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EXPERIMENTAL ARTICLES

Taxonomic Composition of Bacteria Associated with Cultivated Mollusks *Crassostrea lugubris* and *Perna viridis* and with the Water of the Gulf of Nha Trang Lagoon, Vietnam

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Abstract—One hundred and four strains of heterotrophic bacteria have been isolated and characterized from two species of bivalve mollusks cultivated in the Gulf of Nha Trang (Vietnam) and from the water of a mariculture farm. The isolates have been identified on the basis of morphological, physiological, biochemical, and chemotaxonomic properties, as well as by the content of G+C bases in DNA. In the microflora of mollusks, *Vibrio alginolyticus* was predominant; the pathogenic species *V. harveyi* and *V. splendidus* were found as well. Staphylococci and bacilli occupied the second place in abundance after vibrios. In addition, coryneforms and enterobacteria, as well as *Pseudomonas* spp. and *Pseudoalteromonas* spp., were revealed. The composition of the water microflora was more diverse as compared with the microflora of mollusks. In the water, *Bacillus* spp., *Vibrio* spp., and *Pseudomonas* spp. were predominant. *Brevibacterium* spp. and other coryneform bacteria, as well as enterobacteria, occurred in significant amounts. In addition, *Pseudoalteromonas* spp., *Marinococcus* sp., *Halobacillus* sp., *Shewanella* sp., *Sulfitobacter* sp., and bacteria of the CFB cluster were noticed. The presence of pathogenic and conditionally pathogenic bacterial species in the water and mollusks is probably the reason for the high death rate of cultivated animals at the mariculture farm.

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Key words: bacterial communities, fatty acids, mollusks, mariculture farm.

The role of cultivated hydrocoles as a source of valuable and relatively inexpensive protein is rapidly increasing worldwide. Bivalve mollusks, including mussels and oysters, are filter feeders pumping huge amounts of water, up to 7.5 l/h, through their bodies [1] to provide sufficient food and oxygen. Cultivated organisms transform their habitats by changing the biota and substrate composition [2]. They release quite a number of nutrients into the water and accumulate the pollutants associated with solid particles. Artificially grown invertebrates may accumulate bacterial pathogens and act as a reservoir of vibrios which are dangerous for humans and hydrocoles [3]. Many vibrio species that inhabit marine environment are pathogenic both for humans and for sea animals and cause great economic damage to mariculture farms [4]. As a result, bacteriological monitoring at mariculture farms is necessary.

The goal of this work was to determine the composition of the heterotrophic microflora of cultivated mollusks: oyster *Crassostrea lugubris* and green mussel *Perna viridis*, as well as the microflora of ambient water, and to reveal possible pathogens of invertebrates.

MATERIALS AND METHODS

Animal and water samples were taken in January 2005 in the lagoon of the mariculture farm in the Gulf of Nha Trang (Vietnam), at four stations with the following characteristics:

Station 1. The central part of the lagoon not used for mariculture. Depth: 4 m.

Station 2. Straight-line distance from the shore: 70 m. Depth: 0.6–0.8 m. Oyster boxes were located through the whole depth of the water column.

Station 3. Distance from the shore: 150 m. Depth: 2 m. Oyster and mussel beds were 0.5–1.0 m deep.

Station 4. Distance from the shore: 1000 m. Depth: 2 m. Mussel beds were 0.5–1.0 m deep.

The animals taken from stations 2–4 were placed into sterile plastic bags. Water samples were taken by a bathometer from the surface and near the bottom at each station, except for station 2, where the depth was

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less than 1 m, and transferred into sterile test tubes. The samples were transported to the laboratory on ice. The animals were dissected, their soft tissues were weighed, and the tissues of a total sample of ten animals were aseptically ground in a mortar to homogeneity.

