Antibiotic resistance in bacterial communities of the oyster *Crassostrea rivularis* from different salinity zones in Qinzhou Bay, Beibu Gulf, China

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The oyster is one of the most abundantly harvested shellfish in the world. To explore the impact of salinity on antibiotic-resistant bacteria (ARB) and the microbial community associated with farmed oysters, oysters were taken from high-, medium-, and low-salinity zones (labeled HS, MS, and LS, respectively) in Qinzhou Bay of Beibu Gulf, China. ARB were tested with the Kirby–Bauer method. Species of ARB were confirmed by 16S rDNA analysis. Microbial communities were analyzed by high-throughput sequencing technology. The results indicate that HS-derived ARB (>60%) resisted β-lactams and aminoglycosides and that LS-derived strains resisted macrolide and tetracyclines. All strains resisted 4 or more antibiotics. A total of 542 operational taxonomic units were detected in the samples, with *Shewanella*, *Vibrio*, and *Endozoicomonas* being the dominant genera (>80%), although distributed differently among the different salinity samples. The oyster microbial richness ranked as MS > LS > HS. This study provides an important reference for future efforts to explain factors or mechanisms underlying correlations between ARB, the microbiome, and salinity and thus the potential health of oysters in this region.

**Keywords:** Salinity, Antibiotic, Resistance, Microbiome, *Crassostrea rivularis*

1. Introduction

*Crassostrea rivularis* is one of the important oysters in China. It is a marine invertebrate of the family Ostreidae (Mollusca, Bivalvia) and is mainly distributed in the estuaries and coastal zones of South China (Wang et al., 2014). Oysters are well known as filter feeders and, thus, have a significant role in the marine environment, with a close relationship to the aquatic environment. During the breeding process, oysters absorb antibiotics, microplastics, heavy metals, and other pollutants from the water and, therefore, have often been used as indicators of environmental pollution (Allen et al., 2010). Moreover, oysters contain many microbes throughout their bodies, including bacteria, fungi, archaea, viruses, and small protists. Dynamic changes in the microbial composition and structure in oysters result in invasion by pathogenic microorganisms, negatively impacting the health of their host (Wang et al., 2018). In recent decades, antibiotics have been used to restrict the growth of bacteria, but emerging resistance has limited their effectiveness (Cao et al., 2020). Antibiotics remain the primary means of controlling bacterial diseases in aquaculture (Wang et al., 2014, 2015). However, the ample use of antibiotics in human and veterinary medicine has led to widespread antibiotic resistance (Voigt et al., 2020). When antibiotics enter an aquatic environment, they can impact the microbial community throughout the aquatic ecosystem (Yang et al., 2018). Antibiotics have been shown to influence the intestinal bacterial composition of oysters with negative effects on the health of the hosts (Devine et al., 2013; Pan and Yu, 2013). In some cases, bacteria have evolved the ability to degrade antibiotics as a homeostatic response to stress (Allen et al., 2010; Grenni et al., 2018). In addition, antibiotics have been reported to alter the phylogenetic structure of a microecosystem, increasing the number of antibiotic-resistant organisms, and disturbing ecological functions in that microecosystem (Chang and He, 2010). The increasing incidence of antibiotic-resistant bacteria (ARB) in watersheds has become a global concern, with ARB being reported to be more prevalent among bacteria from mollusks than from the water itself (Bighiu et al., 2019).

To minimize or even avoid the use of antibiotics for bacterial disease prevention, understanding the structure

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of a bacterial community which maintains a dynamic balance in healthy hosts is important. Once this balance is changed, fatal infections can occur, as reported in oysters (Sakamoto et al., 2018). Variation in the bacterial composition of oysters is determined by habitat conditions, such as salinity, trophic level, and the presence of antibiotics (Sullam et al., 2012; Lokmer and Wegner, 2014). When oysters were exposed to low-salinity (LS) or high-salinity (HS) conditions, the proportion of pathogenic bacteria increased, whereas the proportion of symbiotic or beneficial bacteria decreased (Zhang et al., 2016). During the La Niña portion of El Niño-Southern Oscillation, decreased rainfall was reported to lead to increasing salinity, triggering a rise in the prevalence and intensity of dermo disease, which increases oyster mortality (Soniat et al., 2012). The frequent deaths of oysters during the summer have also been related to HS (Lewis et al., 1996; Richards et al., 2015). However, the relationship between salinity and oyster microbial community structure, in addition to the impacts of antibiotics, has not been a focus of research.

In this study, we investigated the microbial community of Crassostrea rivularis oysters farmed in different salinity zones and determined the ARB sampled from those oysters. To our knowledge, the results are the first to reveal ARB from oysters under different salinity levels, as well as their associated bacterial communities.

