Spoilage of refrigerated *Litopenaeus vannamei*: eavesdropping on *Acinetobacter* acyl-homoserine lactones promotes the spoilage potential of *Shewanella baltica*

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Abstract *Shewanella baltica* and *Acinetobacter* are among the predominant spoilage bacteria in refrigerated shrimp (*Litopenaeus vannamei*). *S. baltica* are incapable of producing acyl-homoserine lactone (AHL) quorum sensing signals, but can respond to environmental AHLs. In this paper, *Acinetobacter* was found to produce three AHLs, i.e. *N*-butanoyl-L-homoserine lactone (C4-HSL), *N*-(3-oxo-hexanoyl)-L-homoserine lactone (O-C6-HSL) and *N*-(3-oxooctanoyl)-L-homoserine lactone (O-C8-HSL), according to thin-layer chromatography using the bioreporter *Agrobacterium tumefaciens* A136. The agar diffusion and β -galactosidase assays revealed that *S. baltica* could eavesdrop on these three AHLs from *Acinetobacter*. Eavesdropping on *Acinetobacter* AHLs especially C4-HSL was found to boost the growth of *S. baltica* particularly under nutrient limiting conditions (up to 40-fold increase) in the co-culture experiments. The azocasein assay revealed that *S. baltica* produced fourfold more extracellular proteases in response to *Acinetobacter* AHLs. As demonstrated by the biofilm crystal violet staining assay and confocal laser scanning microscopy, eavesdropping also decreased the biofilm-forming capacity of *Acinetobacter*. By inoculation of *S. baltica* and *Acinetobacter* onto surface-sterilized shrimp, eavesdropping was found to endow a growth advantage to *S. baltica* in vivo, resulting in

a 0.5 day shortened shelf life of shrimp according to total volatile basic nitrogen levels and sensory analysis. Overall, the AHL-dependent eavesdropping increased the spoilage potential of *S. baltica*, providing a fresh perspective on the spoilage process of refrigerated *L. vannamei*, and this may inspire the development of novel preservation techniques in the future to further reduce post-harvest loss of shrimp.

Keywords Shrimp · Spoilage · Eavesdropping · Quorum sensing · Extracellular proteases · Biofilm

Introduction

Shrimp is a nutritious seafood with high acceptability around the world. *Litopenaeus vannamei*, the predominant species of farmed shrimp, accounts for about 80% of global aquaculture production of shrimp in 2014 (FAO 2017). The prime quality of shrimp is usually maintained by refrigerated or iced storage during distribution or in the market. *Shewanella* are considered as strong spoilers of chilled seafood (Svanevik et al. 2015; Zhang et al. 2016), and produce a variety of off-odors (e.g. amines, sulfur compounds, and indoles) due to their remarkable versatility in electron acceptors and metabolic pathways (Ólafsdóttir 2005). *Shewanella* have been described as specific spoilage organisms of refrigerated *L. vannamei* with *S. baltica* (*Sb*) and *S. putrefaciens* accounting for 19.2 and 17.3% of the microbiota, respectively (Zhu et al. 2015). *Acinetobacter* sp. (*Ab*) are among the predominant bacteria in spoiled chilled seafood, but are not regarded as important spoilers because of their inability of producing extracellular lipases, hydrogen sulfide and trimethylamine (Rawat 2015). *Ab* have been found to account for 11.5% of the microbiota of spoiled refrigerated *L. vannamei* (Zhu et al. 2015).

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Quorum sensing (QS) is an intercellular communication mechanism that allows bacteria to regulate collective behaviors (e.g. virulence, bioluminescence, biofilm formation, and swarming) in response to alternations of cell density or species composition (Papenfort and Bassler 2016). QS is operated by production and sensing of signaling molecules (called autoinducers) that, at threshold concentrations, collectively change global patterns of gene expression. Gram-negative and -positive bacteria utilize acetylated homoserine lactones (AHLs) and oligopeptides as autoinducers, respectively. Autoinducer-2 is a QS signal universally found in both Gram-negative and -positive bacteria. Some Gram-negative bacteria, especially marine microorganisms, also employ cyclic dipeptides (diketopiperazines) as a kind of QS signal (Gu et al. 2013).

