‰ were maintained.

Larval tanks (1000 L) containing 5 larvae l^{-1} were reared following the protocol described by Iglesias et al. (2004). In this study, paralarvae were fed with *Artemia* nauplius or *Artemia* nauplius complemented with *Maja brachydactyla* zoeas or freeze-dried feed (made from different species of crustaceans).

Water samples (1 ml) were serially diluted in sterile seawater and spread out (0.1 mL) over plates of Tryptic Soy Agar supplemented up to 1% (w/v) NaCl (TSA-1) and Thiosulphate-Citrate-Bile-Sucrose (TCBS) in duplicate. Colonies were counted after 4 days of incubation at 22 °C, and the results expressed in colony-forming units (CFU) per ml. Whereas TSA-1 was used for total heterotrophic bacteria counts, TCBS was for presumptive *Vibrio* ones. The last one was used since *Vibrio* species are a significant component of culturable marine bacterial populations (Thompson et al. 2004; Montes et al. 2006; Beaz-Hidalgo et al. 2008; Guisande et al. 2008).

Colonies of each morphology from TSA-1 or TCBS were picked off and spread on TSA-1 to obtain pure cultures, and then inoculated on Tryptic soy broth with 1% (w/v) NaCl and 15% (v/v) of glycerol for their conservation at −80 °C.

8.2.1.2 Microbial Counts

Total heterotrophic bacteria associated with the octopus cultures are shown in Table 8.1. The results showed that

Fig. 8.1 *O. vulgaris* paralarvae reared in aquarium conditions. **a** Histological section of the connective tissue of paralarvae infected by bacteria (arrow). **b** Detail showing the strong septicemia observed in the whole paralarvae. H&E stain. Images **a** and **b** by courtesy of Dr. R. Fernández-Gago. *Scale bars* **a**, **b** 20 µm
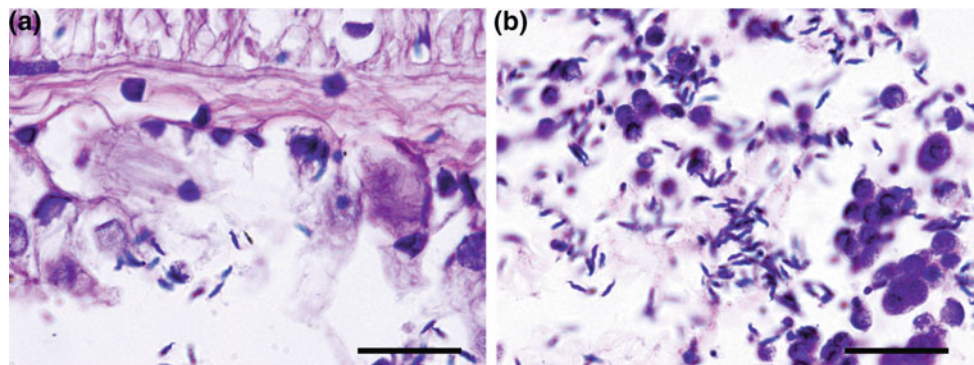
Table 8.1 Bacterial counts and species identified in different paralarval stages of *O. vulgaris* hatchery

	Experiment 1			Experiment 2					Experiment 3		Experiment 4
	WIH1	WOA	ATW	ZTW	PTW + AZ	PTW + DA	PTW + DAZ	DAWT	PTW + A	PTW + ACF	WIH2
Culturable bacteria counts											
Total heterotrophic (TSA-1) (CFU/ml)	7×10^2	–	1×10^4	1.3×10^4 ($\pm 7 \times 10^3$)	2×10^4 ($\pm 5 \times 10^3$)	4×10^2 ($\pm 3 \times 10^2$)	1.5×10^2 ($\pm 5 \times 10^1$)	1×10^1	7.5×10^5 ($\pm 2 \times 10^5$)	4×10^6 ($\pm 7 \times 10^3$)	1.8×10^4 ($\pm 7 \times 10^3$)
Presumptive Vibrios (TCBS) (CFU/ml)	–	–	4.8×10^2 ($\pm 2.1 \times 10^2$)	2.5×10^2 ($\pm 1.5 \times 10^2$)	9×10^2 ($\pm 1 \times 10^2$)	1.9×10^2 ($\pm 1 \times 10^2$)	1×10^2	1×10^1	5.8×10^4 ($\pm 1.5 \times 10^3$)	7×10^5 ($\pm 5 \times 10^4$)	4.8×10^3 ($\pm 2 \times 10^3$)
Species identified											
Splendidus Clade											
V. atlanticus/V. tasmaniensis						+(32)	+(23)		+(1, 2, 3, 8, 13)	+(4, 5, 7)	+(25, 38)
V. gigantis/V.crassostreae/V.pomeroyi					+(34)	+(31, 35, 36)	+(33)		+(6, 14)	+(11)	
V. gallaecicus				+(39)							
V.hemicentroti/V.lentus									+(10)		+(26, 28, 29)
V. alginolyticus			+(27)								
V. neptunius					+(30)						
V. proteolyticus			+(18, 19)	+(21)	+(12)						
P. fluorescens			+(17, 20)		+(16)						
Pa. undina										+(24)	
Sh. marinintestina/Sh. sairae/Sh. schelegeliana									+(9, 22)	+(15)	

In brackets SE for bacteria counts and strain for species identified. *WIH1* seawater inside the hatchery filtered until 1 μm; *WOA*: seawater outside *Artemia* culture; *ATW Artemia* tank water; *ZTW* Zoeae tank water; *PTW + AZ* octopus paralarvae tank water mixed with *Artemia* and zoeae; *PTW + DA* octopus paralarvae tank water mixed with disinfected *Artemia*; *PTW + DAZ*. octopus paralarvae tank water mixed with disinfected *Artemia* and zoeae; *DAWT* disinfected *Artemia* water tank; *PTW + A* octopus paralarvae tank water tank; *PTW + ACF* octopus paralarvae tank water mixed with *Artemia* and freeze-dried food; *WIH2* seawater inside the hatchery filtered until 20 μm and treated with low wattage UV

there are strong evidences that the food (live or freeze-dried) supplied to the larvae constitutes the main source of the bacteria associated to the cultures of the octopus larvae.