For inoculation on bacterial media, soft tissue homogenate was diluted 100-fold with sterile seawater. TCBS agar, Endo agar, and Chapman medium were inoculated with 0.1 and 1 ml of the homogenate per plate; Youschimizu–Kimura (Y–K) medium [5] was inoculated with 0.1 ml of the homogenate from serial dilutions. The quantity of heterotrophic bacteria was determined on Y–K medium by inoculation of 0.1 ml of nondiluted seawater and serial dilutions of each sample, as well as the homogenate of mollusks. Inoculated plates were kept for two days at 35°C for the first three media and up to two weeks at room temperature for Y–K medium. The plates were examined daily, the number of grown colonies was counted, and the colonies of all morphotypes were subcultured by stabbing to new plates with appropriate media for further reinoculations to obtain a pure culture of bacterial isolate.

The colony morphology of the isolated cultures was studied on the isolation medium, RPA, and Y–K medium using an MBS-10 stereomicroscope. The motility of bacteria in cultures was tested in a living state using a dark-field condenser. Cell morphology and cell wall type were studied in gram-stained preparations.

The investigated properties of the isolated strains included the presence of lysine and ornithine decarboxylases and arginine dihydrolase determined according to Moller and by means of SIP (System Indicator Paper) for the identification of vibrios, and by means of SIP only for the identification of bacteria from the family *Enterobacteriaceae.* Production of indole, acetylmethylcarbinol, glucose oxidation and fermentation by Hugh–Leifson, reaction with Simmons citrate, reduction of nitrates, and the presence of catalase and cytochrome oxidase were tested. The ability to degrade lactose, sucrose, arabinose, mannose, mannitol, and inositol with the production of acid was studied using Giss medium and SIP. The ability to produce gelatinase and amylase was assayed on an agar containing the appropriate substrate. Resistance to ampicillin, polymyxin, oxacillin, streptomycin, benzene penicillin, and vibriostatic agent 0-129 was determined by diffusion into agar, using paper discs saturated with the corresponding antibiotic. Cultures' requirements for Na^+ ions were tested on media without NaCl and with 3, 6, 8, 10, 12.5, 15, or 20% NaCl. DNA was isolated from the cells and the G+C content of the DNA was determined according to the methods used previously [6]. The isolates were identified by the total of the above characteristics using the manual [7] and the keys for identification of vibrios [8]. The strains of the isolated cultures were preserved in test tubes with 20% glycerol in seawater at –75°C. Based on the study of the morphological, cultural, and some biochemical properties, all of the bacterial isolates were divided into groups so that each group contained strains with identical sets of phenotypic characteristics. One strain was taken at random from each group of isolates for the analysis of bacterial fatty acids, according to the methods used previously [9]. The species affiliation of some strains was defined by sequencing of the 16S rRNA of bacterial isolates.

RESULTS

One hundred and four heterotrophic bacteria were revealed as pure cultures from the cultivated mollusks and from the water samples (42 and 62 strains, respectively), isolated, and characterized. Two isolates have not been identified so far. Morphological, cultural, biochemical, and chemotaxonomic characteristics of bacterial isolates were taken into account for their identification. Vibrios, bacilli, flavobacteria, pseudomonads, staphylococci, and bacteria of the *E. coli* group were identified by a set of characteristics previously described by us in our work on the characterization of the microflora of corals from the Gulf of Nha Trang [9]. Since both studies were carried out in the same region and the microflora compositions of the relevant subjects were identical for some taxa, we considered it possible in the present work to characterize the properties of only those bacteria which have not been isolated previously from the hydrocoles and water of the Gulf of Nha Trang.

Since vibrios comprise both pathogenic and nonpathogenic species, identification of *Vibrio* bacteria to the species level was particularly significant for our study. Hence, the data on the phenotypic properties of vibrios are given in a separate table. The entire species spectrum of vibrios was isolated using thiosulfate-citrate-bile-sucrose agar (TCBS agar). TCBS agar underestimates the density of vibrios population as compared with marine agar (MA) but reveals minor *Vibrio* species [10]. The bacteria *V. alginolyticus* were characterized by the absence of arginine dihydrolase; the ability to produce acid from sucrose, mannose, and mannitol; the absence of growth without Na^+; and growth on the media with up to 10% NaCl (Table 1). The DNA G+C content of this species varied insignificantly (45.2 to 45.6 mol %). *V. parahaemolyticus* was determined by the absence of growth on a medium with 10% NaCl and the inability to produce acid from sucrose. *V. vulnificus* produced acid only from mannose, possessed lysine and ornithine decarboxylase, and did not grow on the media with NaCl above 8%. *V. splendidus* had no arginine dihydrolase, lysine, or ornithine decarboxylase; produced no acid from sucrose and arabinose; and did not grow on the medium with NaCl content above 6%. *V. harveyi* had no arginine dihydrolase, did not produce acid from arabinose, and also did not grow on the media with NaCl content above 6%. Three major fatty acids, 16:0, 16:1(n-7), and 18:1(n-7), comprised 67–77% of the total acids, with 40% of 16:1(n-7). Various minor components included mainly saturated and monounsat-