2. Materials and methods

2.1. Sample collection and screening, isolation, and purification of antibiotic-resistant strains

In June 2018, oysters (n = 30, 3 parallel groups, 10 oysters in each group, the average shell length approximately 8 cm) were taken from each of 3 salinity zones in Qinzhou Bay, Beibu Gulf: HS of 22‰, medium salinity (MS) of 15‰, and LS of 5‰. The geographical positions were as follows: HS zone, N 21°37′, E 108°53′; MS zone, N 21°39′, E 108°51′; and LS zone, N 21°42′, E 108°50′ (Figure 1). Approximately 0.5 g of oyster intestines from each group of 10 oysters (3 groups per site) were dissected and mixed with 1 mL of sterile physiological saline to be homogenized. The homogenate was then serially diluted into sterile physiological saline, resulting in three dilutions: 10⁻¹, 10⁻², and 10⁻³. From each dilution, a volume of 100 μL was added to each Mueller–Hinton agar (MHA) medium. Three replicates were then coated with each dilution and placed in an incubator (28°C) for 24 h. To determine the percentage of heterotrophic bacteria resistant to different antibiotics, an additional volume of 100 μL of each serial dilution (in triplicate) was smeared on MHA plates supplemented with the following 7 antibiotics, which were based on preliminary background investigations (Wang et al., 2014, 2015): gentamicin (GEN; 16 μg mL⁻¹), furazolidone (FUR; 4 μg mL⁻¹), rifampicin (RIF; 4 μg mL⁻¹), ciprofloxacin (CIP; 4 μg mL⁻¹), enrofloxacin (ENR; 8 μg mL⁻¹), chloramphenicol (CHL; 32 μg mL⁻¹), or sulfamethoxazole-trimethoprim (SMZ-TMP; 16/4 μg mL⁻¹). The plates were then placed in an incubator at 28°C for 72 h, and resulting colonies were purified and identified by blasting the 16S rDNA sequence (Section 2.2). Synchronously, the rest of the homogenate (from 10 oysters) was frozen quickly with liquid nitrogen for subsequent bacterial community analysis by high-throughput sequencing (Section 2.5).

2.2. Extraction of bacterial genomic DNA and polymerase chain reaction (PCR) identification of 16S rDNA

Total DNA from the isolated antibiotic-resistant strains was extracted by using a kit for PCR amplification. For the PCR reaction system, TaqMaster Mix was used, with the primers 8F (5′-GGTTACCTTGTTACGACTT-3′) and 1492 R (5′-AGAGTTTGATCCTGCTCAG-3′). PCR was performed under the following conditions: an initial denaturation step at 95°C for 40 s, subsequent annealing at 55°C for 40 s, extension at 72°C for 45 s, 35 cycles, and a final extension step at 72°C for 10 min. The products were detected by 1.2% agarose gel electrophoresis and then purified and sequenced. The resulting sequences were subjected to Blast analysis in GenBank.

2.3. Kirby–Bauer (K–B) diffusion method and calculation of ARB

Antibiotic susceptibility tests of the identified colonies were performed in triplicate using 20 antibiotic discs as follows: FUR (10 μg), vancomycin (VAN; 30 μg), RIF (5 μg), clindamycin (CLI; 10 μg), amoxicillin (AMO; 10 μg), penicillin (PEN; 10 U), kanamycin (KAN; 30 μg), tobramycin (TOB; 10 μg), tetracycline (TET; 30 μg), streptomycin (STR; 10 μg), GEN (10 μg), florfenicol (FLO; 10 μg), erythromycin (ERY; 30 μg), SMZ-TMP (23.75/1.25 μg), doxycycline (DOX; 30 μg), norfloxacin (NOR; 10 μg), CIP (5 μg), ofloxacin
(OFI; 5 μg), ENR (10 μg), and neomycin (NEO; 10 μg). All isolates were tested for antimicrobial sensitivity using the disk diffusion method with MHA culture plates for 24–48 h in the thermostatic biochemical incubator (28°C), with diameters of the bacteriostatic zone measured by Vernier caliper. All procedures and corresponding results were interpreted according to the guidelines of the Clinical Laboratory Standards Institute (National Committee for Clinical Laboratory Standards, 2010) and the literature (Wang et al., 2014, 2015). The strains used for quality control were Escherichia coli ATCC25922 and Staphylococcus aureus ATCC25923.