A significant percentage (50–75%) of total heterotrophic bacteria grew in TCBS, and as it was confirmed in this study, most of them were included in genus *Vibrio* (Table 8.1). Their counts remained uniform in most of the culture conditions studied. The lowest value was detected in the cultures treated with disinfectant, whereas the highest was detected in that containing *Artemia* mixed with freeze-dried feed. These results are in agreement with those of scallop larval culture, where concentrations of *Vibrio* varied only between 10^2 and 10^3 cels ml^{-1} (Nicolas et al. 1996). However, higher counts from a culture water of *Artemia* (10^3–10^4 cfu ml^{-1}) were detected in early times of the culture period (Verschuere et al. 1997). Variations in *Vibrio* counts are frequent in hatcheries. In fact, an increase of *Vibrio* counts associated to clam's hatchery was previously found after the rise of environmental temperature (Mechri et al. 2012).

Finally, in Experiment 4, the total heterotrophic bacteria and the presumptive *Vibrio* were increased in 2-log or 3-log units, respectively, with respect at those from water inside the hatchery used for larval culture. Variations in the treatment of the seawater (mainly due to filtration) could explain these results.

8.2.2 Characterization of Culturable Bacteria Associated with Octopus Paralarvae Culture

To carry out the characterization of bacteria associated with the different culture conditions studied, a total of 38 strains corresponding to the colonies that had different morphologies on TSA-1 or TCBS were selected. For their characterization, primary and secondary identification biochemical tests and PCR with specific primers were performed, but the final identification and/or assignation to closely related species was performed by sequencing of 16S rRNA gene.

All strains were characterized by phenotypical tests and only 30 by sequencing of 16S rRNA gene. The closest-neighbouring species, which shared a similarity value in the 16S rRNA sequences of $\geq 98\%$, were used to identify the isolates. Molecular identification was assigned to the remaining strains (8), when the strains identified by 16S rRNA and the unidentified ones shared the same phenotypical profile.

8.2.2.1 Phenotypical Characterization
Firstly, the strains were phenotypically characterized by using a set of primary identification tests: gram staining, motility, oxidase, catalase, oxidative or fermentative glucose metabolism on O/F Basal Medium (O/F), growth ability at 0% NaCl or in TCBS and susceptibility to O129 vibriostatic agent (2, 4-diamino-6, 7-di-isopropylpteridine phosphate; 150 μg/disc).

These assays indicated that all the strains were gram-negative motile rods and that they were positive for all tests except for being able to grow at 0% NaCl and the glucose metabolism. The two last tests allowed discriminating three groups of strains; (1) strains showing aerobic/anaerobic glucose metabolism, being all of them unable to grow at 0% NaCl (facultative anaerobes); (2) strains without aerobic/anaerobic glucose metabolism, being all of them unable to grow at 0% NaCl; (3) strains without oxidative/fermentative glucose metabolism (aerobes), but with ability to grow at 0% NaCl.

All these tests presumptively grouped all facultative anaerobes in genus *Vibrio*. All of them were able to grow well on TCBS and allowed discriminating between strains with ability to use sucrose (Table 8.2). Although, this medium is used for the isolation of *Vibrio* spp., it is also possible, but with poor growth, for other genera (Guisande et al. 2004). In fact, this was the case for our bacteria identified as *P. fluorescens*.

In order to confirm this presumptive identification, secondary biochemical tests were performed for the anaerobic facultative group by using the commercial phenothypic test API 20E (BioMerieux) (Table 8.2). This system is frequently used for the identification of fish pathogens, since its database includes an important number of them. However, it is well known that several reactions, among them decarboxylases [arginine dehydrolase (ADH), lysine decarboxylase (LDC), ornithine decarboxylase (ODC)], must be compared with conventional biochemical tests, since if there are differences, data from the last are preferable as reference (Buller 2004; Popovic et al. 2007). In addition, they were also included in this study, and they were determined as described by Montes et al. (1999). Presence of *Vibrio* was confirmed by the system, and its database allowed determining that the strains were closely related to *V. alginolyticus, V. proteolyticus* and *V. splendidus.*

It is well known that *V. splendidus* is phylogenically closely related to other species all of them termed as *V. splendidus*-related group (Splendidus clade), which is the most diverse among *Vibrionales* (Sawabe et al. 2007). API 20E was unable to discriminate these species since strains included in the same group showed variable biochemical profiles (Table 8.2). It is also difficult by using numerical taxonomy or molecular methods (Lago et al. 2009; Revised by Oden et al. 2016).

8.2.2.2 Molecular Characterization
In a previous study, the combined specificity of *V. tasmaniensis* (VTS/VT) and *V. splendidus* (VTS/VS) primer sets offered the best coverage (86%) in terms of separating

Table 8.2 Characterization and Identification of *Vibrio* strains associated with of *Octopus vulgaris* hatchery

	Groups identified by sequencing assignment[a]				*V. alginolyticus*	*V. neptunius*	*V. proteolyticus*
	Splendidus clade						
	V. atlanticus/ V. tasmaniensis	*V. gigantis/ V. crassostreae/ V. pomeroyi*	*V. gallaecicus*	*V. hemicentroti/ V. lentus*			
Tests to identify strains							
Sequence (16S rRNA) identity (%) with the closest neighbour	≥ 98.7%	≥ 99.5%	99.8%	>98.5%	99.9%	99.9%	99.9%
Amplification with specific primers							
VTS/VT (*V. tasmaniensis*)	+(10/12)	+(4/8)	+	+(1/4)	−	−	−
VTS/VS (*V. splendidus*)	+(4/12)	+(4/8)	−	−	−	−	−
VN1/VN2 (*V. neptunius*)	−	−	−	−	−	+	−
Biochemical characteristics							
TCBS (sucrose +)[b]	+(8/12)	+(4/8)	−	−	+	+	−
TCBS (sucrose −)[b]	+(4/12)	+(4/8)	+	+(4/4)	−	−	+(3/3)
Arginine dihydrolase (ADH)[b]	+	+	+	+	−	+	+
Lysine decarboxylase (LDC)[b]	−	−	−	−	+	−	+
Ornithine decarboxylase (ODC)[b]	−	−	−	−	+	−	−
ß-galactosidase[c]	+(4/8)	+(7/8)	−	+	−	−	−
Gelatinase[c]	+(5/8)	+	−	+	+	+	+
Citrate utilization[c]	−(5/8)	−	−	−	−	+	+
Voges Proskauer[c]	−	−	−	−	+	−	+
NO_2 production[c]	+	+	+	+	+	−	+
Fermentation/Oxidation[c]							
Amygdalin	+	+(5/8)	+	+(1/2)	+	+	−
Arabinose	−(7/8)	−	−	−	−	−	−
Inositol	+	+(7/8)	+	+	+	−	+
Melibiose	−(6/8)	−(7/8)	−	−	−	−	−
Rhamnose	+(5/8)	−(5/8)	−	−	+	−	−
Sorbitol	+(7/8)	+(6/8)	−	+(1/2)	+	−	+
Sucrose	+(5/8)	+(6/8)	−	−	+	+	+(2/3)

