Stress Response of Vibrio parahaemolyticus and Listeria monocytogenes Biofilms to Different Modified Atmospheres

Hui Qian¹, Wei Li¹, Linxia Guo¹, Ling Tan¹, Haiquan Liu¹,²,³,⁴, Jingjing Wang¹,²,³⁎, Yingjie Pan¹,²,³ and Yong Zhao¹,²,³⁎

¹ College of Food Science and Technology, Shanghai Ocean University, Shanghai, China, ² Laboratory of Quality and Safety Risk Assessment for Aquatic Products on Storage and Preservation (Shanghai), Ministry of Agriculture, Shanghai, China, ³ Shanghai Engineering Research Center of Aquatic-Product Processing and Preservation, Shanghai, China, ⁴ Engineering Research Center of Food Thermal-Processing Technology, Shanghai Ocean University, Shanghai, China

The sessile biofilms of Vibrio parahaemolyticus and Listeria monocytogenes have increasingly become a critical threat in seafood safety. This study aimed to evaluate the effects of modified atmospheres on the formation ability of V. parahaemolyticus and L. monocytogenes biofilms. The stress responses of bacterial biofilm formation to modified atmospheres including anaerobiosis (20% carbon dioxide, 80% nitrogen), micro-aerobiosis (20% oxygen, 80% nitrogen), and aerobiosis (60% oxygen, 40% nitrogen) were illuminated by determining the live cells, chemical composition analysis, textural parameter changes, expression of regulatory genes, etc. Results showed that the biofilm formation ability of V. parahaemolyticus was efficiently decreased, supported by the fact that the modified atmospheres significantly reduced the key chemical composition [extracellular DNA (eDNA) and extracellular proteins] of the extracellular polymeric substance (EPS) and negatively altered the textural parameters (biovolume, thickness, and bio-roughness) of biofilms during the physiological conversion from anaerobiosis to aerobiosis, while the modified atmosphere treatment increased the key chemical composition of EPS and the textural parameters of L. monocytogenes biofilms from anaerobiosis to aerobiosis. Meanwhile, the expression of biofilm formation genes (luxS, aphA, mshA, oxyR, and opaR), EPS production genes (cpsA, cpsC, and cpsP), and virulence genes (vopS, vopD1, vcrD1, vopP2β, and vcrD2β) of V. parahaemolyticus was downregulated. For the L. monocytogenes cells, the expression of biofilm formation genes (flgA, flgU, and degU), EPS production genes (mo2554, lmo2504, iniA, mliB), and virulence genes (vopS, vopD1, vcrD1, vopP2β, and vcrD2β) was upregulated during the physiological conversion. All these results indicated that the modified atmospheres possessed significantly different regulation on the biofilm formation of Gram-negative V. parahaemolyticus and Gram-positive L. monocytogenes, which will provide a novel insight to unlock the efficient control of Gram-negative and Gram-positive bacteria in modified-atmosphere packaged food.

Keywords: stress response, Vibrio parahaemolyticus, Listeria monocytogenes, modified atmospheres, biofilms, extracellular polymeric substance, gene expression
INTRODUCTION

*Vibrio parahaemolyticus* and *Listeria monocytogenes* are important facultative and food-borne pathogens in the food industry (Xu et al., 2015; Vazquez-Boland et al., 2017; Chimalapati et al., 2018). In 2014, 605 cases of *V. parahaemolyticus* infections in the United States were reported according to the Cholera and Other Vibrio Illness Surveillance (Centers for Disease Control and Prevention [CDC], 2014). Meanwhile, 675 listeriosis cases were reported in 47 states and the District of Columbia (Centers for Disease Control and Prevention [CDC], 2014). In China, 71 outbreaks of *V. parahaemolyticus* in Zhejiang Province were monitored from 2010 to 2014, resulting in 933 illnesses and 117 hospitalizations (Chen et al., 2017). For listeriosis, 253 invasive cases are reported from 2011 to 2016 in 19 provinces (Li W. et al., 2018).

In natural environments, the formation of bacterial biofilms is the prevailing microbial lifestyle (Wattnick and Kolter, 2000). Biofilms are complex communities of microorganisms attached to surfaces and enclosed in firm three-dimensional, multicellular, complex, and self-assembled extracellular polymeric substances (EPS) [exopolysaccharides, proteins, extracellular DNA (eDNA), etc.] (Lamas et al., 2016; Han et al., 2017). EPS immobilizes biofilm cells, which facilitates their intense interactions including intercellular communication, horizontal gene transfer, and the formation of synergistic microconsortia (Flemming and Wingender, 2010). In addition, EPS contributes to the initial attachment and nutrient capture of bacteria and the integrity of the biofilm structures (Han et al., 2017; Tan et al., 2018). Meanwhile, EPS has been demonstrated to have good resistance to different environmental stresses including disinfectants, desiccation, salinity, temperature, heavy metal pollution, etc. (Xue et al., 2012; Blanco et al., 2019).

During recent decades, there is increasing evidence indicating that biofilms are involved in contaminating food processing equipment and food products (Brooks and Flint, 2008), including *V. parahaemolyticus* and *L. monocytogenes*. For *V. parahaemolyticus*, it can form biofilms on various biotic or abiotic surfaces such as oysters, stainless steel (SS), etc. (Mizan et al., 2016). Moreover, the pathogenic strains of *V. parahaemolyticus*, on average, formed more biofilm than non-pathogenic strains at all tested temperatures (Song et al., 2016). Additionally, *V. parahaemolyticus* formed the highest amount of biofilms at 2% NaCl and the least biofilm at 5% NaCl (Mizan et al., 2018). For *L. monocytogenes*, when bacteriocin from *L. plantarum* ST8SH, vancomycin (antimicrobial), propolis (a natural antimicrobial product), and EDTA (chelating agent) are used individually or combined, the formation of bacterial biofilms will be inhibited in different degrees (Todorov et al., 2018). Different strains differ in their biofilm formation ability, which is closely linked with the resistance of *L. monocytogenes* to antimicrobials in food processing environments (Pan et al., 2009; van der Veen and Abee, 2010). The study suggests that EDTA influences biofilm formation by affecting the initial adherence of *L. monocytogenes* onto abiotic surfaces (Chang et al., 2012).

For *Vibrio*, extracellular nucleases (Xds and Dns) control the level of eDNA and are involved in multiple processes including the development of a typical three-dimensional biofilm structure (Seper et al., 2011). The exogenous addition of extracellular flagellin-homologous proteins (rFHPs) significantly increased the biofilm formation of *V. parahaemolyticus*, reaching ~3.8-fold compared to control (Jung et al., 2019). For timely expression of EPS under specific conditions, bacterial cells utilize diverse signal recognition systems and subsequent regulatory mechanisms, such as quorum sensing and cyclic di-GMP (c-di-GMP) (Jung et al., 2019). CpsQ is a c-di-GMP-binding transcription factor that activates the expression of capsular polysaccharide (CPS) genes, thereby inducing biofilm development in *V. parahaemolyticus* (Zhou et al., 2013). For *L. monocytogenes*, all genes of the pssA-E operon and a separately located pssZ gene are required for exopolysaccharide production. Under environmental conditions, eDNA of *L. monocytogenes* is released by lysate bacteria, which enhances the initial attachment and formation of biofilm (Colagorgi et al., 2016). Meanwhile, the bacterial surface or extracellular proteins have been shown to be responsible for the induction of biofilm formation (Kayal and Charbit, 2006; Franciosa et al., 2009; Colagorgi et al., 2016).

The expression of genes related to bacterial biofilm plays a key role in biofilm formation. Flagella and other filamentous structures regulated by related genes have clearly been shown to contribute to the initial approach, attachment, and efficient biofilm formation on surfaces (Kirov, 2003). The bacterial gene expression regulated by quorum sensing is caused by small molecules called autoinducers, allowing bacteria to adapt efficiently to environmental conditions during growth (Lamas et al., 2016). For *V. parahaemolyticus*, the quorum-sensing genes including *opaR* and *luxS* play a critical role in the biofilm formation. Additionally, the *cpsA* and *cpsC* gene-regulated secretion of CPS is associated with the adhesion ability of *V. parahaemolyticus* (Hsieh et al., 2003).