The calculation formulas are as follows. Detection rate (% of antibiotic-resistant strains = (quantity of strains on MHA plates with single antibiotic/quantity of strains on MHA plates with no antibiotic) × 100. Antibiotic-resistant rate (% of strains resistant to antibiotic with K-B method/total quantity of tested strains) × 100.

2.4. Cluster typing of ARB

SPSS version 21 software was used for the cluster analysis of isolated strains. A systematic clustering method was used to classify bacteria by taking the diameter of the antibiotic zone as the variable. The squared Euclidean distance was used to represent the congener relationship of ARB.

2.5. High-throughput sequencing and biosignal analysis

The oyster samples were sent to Guangzhou JiRui Gene Technology Co. Ltd. (China) for extraction of DNA and PCR amplification by using the Illumina MiSeq Sequencing platform. PCR was performed from V3–V4 variable regions of 16S rDNA to identify the bacteria taxonomically. The variable region sequences of 16S rDNA were analyzed by constructing a high-throughput sequencing library in order to identify the composition and abundance of bacteria in the visceral mass from the oysters.

To obtain higher quality and more accurate results of bioinformatics, the sequencing data were optimized using the analysis software Cutadapt (V1.9.1), Vsearch (1.9.6), and Qiime (1.9.1). The 2 sequences were aligned and spliced according to overlapping regions and regions of the alignment. The metric calculation was performed using QiIME software.

Random sampling of sequences was used to construct rarefaction curves based on the number of sampled sequences and the number of represented operational taxonomic units (OTUs), with Qiime (1.9.1) and R language. To further explore the dominant bacteria at each level between HS and MS samples, we used linear discriminant analysis effect size (Lefse; Tharwat et al., 2017). The main analysis steps of Lefse involved use of (1) the Kruskal–Wallis rank sum test to detect all characteristic species and significant species abundance differences between different groups, (2) the Wilcoxon rank sum test to test whether all subspecies of the significantly different species obtained in the previous step converged to the same taxonomic level, and (3) linear discriminant analysis to obtain the final differential species (i.e., biomarker).

3. Results

3.1. Antibiotic-resistant strains in oysters from the HS and LS zones

ARB were screened on MHA medium containing 7 common antibiotics. Of the 27 strains obtained, 16 strains were isolated from oysters in the HS zone and 11 strains were isolated from oysters in the LS zone (Table 1). The HS-derived strains were mainly species of Vibrio (29.4%), Acinetobacter (23.5%), and Enterobacter (23.5%), whereas the LS-derived strains were mainly species of Tenacibaculum (27.3%), Pseudomonas (27.3%), and Acinetobacter (18.1%). The HS-derived antibiotic-resistant strains detected and isolated with the MHA medium, which contained various single antibiotics, were resistant to GEN (0.20%), RIF (0.08%), CIP (0.02%), and FUR (0.78%); Figure 2). The LS-derived strains were resistant to CHL (0.02%), GEN (0.56%), and RIF (0.10%; Figure 2).

When tested for antibiotic resistance by the K-B method, the HS-derived strains were resistant to PEN, AMO, VAN, GEN, KAN, TOB, CHL, RIF, and FUR, at rates greater than 60%. LS-derived strains were resistant to PEN, AMO, VAN, CHL, TET, RIF, and FUR at similar rates (>60%). The strains were particularly resistant to FUR and VAN, with resistance rates well above 90%. HS-derived strains that resisted PEN, AMO, VAN, GEN, KAN, TOB, CLI, RIF, and FUR were more than 60%, whereas LS-derived strains that resisted PEN, AMO, VAN, CLI, TET, RIF, and FUR were more than 60% (Figure 3).

Tests for multiple antibiotic resistance revealed that each of the isolates was resistant to at least four antibiotics (Table 2, Figure 4). Among these bacteria, the strains labeled HS4 and HS5, which had been isolated from the plates containing FUR, seemed to be the “super strains.” HS4 resisted all 17 antibiotics tested: AMO, FLO, VAN, GEN, KAN, TOB, NEO, NOR, OFL, CIP, ERY, CLI, TET, DOX, RIF, SMZ-TMP, and FUR, whereas HS5 was resistant to 14 antibiotics: FUR, CLI, RIF, AMO, PEN, KAN, TOB, STR, GEN, NEO, ERY, NOR, CIP, and ENR.