[a]Multiple alignment of sequences was created by ClustalW in Genious Editor version 12.0.6 (Biomatters; http://www.geneious.com/). This included 1.138 positions after the removal of ambiguous ones. A phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) version 6 (Tamura et al. 2013). This was performed using the neighbour-joining method and Tamura–Nei distance model, with the calculation of cluster stability by bootstrap analysis with 1000 replicates. All available species of each genus closest to studied strains were selected

[b]Tests performed by using conventional biochemical tests

[c]Tests performed by using API 20E. All strains were negative for H_2S production, hydrolysis of urea or presence of tryptophane deaminase; and they were positive for indole production and fermentation/oxidation of D-glucose or D-mannitol (all of them performed by using API 20E). In brackets number of positive or negative strains of all assayed

several of the species included in the Splendidus clade, and from other *Vibrio* species (Lago et al. 2009). In this study, a positive amplification was shown in all strains which were characterized in the Splendidus clade, except in six (13, 26, 28, 29, 31, 33). In fact, from the positive ones, 84% (16/19) and 42% (8/19) of strains reacted with VTS/VT and VTS/VS

primer sets, respectively (Table 8.2). Negative amplification was shown by the remaining strains characterized as *Shewanella* (*Sh.*) or *Pseudomonas*; on the contrary, a positive amplification with the primer set VTS/VT was shown by strain 24, which was identified by 16S rRNA gene analyses as *Pseudoalteromonas* (*Pa.*) *undina*. Furthermore, a

V. neptunius primer set was highly specific, showing only cross-reaction with *V. parahaemolyticus* species from 44 tested species (Lago et al. 2009). Similarly, in this study only one strain, which was identified by 16S rRNA gene analyses as *V. neptunius*, was positive (Table 8.2). Since these primer sets were developed, the number of species included in the Splendidus clade has increased (revised by Oden et al. 2016). In this study, it is shown that they have cross-reaction with them. Nevertheless, these results revealed that the primer sets are a valuable tool for a fast detection of Splendidus clade, and to separate this clade from other *Vibrio* species.

Additionally, a sequencing of 16S rRNA gene analyses was performed according to the method described by Guisande et al. (2008), in order to confirm all these results. The strains assigned to each phylogenetic group are shown in Table 8.1, and the sequence identity (%) with the closest neighbour in Table 8.2. These results allowed to confirm that 81% (31 of a total of 38 strains) of strains were facultative anaerobes, and the remaining was aerobic. Among facultative anaerobic bacteria, the dominant genus identified was *Vibrio* (100%). The highest number (63.2%) of *Vibrio* strains was included in Splendidus clade, and they were assigned to different groups depending on their closest similarity with species (Table 8.2). In addition, one strain was identified as *V. gallaecicus*.

The 16S rRNA gene analyses allowed a more complete identification since *V. gallaecicus, V. neptunius, V. proteolyticus, Pa. undina* and *P. fluorescens* were identified. Furthermore, several strains were shown more closely related to specific species of Splendidus clade, *V. alginolyticus* or *Shewanella*.

8.2.3 Pathogenicity of Culturable Bacteria Associated with Octopus Paralarvae Culture

The Splendidus clade is the most abundant in marine samples associated with several marine animals, water column, occurring in bacterioplankton and sediments (revised by Pérez-Cataluña et al. 2016), and also are part of regular components of farmed aquatic animal microbiota (Montes et al. 2003; Guisande 2004; Baez et al. 2008; Kwan and Bolch 2015; Oden et al. 2016).

In this study, these strains were the most abundant in all culture conditions analysed, except in the conditions of Experiment 1, where dominant groups were strains identified as *V. proteolyticus, V. alginolyticus* or *P. fluorescens*. Similarly, *V. alginolyticus* was also the predominant species associated with clam or gilthead sea bream hatcheries (Snoussi et al. 2006; Mechri et al. 2012); *P. fluorescens* was also found associated with the sea bream hatchery, and it was one of the autochthonous denitrifying bacterium isolated

from marine biofilters at a recirculation aquaculture system (Snoussi et al. 2006; Borges et al. 2008). Other species of *Pseudomonas* were also associated with oyster larvae (Farto et al. 2006) and with *Artemia* culture (Verschuere et al. 1997). Finally, *Shewanella* or *Pa. undina* were also identified associated with culture water containing octopus paralarvae and commercial feed or *Artemia* of this study. These groups were also previously found from healthy oyster cultures (Farto et al. 2006).

It is well known that several of the groups isolated in this study may have a role in larval mortality in cultures. In fact, several bacterial included in the Splendidus clade are considered pathogenic for bivalve molluscs (oyster, clam, scallop, mussel) (Revised by Beaz-Hidalgo et al. 2009; Revised by Kwan and Bolch 2015; Vanhove et al. 2015; Cheikh et al. 2016) and also cephalopod molluscs (Farto et al. 2003; Iehata et al. 2016). Similarly, *V. alginolyticus* was pathogenic for carpet shell clam larval and juvenile (Gómez-León et al. 2005; Mechri et al. 2015), fish (Liu et al. 2004; Zheng et al. 2017), crustacean (Jayaprakash et al. 2006; Xu et al. 2013), scallop (Riquelme et al. 1996), red abalone (Anguiano-Beltrán et al. 1998), associated with infected Chilean octopus eggs (Iehata et al. 2016), with ability to cause skin lesions to young octopus (Hanlon and Forsythe 1990), and isolated from skin ulcers and/or different organs of cultured cuttlefish (Sangster and Smolowitz 2003). *V. proteolyticus* has been previously identified as part of the *Vibrio* consortium isolated from diseased corals (Cervino et al. 2008), and were identified as pathogens of *Artemia* spp. (Verschuere et al. 2000). Another pathogen described for *Artemia* sp. is *V. neptunius* (Verschuere et al. 2000; Austin et al. 2005), being also for oyster (Prado et al. 2005; Guisande et al. 2008). There are several strains of *Pa. undina* described as biological controllers of virus (Maeda et al. 1997), however, other were pathogens for gilthead sea bream or scallop (Pujalte et al. 2007; Sandaa et al. 2008). The genus *Pseudoalteromonas* was also previously found associated with infected Chilean octopus eggs (Uriarte et al. 2011; Iehata et al. 2016).