The stress response (such as oxygen starvation stress, etc.) is an adaptive strategy for bacteria, which allows them to rapidly cope with changing environmental conditions and ensure their survival (Schimmel et al., 2007; Alvarez-Ordonez et al., 2015). When the modified atmospheres act on bacteria, they are able to maintain viability by regulating certain genes (Alvarez-Ordonez et al., 2015). Previous studies found that *V. parahaemolyticus* and *L. monocytogenes* could grow under aerobic and anaerobic conditions (Gomez-Gil et al., 2003; Valimaa et al., 2015). However, the modified atmosphere packaging (MAP) also dramatically reduced the growth of Gram-negative bacteria including *Vibrio* spp. (Paludan-Muller et al., 1998). In addition, the Gram-positive *L. monocytogenes* were inhibited under all MAP compositions [(1): 40% CO₂/55% N₂/5% O₂, (2): 60% CO₂/40% N₂, and (3): 50% CO₂/50% N₂] (Arvanitoyannis et al., 2011). Therefore, the modified atmospheres are an important way of affecting the growth of Gram-negative and Gram-positive bacteria.

The biofilm formation ability of *V. parahaemolyticus* and *L. monocytogenes* in multiple conditions (temperatures, nutrients, contact surface, or pH) has been well studied in previous studies (Di Bonaventura et al., 2008; Nilsson et al., 2011; Bonsaglia et al., 2014; Song et al., 2016). However, few studies paid attention to the stress responses of the biofilm.
formation of *V. parahaemolyticus* and *L. monocytogenes* to the modified atmospheres. On this basis, the aim of this study was to evaluate the biofilm formation of *V. parahaemolyticus* and *L. monocytogenes* under the stress of different modified atmospheres including anaerobiosis (20% carbon dioxide, 80% nitrogen), micro-aerobiosis (20% oxygen, 80% nitrogen), and aerobiciosis (60% oxygen, 40% nitrogen), by determining the changes in the EPS, structural parameters, and the related regulatory genes. The present study will provide a novel insight to unlock the efficient control of Gram-negative and Gram-positive bacteria in modified-atmosphere packaged food.

**MATERIALS AND METHODS**

**Bacterial Strains and Culture Conditions**

Four-strain cocktails of *V. parahaemolyticus* strains (VP-S36, VP-39, VP-49, and VP-54) were used (Li et al., 2017). Meanwhile, four-strain cocktails of *L. monocytogenes* strains (NO.12, NO.14, NO.51, and NO.66) were used. All of the genotypes and origins of each strain are listed in **Table 1**. Strains of *V. parahaemolyticus* were stored at −80°C in tryptic soy broth (TSB, Land Bridge Technology, China) with 50% (v/v) glycerol. And the four *L. monocytogenes* strains in Brain Heart Infusion (BHI) with 50% glycerol were stored at −80°C. Before every experiment, frozen cells of *V. parahaemolyticus* were streaked on thiosulfate citrate bile salts sucrose agar (TCBS agar, Land Bridge Technology, Beijing, China) and with shaking at 200 r/min. Subsequently, the cultures of *V. parahaemolyticus* and *L. monocytogenes* were diluted by TSB thermoplastic, respectively. Subsequently, the 24-well plates of *V. parahaemolyticus* cocktail were incubated at 25°C statically to form biofilms for 48 h, and the *L. monocytogenes* cocktail was incubated at 37°C statically to form biofilms for 72 h.

**Biofilm Formation in Different Modified Atmosphere Conditions**

Biofilm formation of *V. parahaemolyticus* and *L. monocytogenes* cocktails was carried out as described previously (Song et al., 2016; Han et al., 2017) with minor modifications. Static biofilms were grown in 24-well polystyrene microtiter plates (Sangon Biotech Co., Ltd., Shanghai, China) under three different atmosphere conditions: anaerobiosis (20% carbon dioxide, 80% nitrogen), micro-aerobiosis (20% oxygen, 80% nitrogen), and aerobiciosis (60% oxygen, 40% nitrogen), and then sealed with thermoplastic, respectively. Subsequently, the 24-well plates of *V. parahaemolyticus* cocktail were incubated at 25°C statically to form biofilms for 48 h, and the *L. monocytogenes* cocktail was incubated at 37°C statically to form biofilms for 72 h.

**Crystal Violet Staining Method**

Biofilm production was quantified by using the crystal violet staining method as described previously (Han et al., 2017; Tan et al., 2018) with some modifications. After incubation, the supernatant of the plates’ wells was discarded, these plates were gently washed three times with 1 ml of 0.01 M phosphate buffer (PBS, pH 7.4) to remove planktonic cells, and then the microtiter plates were put into the electric blast drying oven for about 20 min and stained with 1 ml of 0.1% (w/v) crystal violet (Sangon Biotech Co., Ltd., Shanghai, China) for 30 min at room temperature. The plates were then gently washed three times with PBS (Sangon Biotech Co., Ltd., Shanghai, China) to remove excess crystal violet. The next step was to dissolve the crystal violet-stained biofilm with 1 ml 95% ethanol for 30 min. Biofilm was solubilized using 1 ml of 95% ethanol (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) for 30 min at room temperature. The optical density of each well was measured at a wavelength of 600 nm (Han et al., 2017). All assays were performed in triplicate in three independent experiments. And at least 12 wells were tested in each experiment.

**Visualization of the Biofilms Using Confocal Laser Scanning Microscopy**

Before the experiment of cultivating biofilm, a sterile glass slide (1.4 cm in diameter) also needed to be placed into the wells of the 24-well microtiter plates. The culture process of biofilms is as described above. The biofilms of *V. parahaemolyticus* and *L. monocytogenes* cocktails for confocal laser scanning microscopy (CLSM) were pre-treated based on the method of Han et al. (2017). Subsequently, the next step is to add the corresponding bacterial solution and fresh culture medium and

---

**Table 1A** | The genotype and origins of *Vibrio parahaemolyticus* used in this study.

| Strains | Genotype | Origin |
|---------|----------|--------|
| VP-S36  | tdh+/tdh+/tdh+ | Shrimp |
| VP-C39  | tdh+/tdh+/tdh+ | CDC    |
| VP-C49  | tdh+/tdh+/tdh+ | Clinical isolation |
| VP-C54  | tdh+/tdh+/tdh+ | Clinical isolation |

**Table 1B** | The genotype and origins of *Listeria monocytogenes* used in this study.

| Strains | Genotype | Origin               | Number |
|---------|----------|----------------------|--------|
| NO.12   | 1/2a     | Raw pork             | 4bLM   |
| NO.14   | 1/2a     | Human                | ATCC 17644 |
| NO.51   | 4b       | Spinal fluid of child with meningitis | ATCC 193932 |
| NO.66   | 4b       | Human                | ATCC 19115 |
then incubate them following the previous experiment of biofilm formation conditions. After static incubation, the biofilms of *V. parahaemolyticus* and *L. monocytogenes* formed on the sterile glass, and then the plates were washed three times with 1 ml of 0.01 M PBS. Next, the plates were fixed with 4% glutaraldehyde (Sangon Biotech Co., Ltd., Shanghai, China) at 4 °C for 30 min. Then, the biofilms were gently rinsed three times with 1 ml of 0.01 M PBS. Therefore, after the above operation, SYBR Green I (Sangon Biotech Co., Ltd., Shanghai, China) was used to stain the biofilms at room temperature and dark conditions for 30 min. Before removing the sterile glass from the plate, the plate needs to be washed three times with 1 ml of 0.01 M PBS. It may take about 20 min to air-dry the sterile glass before the end of the experiment. All microscopy images were captured and acquired using the CLSM machine (LSM710, Carl Zeiss AG, Germany). A 40 × objective was used to monitor SYBR Green I fluorescence excited at 488 nm and emitted at 500–550 nm. Then the most representative place was scanned to provide a stack of horizontal planar images with a z-step of 1 μm. In order to compare the change of the biofilms under different modified atmospheres, the CLSM images were performed by using the ISA-2 software (Professor Haluk Beyenal, Montana State University, United States) to determine the structural parameters [such as biovolume, mean thickness, bio-roughness, and textural entropy (TE)] of biofilms (Beyenal et al., 2004b). For each sample, the representative images of nine separate sites on the glass slide were randomly acquired.