Table 1. Phenotypic properties of some *Vibrio* spp. strains

Test	Vibrio species				
	V. alginolyticus	*V. parahaemolyticus*	*V. vulnificus*	*V. splendidus*	*V. harveyi*
Oxidase	+	+	+	+	+
O/F test	+/+*	+/+	+/+	+/+	+/+
Motility	+	+	+	+	+
Arginine dihydrolase	–	–	–	–	–
Lysine decarboxylase	+	+	+	_	+
Ornithine decarboxylase	V**	V	+	_	+
Acid from:					
lactose	–	–	–	–	–
sucrose	+	–	–	–	+
arabinose	–	–	–	–	–
mannose	+	+	+	+	+
mannitol	+	V	–	+	+
inositol	–	–	–	–	–
Indole	+	+	+	+	+
Nitrate reductase	+	+	+	+	+
Growth on NaCl, %					
0	–	–	–	–	–
6	+	+	+	+	+
8	+	+	–	–	–
10	+	–	–	–	–
Amylase	+	+	+	+	+
Gelatinase	+	+	+	+	+
Sensitivity to:					
0-129	+	+	+	+	+
ampicillin	–	–	–	–	–
polymyxin	+	+	+	+	+
G+C content in DNA, mol %	45.2–45.6	47.0–47.9	46.3	45.8	48.8

* O/F test: oxidation/fermentation of glucose on Hugh–Leifson medium.

** V, characteristic variable depending on strain.

urated acids with a straight chain. On the basis of these distinguishing features, the strains under study could be identified as vibrios [11].

Marinococci had the morphological characteristics typical of gram-positive cocci, as well as bright colony pigmentation; they exhibited tolerance to high Na^+ content in the medium, possessed no cytochrome oxidase, and did not utilize sugars; mol % G+C was 46.3 (Table 2). The above features made it possible to differentiate *Marinococcus* sp. from other marine gram-positive coccoid bacteria.

Brevibacteria were characterized by the "rod–coccus" cycle and differed from other coryneform bacteria mostly in their chemotaxonomic characteristics (Table 3). The four analyzed strains had a similar fatty acid composition with a simple set of components, dominated by branched odd acids: *antheiso*-15:0 and *antheiso*-17:0 (up to 90% of the total). The above features of the fatty acid composition allow these strains to be classified in the genus *Brevibacterium*.

Halobacteria on Y–K medium formed lemon-yellow colonies tightly ingrown into the agar; in smear preparations they were revealed as gram-positive large rounded rods. Cytochrome oxidase and nitrate reductase were absent; they did not grow on the media without NaCl and were resistant to high salt contents in the medium. The taxonomic status of these bacteria was confirmed by the results of the fatty acid analysis of cell lipids (Table 3). More than 93% of all fatty acids were components with a branched chain, the major being *iso*-14:0, *iso*-15:0, and *antheiso*-15:0 (84.0%). These fatty acids are typical of many bacilli; however, the

Table 2. Phenotypic properties of heterotrophic bacteria associated with mollusks and water, Gulf of Nha Trang, Vietnam