As shown in Figure 4, based on the diameter of the bacteriostatic zone, the resistant bacteria divided into 4 groups (Groups I–IV) using a systematic clustering method. Interestingly, HS-derived strains (HS1–HS16) clustered in Groups I and II. Group I subdivided into four subgroups (i–iv), which included 5, 4, 3, and 2 types of antibiotics, respectively. Strains clustered in subgroup ii belonged to the genus Vibrio and showed resistance to FUR, PEN, KAN, GEN, and TOB. Strains clustered in subgroup ii and iii belonged to the genus Enterobacter and were resistant to FUR, VAN, RIF, CLI, and TOB. Strains clustered in subgroup i were Acinetobacter spp., and they were resistant to FUR, VAN, RIF, CLI, AMO, PEN, KAN, TOB, GEN, NEO, and ERY. LS-derived strains clustered mainly in Groups III and IV and contained a variety of antibiotics with large differences among subgroups. Strains clustered in Groups III and IV mainly belonged to the genera Pseudomonas and Tenacibaculum. In sum, most strains were resistant to FUR, VAN, CLI, and AMO, although they were different bacterial species. Thus, the antibiotic of the isolates seemed to be independent of taxonomy and more closely related to salinity.
3.2 Microbial communities in different salinity zones

The microbial diversity of the oyster samples from the different salinity zones was also analyzed in depth. Saturating rarefaction curves indicated that the amount of sequencing data obtained for each sample was sufficient to represent the species richness of the microbial communities present in the oysters (Figure 5). The total number of OTUs obtained was 542. The average Shannon indices, reflecting both species abundance and evenness in the communities, were 4.07, 4.92, and 4.59 for the HS-, MS-, and LS-derived samples, respectively (Table 3), where the larger the index value, the greater the species abundance. The coverage of all of the samples was >99.8%, indicating that the sample library was adequate.

The Ace and Chao indices indicated a significant negative correlation between salinity and bacterial richness ($r = -0.96$, $P < 0.01$). LS-derived samples were richer in microbiota than samples from the other salinity zones, with the highest bacterial indices of 145 for both Ace and Chao1 (Table 3).

After merging the highest levels of taxa between groups of samples from the different salinity zones, a heat map of the 30 most abundant genera was developed and analyzed (Figure 6). The HS-derived and MS-derived samples first clustered in a group, indicating the similarity of their bacteria, which then clustered with the LS-derived samples.

The bacteria classified from the oysters were mainly *Shewanella*, *Vibrio*, *Endozoicomonas*, *Arcobacter*, *Mycoplasma*, *Pseudoalteromonas*, *Aeromonas*, *Escherichia-Shigella*, and *Bacteroides* (Figure 7). The *Shewanella*
Figure 2. Detection rate of antibiotic-resistant strains from oysters farmed in high-salinity and low-salinity zones. The antibiotics tested were enrofloxacin (ENR), chloramphenicol, gentamicin, rifampicin, sulfamethoxazole-trimethoprim (SMZ-TMP), ciprofloxacin, and furazolidone. The detection rate (%) of strains resistant to ENR or SMZ/TMP was zero. Error bars indicate standard deviation of the mean (n = 3). DOI: https://doi.org/10.1525/elementa.2020.00095.f2

Figure 3. Antibiotic resistance rates of bacterial strains in oysters from high-salinity and low-salinity zones. The tested antibiotics are penicillin, amoxicillin, flufenicol, vancomycin, streptomycin, gentamicin, kanamycin, tobramycin, neomycin, norfloxacin, ofloxacin, ciprofloxacin, enrofloxacin, erythromycin, clindamycin, tetracycline, doxycycline, rifampicin, sulfamethoxazole-trimethoprim, and furazolidone. DOI: https://doi.org/10.1525/elementa.2020.00095.f3
content in the oyster samples appeared to decline with decreasing salinity levels: HS-derived (34.7%) > MS-derived (19.0%) > LS-derived (9.4%), as did *Vibrio* content (Figure 7). In contrast, another genus, *Endozoicomonas*, had a much higher proportion in LS-derived oysters (23.2%) than in HS- or MS-derived oysters, while the percentage of *Pseudoalteromonas*, *Escherichia-Shigella*, and *Mycoplasma* was higher in MS-derived oysters than in HS- or LS-derived oysters.

The top five different genera between HS-derived and LS-derived oyster samples were *Endozoicomonas, Flavobacterium, Pseudoalteromonas, Shewanella*, and *Vibrio*. Compared to LS-derived oyster samples, the relative abundance of *Shewanella* and *Vibrio* was significantly higher in HS-derived oysters (*P* < 0.05), and *Endozoicomonas, Flavobacterium, and Pseudoalteromonas* in LS-derived oysters were much higher than those in HS-derived oysters (*P* < 0.05; Figure 8a). The top five different genera between