### Visualization of the Biofilms Using Scanning Electron Microscopy

The method of culturing bacterial biofilms is similar to that described above. The biofilms of *V. parahaemolyticus* and *L. monocytogenes* cocktails for CLSM were pre-treated based on the method of Han et al. (2017). Next, the plates were incubated to form bacterial biofilms. After static incubation, the biofilms were washed three times with 1 ml of 0.01 M PBS and then were fixed overnight with 2.5% glutaraldehyde at 4 °C. Next, the biofilms were gently washed three times with 1 ml of sterile 0.01 M PBS. Then, the biofilms were dehydrated in an ascending acetonitrile series (30, 50, 70, 80, 90, and 100% twice for 10 min each). Samples were then dried and finally coated with a turbomolecular pumped sputter coater (Q150T ES PLUS, Quorum, United Kingdom). Subsequently, extreme-resolution images were randomly acquired.

### EPS Extraction and Chemical Analysis

Extracellular polymeric substance, which consists of bacterial biofilms, was extracted using the sonication method (Liu et al., 2007; Gong et al., 2009; Han et al., 2017). Firstly, the density of the planktonic cells of the suspended cultures was measured by a BioTek microplate reader at OD595nm. Then, the planktonic cells were removed and discarded after washing three times with 1 ml of 0.01 M PBS. Afterward, the mature biofilms were collected and scrapped in 1 ml 0.01 M KCl solution. The cells were sonicated by a sonicator (VCX 500, SONICS, Newtown, CT, United States) for four cycles of 5 s of run and 5 s of pause at a power level of 3.5 Hz. Afterward, the sonicated suspensions were centrifuged at 4°C, 4000 × g for 10 min, and the supernatant was then filtered through a 0.22 mm membrane filter (Sangon Biotech Co., Ltd., Shanghai, China). The amounts of protein, carbohydrate, and eDNA in the filtrate were analyzed. The eDNA was quantified by the Quant-it™ PicoGreen® dsDNA Reagent and Kits (Life Technologies, Shanghai, China) according to the manufacturer’s instructions (Grande et al., 2015). For extracellular protein, it was quantified by the Stable Lowry Protein Assay Kit (Sangon Biotech Co., Ltd., Shanghai, China), and bovine serum albumin (BSA) was used as a protein standard to perform the calibration curve, which contains slightly more modifications compared with the Lowry method. A certain amount of extracellular polysaccharide in the filtrate was quantified by the phenol–sulfuric acid method (Kim and Park, 2013) and expressed as OD_{490nm}/OD_{595nm}. Each experiment was carried out at least three times.

### Expression of Biofilm Formation, EPS, and Virulence-Related Genes

After the treatment of *V. parahaemolyticus* and *L. monocytogenes* biofilm under three modified atmospheric conditions, the total RNA was extracted using the Bacteria RNA Extraction Kit (Vazyme Biotech Co., Ltd., Nanjing, China) and quantified using a BioTek microplate reader. Total RNA was then resuspended in 50 μl of diethyl pyrocarbonate (DEPC)-treated water. RNA purity and integrity were assessed according to a previous study (Kim et al., 2016). Complementary DNA (cDNA) was reversely transcribed from 2 μl of total RNA using a HiScript® III RT SuperMix for qPCR (+ gDNA wiper) (Vazyme Biotech Co., Ltd., Nanjing, China). The qRT-PCR reactions were executed via a ChamQTM Universal SYBR® qPCR Master Mix and carried out by a 7500 Fast Real-Time PCR system (Applied Biosystems, Waltham, MA, United States). All the primers were synthesized by Sangon Biotech (Shanghai, China). All qPCR reactions were performed in a total volume of 20 μl containing 10 μl of 2 × ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd., China), 0.4 μl of 10μM forward primer, 0.4 μl of 10 μM reverse primer, 2 μl of cDNA, and 7.2 μl of deionized water. Cycling parameters for qPCR included an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, and primer extension at 72°C for 30 s. The fluorescent products were detected after the extension step of each cycle. The changes in relative gene expression were calculated with the 2^{−ΔΔCT} method. Primers used in this study for the detection of *V. parahaemolyticus* and *L. monocytogenes* are listed in Table 2. Genes 1–14 in Table 2 belong to *V. parahaemolyticus*, and genes 15–26 belong to *L. monocytogenes*. Each experiment was carried out at least three times.

### Statistical Analysis

The experimental data were expressed as the mean ± standard deviation. Analysis of one-way ANOVA was used to compare the
### TABLE 2 | Primer sequences of the RT-qPCR assay.

| Number | Gene   | Primer name | Primer sequence (5′–3′) | References       |
|--------|--------|-------------|-------------------------|------------------|
| 1      | recA   | recA-F      | GGTAGTAGAAAAAGCGGCTGT   | Ma et al., 2015  |
|        | recA-R |             | GCAAGTGTTCTGTTGTTAAG    |                  |
| 2      | oxyR   | oxyR-F      | TCGTCAAGTCCAGGGAAGG     | Chung et al., 2016 |
|        | oxyR-R |             | TGGTGCCGAGTAACTAG       |                  |
| 3      | aphA   | aphA-F      | ACAACCCACCGTGTTGAG      | Wang et al., 2013 |
|        | aphA-R |             | GTGTAAGGGCGTTGTTAAG     |                  |
| 4      | luxS   | luxS-F      | CATCTCAACCCGACTAAACC    | Zhang et al., 2016 |
|        | luxS-R |             | ACTCTGTTTCACTGAGTTAAG   |                  |
| 5      | opaR   | opaR-F      | TGTCTTACCAACCGACTAAACC | This study       |
|        | opaR-R |             | GCTCTTACCACTGAGTTAAG    |                  |
| 6      | mshA   | mshA-F      | GGTTCGCTGTTAAGCTAGCAG   | Shime-Hattori et al., 2006 |
|        | mshA-R |             | CGTCAAGAATGTCGCGC       |                  |
| 7      | cpsA   | cpsA-F      | AGGCGACAACGAAGAAATGCG   | This study       |
|        | cpsA-R |             | CCACTCATTACCGAGGTTGCG   |                  |
| 8      | cpsC   | cpsC-F      | TCGAGCACGAGCTACGATAAGGA| This study       |
|        | cpsC-R |             | AACGCTGAGCCGCACTTCAA    |                  |
| 9      | cpsR   | cpsR-R      | CCAAATGAGGAAACCGAGTCA   | This study       |
|        | cpsR-R |             | AGCAAACAGCAAAGAAGGTAT   |                  |
| 10     | vcrD1  | vcrD1-F     | AAGGTAGTGCGCCCGGTCCGT   | Ding et al., 2016 |
|        | vcrD1-R|             | AAGCAGCGACGACACGAAAT    |                  |
| 11     | vopS   | vopS-F      | TAGAACCGGATTCGGTGGCC    | Ding et al., 2016 |
|        | vopS-R |             | TTACCGGAGCTCTTGCAGCG    |                  |
| 12     | vopD1  | vopD1-F     | GCGGCGGTCGATAGAAGGCAA   | Ding et al., 2016 |
|        | vopD1-R|             | AAGCTCACCGCTCGACAGTG    |                  |
| 13     | vcrD2p | vcrD2p-F    | AAGAAGGTTGTGGGACGAAAGCG| Ding et al., 2016 |
|        | vcrD2p-R|            | CCCTACGAGGCCAGTTGAGA    |                  |
| 14     | vopP2p | vopP2p-F    | AGAGGCGGTTGCAAATGCTG    | Ding et al., 2016 |
|        | vopP2p-R|            | ACCTCCGCAACCCTAAGTCCA   |                  |