Then, although there is an absence of published data available from octopus hatcheries, the culturable species observed are comparable to those reported previously from other marine hatcheries. This diversity resembles that of coastal seawater (Thompson et al. 2005), and several of them can cause mortality in hatchery production. Previous studies have shown that differences in dominance of a specific group can be explained by variations in interactions within the total bacterial community due to alterations in environmental conditions of cultures. Those changes can encourage dominance and diversity of more virulent species (Kwan and Bolch 2015). So, in our study, all cultures of octopus larvae analysed showed similar levels of mortality, and also a

genetically diverse community, but different between Experiment 1 and Experiments 2–3.

Further studies are needed to understand the equilibrium among a community of microorganisms, and how changes in husbandry practices select for more virulent genotypes.

8.3 Potential Pathogenic Bacteria for Juvenile and Adults

Several bacteria have been associated with diseased cephalopods (octopuses, squids and cuttlefish). Although most of them have been isolated from skin lesions, they were also from sterile organs or fluids (gill heart, reproductive organs, haemolymph) and others (eye). Particularly, mainly several species of *Vibrio*, such as *V. alginolyticus, V. parahaemolyticus, V. splendidus* or *V. lentus*, and recently *Photobacterium (Ph.) swingsii* and *L. garvieae* have been reported. This fact confirms their potential as invaders, and also the positive septicaemia described in several cases, which is associated with advanced stages of infection. The halting of disease progression and the promotion of healing, after antibiotic treatment of several animals, also show a role of bacteria in diseases of animals (Hanlon and Forsythe 1990; Sherrill et al. 2000; Sangster and Smolowitz 2003). However, most of bacteria were not confirmed as the cause of death of animals, and it is uncertain if they were the initial cause of infection. In this section, a brief reference to those bacteria not confirmed as the causative agent of death and/or the initial cause of infection are included.

8.3.1 Miscellaneous Bacteria Associated with Skin Lesions

There are several studies showing a variety of bacteria with ability to colonize skin lesions in octopus maintained in captivity. Crowding and increased contact among cultured octopus are proposed as the more probable cause of physical injury, developing into ulcerations due to colonization of bacteria, which would induce a fatal infection affecting multiple organs in the same individual, if untreated. Similarly, this would be the process for wild-caught squids maintained in the laboratory or cuttlefish maintained during a 12-year period in exhibition aquariums. Furthermore, several captured wild octopuses displayed skin lesions, which became worse under laboratory conditions (Hanlon and Forsythe 1990; Sherrill et al. 2000; Farto et al. 2003).

The clinical signs most frequently described for octopus include skin ulcers in the head through which bacteria penetrate, progressing into an advanced infection stage showing deep wounds in arms or head mantle of octopus (Hanlon and Forsythe 1990; Farto et al. 2003).

Previously, until 36 bacteria species had been isolated from skin ulcers of diseased octopuses or squids, most of them from cultured conditions. Particularly, the majority corresponded to gram-negative bacteria. From these, the mainly ones were different species from the genus *Vibrio* with ability to cause vibriosis such as *V. anguillarum, V. alginolyticus, V. parahamolyticus* and *V. splendidus*. Different species of *Pseudomonas* and *Aeromonas*, and presumptive *Cytophaga*-like strains were also detected. Furthermore, gram-positive bacteria were also detected such as *Bacillus* and *Staphylococcus* (Hanlon and Forsythe 1990; Farto et al. 2003; Tsai et al. 2012; Fichi et al. 2015). However, only *V. alginolyticus* or *V. lentus* have been confirmed with ability to cause lesions in octopus (Hanlon and Forsythe 1990; Farto et al. 2003). All these studies have also shown that is frequent that skin lesions contain different species of bacteria simultaneously.

A brief description of several bacteria indicated in this section is shown below.

Vibrio alginolyticus and *Vibrio parahaemolyticus*

Both *V. alginolyticus* and *V. parahamolyticus* are motile gram-negative bacteria in Vibrionaceae family, Gammaproteobacteria class, which have been isolated frequently from marine and coastal waters throughout the world. Particularly, in a clam hatchery located in the Mediterranean coast has been shown that *V. alginolyticus* strains were the most dominant, and *V. parahaemolyticus* strains represent only 2% of all Vibrionaceae isolated (Mechri et al. 2012). Both have been associated with marine organism's diseases, and molluscs among them. This is detailed for *V. alginolyticus* in Sect. 8.2 (Pathogenicity). Furthermore, an exhaustive description of infection stages of this species was previously reported for cultured sepoids, suggesting, in addition to skin lesion, other routes of entrance in animals (Sangster and Smolowitz 2003). On the other hand, in diseases affecting small abalone, Spanish toothcarp and shrimp was isolated *V. parahaemoyticus* (Alcaide et al. 1999; Liu et al. 2000; Choi et al. 2017).

Both species were also isolated from skin ulcers of *O. joubini*, but only *V. alginolyticus* was proved with ability to produce ulcers in this species (1 g) at concentrations of 10^6 CFU ml^{-1}, after performing an incision on mantle in order to provide an invasion site for bacteria (Hanlon and Forsythe 1990). Furthermore, both species were also isolated from skin lesions of cultured *O. vulgaris* (Fichi et al. 2015). Small and larger lesions were detected (Fig. 8.2), showing a white appearance similar to those previously described (Hanlon

Fig. 8.2 White skin lesion on head in an adult female of *O. vulgaris* cultured in Italy

and Forshyte 1990; Farto et al. 2003). Moreover, it was also isolated from gill heart, evidencing their potential as invader. Similarly, it was shown for *V. parahaemolyticus* in squids and cuttlefish being isolated from haemolymph and other organs (Hanlon and Forshyte 1990; Sangster and Smolowitz 2003). In addition, to bacterial species, *Aggregata octopiana* oocysts and betanodavirus were also found in the same skin lesion (Fichi et al. 2015). Co-infection was previously shown as an enhancing factor for bacterial invasion in fish (Rodríguez-Quiroga et al. 2016).

