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Evaluation of the spoilage potential of bacteria isolated from spoiled cooked whole tropical shrimp (*Penaeus vannamei*) stored under modified atmosphere packaging

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Abstract:

The spoilage potential of isolates belonging to five bacterial groups/species (*Shewanella baltica, Carnobacterium maltaromaticum, Aeromonas salmonicida*, *Vibrio* sp., "other *Gamma-Proteobacteria*" [containing one strain of *Pseudoalteromonas* sp. and one strain of *Psychrobacter* sp.]) isolated from spoiled cooked and whole tropical shrimp stored under modified atmosphere packaging (MAP) was evaluated by inoculation into ionized cooked and peeled tropical shrimp followed by storage for 32 days at 8°C. Microbial growth and sensory changes were monitored during the storage period. The major spoilage bacterial isolate groups were *C. maltaromaticum* and *S. baltica*. In order to characterize their spoilage potential further and to study the effect of their interactions, each of these two specific spoilage organisms (SSO) and one mixed-culture, *C. maltaromaticum*/*S. baltica,* were tested using a combination of complementary methods: molecular (PCR-TTGE), sensory, chemical, and conventional microbiological analyses. It was concluded that, in the mixed-culture-inoculated samples, both species groups imposed their spoilage characteristics.

Highlights

► Spoilage potential of 5 bacterial groups isolated from spoiled cooked whole shrimp. ► Specific spoilage organisms were identified among these bacterial groups. ► Characterization of spoilage potential and bacterial interactions of 2 dominant spoilers. ► In co-culture, each bacterial group imposed its spoilage characteristics.

Keywords : Spoilage potential ; bacterial interaction ; Specific Spoilage Organism ; cooked tropical shrimp ; volatile compounds

1. Introduction

Prawn and shrimp are the most important products from aquaculture: more than 3.7 million tons were produced in 2010 with a value of more than 16 billion US dollars. Moreover, *Penaeus vannamei*, also called white leg shrimp, is the species most reared with about 2.7 million tons in 2010 representing 11 billion US dollars on its own (FAO Fisheries Statistics 2012). The current market trend is for the processing of value-added products like cooked shrimp, which is very popular and widely sold in supermarkets as a chilled ready-to-eat product under modified atmosphere packaging (MAP). Shrimp quality is essential to maintain not only product value but also the reputation of the farmer, processor and country (Bari *et al.*, 2011).

The spoilage bacterial species of a packaged product depend on its endogenous microbiota, the processing undergone, the type of packaging (MAP, vacuum, aerobic, etc.) and storage temperature.

While Nordic shrimp (*Pandalus borealis*) are cooked directly after catching and then frozen (sometimes after being peeled), tropical shrimp (*Penaeus* sp.) are generally frozen immediately after catching and sold to the processor (Leroi and Joffraud, 2011). It has been known for a long time that processing practices influence microbial count and shrimp microbiota composition. Cooking is the major step to reduce shrimp microbial load but peeling and brining lead to a recontamination dominated by Gram-positive bacteria (Harrison and Lee, 1968). Gram-positive bacteria such as lactic acid bacteria (*Carnobacterium* sp, *Enterococcus* sp., etc.) and *Brochothrix thermosphacta* have been identified as the major spoilage flora of MAP brined and drained cooked shrimp and non-drained MAP cooked peeled shrimp (Dalgaard *et al.*, 2003; Mejlholm *et al.*, 2005; Jaffrès *et al.*, 2009).

At the onset of spoilage, the product contains some bacteria that are involved in spoilage, the so-called specific spoilage organisms (SSO), and others that grow without

causing unpleasant changes. To identify the SSO among the bacterial groups present at the time of spoilage, the impact of bacterial isolates on the chemical and sensory characteristics obtained on inoculated products should be compared to those of naturally spoiled products. These experiments enable the spoilage potential of microorganisms to be determined, i.e. their ability to produce metabolites resulting in off-odours or off-flavours (Gram *et al.*, 2002; Dalgaard, 2006).

Enhancing our knowledge of the SSO for specific seafood products will lead to the development of better detection methods, shelf-life predictions and preservation techniques, thereby reducing losses due to spoilage and improving seafood quality (Dalgaard, 2000).