Characteristics	Microorganisms							
	Bacillus	*Brevibacterium*	*Pseudoalteromonas*	*Marinococcus*	*Flavobacterium*	*Shewanella*	*Halobacillus*	*Sulfitobacter*
Gram staining	+	+	–	+	–	–	+	–
Cell morphology*	r/r	i/r, c	r, c/r	c	r	r	r	r
Motility	+	–	+		–	+	–	–
Pigment**	w, y	y, o, w	y, w	y	–	–	y	y
Oxidation/fermentation Hugh–Leifson medium***	O, F +/+, –/–	O –/–	O –/–, +/–	O –/–	O –/–	O +/–	O –/–	O –/–
Oxidase	+, –	–	+	–	–	+	–	+
Catalase	+	n/t****	+, –	+	+	+	+	+
Growth on NaCl, %	–	–	–	–	–	+	–	–
3	+	+	+	+	+	+	+	+
6	+	+	+	+	+	–	+	+
9	+, –	+	+, –	+	–	–	+	–
12.5	+, –	+, –	–	+	–	–	–	–
Nitrate reductase	+, –	+, –	+, –	–	–	+	–	–
Arginine dihydrolase	n/t	+	–	–	n/t	–	n/t	–
Lysine decarboxylase	n/t+	n/t	n/t	–	n/t	–	n/t	–
Ornithine decarboxylase	n/t	n/t	n/t	–	n/t	+	n/t	–
Indole	–	n/t	n/t	–	+	–	–	–
Urease	n/t	n/t	n/t	–	–	n/t	n/t	–
Sensitivity to:								
0-129	n/t	–	–	+	–	–	–	n/t
polymyxin	n/t	n/t	+	n/t	+	+	–	–
penicillin	–	n/t	+, –	n/t	+	+	+	+
ampicillin	–	n/t	+, –	n/t	+	n/t	+	+
oxacillin	–	n/t	+, –	n/t	+	n/t	–	+
streptomycin	n/t	n/t	–	n/t	+	n/t	+	+
Gelatinase	–, +	+	n/t	–	+	+	n/t	+
Amylase	n/t	+	n/t	+	+	+	n/t	–
Acid from:								
glucose	+, –	–	+, gas	–	–	+	–	–
mannose	n/t	–	+	–	–	–	n/t	n/t
arabinose	+, –	–	+	+	–	–	n/t	n/t
sucrose	n/t	–	+	–	–	+	n/t	n/t
lactose	n/t	–	+	–	–	–	n/t	n/t
G+C content in DNA, mol %	36.3–47.5	66.5–67.3	41.7–51.2	46.3	32.5	51.7	41.9–50.0	67.1

* r, rods; c, cocci; r/r, regular rods; c/r, curved rods; i/r, irregular rods.

** w, white; y, yellow; o, orange; "–", without pigment.

*** O, oxidative and F, fermentative type of metabolism; oxidation/fermentation of glucose on Hugh–Leifson medium.

**** nt, not tested.

Table 3. Composition of fatty acids in individual strains of the heterotrophic bacteria isolated from mollusks and water of the Gulf of Nha Trang in % of total

Fatty acid	*Vibrio*					*Brevibacterium*				*Halobacillus*	*Sulfitobacter*
	498	499	503	505	534	486-ii	487-ii	513	526	515	519
12:0	3.3	2.4	2	2.5	4.0						
12:1											1.5
13:0-*i*	1.0	1.1	0.3	0.6	2						
13:0		0.3			0.4						
14:0-*i*	1.1					0.2				17.2	
14:0	7.1	5.2	2.9	3.9	7.4					1	
14:1	1.1	0.5	0.3		1.2						
15:0-*i*	1	1			1.5	4.7	4.9	3.2	3.3	21.0	
15:0-*ai*	0.3					47.1	40.8	34.8	41.3	45.4	
15:0	2.2	2.7	2.3	2.3	3.4	0.7	0.7	0.6	0.6	1	
15:1(n-8)		0.8									
16:0-*i*	5.7	1.3	0.5	0.6	0.3	3.7	3.9	3.0	3.2	2.3	
16:0-*ai*										3.6	
16:0	17.9	10.5	16.7	18.2	15.2					1.6	0.6
16:1(n-9)										2.9	
16:1(n-7)	33.2	42.6	38.7	38.2	32.5						0.4
17:0-*i*	1.3	1.5	0.6	0.6	3.2	0.8	0.9	0.7	0.7		
17:0-*ai*	0.7	0.6	0.7	0.5	0.5	38.0	41.1	41.8	41.7	2.4	
17:1-*ai*	0.6	0.5	0.6	0.6	1.8					1.6	
17:0	1.5	1.8	2.1	2.0	2.3	0.5	0.5	0.6	0.6		1.2
17:1(n-8)	1.5	2.9	3.0	2.4	2.1						
17:1(n-6)	0.4	0.9	0.8	0.6	1						
18:0	0.3		0.6	0.7	0.3						3.7
18:1(n-7)	18.2	21.6	21.3	19.3	19.2						91.2
18:2											1.3
14:0-3OH			0.3		0.5						