Additionally, both species can infect humans. Whereas, the infections of *V. alginolyticus* are frequently associated with wound infections, external otitis or cellulitis, acute gastroenteritis are caused by *V. parahamolyticus*. The strains of *V. parahaemolyticus* affecting humans have the ability to produce a thermostable direct haemolysin (TDH) and/or a thermostable-related haemolysin (TRH). To date, these toxins have not been produced by most of strains associated with diseases in aquatic animals. On the other hand, there are limited studies on ability of human clinical isolates to cause diseases in marine organisms, but was proved that human clinical or environmental strains were infectious for both humans and abalones (Lee et al. 2003). On the contrary, recently, the lack of effectiveness in causing disease in a shrimp host was proved for human clinical strains (Choi et al. 2017). Differences between strains of *V. alginolyticus* affecting humans or marine organisms are still unavailable.

Both species swarm across TSA-1% and completely covers the plate in 24 h at 22 °C. Differences in colour of colonies are shown in TCBS, and they are yellow and green for *V. alginolyticus* and *V. parahaemolyticus*, respectively. Photographs of culture and microscopic appearance of organisms are detailed in texts such as Buller (2004).

They are also halophilic. Although most of them show an optimum growth with NaCl concentrations of 2–4% of NaCl, particularly, *V. alginolyticus* is more tolerant to higher and lower concentrations. In fact, there is a well growth up to 8% of NaCl and 40% of strains were described with ability to grow also at 10% NaCl. On the contrary, *V. parahamolyticus* exhibits poor growth in media with 6% NaCl and 10% NaCl is inhibitory (Mechri et al. 2015). Moreover, both are able to growth between 15 and 42 °C. Although they can live in a wide range of temperatures their growth is favoured by the increase of temperatures, and for example, in *V. parahamolyticus* their outbreak of diseases has been associated with thermal induction (Liu et al. 2000; Huang et al. 2001).

A fast preliminary identification is proposed through the number of steps described in Sect. 8.2 (Characterization of culturable bacteria). Other commercial identification systems (API ZYM, API 20NE, etc.) were also proposed for their identification. Although several of primers developed have cross-reaction with both species, specific primers were also proposed. Particularly, for *V. alginolyticus* that corresponding to the heat shock protein 40, and for *V. parahamoltycus* PCR that detects a fragment (including a non-coding region and a phosphatidylserine synthetase gene) termed R72H (Buller 2004; Mechri et al. 2015).

Sensitivity or resistance at the same antibiotic was shown by strains of both species depending on their origin. Both are resistant to a wide range of antibiotics commonly used in aquaculture (ampicillin, erythromycin, tetracycline, streptomycin and chloramphenicol). Flumequine and streptomycin are promising candidates for therapy against *V. alginolyticus* and *V. parahaemolyticus*, respectively (Mechri et al. 2015). Alternative promising measures to antibiotics to prevent the diseases caused by these species such as the use of anti-biofilm agents (palmitic acid, nanoparticles) were recently proposed (Chari et al. 2017; Santhakumari et al. 2017). Another hopeful treatment are the peptides produced by *Bacillus subtilis* which were proved with antimicrobial activity against both species, and in addition, had a protective effect against *V. parahaemolyticus* when they were incorporated into the diet of shrimps (Cheng et al. 2017). Water treatment by using photolysis therapy was effective in controlling *V. parahamolyticus* (Malara et al. 2017).

Vibrio splendidus-related group (Splendidus clade)

Splendidus clade, *Vibrionaceae* family, Gammaproteobacteria class, consists of a group of species phylogenically closely related with *V. splendidus*. To date, the species are: *V. artabrorum, V. atlanticus, V. crassostreae, V. celticus, V. cortegadensis, V. cyclitrophicus, V. chagasii, V. fortis, V. gallaecicus, V. gigantis, V. hemicentroti V. kanaloae, V. lentus, V. pomeroyi, V. pelagius, V. splendidus, V. tasmaniensis* and *V. toranzoniae*. Recently, it has been proposed the exclusion of 5 species (*V. atlanticus, V. cortegadensis, V. chagasii, V. fortis, V. pelagius*), but still they
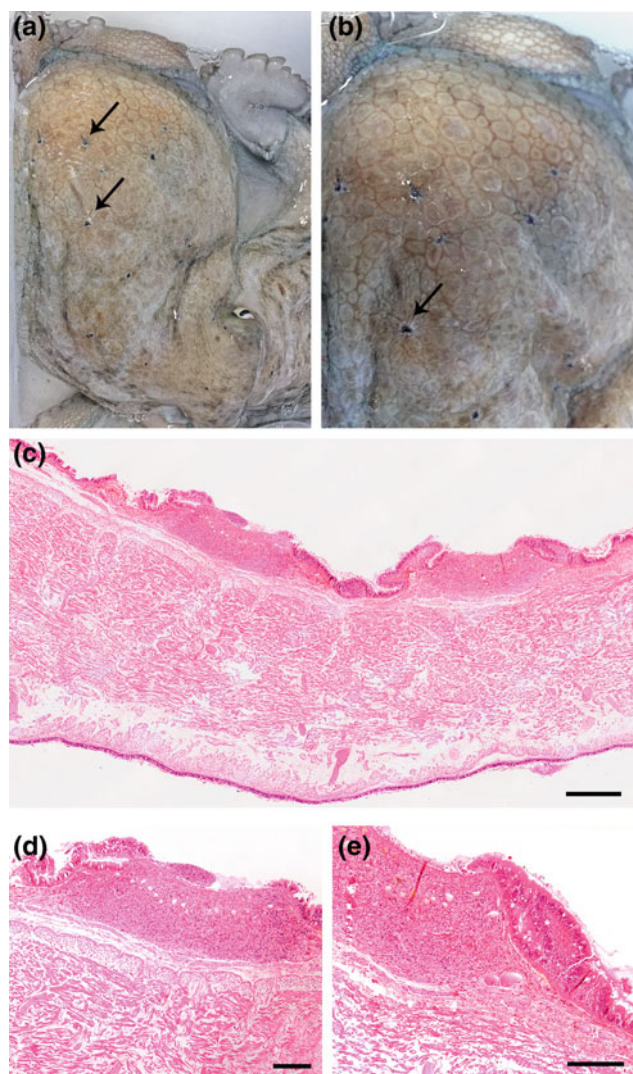
Fig. 8.3 *O. vulgaris* from maintained in aquarium conditions. **a** Macrophotography of the dorsal mantle showing small black spots on the skin. **b** Detail of small ulcers surrounding the black spots. **c–d**. Histological sections of the skin lesions showing ulceration and loss of skin epithelium (arrows) (**c**). Dense aggregations of bacteria, haemocyte infiltration and inflammation of the infected area (arrow heads) H&E stain. *Scale bars* **c**, 500 μm; **d**, **e** 200 μm