Autoinducers, which usually differ for different bacterial species, seem to be a private message among cells sharing evolutionary interests. However, in multispecies communities, where autoinducers in nature can be detected by any cell, eavesdropping (i.e. where bacteria sense autoinducers not produced by themselves) may occur. The ability to eavesdrop may exert positive or negative effects on bacterial survival depending on habitat conditions and competitors (Diggle et al. 2007; Miller et al. 2016). *Shewanella* in refrigerated *L. vannamei* have been found to harbor an incomplete AHL-dependent QS circuit (Zhu et al. 2015). They are incapable of producing AHLs due to the lack of an AHL synthetase like LuxI and LuxM, but can sense these molecules via a LuxR-type receptor protein. *Ab*, which are also among the predominant bacteria in spoiled refrigerated shrimp, have been reported to use AHLs as QS signaling molecules (Bhargava et al. 2010). *Sb* thus may eavesdrop on *Ab* AHLs during spoilage of refrigerated *L. vannamei*, which may confer an important advantage for *Sb* to evolve into specific spoilage organisms during refrigerated storage of *L. vannamei*.

The present study evaluated the ability of *Sb* to eavesdrop on *Ab* AHLs, and investigated the effects of eavesdropping on bacterial growth, extracellular proteolytic activities and biofilm formation in the co-cultures of *Sb* and *Ab*. The in vivo effects of eavesdropping were also examined by inoculation of *Sb* and *Ab* onto surface-sterilized shrimp.

Materials and methods

Materials and bacterial strains

Live shrimp (*L. vannamei*) were transported from a local farm (Qingdao, China) in oxygenated water to the laboratory. Shrimp were washed two times with running water before being stunned with ice. Shrimp of uniform weight

and length (average weight: 11.8 ± 1.9 g; average length: 10.5 ± 1.5 cm) were used, and were randomly divided into four groups. Plate count agar, iron agar, and Luria–Bertani (LB) medium were provided by Haibo Biotechnology Co., Ltd. (Qingdao, China). Azocasein (#11610), fluorescein isothiocyanate-concanavalin A (FITC-conA, #C7642), *N*-butyryl-DL-homoserine lactone (C4-HSL, purity $\geq 97\%$, #09945), *N*-(3-oxohexanoyl)-L-homoserine lactone (O-C6-HSL, purity $\geq 98\%$, #K3007), *N*-(3-oxooctanoyl)-L-homoserine lactone (O-C8-HSL, purity $\geq 97\%$, #O1764) and X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside, purity $\geq 98\%$, #B4252) were purchased from Sigma-Aldrich Co. (Shanghai, China). RP-18 modified silica gel 60 chromatographic plates coated with F254 fluorescent indicator (#105559) were purchased from Merck (Darmstadt, Germany). Other chemicals used in this study were of analytical grade.

Agrobacterium tumefaciens A136 (pCF218)(pCF218) was a kind gift from Dr. Zhong (Nanjing Agricultural University, Nanjing, China). *Sb* and *Ab* strains were previously isolated from spoiled *L. vannamei* under refrigerated storage by our group, and identified by 16S rRNA sequencing. All bacteria were routinely grown in LB medium at 30 °C.

In vitro growth assay

LB medium and tenfold diluted LB medium were used in the in vitro growth assay according to Moslehi-Jenabian et al. (2011). Mid-logarithmic bacterial cells were inoculated into fresh medium at a concentration of 10^5 colony-forming unit (CFU)/mL. To inoculate the co-culture, *Sb* and *Ab* were mixed at the CFU ratio of 1:1 or 1:5 in sterile phosphate buffer saline (PBS) before inoculation. Exogenous AHLs were added as follows: C4-HSL, 10 μ M; O-C6-HSL, 10 μ M; and O-C8-HSL, 1 μ M. Cultures were incubated at 30 °C with shaking (160 r/min) for 12 h. Bacterial counts of *Sb* and *Ab* were determined on iron agar as black and milky colonies, respectively.

Assays for AHLs

The agar diffusion assay

Agrobacterium tumefaciens A136, *Sb* and *Ab* were streaked in parallel on LB agar plates overlaid with 50 μ g/mL X-Gal (Morohoshi et al. 2005). The plates were then incubated at 30 °C for 24–48 h. Induction of the bioreporter was indicated by the development of blue coloration of the *A. tumefaciens* A136 streak.