Fatty acid	*Pseudoalteromonas*	*Pseudomonas*		*Staphylococcus*			*Bacillus*				*Acinetobacter*
	452	532	492	464	465	463	451	529	485	485pr	446
12:0	2.9	2.2									6.2
12:1	2.3										
13:0-*i*										1.2	
13:0	1.1										
13:1	3.3										
14:0-*i*				1	1	2.1	1		1	1.3	
14:0	0.7	3.7	9.3						0.5	0.6	
15:0-*i*				22.8	6.3	30.8	8.7	0.3	48.7	48.2	
15:0-*ai*				41.6	53.5	47.3	50.0	37.4	33.9	32.3	
15:1-*ai*									0.9	1.2	
15:0	4.9	1.4	3.0		0.7		1.2	0.6	0.6	0.8	
15:1(n-8)	3.9										
16:0-*i*				0.5	2.8		2.3	11.9	1.9	1.7	
16:0-*ai*	2.6			0.8			4.4		0.5	0.8	
16:0	5.1	29.5	30.9	0.4	2.5		2.3	0.6	0.8	0.9	10.3
16:1(n-9)		0.4	0.7	0.7			0.9			1	1.9
16:1(n-7)	22.7	16.2	24.0		0.6		2.0				28.6
17:0-i				5.0	4.5	3.4	2.8		1.9	2.5	1.6
17:0-*ai*				10.1	17.1	6.8	16.3	46.6	3.5	5.2	
17:1-*ai*				5.4		3.3	4.9			0.9	7.3
17:0	5.6	1.1	0.5					0.8			
17:1(n-8)	29.4		0.6								
17:0cy	1.3	14.9	7.6								
18:0		0.5	0.3	0.3	3.5			0.5			2.1
18:1(n-9)											35.8
18:1(n-7)	3.7	22.1	15.2		1.5						6.3
14:0-3OH		1.8	1.4								
19:0cy	1.3	3.9							0.8	0.3	

revealed ratio of these components is specific to representatives of the genus *Halobacillus*.

Sulfitobacter spp. were detected solely on the basis of chemotaxonomic characteristics. Two main features that easily distinguish sulfitobacters from other bacteria are an unusually high level of cis-vaccenic acid 18:1(n-7), which amounts to over 90% of the total acids, and the presence of acid 18:2, unusual for bacteria.

Shewanellas had cytochrome oxidase and ornithine decarboxylase, fermented glucose, and did not need Na^+ for their growth; their G+C content was 51.7 mol % (Table 2). The fatty acid composition of one analyzed strain was quite close to that described for *Shewanella* species [12]. It was characterized by a high proportion of acids 16:0, 16:2(n-7), and 18:1(n-7) and a noticeable concentration of branched acids. Some *Shewanella* species are known to produce eicosapentaenoic acid 20:5(n-3). However, it was absent in the strain under study.

Pseudoalteromonads were characterized by the presence of cytochrome oxidase and gelatinase, a lower mol % value of G+C content in DNA (41.7–51.2) than in pseudomonads, and a specific spectrum of antibiotic resistance. The typical distribution of fatty acids was shown in the *Pseudoalteromonas* strain characterized by diversity of fatty acid components with the prevalence of acids 16:1(n-7), 17:0, and 17:1(n-8) and a significant share of acids C12, C13, C14, C15, and C19.