form a clade composed of at least 13 species (Oden et al. 2016).

Most of them have been associated with marine organism's diseases, including molluscs (Sect. 8.2). In wild-caught *O. vulgaris,* without apparent damage, we frequently detected small black spots on their dorsal mantle, and in some occasions small ulcers surrounding the spots (Fig. 8.3a, b). Bacteria isolated, which were identified by using sequencing of 16S rRNA gene, were closely related to *V. splendidus/V. atlanticus/V. kanlaloae*. However, when octopus were kept in the aquarium tanks, the ulcerations were much more evident and spread infection to head and

arms, and also to other conspecifics sharing the tank. Death was not observed in any of animals at least for four months.

Histopathological lesions were characterized by dense aggregations of bacteria, ulceration, loss of skin epithelium, and haemocyte infiltration and inflammation of the infected area (Fig. 8.3c, d).

Splendidus clade includes gram-negative rods, motile and facultatively anaerobic bacteria with ability to growth on TCBS, ability to produce acid from a wide variety of substrates, and also with facility to grow by using an extensive number of substrates as sole carbon source. These lasts phenotypic features are the mainly basis to differentiate the species within Splendidus clade, but it is still difficult since variations in the same phenotypic test are reported among different strains included in the same species and different research groups.

A fast preliminary identification is proposed through the number of steps described in Sect. 8.2 (Characterization of culturable bacteria). Firstly, a phenotypical characterization with primary identification tests including the decarboxylases (ADH, LDC, ODC). Secondly, since Splendidus clade, is the most abundant in marine environment, the use primers VTS/VT and VTS/VS primer sets are a valuable tool for a fast detection of Splendidus clade. Finally, the 16S rRNA gene analyses are also a valuable tool to resolve distinct genera, but it is insufficient to discriminate closely related species such as those included in the Splendidus clade. If a final identification will be necessary, then a multilocus sequence analysis (MLSA) technique, which is based on the sequencing of multiple housekeeping genes, should be applied (Pérez-Cataluña et al. 2016; Oden et al. 2016).

The susceptibility of *V. splendidus* to several antibiotics was recorded (chloramphenicol, flumequine, nitrofurantoin, nifurpirinol, oxonic acid and potentiated sulphonamide), and their use was successful for the treatment of infection in fish (Revised by Austin et al. 2007). Chloramphenicol and gentamycin were also highly effective in the treatment of squids or cuttlefishes (Hanlon and Forsythe 1990). Recently, other alternative strategies to control an outbreak have been effective, such as the use of a combination of different phages (Li et al. 2016). Treatments to control disease caused by other species of Splendidus clade are still unavailable.

8.3.2 Miscellaneous Bacteria Associated with Eye Damages or Associated Tissues

Previously, several *Vibrio* species were proved as invaders of squid eyes, having hardly affected the cornea. Particularly, *V. anguillarum*, *V. charchariae* or *V. harveyi* were cultured from the posterior chamber of squids. Furthermore, *Micrococcus* sp. was also detected in the vitreous humour, the posterior lens surface and the haemolymph of affected

Fig. 8.4 Retrobulbar lesion in an adult male of *O. vulgaris* cultured in Italy: **a** White fluid material leaked from the retrobulbar lesion after the removal of the ocular bulb. **b** Retrobulbar-confined cavity (2–3 cm diameter) excised from the head
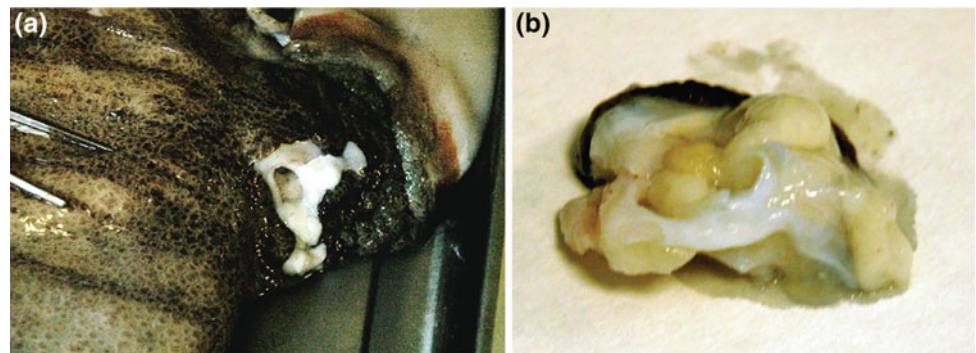
squids. Authors proposed a primarily involving damage to the cornea, which would be previous to the infection (Hanlon and Forsythe 1990). Recently, *Ph. swingsii* and *L. garvieae* were isolated from a white fluid material leaked from a retrobulbar lesion after the removal of the ocular bulb in an adult male common octopus. Until removing the ocular bulb, no gross lesion was observed. The lesion appeared as a confined cavity of 2–3 cm diameter (Fig. 8.4a, b).