The β -galactosidase assay

AHLs were quantified by assaying β -galactosidase induction in cellular lysate of *A. tumefaciens* A136. The cell-free culture supernatant was prepared by centrifugation ($10,000g \times 5$ min) and $0.22\text{-}\mu\text{m}$ filtration. The cell-free shrimp extract was prepared as described by Blana and Nychas (2014). The cellular lysate of *A. tumefaciens* A136 was prepared as described by Kawaguchi et al. (2008). The cell-free culture supernatant or the cell-free shrimp extract was mixed with the cellular lysate of the bioreporter and $20\text{ mM KH}_2\text{PO}_4$ (v:v:v = 1:1:2), and the mixture was added into wells of a 48-well plate, followed by the addition of X-Gal at a final concentration of $500\text{ }\mu\text{g/mL}$. The plate was then cultured at $30\text{ }^\circ\text{C}$ for 3 h and the absorbance at 635 nm (OD_{635}) was determined with a microplate reader (Bio-tek, Vermont, USA). The sterile LB medium was used as the negative control. The β -galactosidase activity (U) = OD_{635} of the sample/ OD_{635} of the negative control.

Thin-layer chromatography (TLC)

AHLs were preliminarily identified by reversed-phase TLC based on their differences in molecular weight and side-chain property (Morohoshi et al. 2005). AHLs in the cell-free culture supernatant was extracted using acidified ethyl acetate (5.0 mL acetic acid per liter). The extract was rotary evaporated until dry, and re-dissolved in a small volume of methanol. AHLs were then identified using reversed-phase C18 TLC plates (silica gel 60 F254) as described by Rasch et al. (2005). Briefly, after samples were spotted onto the plate and separated with methanol–water ($60:40$, v/v), AHLs were located as blue spots using the bioreporter *A. tumefaciens* A136. The standards used were as follows: C4-HSL, 1 mM ; O-C6-HSL, $1\text{ }\mu\text{M}$ and O-C8-HSL, 100 nM .

Biofilm assays

Biofilm matrix was quantified by crystal violet-staining according to Zhu et al. (2016). Bacterial suspensions in LB medium were transferred into the wells of a 48-well plate (Nunc, Rochester, NY, USA). The plate was then incubated without shaking at $30\text{ }^\circ\text{C}$ for 36 h. After the removal of contents in the wells, modified sterile peptone water (1 g/L peptone and 8.5 g/L NaCl) was added in a gentle way to remove loosely attached cells. The adherent biofilms on the surface were stained with 0.1 g/L crystal violet for 15 min or 10 mg/L fluorescein isothiocyanate-concanavalin A in the dark for 30 min, followed by washing with sterile PBS for three times. Crystal violet-stained biofilms in microplate wells were re-solubilized using 95% ethanol, and

the absorbance at 570 nm (OD_{570}) in each well was determined using a microplate reader (Bio-tek, Vermont, USA). In order to measure thickness and compactness of the biofilm, fluorescein isothiocyanate-concanavalin A-stained biofilm matrixes were observed under a LSM 510 confocal laser scanning microscope (Zeiss, Jena, Germany) according to Ivnitsky et al. (2007).

Proteolytic activity assay

Extracellular proteolytic activity was determined spectroscopically using the azocasein method as described by Olajuyigbe and Ajele (2008). The bacterial cultures removed from wells of the plate in the biofilm assay were centrifuged ($10,000g \times 5$ min) with the supernatants used as crude enzyme solutions. The crude enzyme solution was mixed with equal volume of the substrate solution (20 g/L azocasein in 50 mM sodium phosphate buffer, pH 7.2), and the mixture was incubated at $30\text{ }^\circ\text{C}$ for 30 min. To stop the reaction, equal volume of 0.5 M trichloroacetic acid was added, and the mixture was then centrifuged at $10,000\text{ g}$ for 5 min to remove the precipitate. The supernatant was mixed with equal volume of 1.0 M NaOH, and the absorbance at 366 nm (OD_{366}) of the mixture was measured using a microplate reader (Bio-tek, Vermont, USA). The extracellular proteolytic activity, one unit of which was defined as the amount of enzyme that produced an absorbance change of 0.01 at 366 nm , was calculated as follows: the proteolytic activity = $(\text{OD}_{366}$ of the sample – OD_{366} of the negative control)/0.01.