Microbacterium shleiferi, Serratia marcescens, Staphylococcus sciuri were identified by the results of 16S rRNA sequence (unpublished data). Staphylococci were characterized by the prevalence of branched iso- and antheiso-fatty acids: C15 and C17. The variability of the fatty acid composition of three strains under study may point to their affiliation with different species. The fatty acid profiles of strain 492 and 532 demonstrated features typical of pseudomonads [9]. These included a combination of common acids 16:0, 16:1(n-7), and 18:1(n-7) (about 70% in total), and cyclic acids 17:0cy and 19:0cy (up to 18.8%). Noticeable differences in the ratio of fatty acids support classifying the strains under study as different species. The distinguishing feature of the group of five strains was the presence of high concentrations of hydroxy acids (about 20% in total), such as *iso*-15:0-3OH and *iso*-17:0-3OH, as well as of branched acids, particularly *iso*-15:0 and *iso*-15:1 (27 and 29%, respectively). Taking into account these peculiarities, they were ascribed to the *Cytophaga–Flavobacterium–Bacteroides* cluster.

The numbers of heterotrophic bacteria in oysters and mussels determined on Y–K medium were on average 6.4×10^6 and 7.12×10^6, respectively; in the water, it was lower and varied within 1.8×10^4 to 5.3×10^4 cells/ml. The data on the distribution of heterotrophic bacteria in mollusks and water showed that vibrios dominated in all hydrocole samples as compared with other bacteria (Table 4). The exception was oyster samples taken near the bottom at station 2, where staphylococci were predominant. Among the vibrio strains isolated from oysters and green mussels, 87% belonged to the species *V. alginolyticus*; in addition, there was one strain which we did not succeed in identifying to the species level. *V. harveyi* and *V. splendidus* consituted an essential share.

Table 3. (Contd.)

Fatty acid	*Flavobacterium*				
	417	425	426	427	488
13:0-i	0.3				0.8
14:0	0.4	1.8	2.9	1.9	0.3
15:0-*i*	36.4	16.6	18.7	14.3	27.1
15:0-*ai*	5.2	2.8	1	2.2	2.2
15:1-*i*	25.8	14.8	17.4	15.2	29.2
15:0	11.5	18.3	13.7	15.2	11.5
15:1(n-8)	1.0				0.6
15:1(n-6)	1.2	2.8	2.0	3.2	0.6
16:0-*i*		1.1			0.5
16:0	0.5	1.4	2.0	1.0	1.0
16:1(n-7)	0.2	12.7	12	12	1.9
12:0-3-OH		1.7	1.1	2.1	2.
15:0-*i*-2OH	4.3	2.5	5.8	3.8	2.8
14:0-3-OH		1.4	1.4	2.1	
15:0-*i*-3OH	8.4	6.1	6.5	8.7	2.9
15:0-3-OH		1.9		2.3	
16:0-*i*-3OH	1.0	6.4	3.2	7.3	0.8
16:0-3OH		1.7		1.9	0.6
17:0-2OH	1.3				
17:0-*i*-3OH	2.7	5.9	10.4	6.9	12.5

Besides vibrios, the studied mollusks showed a high content of *Staphylococcus* spp., particularly in the oysters grown near the bottom: up to 0.12×10^2 cells/ml. In mussels, this number reached 1.08×10^2–1.32×10^2 cells per 1 g of wet tissue. As was stated above, staphylococci dominated in the microflora of oysters from the near-bottom boxes of station 2. Strain 466, which was selected at random from the colonies on Chapman medium with the predominant morphotype, was identified on the basis of 16S rRNA sequencing data as *Staphylococcus sciuri* (unpublished data). Besides the prevalent vibrios and staphylococci, mollusks were shown to carry bacilli, coryneform bacteria, pseudomonads, pseudoalteromonads, and bacteria of the *E. coli* group. Oysters were contaminated by substantial amounts of bacilli, while enterobacteria contaminated both oysters and mussels. The ratio of gram-negative to gram-positive bacteria in the microflora of cultivated mollusks varied from 1 : 1 to 3 : 1, but gram-negative microflora were predominant.