Several studies have focused on MAP cooked and peeled tropical shrimp spoilage or MAP brined and drained shrimp spoilage (Dalgaard, 1995; Dalgaard *et al.*, 2003; Mejlholm *et al.*, 2005; Laursen *et al.*, 2006; Jaffrès *et al.*, 2009; Noseda *et al.*, 2010; Jaffrès *et al.*, 2011; Noseda *et al.*, 2012) but this present study is the first to investigate the spoilage potential of bacteria isolated from whole tropical shrimp.

Our aim was to identify the SSO dominating MAP stored cooked whole tropical shrimp (*Penaeus vannamei*) and to highlight the interactions between these species. The ability to spoil was investigated for different bacterial groups previously isolated from whole spoiled cooked tropical shrimp; and then the spoilage potential of the strongest spoiling bacterial isolate groups was further characterized.

2. Materials and Methods

This study was conducted in two steps. In the first step, five different bacterial groups previously isolated from spoiled whole tropical shrimp were inoculated in a shrimp model

system by monitoring bacterial and sensory changes throughout the storage period. In the second step, to investigate the interaction among spoilage bacteria, binary cultures combining the two main spoilage bacterial isolates were inoculated into the shrimp model system and characterized by molecular (PCR-TTGE), sensory, chemical, and conventional microbiological analyses.

2.1. Bacterial isolates

All isolates tested were previously isolated from four different batches of spoiled cooked whole tropical shrimp obtained from a local plant and packaged under MAP (50% CO_2/50% N_2) and stored according to the shelf-life validation protocol adopted by the company (8 days at 2°C then 6-8 days at 8°C). All isolates were identified by 16S rRNA gene partial sequencing (700 bp) and found to belong to different genera or species distributed in five different bacterial groups: *Shewanella baltica, C. maltaromaticum, Aeromonas salmonicida, Vibrio* sp., "other *Gamma-Proteobacteria"* (containing one strain of *Pseudoalteromonas* sp. and one strain of *Psychrobacter* sp.*)*. Each group was represented by 2 to 6 isolates of the same species depending on the group (except for the "other *Gamma-Proteobacteria*" group which was composed of two different species) (Table 1).

2.2. Challenge tests

Cooked and peeled tropical shrimp from Colombia (South America) (about 7 kg) were cooked by the manufacturer and directly transported to the laboratory. They were packaged under vacuum in 1 kg bags, frozen at -80°C then sterilized and thawed before the experiment as described by Macé *et al* (2013). Isolates were pre-cultured individually in brain heart infusion broth (BHI) (Difco Laboratories, Detroit, MI, USA) at 20°C until their maximal concentration (7-9 log (CFU g^{-1})) was reached (1-3 d). Cultures of several isolates belonging

to the same species were pooled in a sterile vial and diluted in sterile peptone water (0.85% NaCl and 0.1% peptone), in order to achieve an inoculation mixture containing 4 log ($CFU.ml^{-1}$). Each inoculation mixture (36 mL) was sprayed onto a batch of approximately 1.2 kg of the shrimp model system placed in a laminar flow hood to reach an inoculated level of about 3 log ($CFU.g^{-1}$). A control was prepared by inoculating the ionized shrimp matrix with sterile water. Each batch of inoculated shrimp and the non-inoculated control were placed in 5 plastic trays, each containing ~240 g portions (one for each analysis date), and packaged under modified atmosphere (50% CO_2 and 50% N_2) using a Multivac T 200 machine (Hagenmüller, Wolfertschwenden, Germany) and a low gas permeability film (low density polyethylene, LDPE, LINPAC Plastics, permeability: $O_2 < 5$ cm^3/m^2.24 h.bar, $CO_2 < 25$ cm^3/m^2.24 h.bar). All batches of inoculated shrimp and the control were stored at 8°C for 32 days. Sensory and chemical analyses were carried out after 1, 8, 15, 26 and 32 days. Microbiological analyses were performed for up to 15 days to verify that all the groups were well implanted on the matrix.

For the next stage (named further characterization of spoilage potential), two isolate groups determined as being the main spoilers by the preliminary characterization step were inoculated singly or in co-cultures. Cultures were inoculated as previously described on the pauci-microbial shrimp model system to achieve initial levels of 3 log ($CFU.g^{-1}$). A non-inoculated control was also prepared. Each different batch of shrimp and the control were divided into 18 plastic trays (around 200 g portions) and packaged as described before. All batches were stored at 8°C for 18 days. After 1, 4, 8, 11, 15 and 18 days, samples were subjected to sensory, chemical and microbiological analyses.