### Table 2. Antibiotics of the 27 strains isolated from oysters in the high-salinity (HS) and low-salinity (LS) zones. DOI: https://doi.org/10.1525/elementa.2020.00095.t2

| Strain Number | Species Designations | Antibiotics Resisted | Antibiotic |
|---------------|----------------------|----------------------|------------|
| HS2           | *Acinetobacter sp.*  | 9                    | VAN/FUR/RIF/PEN/CLI/AMO/TOK/KAN/FLO |
| HS3           | *A. calcoaceticus*   | 12                   | VAN/FUR/RIF/PEN/CLI/AMO/TOK/ERY/KAN/NEO/GEN/TET |
| LS4           | *A. venetianus*      | 9                    | VAN/FUR/RIF/PEN/CLI/AMO/TOK/TET/FLO |
| HS4           | *A. venetianus*      | 17                   | VAN/FUR/RIF/CLI/AMO/TOK/ERY/KAN/NEO/GEN/DOX/TET/SMZ-TMP/CIP/NOR/FLO/OFL |
| HS16          | *A. johnsonii*       | 8                    | VAN/FUR/RIF/PEN/CLI/AMO/TOK/KAN |
| LS7           | *A. johnsonii*       | 11                   | VAN/FUR/RIF/PEN/AMO/STR/KAN/NEO/DOX/TET/CIP |
| HS6           | *V. harveyi*         | 5                    | VAN/FUR/TOK/KAN/GEN |
| HS7           | *V. harveyi*         | 8                    | VAN/FUR/PEN/AMO/TOK/STR/KAN/NEO |
| HS8           | *V. harveyi*         | 8                    | VAN/FUR/RIF/KAN/NEO/GEN/SMZ-TMP/NOR |
| HS9           | *V. harveyi*         | 4                    | VAN/KAN/NEO/GEN |
| LS2           | *V. nereis*          | 9                    | VAN/FUR/RIF/CLI/TOK/KAN/NEO/GEN/NOR |
| HS10          | *V. vulnificus*      | 7                    | VAN/FUR/TOK/STR/KAN/NEO/GEN |
| HS11          | *Enterobacter sp.*   | 9                    | VAN/FUR/RIF/PEN/CLI/AMO/TOK/ERY/KAN |
| HS12          | *Enterobacter sp.*   | 6                    | VAN/FUR/CLI/TOK/KAN/GEN |
| HS13          | *E. asburiae*        | 10                   | VAN/FUR/RIF/PEN/CLI/AMO/TOK/ERY/STR/GEN |
| HS14          | *E. asburiae*        | 10                   | VAN/FUR/RIF/PEN/CLI/AMO/TOK/ERY/STR/GEN |
| HS15          | *E. cloacae*         | 9                    | VAN/FUR/PEN/KAN/AMO/ERY/STR/GEN |
| LS9           | *Flavobacterium beibuense* | 9 | VAN/FUR/PEN/CLI/AMO/TOK/STR/SMZ-TMP |
| HS5           | *Microbacterium sp.* | 14                   | FUR/RIF/PEN/CLI/AMO/TOK/ERY/KAN/NEO/GEN/CIP/NOR/ENR |
| LS3           | *Pseudomonas pseudoalcaligenes* | 10 | VAN/FUR/RIF/PEN/CLI/AMO/ERY/DOX/TET/SMZ-TMP |
| LS5           | *P. pachastrellae*   | 8                    | VAN/FUR/RIF/PEN/CLI/AMO/ERY/TET |
| LS6           | *P. pseudoalcaligenes* | 11 | VAN/FUR/RIF/PEN/CLI/AMO/ERY/STR/DOX/TET/FLO |
| HS1           | *Ochrobactrum tritici* | 7 | VAN/FUR/RIF/PEN/CLI/AMO/TOK |
| LS1           | *Staphylococcus saprophyticus* | 8 | VAN/FUR/CLI/STR/KAN/NEO/DOX/TET |
| LS10          | *Tenacibaculum sp.*  | 8                    | VAN/FUR/RIF/PEN/CLI/AMO/STR/TOK |
| LS8           | *T. aestuarii*       | 11                   | VAN/FUR/RIF/PEN/CLI/AMO/STR/KAN/NEO/GEN/TOK/STR/SMZ-TMP/FLO |
| LS11          | *T. aestuarii*       | 4                    | VAN/FUR/RIF/CLI |

VAN = Vancomycin; FUR = furazolidone; RIF = rifampicin; PEN = penicillin; CLI = clindamycin; AMO = amoxicillin; TOB = tobramycin; ERY = erythromycin; KAN = kanamycin; FLO = florfenicol; NEO = neomycin; GEN = gentamicin; DOX = doxycycline; TET = tetracycline; SMZ-TMP = sulfamethoxazole-trimethoprim; CIP = ciprofloxacin; NOR = norfloxacin.
HS-derived and MS-derived oyster samples were Aeromonas, Arcobacter, Pseudoalteromonas, Shewanella, and Vibrio. The relative abundance of Aeromonas, Shewanella, and Vibrio was significantly higher in the HS-derived oysters than in the MS-derived oysters ($P < 0.05$); however, the relative abundance of Arcobacter and Pseudoalteromonas in MS-derived samples was much higher (Figure 8b).