### TABLE 2 | Continued

| Number | Gene   | Primer name | Primer sequence (5′–3′) | References       |
|--------|--------|-------------|-------------------------|------------------|
| 15     | Gap    | Gap-F       | AAAAGCTGCGGCTAAGGTTG    | Mattilä et al., 2011 |
|        |        |             | TTTCAAGTTGCTATTGAAAGCG |                  |
| 16     | flaA   | flaA-F      | GAAGGCGATGACCGAAGCGA    | This study       |
|        |        |             | GCAAGACACAGCAGCGTCACT   |                  |
| 17     | flgE   | flgE-F      | CACGAGGTTCCCGACTTC      | This study       |
|        |        |             | CGGGCCGTGTAACTGCTG      |                  |
| 18     | degU   | degU-F      | GGAAAGTGAAAGCTTATGGCC   | This study       |
|        |        |             | ACTGGGCGGAATTGTTGAG     |                  |
| 19     | invA   | invA-F      | AAATGCACTAAGCTCACATTG   | This study       |
|        |        |             | TGGTTGATGCTAGAACTTGA    |                  |
| 20     | plcA   | plcA-F      | TTACCGGAGAACCGGACAT     | This study       |
|        |        |             | CCGCTACCGGCAGCCGGGA    |                  |
| 21     | lmo2504| lmo2504-F   | GCTTGAGACGGCTG          | This study       |
|        |        |             | TGGCTTCAACCGGGC         |                  |
| 22     | lmo2554| lmo2554-F   | AAAGGCGAGGCGGACTTTGCG  | This study       |
|        |        |             | ACATTCCCGACAGCAGTG      |                  |
| 23     | rmlB   | rmlB-F      | TATTTGCTGAGAAAGCGG     | This study       |
|        |        |             | GGTTGCGGTGTTGAG         |                  |
| 24     | actA   | actA-F      | GCGGAAAGAGTCACTTGCG     | This study       |
|        |        |             | CTGTTGCGGTGTTGAG         |                  |
| 25     | prfA   | prfA-F      | TAACCGAATGGCGCCCAAC     | This study       |
|        |        |             | CCACAAGCGCGGGCAAAT      |                  |
| 26     | hly    | hly-F       | TGGAAACTCGCGCAGCTCA     | This study       |
|        |        |             | QAACCTTACAAGTCC         |                  |

Value differences (P < 0.05) using SPSS 17.0 (SPSS Inc., Chicago, IL, United States).

**RESULTS**

The effects of different modified atmospheres against the biofilm formation of *V. parahaemolyticus* and *L. monocytogenes* cocktails...
are shown in Figure 1. The crystal violet staining assay indicated that the biofilms of *V. parahaemolyticus* were reduced from 1.66 (OD_{600nm}) under anaerobiosis to 1.52 (OD_{600nm}) under micro-aerobiosis, and further significantly (*P* < 0.05) decreased to 0.82 (OD_{600nm}) under aerobic conditions. However, the biofilms of *L. monocytogenes* treated by modified atmospheres were significantly (*P* < 0.05) increased from 1.12 (OD_{600nm}) under anaerobiosis to 1.52 (OD_{600nm}) under micro-aerobiosis, and further significantly (*P* < 0.05) increased to 2.15 under aerobic conditions. All these facts implied that the modified atmospheres produced completely opposite effects on the biofilm formation of *V. parahaemolyticus* and *L. monocytogenes*.

In addition, the CLSM was used to perform *in situ* characterization of *V. parahaemolyticus* and *L. monocytogenes* biofilms under different modified atmospheres. As shown in Figure 2I, the biofilms of *V. parahaemolyticus* presented a compact and structured biofilm architecture under anaerobiosis. However, the biofilms became slightly loose and presented unevenly dispersed structures under micro-aerobiosis (Figure 2II). Much sparser and lower amounts of biofilms were observed under aerobic conditions (Figure 2III). For the biofilms of *L. monocytogenes*, they displayed sparser and looser biofilms with a patchy coverage on the contact surface under anaerobiosis (Figure 2IV). Nevertheless, the biofilms presented an increased amount and compact structures under micro-aerobiosis (Figure 2V). Furthermore, the biofilms displayed more compact and dense structures under aerobic conditions (Figure 2VI).

Scanning electron microscopy (SEM) is used to directly observe biofilms (Pan et al., 2016). As shown in Figure 3I, a layer of mature biofilms of *V. parahaemolyticus* with well-organized network structures was observed under anaerobic conditions, and bacterial biofilm cells were closer to each other. However, few bacterial cells aggregated, and the ordered biofilm structures were not found under micro-aerobiosis (Figure 3II). Even much fewer cells were sporadically scattered on the contact surface under aerobic conditions (Figure 3III). The biofilms of *L. monocytogenes* also exhibited an opposite trend of structure changes compared with *V. parahaemolyticus* (Figures 3IV–VI). Based on above results, the modified atmospheres especially for aerobic conditions significantly inhibited the biofilm formation of *V. parahaemolyticus*, while the anaerobiosis greatly restrained the biofilm formation of *L. monocytogenes*.

The effects of modified atmospheres on the production of EPS including eDNA, protein, and polysaccharide were examined in the biofilms of *V. parahaemolyticus* and *L. monocytogenes* (Figure 4). Overall, the eDNA and protein of *V. parahaemolyticus* biofilms were greatly reduced from anaerobic to aerobic conditions. However, the extracellular polysaccharide was increased. In detail, the eDNA of EPS was significantly (*P* < 0.05) reduced from 14.3 µg/ml under anaerobiosis to 0.08 µg/ml (89%) under aerobic conditions (Figure 4A). Meanwhile, the extracellular protein of EPS was markedly (*P* < 0.05) reduced from 14.3 µg/ml under anaerobiosis to 5.0 µg/ml (65%) under aerobic conditions (Figure 4B). Conversely, the extracellular polysaccharide of EPS in *V. parahaemolyticus* biofilms was slightly increased from 1.02 to 1.60 (OD_{490nm}/OD_{595nm}) (Figure 4C).

For the biofilms of *L. monocytogenes*, the eDNA and protein were dramatically elevated from anaerobic to aerobic conditions. However, the extracellular polysaccharide was reduced from anaerobic to aerobic conditions. In Figure 4A, the eDNA content of EPS was significantly (*P* < 0.05) increased from 0.32 µg/ml under anaerobiosis to 0.93 µg/ml (73%) under aerobic conditions. Similarly, the extracellular protein of EPS was markedly (*P* < 0.05) increased from 6.42 µg/ml under anaerobiosis to 24 µg/ml (66%) under aerobic conditions. Conversely, the extracellular polysaccharide of EPS in *L. monocytogenes* biofilms was gradually reduced from 1.53 under anaerobiosis to 0.93 under aerobic conditions.
Vibrio parahaemolyticus possesses the ability to form biofilms and secrete endotoxin, which can lead to serious diseases, and L. monocytogenes can also form biofilms and cause terrible listeriosis by secreting exotoxin such as listeriolysin O (LLO) (Gekara et al., 2008; Hamon et al., 2012; Wang et al., 2015). In this study, the regulatory genes of biofilm formation (luxS, aphA, mshA, oxyR, and opaR) and EPS production genes (cpsA, cpsC, and cpsR) of V. parahaemolyticus were selected to determine the effects of the modified atmospheres on their transcriptional levels. In Figure 6A, the expression levels of the biofilm formation genes and EPS production genes were slightly downregulated by aerobiosis; however, all the genes were significantly (P < 0.05) upregulated under anaerobiosis. For the L. monocytogenes cells, the gene expression levels of the biofilm formation (flgA, flgE, and degU) and EPS production (Imo2554, Imo2504, inlA, rmlB) were significantly (P < 0.05) downregulated under the anaerobiosis, except inlA and rmlB (Figure 6C). However, all the above genes were slightly upregulated by aerobiosis, except the obviously upregulated flgA.

To investigate the effects of modified atmospheres on the expression levels of virulence genes, the genes including vopS, vopD1, vcrD1, vopP2β, and vcrD2β of V. parahaemolyticus and actA, plfA, hly, and plcA of L. monocytogenes were evaluated by RT-qPCR. For V. parahaemolyticus (Figure 7A), the expression levels of all the virulence genes were downregulated under aerobiosis, reaching 1.88, 1.30, 1.96, 1.14, and 1.10 under aerobiosis (OD490nm/OD595nm). It was concluded that the modified atmosphere treatment inhibited the biofilm formation of V. parahaemolyticus, and the inhibition degree values could be ranked as eDNA > extracellular proteins > extracellular polysaccharide; however, the modified atmosphere treatment enhanced the biofilm formation of L. monocytogenes, and the enhancement degree values could be ranked as extracellular proteins > eDNA > extracellular polysaccharide.