2.3. Enumeration of inoculated isolates

148 At each sampling date of the preliminary characterization, one tray of the different
149 batches was used for microbiological count using Brain Heart Infusion Agar medium (BHI) as
150 described by Macé *et al* (2013).
151 For further characterization of the spoilage potential, three trays of the different
152 batches were used for microbiological analysis at each sampling date. From each package, a
153 10 g portion was aseptically weighed and the three portions were pooled, treated and
154 enumerated as described previously on Brain Heart Infusion Agar medium and pour-plated on
155 Iron Agar (IA). For co-cultured groups, the species were distinguished on IA plates by their
156 morphological and coloration differences: *S. baltica*, which is an H_2S-producing bacterium,
157 produces black colonies on IA.
158

159 **2.4. Sensory analysis**

160 Sessions were performed in individual partitioned booths, as described in the
161 procedure NF V-09-105 (AFNOR, 1987), equipped with a computerized system (Fizz,
162 Biosystèmes, Couternon, France). All the samples were frozen after microbiological analysis
163 and kept frozen at –80°C until sensory evaluation. An experimental design was constructed
164 for the sensory sessions in order to balance for contrast effects (bacterial groups and time of
165 storage). Principal component analysis (PCA) with standardization was performed on the
166 means of the scores for each sensory descriptor and spoilage intensity for further
167 characterization. Multivariate data processing was carried out with Uniwin Plus 6.1 software
168 (Sigma Plus).
169 Sensory analysis was carried out by an internal panel of IFREMER, experienced in the
170 evaluation of seafood, especially of tropical shrimp (Jaffrès *et al.*, 2009 and 2011). For each
171 assessor, portions of 3-4 shrimp were placed in plastic containers with lids to keep the odours
172 intact.

Nine trained panellists, selected according to their sensory capacities, participated in the preliminary characterization of spoilage potential. One tray per batch was opened. Panellists scored the spoilage level on a continuous scale from 0 to 10 and chose two main characteristic odours from the following descriptors selected during preliminary sessions: rice, crustacean, surimi, marine/iodine, milky, butter/caramel, floor cloth, pyrrolidine, dirt/mould, amine, acid, sour/fermented, feet/cheese, cabbage/sulphur, nothing. The products were considered strongly spoiled when they reached a score of 6.

For further characterization of the spoilage potential, three trays per batch were used and 12 trained panellists participated in this experiment. A quantitative descriptive analysis (QDA) described by (Stone and Sidel, 2004) was used to determine the sensory profile (ISO, 2003) of each batch inoculated with each species and co-cultured species. First, panellists had to score the spoilage level on a continuous scale from 0 to 10 and then using the following appropriate odour descriptors considered pertinent during the previous step: overall intensity, rice, crustacean, milky, butter/caramel, pyrrolidine, amine, acid/ vinegar, sour/fermented, feet/cheese, cabbage/sulphur.

A two-way analysis of variance was applied to the panellists' scores for spoilage intensity with products (strain) and panellists as independent factors. Significant differences between means were determined using Duncan's multiple range test ($p < 0.05$) (Fizz software).

2.5. Chemical analysis

Total Volatile Basic Nitrogen (TVBN) measurements were only performed in the further characterization stage. At each sampling date, 150 g of inoculated cooked and peeled tropical shrimp were analysed as described by Macé *et al* (2013).

198 **2.6. Analysis of volatile compounds**

199 During further characterization of spoilage potential, at day 1 and day 18, volatile
200 compounds produced by the non-inoculated control sample and the *S. baltica*-inoculated
201 sample were analysed using a gas chromatography device, model GC 7890A, Agilent
202 (Agilent Technologies, Santa Clara, USA) coupled with a mass spectrometer 5975 C VL
203 (Agilent) and flame ionization detector after solid-phase microextraction (SPME/GC−MS–
204 FID). The extraction and injection processes were performed automatically using an
205 autosampler MPS 2 (Gerstel, Mülheim, Germany). Briefly, volatile compounds of 5-g
206 portions of shrimp sample were analysed as described by Jaffrès *et al* (2011) with some
207 adjustments: vial heating for 40 min (instead of 50 min), Stable Flex™ exposure in the
208 headspace of the vial for 30 min (instead of 25 min) and maximum oven temperature of
209 240°C (instead of 280°C).