Shrimp spoilage assays

Sterilized shrimp meat (at least 5 kg) was prepared as described by Zhu et al. (2017). Mid-logarithmic bacterial cells suspended in sterile PBS were sprayed on surface-sterilized shrimp for inoculation at a level of 10^4 – 10^5 CFU/g. For co-inoculation, the PBS suspensions of *Sb* and *Ab* were combined at the CFU ratio of 1:1 or 1:5 before spray-inoculation. Sterile PBS was as the negative control. Samples were stored at $4\text{ }^\circ\text{C}$ for 5 days. Bacterial counts were determined on iron agar, and H_2S -producing bacteria were selectively enumerated as black colonies. Total volatile basic nitrogen (TVB-N) in samples was assayed using steam distillation with a Kjeldahl distillation apparatus and titration (Botta et al. 1984), and its levels were expressed as $\text{mg}/100\text{ g}$ of shrimp meat.

Sensory analysis was performed by 15 experienced panelists according to a conventional profile (ISO 13299 2003). Panelists were trained as described by Stohr et al. (2001). Samples were served to the panelists in plastic vessels with lids. Spoilage levels were scored from 0 to 10 based on texture, color and odor. An average score of 6 was

defined as the sensory rejection point. The panelists also chose two main characteristic odors from the following descriptors: fresh/original, grass, nothing, fishy, cabbage, acid/vinegar, sour/fermented, butter/caramel, amine, sulphur, indole/feet (Macé et al. 2014). The frequencies obtained for each descriptor by all the panelists were recorded for each group, on each analysis day. To examine the correlation between characteristic odors and inoculated bacteria, principal component analysis (PCA) was performed on the means of the frequencies for each sensory descriptor.

Statistical analysis

All the experiments were repeated at least three times in this study. Data were presented as the means $\pm$ standard deviations. Statistical analyses were done using SPSS software version 19.0 (SPSS, Inc., Chicago, USA) and OriginPro 7.0 software (OriginLab Co., Northampton, USA). The mean differences were compared by one-way ANOVA with least significant difference (LSD).

Results and discussion

Production and eavesdropping of *Ab* AHLs

Ab have been shown to possess a LuxI/LuxR-type QS system utilizing AHLs as their QS signals (Bhargava et al. 2010). As shown in the agar diffusion assay using an AHL-specific bioreporter *A. tumefaciens* A136 (Fig. 1a), *Ab* induced the expression of β -galactosidase activity (blue coloration), suggesting their ability to secrete AHLs. *Sb* were not able to induce *A. tumefaciens* A136 to produce the blue color (Fig. 1a), but seem to block the agar diffusion of *Ab* AHLs (Fig. 1b). As demonstrated by the β -galactosidase assay, the AHL activity of *Ab* increased substantially until the early stationary phase, and then declined rapidly during the middle to late stationary phase (Fig. 1c). However, there was no AHL activity in the co-culture of *Sb* and *Ab* with an inoculation ratio of 1:1 (Fig. 1d). These results suggest that *Sb* could deplete ambient AHLs derived from *Ab*.

Morohoshi et al. (2008) reported that *Shewanella* sp. strain MIB015 encoded AHLs-acylases to degrade ambient AHLs. However, no AHL-acylase was detected in either culture supernatants or cell lysates of *Sb* in the present study (data not shown). Based on the shapes and retention factors of the blue spots on the TLC plate (Fig. 1e), *Ab* produced at least three detectable AHLs, i.e. C4-HSL, O-C6-HSL and O-C8-HSL. Our previous study has found that all of these three AHLs could be sensed by *Sb* via the LuxR receptor (Zhu et al. 2015). Thus eavesdropping on

Ab AHLs by *Sb* should account for the blockage of AHL diffusion in the agar diffusion assay (Fig. 1b) and the lowered level of AHL activity in the 1:1 co-culture (Fig. 1d). To our knowledge, this is the first report on the ability of *Shewanella* to eavesdrop on AHLs from habitat competitors. Eavesdropping may be an important communication strategy for *Shewanella* to win out as specific spoilage microorganisms in seafood, and to test this hypothesis, we evaluated the effect of eavesdropping on spoilage potential of *Sb* in the following sections.