Subsequently, the morphological and structural changes of the biofilms of V. parahaemolyticus and L. monocytogenes were analyzed by ISA software (Figure 5). The quantitative image analysis (QIA) of V. parahaemolyticus biofilms revealed that the biovolume of biofilms was greatly (P < 0.05) decreased from 13.49 × 10^5 μm^3 under anaerobiosis to 8.62 × 10^5 μm^3 under aerobiosis (Figure 5A). The biofilm thickness was significantly (P < 0.05) reduced from 16.72 μm under anaerobiosis to 9.65 μm under aerobiosis (Figure 5B). Meanwhile, the bio-roughness was significantly (P < 0.05) increased from 0.46 under anaerobiosis to 1.00 under aerobiosis (Figure 5C). Meanwhile, the TE was slowly increased from 7.16 under anaerobiosis to 7.40 under aerobiosis (Figure 5D).

For L. monocytogenes, the biovolume of its biofilms was significantly (P < 0.05) increased from 11.41 × 10^5 to 20.58 × 10^5 μm^3 during the physiological conversion from anaerobiosis to aerobiosis (Figure 5A). The biofilm thickness was dramatically (P < 0.05) increased from 11.63 to 19.63 μm (Figure 5B). Meanwhile, its bio-roughness was markedly (P < 0.05) improved from 0.49 under anaerobiosis to 0.27 under aerobiosis (Figure 5C). The TE was gradually decreased from 7.82 under anaerobiosis to 7.59 under aerobiosis (Figure 5D).

The expression levels of all the virulence genes were downregulated under aerobiosis, reaching 1.88, 1.30, 1.96, 1.14, and 1.10 under aerobiosis (OD490nm/OD595nm). It was concluded that the modified atmosphere treatment inhibited the biofilm formation of V. parahaemolyticus, and the inhibition degree values could be ranked as eDNA > extracellular proteins > extracellular polysaccharide; however, the modified atmosphere treatment enhanced the biofilm formation of L. monocytogenes, and the enhancement degree values could be ranked as extracellular proteins > eDNA > extracellular polysaccharide.

Subsequently, the morphological and structural changes of the biofilms of V. parahaemolyticus and L. monocytogenes were analyzed by ISA software (Figure 5). The quantitative image analysis (QIA) of V. parahaemolyticus biofilms revealed that the biovolume of biofilms was greatly (P < 0.05) decreased from 13.49 × 10^5 μm^3 under anaerobiosis to 8.62 × 10^5 μm^3 under aerobiosis (Figure 5A). The biofilm thickness was significantly (P < 0.05) reduced from 16.72 μm under anaerobiosis to 9.65 μm under aerobiosis (Figure 5B). Meanwhile, the bio-roughness was significantly (P < 0.05) increased from 0.46 under anaerobiosis to 1.00 under aerobiosis (Figure 5C). Meanwhile, the TE was slowly increased from 7.16 under anaerobiosis to 7.40 under aerobiosis (Figure 5D).

For L. monocytogenes, the biovolume of its biofilms was significantly (P < 0.05) increased from 11.41 × 10^5 to 20.58 × 10^5 μm^3 during the physiological conversion from anaerobiosis to aerobiosis (Figure 5A). The biofilm thickness was dramatically (P < 0.05) increased from 11.63 to 19.63 μm (Figure 5B). Meanwhile, its bio-roughness was markedly (P < 0.05) improved from 0.49 under anaerobiosis to 0.27 under aerobiosis (Figure 5C). The TE was gradually decreased from 7.82 under anaerobiosis to 7.59 under aerobiosis (Figure 5D).
times, separately; but all the genes were upregulated under anaerobiosis, reaching 1.84, 1.36, 1.46, 5.30, and 1.31 times, respectively. However, the expression levels of all virulence genes of *L. monocytogenes* were downregulated under anaerobiosis, reaching 2.09, 3.58, 1.76, and 1.66 times, respectively; however, all the genes were upregulated under aerobiosis, reaching 1.37, 1.44, 1.14, and 1.21 times, separately (*Figure 7B*).

DISCUSSION

Previous studies showed that all the tested modified atmospheres were effective in reducing *V. parahaemolyticus* in sea bream fillets compared with air-packaged samples (Provincial et al., 2013a). Meanwhile, Provincial et al. (2013b) found that the modified atmospheres were more effective in decreasing the *Listeria* spp. with the increase of CO$_2$ concentrations from 20% to 30%. Rutherford et al. (2007) also found differences in the amount of *Listeria* on shrimp which were stored in air, vacuum, and map packaging (100% CO$_2$) at 3, 7, and 10°C. Moreover, *V. parahaemolyticus* and *L. monocytogenes* could form bacterial biofilms to survive on food processing surfaces under anaerobiosis or aerobiosis (Voidarou et al., 2011; Valimaa et al., 2015; Perez-Ibarreche et al., 2016; Song et al., 2016). In an early study, the biofilm formation of *Vibrio vulnificus* was reduced due to the lack of oxygen (Phippen and Oliver, 2015). However, *Salmonella* spp. produced the highest amount of biofilm under a CO$_2$-rich atmosphere (Stepanovic et al., 2003). In food products, Wang et al. (2017) reported that the MAP inhibited the growth of *Pseudomonas fragi* strains in meat, and more loose and less bound EPS were produced by *P. fragi* in the modified-atmosphere packaged samples. The present study demonstrated that *V. parahaemolyticus* possessed a stronger ability to develop biofilms under anaerobic conditions in comparison with micro-aerobiosis and aerobiosis (*Figure 1*).

For Gram-positive bacteria, compared to the increased CO$_2$ atmosphere and anaerobic condition, the aerobic atmosphere of methicillin-resistant *Staphylococcus aureus* was not efficient.
in promoting the biomass of biofilms (Ursic and Tomic, 2008). However, the aerobic biofilms of wild-type S. aureus were more robust than that under anaerobic conditions (Hess et al., 2013). Our results showed that the L. monocytogenes biofilms were increased from 1.01 (OD$_{600nm}$) under anaerobiosis to 2.02 (OD$_{600nm}$) under aerobiosis (Figure 1). In addition, the biofilms formed by different Gram-negative Salmonella strains ($n = 30$) exhibited a different stress response to the modified atmospheres, indicating the heterogeneity of the biofilm formation ability of Salmonella strains under the modified atmospheres (Stepanovic et al., 2003).

It is widely reported that high fluorescence intensities observed by CLSM are caused by the aggregation of biofilms in different layers and depths (Webster et al., 2005; Han et al., 2017). Moreover, SEM images can also be used to describe the biofilm morphotypes (Simões et al., 2007). In our study, the results of CLSM and SEM clearly showed that the biofilm cells of V. parahaemolyticus displayed more compact aggregates and well-organized structures under anaerobiosis compared to micro-aerobiosis and aerobiosis (Figures 2, 3). However, it was observed that few aggregated cells and sparser structures of L. monocytogenes biofilms formed with a lower fluorescence intensity under anaerobiosis (Figures 2, 3). Overall, the results of the CLSM and SEM confirmed the results of crystal violet staining.

The EPS consists of about 80% dry mass of the biofilms, mainly including extracellular proteins (3–37%), nucleic acids (9–50%), and polysaccharides (3–21%) (Andersson et al., 2009), which plays a major role in mediating biofilm formation (Liu et al., 2007). It was concluded that the eDNA was essential in the initial establishment of Pseudomonas aeruginosa biofilms and perhaps biofilms formed by other bacteria (Whitchurch et al., 2002). Besides, three matrix proteins contributing to biofilm stability were identified in Vibrio cholerae, which involved in cell–cell and cell–surface adhesion (Yildiz et al., 2014). Up to now, the most descriptive matrix protein was extracellular adhesin.
CdrA, which promoted the aggregate formation through the interaction of extracellular polysaccharide Psl under planktonic conditions and helped to stabilize the matrix and maintain the structural integrity of aggregates (da Silva et al., 2019). In Figure 4, the eDNA and extracellular proteins of the *V. parahaemolyticus* biofilms were significantly (*P* < 0.05) reduced from anaerobiosis to aerobiosis, but the extracellular polysaccharides were gradually increased. However, our results showed that the eDNA and extracellular proteins of the *L. monocytogenes* biofilms were dramatically (*P* < 0.05) increased from anaerobiosis to aerobiosis; conversely, the extracellular polysaccharides were decreased (Figure 4). Our results were supported by the fact that anaerobic incubation decreased the production of extracellular polysaccharide in Gram-negative *Escherichia coli* 0157:H7 (Dewanti and Wong, 1995). It showed that the extracellular proteins and eDNA of Gram-positive *S. aureus* were significantly decreased under anaerobic conditions (Hess et al., 2013). The results demonstrated that the anaerobic conditions induced the highest levels of carbohydrates in biofilms compared to aerobic conditions (Yang et al., 2006). The reported results were highly coincident with the changes of EPS of Gram-positive *L. monocytogenes* in this study.