210 Compounds were identified by comparison of mass spectra with a reference database
211 (Wiley 6.0), and by comparison of mass spectra and linear retention indexes (LRI) with those
212 of standards injected in the same conditions.

213 Mean values of sample peak areas were compared by analysis of variance to identify specific
214 spoilage volatile compounds. ANOVA processing was performed with Statgraphics Plus 5.1
215 software (Statistical Graphics Corp.). Data were reported as log (peak area/g) for each specific
216 compound detected.

217

218 **2.7. Temporal Temperature Gel Electrophoresis (TTGE) analysis on co-cultured**
219 **inoculated samples**

220 The suspension prepared for bacteriological analysis was used to obtain molecular
221 fingerprints from the shrimp matrix inoculated with co-culture isolate groups. Bacterial DNA
222 extraction was performed as described previously by Jaffrès *et al.*, 2009.

Bacterial DNA from the inoculated shrimp matrix was analysed by PCR-TTGE as described previously by Jaffrès *et al.*, 2009. Standardization, analysis and comparison of TTGE fingerprints were monitored using BioNumerics Software, version 6.0 (Applied Maths NV, Sint-Martens-Latem, Belgium) as described by Macé *et al.*, 2012.

3. Results

3.1 Preliminary characterization of spoilage potential

3.1.1. Enumeration of the different bacterial groups

Figure 1 shows the mean growth pattern of the five different bacterial groups. The ionized control was little contaminated, about 0.7 log $CFU.g^{-1}$ at inoculation time, and thus considered a pauci-microbial matrix. The initial counts of the inoculated samples were between 2.6 to 4.2 ($CFU.g^{-1}$). Growth of bacterial groups reached between 5.4 and 8.5 log ($CFU.g^{-1}$) after 8 days of storage. At 15 days of storage, all the bacterial groups rose to levels ranging between 6.3 ("other *Gamma-Proteobacteria*" group) and 9.9 log ($CFU.g^{-1}$) (*C. maltaromaticum*) and were considered well implanted.

3.1.2 Sensory characteristics of inoculated cooked tropical shrimp

Figure 2 presents the spoilage kinetics of the different inoculated samples. Although the results are not always significantly different due to differences between panellist responses, a trend can be observed for each sample. After 1 day of storage, all samples were considered non-spoiled, with a score of about 2 out of 10 (Figure 2). At day 8, the spoilage samples had increased and samples inoculated with two bacterial groups, *C. maltaromaticum* or *A. salmonicida*, were considered strongly spoiled with a grade above 6.

After 15 days, two samples inoculated with *C. maltaromaticum* or *S. baltica* were assessed as strongly spoiled with a score of around 7 or 8, respectively. Those inoculated with

A. salmonicida appeared less spoiled until the end of storage with scores decreasing from 5.5 (15 days) to 4.6 (26 days) and finally to 4.3 (32 days). *Vibrio* sp.- or "other *Gamma-Proteobacteria*"-inoculated samples remained lightly spoiled with a score between 4 and 5. The ionized control sample was not considered spoiled throughout the storage period. Its spoilage score reached 3 at the end of storage, probably due to the remaining endogenous bacteria.

After 8 days of storage, the inoculated batches exhibited different typical characteristic odours. For each bacterial group, the main sample off-odours, as quoted by at least 3 panellists, are listed in Table 2. Sour and cabbage/sulphur were determined as typical odours for *S. baltica*-inoculated samples during all the storage, while sour and feet/cheese characterized *C. maltaromaticum*-inoculated spoiled samples. The main odours of *A. salmonicida*-inoculated samples changed from butter and pyrrolidine during the first 8 days of storage to a mix of several odours like sour, cabbage/sulphur, butter, amine, feet/cheese at the end. Crustacean odour, linked to fresh shrimp, was found at first on *Vibrio* sp.-inoculated samples but then sour, acid and cabbage/sulphur odours prevailed. After 15 days of storage, "other *Gamma-Proteobacteria*"-inoculated samples displayed feet/cheese odours, which were replaced by a sour odour at the end. Throughout storage, non-inoculated sample displayed a mix of typical fresh (crustacean, rice, nothing) and spoiled shrimp odours (cabbage/sulphur, sour/fermented, acid, feet/cheese).