Lactococcus garvieae

This is a gram-positive bacterium, which causes diseases in several aquatic and terrestrial animals (Tsai et al. 2012). In fish, it is responsible for hyperacute haemorrhagic septicaemia, but it has also been identified in bacterial outbreaks in aquatic invertebrates, such as the giant freshwater prawn, *Macrobrchium rosenbergii* (Vendrell et al. 2006; Tsai et al. 2012; Meyburgh et al. 2017). This bacterium has been reported in several species of fish, but it has also detected in other marine animals, such as a bottlenose dolphin, *Tursiops truncatus* (Evans et al. 2006) and a sea turtle, *Caretta caretta* (Fichi et al. 2016). Furthermore, it is considered an emerging pathogen related with handling or ingestion of raw fish and seafood for humans (Gibello et al. 2016). In cephalopods, *L. garvieae* was previously isolated in the muscle of a squid collected in a restaurant as the source of infection in a human endocarditis in Taiwan, but there was the possibility that the squid resulted positive for this bacterium due to a cross contamination with other raw fish (Wang et al. 2007).

L. garvieae, *Streptococcaceae* family, Bacilli class, is an ovoid coccus, not-motile, which grows from 4 to 45 °C, and at 6.5% NaCl (Meyburgh et al. 2017), on several common media. On blood agar and Columbia-colistin-nalixic acid (CNA) agar, the colonies are small, white, alpha haemolytic and catalase negative. Several commercial identification systems, such as API strips, BD Phoenix, the Vitek system or MicroScan are able to identify it, but some strains can be misidentified as *L. lactis* or *Enterococcus* spp. (Gibello et al. 2016). A selective medium to differentiate *L. garvieae*, from

other fish pathogen bacteria, called LG agar, has been developed by Chang et al. (2014). However, some molecular techniques, such PCR amplification of the internal transcribed spacer (ITS) region, sequencing of the 16S rRNA gene, multiplex PCR and DNA microarray have been developed and allow to identify *L. garvieae* (Chang et al. 2014; Gibello et al. 2016; Meyburgh et al. 2017).

The treatment of *L. garvieae* infection in fish is based on lincomycin, oxytetracycline and macrolide antibiotics. A characteristic of this bacterium is the resistance to clindamycin, but resistance to erythromycin, streptomycin, tetracycline, oxytetracycline, florfenicol and some quinolones have also been reported (Gibello et al. 2016).

Photobacterium swingsii

This is a motile gram-negative small coccobacillus in *Vibrionaceae* family, Gammaproteobacteria class. It has been isolated and characterized for the first time from oysters during a vibriosis in Mexico and from the haemolymph of wild spider crabs collected in Canary Islands, Spain, by Gómez-Gil et al. (2011). It grows in TCBS agar, TSA supplemented up 2% NaCl (TSA-2), marine agar, and blood agar in the temperature range of 4–37 °C, and in the salt concentration range from 3 to 6% (Gómez-Gil et al. 2011, Fichi et al. 2015). In TCBS, the colonies of *Ph. swingsii* are green, small, round with smooth border, 2–3 mm diameter, while in TSA-2 and marine agar they are white (Gómez-Gil et al. 2011; Fichi et al. 2015). Some differences were observed among the six strains isolated by Gómez-Gil et al. (2011), regarding the growth conditions and the biochemical reactions.

The strain isolated from octopus grew from 22 to 37 °C and at the 3% salt concentration and it tested positive for oxidase and catalase, and it is possible the identification by API 20 E or API 20NE systems. The identification of *Ph. swingsii* was applied by the sequencing of 16S rRNA gene. By the fact that this bacterium has been isolated from oysters during a vibriosis outbreak and from a retrobulbar lesion in

Fig. 8.5 *O. vulgaris* gills maintained in aquarium conditions. Histological sections of an adult showing rickettsiales-like organisms (arrows) at the base of the gill epithelium with distension of the basal membrane and hypertrophy of the infected cell. H&E stain. *Scale bars* **a** 100 μm; **b** 50 μm
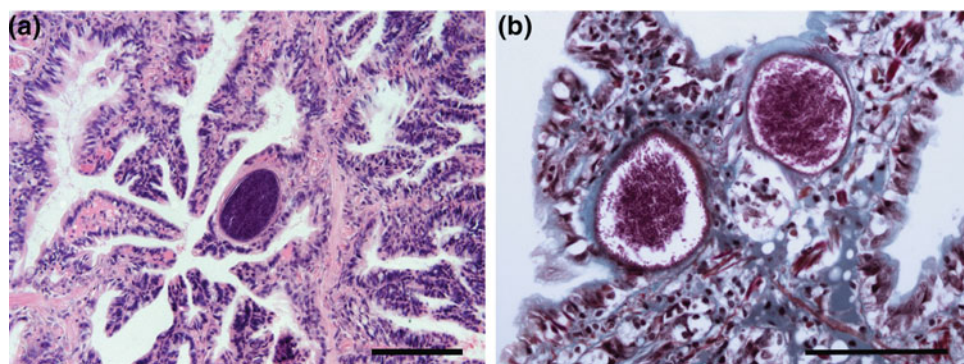
the common octopus, there are strong evidences of its pathogenic role, but this should be confirmed by an experimental infection.

The isolated strain resulted susceptible to the vibrio static O129 at 10 and 150 μg, but no other antibiotic test susceptibility was performed.

8.3.3 Rickettsial-like Organisms (RLO)

RLO are gram-negative bacteria, which have been associated with infection and mass mortalities in several aquatic animals. Particularly, cases of molluscs bivalves have increased since the first description in clams was reported (Azevedo et al. 2006; revised by Romalde and Barja 2010; Ceuta and Boehs 2012; revised by Gollas-Galván et al. 2014). These organisms have been found mainly within the epithelial cells of the mantle, gills digestive gland, connective tissue and hepatopancreas of several molluscs (revised by Gollas-Galván et al. 2014).

Similarly, they were detected in the gills of the common octopus *O. vulgaris*. They were observed like basophilic intracytoplasmatic microcolonies of about 102 μm (70–150) infecting the epithelial cells of the gills (Fig. 8.5a). Invaded host cells became hyperthophic (Fig. 8.5b) and necrosis were occasionally observed (Gestal et al. 1998). No significant harm or signs of disease have been observed in the hosts, since usually a few cells were affected. However, conditions of stress, high animal density or crowding system under intensive rearing increased the occurrence of diseases. Under these conditions, RLO were able to have a detrimental effect on the respiratory gaseous exchange of the octopus and special attention and controls were needed.