Quantitative image analysis was used to characterize the structure of the biofilms (Tan et al., 2018). The variation of the textural parameters reflects the changes of biofilm structure and biofilm adhesion ability (Figures 5A–C). The biovolume represented the total volume of cells (µm³) in the biofilms (Bridier et al., 2010). Meanwhile, the mean thickness (µm) of biofilms was also determined directly from the confocal stack images. In Figures 5A,B, the decrease in biovolume and thickness of biofilms suggested that the total volume of adherent cells was reduced from anaerobiosis to aerobiosis. Bio-roughness offered a metric of variations in biofilm thickness and was an indicator of the superficial biofilm-interface heterogeneity, so the significantly increased bio-roughness suggested that a high variation of biofilm thickness...
The expression of quorum-controlled genes was regulated by a variety of genes. For V. parahaemolyticus, which was necessary for the biofilm formation of V. parahaemolyticus and L. monocytogenes, the expression of oxyR, aphA, opaR, and luxS were downregulated. Such facts indicated that the bacteria decreased their physiological activity and mitigated the expression of essential genes to cut down the energy costs of cells to adapt the changes of environmental conditions (Bayramoglu et al., 2017). Therefore, the results suggested a relationship between the biofilm formation and expression of biofilm and quorum-sensing genes in V. parahaemolyticus (Lamas et al., 2016).

**MshA** is a type IV pilin subunit gene that mediates the adhesion of V. parahaemolyticus to the surface through pili (Shime-Hattori et al., 2006; Aagesen and Hase, 2012). The expression of mshA significantly increased (P < 0.05) under anaerobic conditions but obviously decreased under aerobic conditions, which might suggest decreased functions of type IV pili in the adhesion of V. parahaemolyticus. Meanwhile, the cpsA and cpsC regulated the production and transportation of CPS, and cpsR was required for the exopolysaccharide production of V. parahaemolyticus biofilm (Guvener and McCarter, 2003; Zhang et al., 2018). The downregulation of these genes (cpsA, cpsC, and cpsR) suggested that the aerobic conditions inhibited the production of CPS and exopolysaccharide, which would reduce the adhesion ability and biofilm formation of V. parahaemolyticus on the target surfaces. Likewise, the virulence genes of V. parahaemolyticus are also affected by the modified atmospheres. VcrD1, vopS, and vopD1 are the main virulence factors of V. parahaemolyticus type III secretory system 1 (T3SS1). VcrD1, an inner membrane protein, is a component of T3SS1 in V. parahaemolyticus (Kwon-Sam et al., 2004; Noh et al., 2015). VopS is an effector secreted by T3SS1 during infection and can inhibit actin assembly (Yarbrough et al., 2009). VopD1 is the essential component of the translocation of T3SS1 (Shimohata et al., 2012). VopP2β and vcrD2β are the main virulence factors of V. parahaemolyticus type III secretory system 2 (T3SS2). VopP2β inhibits mitogen-activated protein kinases (MAPK) signal transduction and prevents ATP binding in T3SS2 (Trosky et al., 2007; Tsai et al., 2013). And vcrD2β encodes an

**FIGURE 7** | Fold change normalized to reference gene recA [V. parahaemolyticus (A)] and Gap [L. monocytogenes (B)] in the expression of virulence genes in: anaerobiosis (20% carbon dioxide, 80% nitrogen) and aerobiosis (60% oxygen, 40% nitrogen) relative to micro-aerobiosis (20% oxygen, 80% nitrogen). Error bars represent the standard error. Asterisks represent statistically significant differences (P < 0.05) in fold change relative to micro-aerobiosis.
inner membrane protein of T3SS2 (Kwon-Sam et al., 2004; Tsai et al., 2013). The type III secretion system (T3SS), which consists of T3SS1 (vcrD1, vopS, vopD1) and T3SS2 (vopP2β, vcrD2β), is considered to be an important virulence factor for delivering effectors into host cells (Noh et al., 2015). The downregulation of these genes (VcrD1, vopS, vopD1 VopP2β, vcrD2β) suggested that the aerobic conditions inhibited the expression of these virulence genes, but the anaerobic conditions conversely improved the expression of virulence genes.

For L. monocytogenes, flgA and flgE are flagellum-related genes associated with biofilm formation (Li R. et al., 2018). The degU gene-encoded DegU is essential for flagellar synthesis and bacterial motility in L. monocytogenes (Gueriri et al., 2008; Pieta et al., 2014). It is well known that flagellum-mediated motility plays a predominant role in biofilm formation of L. monocytogenes (Renier et al., 2011). The upregulation of flgA, flgE, and degU (P < 0.05) implied that the aerobic conditions facilitated the initial biofilm formation of L. monocytogenes. However, the downregulation of flgA, flgE, and degU indicated that anaerobic conditions caused a decrease in the ability of biofilm formation. L-rhamnose is widely found in cell walls and capsules of many pathogenic bacteria including L. monocytogenes, which can be regulated by rmlB (Giraud and Naismith, 2000; Eugster et al., 2015). Imo2554 is confirmed to be involved in the glycolipid synthesis of L. monocytogenes, an important cell wall polymer in Gram-positive bacteria (Webb et al., 2009). Imo2504 encodes the binding protein of the cell wall which participates in the biofilm formation (Lourenco et al., 2013). InlA encodes InlA (the bacterial surface protein), which gains invasiveness and invades the L. monocytogenes cells (Pizarro-Cerda et al., 2012). The anaerobic downregulation of rmlB, Imo2554, Imo2504, and inlA indicated that the synthesis of these key proteins was inhibited, indicating the highly decreased physiological activity of L. monocytogenes. Conversely, the aerobic upregulation of these genes suggested the enhanced expression of key proteins and physiological activity of L. monocytogenes which finally strengthened the biofilm formation. For the virulence genes, it was concluded that the pathogenesis was mainly related to the expression of virulence genes (LIPI-1) (Stelling et al., 2010). The virulence genes were located in the virulence island, which is a 9-KB-long gene cluster mainly containing hly, plcA, prfA, and actA (Stelling et al., 2010). In this study, the anaerobic downregulation of hly, plcA, prfA, and actA suggested that the toxicity of L. monocytogenes was decreased, while the aerobic upregulation of these genes indicated the increased toxicity of L. monocytogenes.

CONCLUSION
The modified atmospheres significantly reduced the eDNA and proteins of EPS and negatively altered the biofilm structures of V. parahaemolyticus during the physiological conversion from anaerobiosis to aerobiosis. The modified atmospheres also downregulated the expression of biofilm formation genes (luxS, aphA, mshA, oxyR, and opaR) and EPS production genes (cpsA, cpsC, and cpsR) and virulence genes (vopS, vopD1, vcrD1, vopP2β, and vcrD2β) of V. parahaemolyticus. Conversely, the expressions of biofilm formation genes (flgA, flgU, and degU), EPS production genes (Imo2554, Imo2504, inlA, rmlB) and virulence genes (vopS, vopD1, vcrD1, vopP2β, and vcrD2β) of L. monocytogenes were upregulated during the same physiological conversion. Therefore, the modified atmospheres showed significantly different regulation on the biofilm formation of Gram-negative V. parahaemolyticus and Gram-positive L. monocytogenes. The generated knowledge will facilitate our understanding of the stress response of Gram-negative and Gram-positive pathogens to the modified atmosphere treatment and hence provide an efficient way to control the pathogens in modified-atmosphere packaged foods.

DATA AVAILABILITY STATEMENT
All datasets generated for this study are included in the article.

AUTHOR CONTRIBUTIONS
YZ, JW, YP, and HL conceived and supervised the study. HQ and WL designed and performed the experiments. HQ analyzed the data. LG, LT, JW, and YZ revised the manuscript. HQ wrote the manuscript.