Eavesdropping promoted the growth of *Sb* in the co-culture

Natural environments usually harbor diverse microbial species, and within these communities, bacteria compete with their neighboring rivals for space and nutrients. We used in vitro co-culture systems to investigate interspecies competition between *Sb* and *Ab*. The 1:1 co-culture was found to significantly inhibit the growth of *Sb* rather than that of *Ab* within 12 h of incubation (Fig. 2a). Both *Sb* and *Ab* were not found to produce antimicrobial compounds in the present study (data not shown), so space and nutrients should be the limiting factors for bacterial competition in the co-culture. *Ab* seem to be less prone to competitive inhibition, which might be explained by their well-known ability to survive under nutrient-limiting conditions (Bravo et al. 2016).

The growth of *Sb* was unexpectedly less inhibited by the 1:5 co-culture where as that of *Ab* was even promoted (Fig. 2a). As shown in Fig. 1d, AHL activity of the 1:5 co-culture was significantly higher than that of the blank control ($P < 0.01$), and equaled to about a half of that of the mono-culture of *Ab*. This indicates that *Ab* AHLs in the 1:5 co-culture were in excess of the eavesdropping capacity of *Sb*. Apparently, more AHLs were produced in the 1:5 co-culture compared with the 1:1 co-culture due to the higher proportion of *Ab* inoculated in the former. Exogenous C4-HSL and O-C8-HSL promoted the growth of *Sb*, while no significant effect was observed for exogenous O-C6-HSL (Fig. 2c). C4-HSL was the most abundant AHL in the 1:5 co-culture (Fig. 1e). The growth of *Sb* thus seems to be more profoundly promoted in the 1:5 co-culture where more AHLs especially C4-HSL were produced compared with the 1:1 co-culture. Bacterial growth in LB medium requires extracellular proteases to degrade peptides and peptones. The increased growth of *Ab* in the 1:5 co-culture was possibly due to the greater extracellular proteolytic activity compared with the mono-culture and the 1:1 co-culture as mentioned later (Fig. 3a).

Nutrient conditions have been found to effect QS in *Ab*. *A. calcoaceticus* BD413, which produced four AHL species in minimal media, secreted only three AHLs when

Fig. 1 Production and eavesdropping of AHLs: **a**, **b** agar diffusion assays for AHLs in *Shewanella baltica* (*Sb*) and *Acinetobacter* (*Ab*); **c** the kinetics of growth and β -galactosidase activity of *Ab*; **d** β -galactosidase and **e** thin-layer chromatography assays for *Sb* eavesdropping on *Ab* AHLs. Data are represented as means $\pm$ standard deviations, with different letters (*a*–*f*) indicating significant differences between groups at the $P < 0.05$ level

grown on minimal media supplemented with 0.1% tryptone (Gonzalez et al. 2001). In this study, *Ab* produced three AHLs in the mono-culture and only two AHLs in the 1:5 co-culture (Fig. 1e). In addition, the most abundant AHL in the mono-culture was O-C6-HSL, while that in the 1:5 co-culture was C4-HSL. These changes between the mono-culture and the 1:5 co-culture might be the result of nutrient stress in the latter. The bacterial count of *Ab* in tenfold diluted LB medium, which was used in this study to mimic nutrient limiting conditions, was only one tenth of that in LB medium (Fig. 2a, b), but the AHL activity in tenfold diluted LB medium was more than a half of that in LB medium (Fig. 1d). This indicates that nutrient

limitation stimulates *Ab* to produce more AHLs. A greater proportion of C4-HSL was also found in tenfold diluted LB medium compared with LB medium (Fig. 1e). It thus seems that nutrient competition under the 1:5 co-culture condition played important roles in the increased production of AHLs especially C4-HSL. In addition, the 1:1 co-culture did not inhibit the growth of *Sb* in tenfold diluted LB medium, and on the contrary, a greatly increased (40-fold) growth of *Sb* was observed (Fig. 2b). This demonstrates that the increased production of *Ab* AHLs in tenfold diluted LB medium greatly promoted the growth of *Sb*. In fact, Qazi et al. (2006) reported similar results that AHLs

Fig. 2 *Shewanella baltica* (Sb) and *Acinetobacter* (Ab) counts in **a** LB medium, **b** tenfold diluted LB medium, and **c** LB medium containing exogenous C4-HSL (10 μ M), 3-OXO-C6 (10 μ M) or 3-OXO-C8 (1 μ M) after 12 h of incubation. Data are represented as means $\pm$ standard deviations, with different letters (a–e) indicating significant differences at the $P < 0.05$ level

derived from *Pseudomonas aeruginosa* regulated the growth of *Staphylococcus aureus*.