According to the Lefse analysis used to further explore the dominant bacteria at each level between the HS and MS samples, the results showed that, at class levels, the abundances of Marinifilum, Bdellovibrionales (containing mainly Bacteriovoracaceae), Halobacteriovorax, and Shewanellaceae (containing mainly Shewanella) displayed significant differences ($P < 0.05$) between the HS and MS samples. Marinilum, Bacteriovoracaceae, and Halobacteriovorax were enriched in MS samples, while Shewanellaceae was relatively enriched in HS samples (Figure 9).

4. Discussion
Salinity is an important environmental factor impacting the survival, growth, and distribution of many aquatic organisms (Kumlu et al., 2000). Shellfish, which are
important aquatic animals, can always change their internal environment to adapt to a frequently varying marine environment, such as changing the activity of metabolic enzymes or antioxidant gene expression levels, as in the abalone (Gao et al., 2017). A previous study showed that the expression of several metabolic-related genes of shrimp changed significantly under HS (Gao et al., 2016). Moreover, salinity can significantly influence the microbial community, including microbial antibiotic resistance (Hubbard, 2012; Gonzalez-Martinez et al., 2014; Ng et al., 2014). Salinity has also been reported as an important factor impacting the effectiveness of antibiotics, with salinity treatment reducing both the photolysis and photocatalytic degradation of sulfonamide antibiotics (Yang et al., 2015). At the same time, salinity was shown to correlate negatively with resistant rates of seawater-

**Table 3.** Alpha diversity index analysis of the bacterial community in oysters from different salinity zones. DOI: https://doi.org/10.1525/elementa.2020.00095.t3

| Salinity          | Ace  | Chao1 | Shannon | Simpson | Coverage |
|-------------------|------|-------|---------|---------|----------|
| High (22‰)       | 129  | 131   | 4.07    | 0.88    | 1        |
| Medium (15‰)     | 140  | 140   | 4.92    | 0.93    | 1        |
| Low (5‰)         | 145  | 145   | 4.59    | 0.88    | 1        |

**Figure 6.** The 30 bacterial taxa of highest abundance in oysters from the different salinity zones. The 3 columns indicate samples derived from high-salinity, medium-salinity, and low-salinity zones. Row names are bacterial taxa at the genus level derived from operational taxonomic units based on 16 S rDNA. The tree at the top is the sample cluster tree, and the tree on the left is the taxon cluster tree. Relative values of the taxa are depicted by color intensity. DOI: https://doi.org/10.1525/elementa.2020.00095.f6
Vibrio to KAN, rifampin, and PEN G (Wang et al., 2015). The present study has shown that more than 60% of strains from HS-derived oysters were resistant to β-lactams and aminoglycosides, with a similar number (>60%) from LS-derived oysters being resistant to macrolides and TETs. These results suggest that the bacteria isolated from different salinity zones were resistant to different classes of antibiotics. We also analyzed the antibiograms of bacteria isolated from different salinity zones. Interestingly, these results demonstrated that HS-derived strains cluster in one group while LS-derived strains cluster in another group, providing more evidence that salinity has an important influence on the antibiotic resistance of oyster-residing bacteria. This influence was apparent at the genus level, as the HS-derived antibiotic-resistant strains belonged mainly to Vibrio, Acinetobacter, and Enterobacter, whereas LS-derived strains were mainly species of Tenacibaculum and Pseudomonas. HS has been reported to inhibit the growth of bacteria that carry antibiotic-resistant genes in wastewater bioreactors (Liu et al., 2018), but our results from the natural environment are not consistent with that finding. We found instead that the detection rate of ARB was higher in the HS-derived samples when using a single antibiotic screening method, with Vibrio comprising a larger proportion, which may be due to the halophilism of Vibrio spp. The implication is that oysters cultured in a high-salinity area may suffer a higher risk of dying when disease occurs, especially when the salinity is above 22‰. In a previous study of another aquatic environment with a salinity range of 0‰–12‰, ARB did not correlate with salinity, but the detection of antibiotic-resistant genes appeared to be favored by an intermediate salinity (Bergeron et al., 2016). The relationship between antibiotic resistance of bacteria and salinity in aquatic environments is thus complex. Uncovering factors or mechanisms to explain the available data will require more study in the future.