FUNDING
This research was supported by the National Natural Science Foundation of China (31571917 and 31671779), National Key R&D Program of China (2018YFC1602205 and 2018YFC1602200), Shanghai Agriculture Applied Technology Development Program (T20170404), and Innovation Program of Shanghai Municipal Education Commission (2017-01-00-10-E00056).

REFERENCES
Aagesen, A. M., and Hase, C. C. (2012). Sequence analyses of type IV Pili from Vibrio cholerae. Vibrio parahaemolyticus, and Vibrio vulnificus. Microb. Ecol. 64, 509–524. doi: 10.1007/s00248-012-0021-2
Alvarez-Ordonez, A., Broussolle, V., Colín, P., Nguyen-The, C., and Prieto, M. (2015). The adaptive response of bacterial food-borne pathogens in the environment, host and food: implications for food safety. Int. J Food Microbiol. 213, 99–109. doi: 10.1016/j.ijfoodmicro.2015.06.004

Andersson, S., Dalhammar, G., Land, C. J., and Kuttuva Rajarao, G. (2009). Characterization of extracellular polymeric substances from denitrifying organism Comamonas denitrificans. Appl. Microbiol. Biotechnol. 82, 535–543. doi: 10.1007/s00253-008-1817-3
Arvanitoyannis, I. S., Kargaki, G. K., and Hadjichristodoulou, C. (2011). Effect of several MAP compositions on the microbiological and sensory properties of Graviera cheese. Anaerobe 17, 310–314. doi: 10.1016/j.anaerobe.2011.04.013
Bayramoglu, B., Toubiana, D., and Gillor, O. (2017). Genome-wide transcription profiling of aerobic and anaerobic Escherichia coli biofilm and planktonic cultures. Fems Microbiol. Lett. 364:fnx006. doi: 10.1093/femsle/fnx006
Beyenal, H., Donovan, C., Lewandowski, Z., and Harkin, G. (2004a). Three-dimensional biofilm structure quantification. J. Microbiol. Methods 59, 395–413. doi: 10.1016/j.mimet.2004.08.003

Beyenal, H., Lewandowski, Z., and Harkin, G. (2004b). Quantifying biofilm structure: facts and fiction. Biofouling 20, 1–23. doi: 10.1080/08927014.2004.961908

Blanco, Y., Rivas, L. A., Gonzalez-Toril, E., Ruiz-Bermejo, M., Moreno-Paz, M., Parro, V., et al. (2019). Environmental parameters, and not phylogeny, determine the composition of extracellular polymeric substances in microbial mats from extreme environments. Sci. Total Environ. 650, 384–393. doi: 10.1016/j.scitotenv.2018.08.440

Bonsaglia, E. C. R., Silva, N. C. C., Fernades Júnior, A., Araújo Júnior, J. P., Tsunemi, M. H., and Rall, V. L. M. (2014). Production of biofilm by Listeria monocytogenes in different materials and temperatures. Food Control 35, 386–391. doi: 10.1016/j.foodcont.2013.07.023

Briddel, A., Dubois-Brissonnet, F., Boubetra, A., Thomas, V., and Briandet, R. (2010). The biofilm architecture of sixty opportunistic pathogens deciphered using a high throughput CLSM method. J. Microbiol. Methods 82, 64–70. doi: 10.1016/j.mimet.2010.04.006

Brooks, J. D., and Flint, S. H. (2008). Biofilms in the food industry: problems and potential solutions. Int. J. Food Sci. Technol. 43, 2163–2176. doi: 10.1111/j.1365-2621.2008.01839.x

Centers for Disease Control and Prevention [CDC] (2014). National Enteric Disease Surveillance: COVIS Annual Summary. Available at: https://www.cdc.gov/nationalsurveillance/pdfs/covis-annual-summary-2014-508c.pdf (accessed January, 2015).

Chang, Y. H., Gu, W. M., and McLandorss, L. I. (2012). Low concentration of ethylenediaminetetraacetic acid (EDTA) affects biofilm formation of Listeria monocytogenes by inhibiting its initial adhesion. Food Microbiol. 29, 10–17. doi: 10.1016/j.fm.2011.07.009

Chen, J., Zhang, R. H., Qi, X. J., Zhou, B., Wang, J. K., Chen, Y., et al. (2017). Epidemiology of foodborne disease outbreaks caused by Vibrio parahaemolyticus during 2010-2014 in Zhejiang Province. China. Food Control 77, 110–115. doi: 10.1016/j.foodcont.2017.02.004

Chimalapati, S., de Souza Santos, M., Servage, K., De Nisco, N. J., Dalia, A. B., and Orth, K. (2018). Natural transformation in Vibrio parahaemolyticus: a rapid method to create genetic deletions. J. Bacteriol. 200:e00032-18.. doi: 10.1128/JB.00032-18

Chung, C. H., Fan, S. Y., Yu, S. C., and Wong, H. C. (2016). Influence of oxyR on growth biofilm formation, and mobility of Vibrio parahaemolyticus. Appl. Environ. Microbiol. 82, 788–796. doi: 10.1128/aem.02818-15

Colagiorgi, A., Di Ciccio, P., Zanardi, E., Ghidini, S., and Ianieri, A. (2016). A look inside the Listeria monocytogenes biofilms extracellular matrix. Microorganisms 4:22. doi: 10.3390/microorganisms4030022

da Silva, D. P., Matwuchik, M. L., Townsend, D. O., Reichhardt, C., Lamba, D., Wonzniak, D. J., et al. (2019). The Pseudomonas aeruginosa lectin LecB binds to the exopolysaccharide PAI and stabilizes the biofilm matrix. Nat. Commun. 10:2183. doi: 10.1038/s41467-019-10201-4

Dewanti, R., and Wong, A. C. L. (1995). Influence of culture conditions on growth, biofilm formation, and mobility of Vibrio parahaemolyticus on various food-contact surfaces: relationship with motility and extracellular polymeric substance levels in Salmonella pullorum and analysis of extracellular polymeric substances: comparison of methods and extracellular polymeric substance levels in Salmonella pullorum SA 1685. Environ. Eng. Sci. 26, 1523–1532

Ding, X. Y., Ling-Yun, Q. U., Wang, C., Tian, X. X., Sun, C. J., and Wang, M. Q. (2016). Effect of temperature on T3SS Gene Expression of Listeria monocytogenes by quorum-sensing genes luxM and luxS. Food Microbiol. 75, 1190–1197. doi: 10.1016/j.foodmicro.2015.07.009

Guverri, I., Cyncynatus, C., Dubrac, S., Arana, A. T., Dussurget, O., and Msadek, T. (2008). The DegU orphan response regulator of Listeria monocytogenes autorepresses its own synthesis and is required for bacterial motility, virulence and biofilm formation. Microbiology 154(Pt 8), 2251. doi: 10.1099/mic.0.02008-0

Guo, M. H., Fang, Z. I., Sun, L. J., Sun, D. F., Wang, Y. L., Li, C., et al. (2018). Regulation of thermostable direct hemolysin and biofilm formation of Vibrio parahaemolyticus by quorum-sensing genes luxM and luxS. Curr. Microbiol. 75, 1100–1107. doi: 10.1007/s00284-018-1508-y

Guvener, Z. T., and McCarter, L. L. (2003). Multiple regulators control capsular polysaccharide production in Vibrio parahaemolyticus. J. Bacteriol. 185, 5431–5441. doi: 10.1128/jb.185.18.5431-5441.2003

Hamon, M. A., Ribet, D., Stavr, F., and Cossart, P. (2012). Listeriolysin O: the Swiss army knife of Listeria. Trends Microbiol. 20, 360–368. doi: 10.1016/j.tim.2012.04.006

Han, Q., Song, X., Zhang, Z., Fu, J., Wang, X., Malakar, P. K., et al. (2017). Removal of foodborne pathogen biofilms by acidic electrolyzed water. Front. Microbiol. 8:988. doi: 10.3389/fmicb.2017.00988

Hess, D. J., Henry-Stanley, M. J., Lusczek, E. R., Beilman, G. J., and Wells, C. L. (2013). Anoxia inhibits biofilm development and modulates antibiotic activity. J. Surgical Res. 184, 488–494. doi: 10.1016/j.sjrs.2013.04.049