Table 4. Distribution of heterotrophic bacteria in cultivated oysters, mussels, and ambient water, Gulf of Nha Trang (% of the total number of colonies grown on isolation medium)

Bacterial taxon	Station no., sample											
	2, oysters, surface	2, oysters, bottom	3, oysters	3, mussels	4, mussels	1, water, surface	1, water, bottom	2, water, surface	3, water, surface	3, water, bottom	4, water, surface	4, water, bottom
Bac.	45	–	2	–	–	70	23	–	80	10	30	28
Vibrio	55	34	61	48	47	–	12	–	11	5	19	20
Ps.	–	12	–	–	–	10	58	36	–	50	–	–
PsAlt.	–	–	3	2	–	–	–	–	–	20	30	15
Brev.	–	–	–	–	–	–	–	38	–	–	–	31
Staph.	–	54	28	46	28	–	–	–	–	–	–	–
Ent.	–	–	6	–	25	5	–	–	–	5	–	–
Halob.	–	–	–	–	–	15	–	–	–	–	–	–
Coryn.	–	–	–	4	–	–	7	–	–	5	13	–
Marin.	–	–	–	–	–	–	–	–	–	–	–	3
Flav.	–	–	–	–	–	–	–	–	–	–	–	3
Shew.	–	–	–	–	–	–	–	–	–	–	4	–
Sulf.	–	–	–	–	–	–	–	–	–	–	4	–
Serrat.	–	–	–	–	–	–	–	–	–	5	–	–
Microbac.	–	–	–	–	–	–	–	19	–	–	–	–
n/i	–	–	–	–	–	–	–	7	9	–	–	–

Note: "–", not detected. Symbols: *Bac. – Bacillus*, *Ps. – Pseudomonas, PsAlt. – Pseudoalteromona*s, *Brev. – Brevibacterium*, *Staph. – Staphylococcus*, *Ent.* – enterobacteria of the *E. coli* group, *Halob. – Halobacillus*, Coryn. – coryneforms, *Marin. – Marinococcus*, *Flav. – Flavobacterium*, *Shew. – Shewanella*, *Sulf. – Sulfitobacter*, *Serrat. – Serratia marcescens*, *Microbac. – Microbacterium shleiferi*, n/i, unidentified isolates.

Bacilli dominated in the water of most stations. In the water sample from station 2 containing no bacilli, the dominant position was taken by *Brevibacterium* spp. Other coryneform bacteria were also widely represented in water samples. Halobacteria were found in substantial amounts only in one sample. Among gram-negative heterotrophs, the most numerous were pseudomonads and pseudoalteromonads (which were absent only from the sample of surface water from station 3). Vibrios were widespread in water samples; the majority of them were *V. alginolyticus*; four strains could not be identified to the species level. *V. parahaemolyticus* strains were found in the samples from station 3; *V. vulnificus*, which is pathogenic for a number of sea animals and humans, was found at station 4. The microflora of the water also contained *Microbacterium shleifer*, the quantity of which reached 20% of total colony-forming units per sample. A representative of the family *Enterobacteriaceae, Serratia marcescens*, was found in a sample of near-bottom water from station 3. Single gram-negative *Shewanella* sp., *Flavobacterium* sp., *Sulfitobacter* sp., and gram-positive *Marinococcus* sp. occurred in the water. Bacteria of the *E. coli* group were noticed in minor quantities in two of the seven water samples. On the whole, gram-negative heterotrophic microflora predominated in most of the water samples under study.

DISCUSSION

We have demonstrated the predominance of vibrios in mollusks from the mariculture farm. Since the classical work by Colwell and Liston [13], vibrios are known to be an essential part of the microflora of shellfish, especially oysters. Vibrios prevailed in the heterotrophic microflora isolated from oysters and other edible mollusks grown in Spain [14]. The results of our research are in conformity with the above works. When analyzing the microflora of a macroorganism, one should take into account the dominating *Vibrio* species as well as the physiological state of the animal. It may be assumed that the presence of pathogenic *Vibrio* species in mollusks causes their death upon cultivation. Strain *V. alginolyticus* was prevalent among the vibrios isolated from oysters and green mussels (Table 1). In addition, strain *V. harveyi* was isolated as well. These species are pathogenic for various sea animals, including shellfish [15]. One more pathogenic species,

V. vulnificus, was isolated from water samples from two stations (3 and 4) where mussels were grown. These microorganisms may cause diseases in cultivated mollusks and there is also a risk of their being transferred to humans through the food chain. *V. splendidus* isolated from mussels is known as a pathogen of infection in cultivated oyster *Crassostrea gigas* and causes mass mortality of oysters in France [16]. However, this species was predominant (40%) among other vibrios in healthy oysters in the Mediterranean Sea [10]. We have revealed substantial contamination of cultivated mollusks by *Staphylococcus* spp. However, staphylococci were not found in the water samples. Obviously, mollusks are a reservoir for these microorganisms, providing them with a favorable ecological niche.