Antibiotics administered as prophylactics and therapeutics against infections in both humans and animals are discharged into the water and soil directly or indirectly through wastewater treatment plants (Dolliver and Gupta, 2008). Most oysters live in river estuaries. The geographical locations of the sampling sites in this study can be considered relatively special, given that antibiotics associated with nearby intensive human activities (e.g., hospitals, factories, farming) may be transported to the estuary or coastal area via rivers, although likely in low doses. Importantly, antibiotics pose a risk to ecosystems even at very low concentrations and have biochemical effects on

Figure 7. Relative abundance of bacterial taxa in oysters from the different salinity zones. The 3 columns indicate samples derived from the high-salinity, medium-salinity, and low-salinity zones. Color-coding indicates the relative abundance of each bacterial taxon at the genus level, derived from operational taxonomic units based on 16 S rDNA. DOI: https://doi.org/10.1525/elementa.2020.00095.f7
the microorganisms (Isabel et al., 2009; Huerta et al., 2013). Bacteria, as ubiquitous microbes in the aquatic environment, can acquire antibiotic resistance via horizontal gene transfer or plasmids (Wang et al., 2012a, 2012b). Thus, the spread of ARB from commensal and environmental species to pathogens can occur rapidly (von Wintersdorff et al., 2016). In addition, aquatic bacteria, fish and shellfish pathogens, and human pathogens have been observed to share the same genetic elements and determinants of resistance against quinolones, TETs, and β-lactamases (Boto, 2010). In the present study, screening with a medium containing a single antibiotic yielded

Figure 8. Relative abundance of the five most differentially abundant taxa from the different salinity zones.

The relative abundance of the five genera with the greatest differences between samples is displayed in red for high-salinity oyster samples in (a) and (b) and blue for low-salinity oyster samples in (a) and medium-salinity oyster samples in (b). DOI: https://doi.org/10.1525/elementa.2020.00095.f8
bacterial strains isolated from oysters that showed resistance to multiple antibiotics, with 100% of the isolates resistant to more than four antibiotics, indicating strong multiantibiotic resistance. Furthermore, most of these isolates were resistant to FUR, VAN, RIF, CLI, and AMO, which are important antibiotics used in clinics. Notably, the present results suggest that multi-ARB, of great significance in hygiene and closely related to human health, are widely distributed in the waters and oysters. Several pathogenic bacteria in oysters can infect both humans and oysters, such as *V. parahaemolyticus* and *V. vulnificus* etc. (Zhang et al., 2016; Dickerson et al., 2021). Some pathogenic bacteria show strong resistance to antibiotics, which can be spread among different species through gene-level elements, further threatening the health of aquatic animals and human beings. The ARB and especially the pathogens with multiantibiotic resistance can lead to delayed treatment and increased fatality rate in both humans and farmed animals when cross-contamination happens through the food chain. Hence, antibiotics should be used rationally to reduce antibiotic pollution of the environment.

Microbial community structure associated with oysters has been linked to the season, pH, and temperature (Waldbusser et al., 2011; Petton et al., 2013; King et al., 2019). Maintenance of the microbiota associated with marine animals is a complex process, determined by both environmental factors and host physiology (Dehler et al., 2017). In the current study, we found that salinity impacted the microbial community structure of oysters, as indicated by differences, for example, in the Shannon index for microbial communities in oysters bred in HS, MS, or LS conditions. Previous work in wastewater bioreactors had demonstrated that some members of microbial communities were highly associated with antibiotic-resistant genes at HS but that, overall, ARB were reduced under those conditions (Liu et al., 2018). Salt stress can inhibit many enzymes in microorganisms and affect cellular activity (Song et al., 2018), making the relationship between bacterial richness and antibiotic resistance under different salinity conditions important to confirm. Understanding the ecological niche of ARB and their function in the microbial ecosystem is especially important in an environment where salinity changes and questions arise about the potential emergence or dominance of resistant strains.

The dominant microbes were also distributed differently under the different salinity levels examined. Although communities in the HS- and LS-derived samples were somewhat similar, dominated mainly by *Shewanella* and *Vibrio*, these sample types each had other unique...