Hsieh, Y. C., Liang, S. M., Tsai, W. L., Chen, Y. H., Liu, T. Y., and Liang, C. M. (2003). Study of capsular polysaccharide from Vibrio parahaemolyticus. Infect. nt Immunn. 71, 3329–3336. doi: 10.1128/iai.00622-07

Jung, Y. C., Lee, M. A., and Lee, K. H. (2019). Role of flagellin-homologous proteins in biofilm formation by pathogenic Vibrio Species. Mbio 10:e01793-19. doi: 10.1128/mBio.01793-19

Kaye, S., and Charbit, A. (2006). Listeriolysin O: a key protein of Listeria monocytogenes with multiple functions. Fems Microbiol. Rev. 30, 514–529. doi: 10.1111/j.1574-6976.2006.00021.x

Kim, H. S., and Park, H. D. (2013). Ginger extract inhibits biofilm formation by Pseudomonas aeruginosa PA14. Plos One 8:e76106. doi: 10.1371/journal.pone.0076106

Kim, M. J., Miks-Krajnik, M., Kumar, A., and Yuk, H. G. (2016). Inactivation by 45 +/- 5 nm light emitting diode on Escherichia coli O157:H7, under refrigerated condition might be due to the loss of membrane integrity. Food Control 59, 99–107. doi: 10.1016/j.foodcont.2015.05.012

Kirov, S. M. (2003). Bacteria that express lateral flagella enable dissection of the multifunctional roles of flagella in pathogenesis. Fems Microbiol. Lett. 224, 151–159. doi: 10.1111/j.1574-6967.2003.01045-2

Kwon-Sam, P., Oto, N., Rokuda, M., Myoung-Ho, J., Okada, K., Iida, T., et al. (2004). Functional characterization of two type III secretion systems of Vibrio parahaemolyticus. Infect. Immun. 72, 6659–6665. doi: 10.1128/iai.72.11.6659-6665.2004

Lamas, A., Miranda, J. M., Vaquez, B., Cepeda, A., and Franco, C. M. (2016). Biofilm formation, phenotypic production of cellulase and gene expression in...
Salmonella enterica decrease under anaerobic conditions. Int. J. Food Microbiol. 238, 63–67. doi: 10.1016/j.ijfoodmicro.2016.08.043

Li, H., Tang, R., Lou, Y., Cui, Z., Chen, W., Hong, Q., et al. (2017). A Comprehensive Epidemiological Research for clinical Vibrio parahaemolyticus in shanghai. Front. Microbiol. 8:1043. doi: 10.3389/fmicb.2017.01043

Li, R., Du, W., Yang, J., Liu, Z., and Yousef, A. E. (2018). Control of Listeria monocytogenes biofilm by paenibacterin, a natural antimicrobial lipoepitope. Food Control 84, 529–535. doi: 10.1016/j.foodcont.2017.08.031

Li, W., Bai, L., Fu, P., Han, H., Liu, J., and Guo, Y. (2018). The epidemiology of Listeria monocytogenes in China. Foodborne Pathog Dis. 15, 459–466. doi: 10.1089/fpd.2017.2409

Liu, Y., Li, J., Qiu, X., and Burda, C. (2007). Bacterial activity of nitrogen-doped metal oxide nanocatalysts and the influence of bacterial extracellular polymeric substances (EPS). J. Photochem. Photobiol. A Chem. 190, 94–100.

Lourenco, A., de Las Heras, A., Scortti, M., Vazquez-Boland, J., Frank, J. F., and Gerard, P. (2007). Combined effect of packaging atmosphere and storage temperature on growth of Listeria monocytogenes on ready-to-eat shrimp. Food Microbiol. 24, 731–710. doi: 10.1016/j.fm.2007.03.011

Renier, S., Hebraud, M., and Desvaux, M. (2011). Molecular biology of surface colonization by Listeria monocytogenes: an additional facet of an opportunistic gram-positive foodborne pathogen. Environ. Microbiol. 13, 835–850. doi: 10.1111/j.1462-2920.2010.02378.x

Rutherford, T. J., Marshall, D. L., Andrews, L. S., Coggins, P. C., Schilling, M. W., and Gerard, P. (2007). Combined effect of packaging atmosphere and storage temperature on growth of Listeria monocytogenes on ready-to-eat shrimp. Food Microbiol. 24, 731–710. doi: 10.1016/j.fm.2007.03.011

Schimel, J., Balser, T. C., and Wallenstein, M. (2007). Microbial stress-response physiology and its implications for ecosystem function. Ecology 88, 1386–1394. doi: 10.1890/06-0219

Seper, A., Fengerl, V. H. I., Roier, S., Wolinski, H., Kohlwein, S. D., Bishop, A. L., et al. (2011). Extracellular nucleases and extracellular DNA play important roles in Vibrio cholerae biofilm formation. Mol. Microbiol. 82, 1015–1037. doi: 10.1111/j.1365-2958.2011.07867.x

Shime-Hattori, A., Iida, T., Arita, M., Park, K. S., Kodama, T., and Honda, T. (2006). Two type IV pili of Vibrio parahaemolyticus play different roles in biofilm formation. Fems Microbiol. Lett. 264, 89–97. doi: 10.1111/j.1574-6968.2006.00438.x

Shimohata, T., Mawatari, K., Iba, H., Hamano, M., Negoro, S., Asada, S., et al. (2012). VopB1 and VopD1 are essential for translocation of type III secretion system 1 effectors of Vibrio parahaemolyticus. Can. J. Microbiol. 58, 1002–1007. doi: 10.1139/w12-081

Simões, M. L., Pereira, M. O., Sillankorva, S., Azeredo, J., and Vieira, M. J. (2007). The effect of hydrodynamic conditions on the phenotype of Pseudomonas fluorescens biofilms. Biofouling 23, 249–258.

Song, X., Ma, Y., Fu, J., Zhao, A., and Yong, Z. (2016). Effect of temperature on pathogenic and non-pathogenic Vibrio parahaemolyticus biofilm formation. Food Control 73, 485–491. doi: 10.1016/j.foodcont.2016.08.041

Stelling, C. R. L., Orsi, R. H., and Wiedmann, M. (2010). Complementation of Listeria monocytogenes Null mutants with selected Listeria seeligeri virulence genes suggests functional adaptation of Hly and PrfA and considerable diversification of prfA Regulation in L. seeligeri. Appl. Enviro. Microbio. 76, 5124–5139. doi: 10.1128/ae.03107-09

Stepanovic, S., Cirkovic, I., Majic, V., and Svbac-Vlahovic, M. (2003). Influence of the incubation temperature, atmosphere and dynamic conditions on biofilm formation by Listeria monocytogenes ST8SH and vancomycin, propolis or EDTA for controlling biofilm development of Lactobacillus plantarum. Bioresources 75, 5846–5852. doi: 10.1128/ae.03107-09

Todorov, S. D., de Paula, O. A. L., Camargo, A. C., Lopes, D. A., and Nero, L. A. (2018). Combined effect of bacteriocin produced by Lactobacillus plantarum ST8SH and vancomycin, propolis or EDTA for controlling biofilm development by Listeria monocytogenes. Rev. Argentina Microbiol. 50, 48–55. doi: 10.1016/j.ram.2017.04.011

Treisky, J. E., Li, Y., Mukherjee, S., Keitany, G., Ball, H., and Ort, K. (2007). VopA inhibits ATP binding by acetyylating the catalytic loop of MAPK kinases, J. Biol. Chem. 282, 34299–34305. doi: 10.1074/jbc.M700970200

Tsai, S. E., Jung, K. J., Tey, Y. H., Yu, W. T., Chiuo, C. S., Lee, Y. S., et al. (2013). Molecular characterization of clinical and environmental Vibrio parahaemolyticus isolates in Taiwan. Int. J Food Microbiol. 165, 18–26. doi: 10.1016/j.ijfoodmicro.2013.04.017

Ursic, V., and Tomic, V. M. (2008). Effect of different incubation atmospheres on the production of biofilm in methicillin-resistant Staphylococcus aureus (MRSA) grown in nutrient-limited medium. Curr. Microbiol. 57, 386–390. doi: 10.1007/s00284-008-9211-z

Valimaa, A. L., Tilsala-Timisjarvi, A., and Virtanen, E. (2015). Rapid detection and identification methods for Listeria monocytogenes in the food