Bacterial isolates belonging to *C. maltaromaticum* and *S. baltica* were responsible for the strongest spoilage odours detected and were considered to be the main spoilage bacteria in the product throughout storage. Hence, their spoilage potential was subjected to further characterization. It can be noticed that, despite reaching grade 6 at day 8, *A. salmonicida* was not considered a strong spoiler because its spoilage intensity decreased after 8 days of storage.

3.2 Further characterization of spoilage potential

3.2.1. Spoilage potential of single-species groups

Figure 4 shows the spoilage kinetic trends of the inoculated samples. The ionized control presented weak bacterial counts, from 2.3 to 3.7 log (CFU.g^{-1}), throughout the storage (Figure 3) and was thus considered a pauci-microbial matrix. The control sample was scored "non-spoiled" during the entire storage period (18 days) with scores between 0 and 2 (Figure 4).

The principal component analysis (PCA) presented in Figure 5 was performed on the mean scores of profiling tests and summarizes the evolution of the main odour characteristics. The simultaneous projection of samples and sensory descriptors is shown on the first 1-2 plane. The first axis (64.4% of the information) enables the spoilage level of each sample to be visualized. It divides the typical odours of freshness of non-spoiled products, on the left, from the spoiled samples, on the right, around typical off-odours of cooked tropical shrimp with sour, feet/cheese, etc. The second axis, representing 16.5% of the information, is mainly created by butter, sulphur and pyrrolidine descriptors and enables the visualization of samples presenting the same spoilage characteristics. All the control samples, from day 1 to day 18, are on the left part of this Figure, surrounding the unspoiled inoculated samples and presenting crustacean and rice odours characteristic of freshness.

The results of viable counts of samples inoculated with the two different bacterial isolate groups and their co-culture are shown in Figure 3. *C. maltaromaticum* counts were initially 3.4 log (CFU.g^{-1}) then rapid growth was observed reaching more than 6.5 log (CFU.g^{-1}) after 4 days of storage. At 8 days of storage, *C. maltaromaticum* concentration was 8.9 log (CFU.g^{-1}) and increased to 9.6 log (CFU.g^{-1}) at the end of storage. The sample spoilage score rose to 5.7 after 8 days of storage to reach a maximum of about 6.6 after 11 days before decreasing slowly to 5.4 at the end of storage (Figure 4). These samples exhibited

mainly sour, acid and feet/cheese odours after 11, 15 and 18 days of storage (Figure 5) while the sample at 8 days displayed a butter odour. TVBN production was induced by *C. maltaromaticum* and reached 60 mg-N 100 g^{-1} at the end of storage (Figure 6).

S. baltica were inoculated at around 2 log (CFU.g^{-1}) (Figure 3). A slow growth was observed, reaching about 5.6 log (CFU.g^{-1}) after 8 days of storage and achieved progressively a maximum of 8.7 (CFU.g^{-1}) at the end of storage. The samples were considered unspoiled until the 8^{th} day of storage, lightly spoiled after the 11^{th} day with a score of 4.3 and almost strongly spoiled with a score of 5.9 after 18 days of storage (Figure 4). After 11 days of storage, these samples displayed sulphur, pyrrolidine and amine spoilage odours (Figure 5). An increase in TVBN production was observed with a maximal level of 50 mg-N 100 g^{-1} at the end of storage (Figure 6).

The *S. baltica* group, considered one of the spoiling groups tested here, was included in the volatile compound study using SPME/GC-MS-FID. Approximately 40 compounds were identified in the sample tested. Statistical treatment (p value < 0.05) was used to compare the non-inoculated control sample stored for 1 and 18 days, the newly *S. baltica*-inoculated sample (1 day of storage) and spoiled sample (18 days). Hence, 18 compounds exhibiting a specific behaviour on the spoiled sample tested were determined (Table 3). Among them, 14 were only detected in samples spoiled by *S. baltica*: butanol, isoamylalcohol, 1-hexanol, 2-hexenal, acetic acid, isovaleric acid, isobutyric acid, 2-propanone (acetone), 2-heptanone, 3-hydroxybutanone (acetoin), 2-nonanone, 2-decanone, methyl-P-tert-butyl phenyl acetate and p-menthane. 1-pentanol (amylalcohol), 1-penten-3-ol and methyl mercaptan production increased slightly in *S. baltica*-inoculated samples after 18 days of storage compared to the three other samples (p < 0.05). Ethanol presented a small decrease in the *S. baltica* spoiled samples (p < 0.05).