Figure 9. Analysis of the most differentially abundant taxa between high-salinity (HS) and medium-salinity (MS) samples. Linear discriminate analysis effect size (Lefse) was used to display: (a) taxa elevated in HS samples with a negative linear discriminate analysis (LDA) score (red) and taxa elevated in MS samples with a positive LDA score (green); and (b) a taxonomic cladogram derived from Lefse analysis of 16 S rRNA sequences with relative abundance over 0.5%. Dots and shading with varied colors illustrate the taxon abundance in each group. Yellow dots indicate nonsignificant differences in abundance between zone HS and zone MS. Only taxa meeting an LDA significant threshold of 4 are shown. DOI: https://doi.org/10.1525/elementa.2020.00095.f9
dominant genera; that is, *Aeromonas* in HS-derived samples and *Endozoicomonas* and *Flavobacterium* in LS-derived samples. The dominant microflora in the MS-derived samples differed from the other salinity samples more strongly, as their dominant genera were *Arcobacter* and *Pseudoalteromonas*. *Vibrio* has long been known as an important pathogen causing high mortality in aquatic animals, resulting in the loss of aquacultural stock through diseases caused by *Vibrio* spp. Bivalve mussels often suffer mortality resulting from *Vibrio* infections, which reduce the economic return of such aquaculture farms (Beaz-Hidalgo et al., 2010). Diseases caused by *Vibrio* such as *V. vulnificus* occur as sporadic outbreaks with 10%–20% mortality rates (Fouz et al., 2002; Audemard et al., 2018). By reducing salinity levels from 28‰ to 9.6‰, the mortality of Pacific oyster larvae caused by *V. corallilyticus* could be decreased from 100% to 70.7% and from 100% to 0% for mortality caused by *V. tubiashii* (Richards et al., 2015). The abundance of another common opportunistic pathogen, *Shewanella* spp., also depends on the salt concentration, with the ability of the bacterium to adhere to gills and intestines increasing with increasing salt concentration, thus rendering some species of *Shewanella* conditionally pathogenic to aquatic animals (Beleneva et al., 2007; Linares et al., 2016; Torri et al., 2018).

Microbial communities maintain a dynamic balance in the body of healthy oysters and other hosts. When this balance is altered, induction of fatal infections can be expected (Gramenga et al., 2017). Notably, under severe weather conditions (e.g., severe air temperatures or severe storm activity and mixing of the water column), HS zones in the region of our study are more prone to disease outbreaks (Li et al., 2020). According to reports (Hernroth and Baden, 2018), aquatic animals that are cultivated at too high a density with poor water quality and HS could easily be infected by opportunistic pathogens, such as *Streptococcus*, *Enterococcus*, *Aeromonas*, *Pseudomonas*, *Vibrio*, *Flexibacter*, and *Edwardsiella*. The oysters sampled in the current study all appeared to be healthy, which suggests a dynamic, positive balance between the hosts and their microbial communities in response to their environmental conditions. However, the function or ecological niches of the bacteria should be considered further, especially when diseases or ARB occur.

5. Conclusion
This study analyzed the antibiotic resistance and community structure of bacteria in the oyster, *C. rivularis*, farmed in different salinity zones. The results suggest that salinity has an important influence on the antibiotic resistance of oyster-resident bacteria. Among the antibiotic-resistant strains, those derived from the HS zone belonged mainly to *Vibrio*, *Acinetobacter*, and *Enterobacter*, while those from the LS zone were mainly species of *Tenacibaculum* and *Pseudomonas*. Most of the strains were resistant to FUR and VAN, with detection rates above 90%. HS-derived and LS-derived strains were resistant to different antibiotics, with more than 60% of strains from HS-derived oysters resistant to β-lactams and aminoglycosides, and a similar percentage (>60%) from LS-derived oysters resistant to macrolides and TETs. All strains from the different salinity zones showed strong resistance to multiple antibiotics, with 100% of isolates resistant to at least four antibiotics. Salinity also affected the structure of microbial communities in oysters (with diversity indices being lowest in HS samples), as well as community composition. Despite some similarities, the dominant microbes differed by salinity level. Although comprised mainly of *Shewanella* and *Vibrio*, the dominant populations in HS- and LS-derived samples also contained the unique dominant genera of *Aeromonas* (HS samples) and *Arcobacter* and *Mycoplasma* (LS samples), while MS samples were dominated by *Pseudoalteromonas*, *Aeromonas*, *Escherichia-Shigella*, and *Bacteroides*. These analyses provide a resource for further studies of the impacts of environmental shifts in salinity on ARB and their potential roles in the health of farmed oysters in the region of Beibu Gulf, China, and elsewhere.

Data accessibility statement
All final DNA sequence assembly is available as online supporting material.

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Competing interests
The authors have no competing interests to declare.

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Contributed to conception and design: RXW, BL, JYW.
Contributed to acquisition of data: RXW, BL, LZ, BL, JYW.
Contributed to analysis and interpretation of data: RXW, BL, LZ, HZ.
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