RLO are obligate intracellular organisms, highly fastidious, non-motile, non-spore-forming and highly pleomorphic. Unfortunately, artificial media to isolate these organisms are still unavailable for most of them, being difficult their isolation and characterization. To date, only one species was able to grow on agar plates or broth (revised by Gollas-Galván et al. 2014).

Light microscopic examinations of paraffin sections or indirect immunofluorescence with specific immune serum are the methods used for their identification. Sequencing of 16S, internal transcribed spacer (ITS) or 23S ribosomal DNA sequencing are also strategies used to characterize these bacteria. Moreover, some specific primers set have been developed to identify Rickettsia in salmonids (Mauel et al. 1996).

Limited studies about treatment of RLO to control disease are published. The supply of medicated feed (oxytetracycline and florfenicol) was effective for the treatment against the necrotizing hepatopancreatitis bacterium (NHPB), which is a RLO-affecting crustaceans (Gollas-Galván et al. 2014).

8.4 Pathogenic Bacteria for Adults

In this section, a brief reference to those bacteria confirmed as the causative agent of diseases and mortality in octopus are included.

8.4.1 *Vibrio lentus*

This is a gram-negative bacterium, which was firstly described as an environmental species being associated to reared Mediterranean oyster (Macián et al. 2001), and later also associated with larvae cultures of scallops, and turbot (Le Roux et al. 2004). Studies to assess the diversity of bacteria based on 16S rRNA showed that this species seems to be also associated with mussel or adult turbot hatcheries, and surface of algae (Montes et al. 2006; Wang et al. 2009; Kwan and Bolch et al. 2015). Moreover, it is one of the intestinal autochthonous bacteria from the intestinal tract of the common carp (Chi et al. 2014), and recently it was revealed as a protective agent against vibriosis caused by *V. harveyi* in gnotobiotic sea bass larvae (Schaeck et al. 2016). On the contrary, this species was also associated with lesions in lobsters (Chistoserdov et al. 2005) or octopus. Particularly, in octopus, *V. lentus* was isolated from skin lesions and the gill heart of diseased individual captured from their

Fig. 8.6 *V. lentus* on plates, 48 h, 22 °C. **a** TSA-2. **b** TCBS
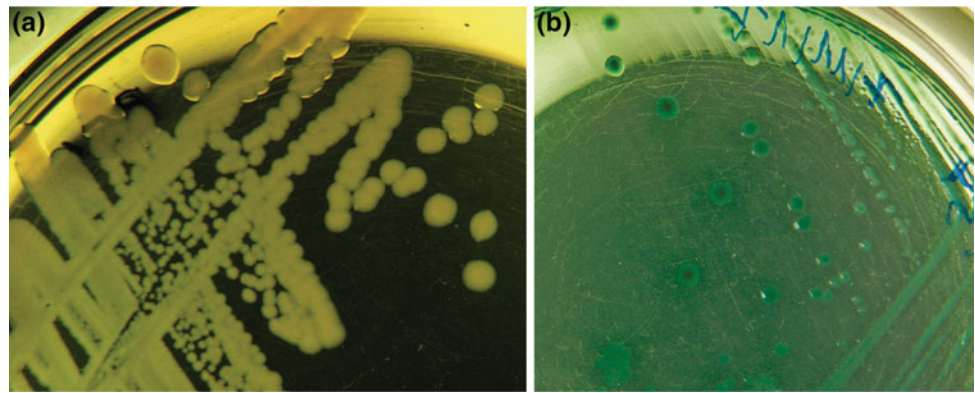
natural marine environment, and it was able to induce both skin lesions and mortality in healthy octopuses (0.5–1 kg) maintained in the laboratory (Farto et al. 2003).

The signs caused by bacteria in octopuses were round hard lesions in the arm or head mantle, which can evolve to skin losing and muscle beneath exposition when the time is advancing. These symptoms were similar to those of stages 3 and 4 of the disease described by Hanlon and Forsythe (1990) for *O. joubini*. In addition, the bacteria was able to colonize gill heart, and induce lesions and mortality after 72 h of exposition, depending on immunity of each individual (Farto et al. 2003).

V. lentus, Vibrionaceae family, Gammaproteobacteria class, is a rod-shaped bacteria, motile, unable to growth at 37 °C or with 7% of NaCl. It shows 0.2–0.4 mm colonies, which are, round, transparent, non-pigmented and unable to swarm after 48 h on TSA-2 at 22 °C (Fig. 8.6a); on TCBS, the colonies are green (Fig. 8.6b). Other phenotypical characteristics were previously described in detail (Macián et al. 2001; Farto et al. 2003).

This species is closely related to other species, which are all included in the Splendidus clade. Specific primers have not been developed, and the number of steps described in Sect. 8.2.1 (Splendidus clade) is suggested for their identification.

V. lentus showed a wide antimicrobial susceptibility pattern, having the highest, with amoxicyllin, cefotaxime, chloramphenicol or piperacillin (Farto et al. 2003). Safer strategies, such as probiotics or others, are still unavailable, for the treatment of infection in octopus.

8.5 Concluding Remarks

To date, different species of *Vibrio* and new potential pathogens (most of them previously reported as causal agents of high losses in hatcheries as well as in natural beds in aquaculture of molluscs) have been shown associated with different stages of cultured or aquarium-maintained cephalopods. However, it is still unknown if most of them are the initial cause of infection.

To understand the equilibrium among a community of microorganisms, and how changes in husbandry practices select for more virulent genotypes are important targets to be addressed in the future.

Prevention techniques are needed to avoid bacterial infections in cephalopods maintained in aquarium systems, which include good welfare practices, cleaning of tanks and control of water and food quality supplied. However, once the presence of the pathogen is confirmed, treatments to combat the infection are needed. To date, most of treatments to control diseases caused by bacteria-affecting cephalopods are based on applying antibiotics. This therapy is becoming more restrictive as a consequence of generating multi-drug resistant pathogens and accumulation of toxic compounds in farmed organisms, causing long-term adverse health effect to humans and other animals. Therefore, special attention should be focussed on the use of alternative methods in order to protect larvae, juvenile and adult cephalopods against pathogens and improve their survival, and also safeguard human health and environment.

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