Some species of staphylococci may cause diseases of hydrocoles. *Staphylococcus epidermidis* was isolated as an etiological agent of an epizooty of cultivated fish [15]. Mainly gram-negative rods with the oxidative type of metabolism are usually isolated from seawater [17], whereas bacilli occur rarely. In water and in free-living oysters from natural population of the Mediterranean Sea, bacilli have been defined as a minor group [10]. The prevalence of *Bacillus* spp. in the water of the examined mariculture farm suggests that in the course of lagoon exploitation, these microorganisms displace the genuinely marine gram-negative bacteria (*Pseudomonas* spp., *Pseudoalteromonas* spp., etc.) typical of non-eutrophicated seawaters. The content of *Pseudoalteromonas* spp. in the mollusks under study was low and similar in both species. This fact may indicate that *Pseudoalteromonas* are obligatory members of microbial communities in mollusks. Our data are in conformity with the results of other researchers [14]. The quantity of pseudomonads in the water was two times higher than in mussels and oysters (Table 4). It has previously been shown that pseudomonads dominated in the oyster *C. gigas* together with vibrios, aeromonads, and acynetobacters, while coryneforms dominated in the water [18]. In opinion of Bianchi [19], gram-positive asporogenous rods, which include, in particular, corineform bacteria, are usually absent in seawater but are repeatedly revealed in bottom deposits. Nevertheless, brevibacteria and other coryneforms were rather widespread in the water of the examined lagoon. Probably, the boxes used for shellfish growing and the small depth (to 2 m) contribute to the stirring of bottom deposits and the distribution into the water column of bacteria typical of bottom deposits.

The bacteria of the *Cytophaga–Flavobacterium–Bacteroides* phylogenetic cluster are the usual microflora of marine invertebrates. They are able to utilize a wide range of macromolecular substrates and possess both oxidative and fermentative types of metabolism [7]. More and more species of this group are associated with the death of various hydrocoles both at mariculture farms and in natural ecosystems [20]. The bacteria of this cluster were absent from the studied mollusk samples and comprised only 3% in water samples.

Shewanella bacteria, widespread in seawater and in different hydrocoles [7], were isolated from lagoon water (Table 4). Some *Shewanella* species may cause diseases in sea animals [21]. *Shewanella* sp. were absent from the studied animal samples, which may be explained either by the low content of these microorganisms in water or by their low competitiveness for colonization surfaces and nutrient sources as compared with other bacteria found in the mollusks under study. Sulfitobacteria, which play an important part in the cycle of organic sulfur and inhabit eutrophic waters of the coastal zone as well as the oligotrophic zone of the open ocean, have been isolated from water but not from animal samples. Marinococci and halobacteria are usual inhabitants of seawater and are regularly diagnosed there in minor quantities [10, 7]. In our samples, they were a minor group as well. Bacteria of the *E. coli* group were regularly detected both in water samples and in mollusk organisms; mussels were contaminated with them to a greater extent. This fact may indicate a continuous flow of these bacteria from the outside, probably with terrigenous water.

Thus, the microflora of lagoon water at the mariculture farm was much more diverse than the microflora of mollusks cultivated, which conforms to the literature data [22]. This fact gives grounds to believe that not all bacterial species from aqueous environment develop in mollusks. Apparently, only microorganisms with a definite set of physiological and biochemical properties are able to colonize animals when coming into contact with their internal surfaces and to join the associative microflora. The revealed pathogenic and conditionally pathogenic bacterial species from mollusks and ambient water are probably the cause of the high mortality of cultivated shellfish and thus indicate the unfavorable ecological situation in the lagoon as a result of its exploitation in mariculture.

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