Eavesdropping affected extracellular proteolytic activity and biofilm formation in the co-culture

Extracellular proteases are essential for bacterial penetration of the muscle during seafood spoilage (Venugopal 1990). *Sb* displayed a much higher (fourfold) extracellular proteolytic activity than *Ab* (Fig. 3a). Although the co-culture in LB medium at both ratios of 1:1 and 1:5 inhibited the growth of *Sb* (Fig. 2a), extracellular proteolytic activities in these co-cultures were significantly higher than that in the mono-culture (Fig. 3a). Our previous study has

reported the increased production of extracellular proteases by *Sb* in the presence of exogenous O-C6-HSL and O-C8-HSL (Zhu et al. 2015). It thus seems that eavesdropping on O-C6-HSL and O-C8-HSL produced by *Ab* stimulated *Sb* to secrete more proteases in these co-cultures. The extracellular proteolytic activity in the 1:5 co-culture was significantly greater than that in the 1:1 co-culture, which is in line with the higher levels of bacterial growth and AHL production in the 1:5 co-culture as mentioned above. In fact, the regulation of extracellular proteolytic activities by the AHL-dependent QS has been previously associated with the spoilage of foods of vegetable and protein origin (Christensen et al. 2003).

Biofilm plays an important role in bacterial resistance to harsh environments, and has been well documented to be controlled by the AHL-dependent QS (Atkinson et al. 2007). *Ab* can thrive under hostile conditions like desiccation, poor nutrition and high antibiotic exposure largely due to their good ability in biofilm formation (Bravo et al. 2016). As shown in Fig. 3b, *Ab* displayed a rather better biofilm-forming capacity than *Sb*, which was in line with the denser and more compact biofilm of *Ab* as observed by confocal laser scanning microscopy (Fig. 3c, d). Biofilm formation in *Ab* have been frequently reported to rely on AHLs (Sarkar and Chakraborty 2008; Kang and Park 2010; Anbazhagan et al. 2012), while C4-HSL, O-C6-HSL and O-C8-HSL have been previously found to inhibit biofilm formation in *Sb* (Zhu et al. 2015). Significantly less biofilm was detected in the 1:1 co-culture compared with the mono-culture ($P < 0.01$) (Fig. 3b), which was in accordance with the results of confocal laser scanning microscopy in Fig. 3d, e. This indicates that *Sb* eavesdropping on *Ab* AHLs could suppress biofilm formation in the co-culture. Significantly more biofilm was detected in the 1:5 co-culture compared with the mono-culture ($P < 0.01$) (Fig. 3b), which agrees with the results of confocal laser scanning microscopy in Fig. 3d, f. As mentioned above, AHLs in the 1:5 co-culture were over supplied to *Sb* eavesdropping, which may reduce the inhibiting effect of *Sb* eavesdropping on biofilm formation of *Ab*. In addition, the above-mentioned higher bacterial density in the 1:5 co-culture compared with the mono-culture can also partially explain the increased biofilm formation in the 1:5 co-culture.

Eavesdropping promoted shrimp spoilage

As shown in Fig. 4a, both *Sb* and *Ab* could proliferate in refrigerated shrimp after inoculation, but *Ab* grew much slower possibly due to its mesophilic property (Ercolini et al. 2009). The AHL activity in the co-inoculated group was about one-third of that in the *Ab* group (Fig. 4c), suggesting that *Ab* AHLs was in excess of the

Fig. 3 Detection of extracellular proteolytic activities and biofilm matrixes of *Shewanella baltica* (*Sb*) and *Acinetobacter* (*Ab*): **a** the azocasein assay; **b** the crystal violet staining assay; **c–f** confocal laser scanning microscopy. Data are represented as means $\pm$ standard deviations, with different letters (*a–d*) indicating significant differences at the $P < 0.05$ level

eavesdropping capacity of *Sb* in the co-inoculated shrimp. Levels of both total bacteria and H₂S-producing bacteria in the co-inoculated group were significantly higher than the other groups from the 2nd day of storage ($P < 0.05$) (Fig. 4a, b). It thus seems that the AHL-dependent eavesdropping also boosted the in vivo growth of *Sb*. In fact, bacterial growth during the spoilage of various foods has been previously found to depend on the AHL-dependent QS (Skandamis and Nychas 2012). Shrimp may be considered as a nutrient-limiting environment, from the perspective that muscle proteins are not readily available nutrients before being hydrolyzed. Extracellular proteases thus can be regarded as the limiting resources for bacterial growth in vivo. The above-mentioned increased production of extracellular proteases by *Sb* in response to *Ab* AHLs,

therefore, can at least partially explain the promotion of in vivo growth of *Sb* by eavesdropping.

TVB-N is one of the most widely used chemical indicators for seafood freshness. Li et al. (2016) reported that exogenous AHLs could stimulate more production of TVB-N in refrigerated turbot (*Scophthalmus maximus* L.). This study also evaluated the effect of AHL-dependent eavesdropping on TVB-N production in vivo. As shown in Fig. 5a, the *Sb* group displayed significantly higher TVB-N levels than the *Ab* group from the 3rd day of storage ($P < 0.05$), while the co-inoculated group showed the highest TVB-N levels from the 2nd day of storage ($P < 0.05$), suggesting that eavesdropping caused more production of TVB-N. A TVB-N level at 25 mg N/100 g has been previously reported as the acceptable limit for shrimp (Okpala et al. 2014). According to the TVB-N

Fig. 4 Enumeration of **a** total bacteria and **b** H₂S-producing bacteria, and **c** detection of the AHL activity in refrigerated shrimp inoculated with *Shewanella baltica* (Sb) or *Acinetobacter* (Ab). Data are represented as means $\pm$ standard deviations, with different letters (a-c) indicating significant differences at the $P < 0.05$ level

levels, the co-inoculated group was judged spoiled about 0.5 day earlier than the *Sb* group. As shown in Fig. 5b, based on the sensory scores, the co-inoculated group was again judged spoiled about 0.5 day earlier than the *Sb* group. Apparently, the co-inoculation promoted shrimp spoilage, which could be attributed to the higher levels of total bacteria and H₂S-producing bacteria in the co-inoculated group as mentioned above.

The main odor characteristics for samples after 1, 3 and 5 days of storage are presented by PCA (Fig. 5c). The first axis (50.2% of the information) enables the storage time (i.e. spoilage level) of each sample to be visualized. The second axis, representing 39.7% of the information, enables the visualization of samples with similar odor characteristics. The *Ab* group and the non-inoculated group

Fig. 5 Evaluation of shrimp spoilage by **a** TVB-N levels, **b** sensory scores as determined by the sensory panel (15 panelists), and **c** main odor characteristics as presented by principle component analysis (A1, A3 and A5 representing samples inoculated with *Acinetobacter* (Ab) and stored for 1, 3 and 5 days; B1, B3 and B5 representing non-inoculated samples stored for 1, 3 and 5 days; C1, C3 and C5 representing samples co-inoculated with *Shewanella baltica* (Sb) and Ab and stored for 1, 3 and 5 days; S1, S3 and S5 representing samples inoculated with *Sb* and stored for 1, 3 and 5 days)

similarly displayed mild spoilage odors like nothing, acid and fishy after 5 days of storage, which indicates that *Ab* had a weak spoilage potential in refrigerated shrimp. The *Sb* group and the co-inoculated group showed quite similar odors like amine, sulphur and feet, the typical unpleasant odors of spoiled seafood, after 5 days of storage, which implies that *Sb* contributed the most part of odor production in the co-inoculated group.

Conclusion

This study discovered the AHL-dependent eavesdropping as a unique communication strategy for *Sb*, and demonstrated that this strategy increased the spoilage potential of *Sb*. Our results provide a fresh perspective on the spoilage process of refrigerated *L. vannamei*. Novel preservation techniques based on chemical or biological disruption of AHL-dependent eavesdropping need to be exploited in the future to further reduce post-harvest loss of shrimp.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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