3.2.2 Spoilage potential of mixed-species group

In the presence of *S. baltica*, *C. maltaromaticum* presented quite similar bacterial growth compared to the monoculture (except that its inoculation level was lower). In the presence of *C. maltaromaticum*, *S. baltica* growth was faster and reached about 7.4 log ($CFU.g^{-1}$) after 8 days of storage (Figure 3).

Co-culture samples gave the maximum production of TVBN with about 63 mg-N 100 g^{-1} observed among all the samples tested in this study (Figure 6).

These samples presented the same spoilage behaviour as *C. maltaromaticum* single-species samples and were strongly spoiled at the same time (Figure 4). However, their characteristic odours were closer to those of *S. baltica*-inoculated samples with sulphur, amine and a stronger pyrrolidine odour, except for the co-inoculated sample at day 8 which presented a butter odour (Figure 5).

TTGE analysis enabled microbiota dynamics to be visualized by examining fingerprints of the dominant bacterial groups evolving during storage in co-culture-inoculated samples. In order to analyse the TTGE patterns, fingerprints of the different samples were compared with those of pure isolates involved in each bacterial group: *C. maltaromaticum*, and *S. baltica*. In fact, these species are present in different seafood products and some of them have already been studied by PCR-TTGE in previous studies (Macé *et al.*, 2012; Macé *et al.*, 2013). Concerning *S. baltica*, two pure isolates were used for assignation because their TTGE profiles present several different bands.

C. maltaromaticum and *S. baltica* co-culture TTGE patterns are shown in Figure 7. Patterns of isolates visualized in any of the samples after 1 and 4 days of storage did not correspond to pure profiles of isolates. They are assumed to be endogenous bacteria present on the ionized sample (data not shown). By comparing band migration positions, *C. maltaromaticum* was assigned in 5 samples after 4, 8, 11, 15 and 18 days of storage. Typical

348 *S. baltica* bands were visualized in 4 samples, from 8 days until the end of storage but
349 presented only faint bands
350
351 **4. Discussion**
352 Contrary to the description of Gram-positive bacteria as the main spoilage organisms
353 in previous studies, here, cooked whole tropical shrimp spoilage microbiota were found to be
354 dominated by *S. baltica* in association with *C. maltaromaticum* (data not shown). It has
355 already been demonstrated that Gram-negative bacteria dominate shrimp microbiota after
356 cooking but before peeling (Harrison and Lee, 1968). Gram-negative bacteria are also a major
357 part of raw shrimp spoilage bacteria stored in ice (Jeyasekaran *et al.*, 2006). A recent study
358 concerning MAP cooked and peeled North Atlantic grey shrimp *(Crangon crangon)* also
359 described H_2S-producing bacteria and LAB as dominant flora at the end of storage (Noseda *et*
360 *al.*, 2012).
361 All bacterial groups present on the spoiled product were not involved in spoilage
362 (Dalgaard, 2000; Gram *et al.*, 2002). For example, in the preliminary characterization of
363 spoilage potential, only two of the five bacterial groups tested were considered to be strong
364 spoilers, namely: *C. maltaromaticum* and *S. baltica* after 8 and 15 days of storage,
365 respectively, at 8°C. A mix of several isolates per species was used to avoid an isolate effect
366 commonly described for spoilage bacteria (Joffraud *et al.*, 2006; Laursen *et al.*, 2006).
367 For each single-species group tested in the preliminary and further characterization
368 steps, the spoilage of the inoculated sample started after a certain bacterial concentration level
369 corresponding to the stationary phase according to the SSO concept (Dalgaard, 2000). *C.*
370 *maltaromaticum*-inoculated samples were spoiled when bacterial counts reached between 8
371 and 9 log ($CFU.g^{-1}$). The same bacterial level was observed for cooked and peeled tropical
372 shrimp and salmon spoilage by *C. maltaromaticum* (Jaffrès *et al.*, 2011; Macé *et al.*, 2013). It

was determined that high concentrations of *S. putrefaciens* (8-9 log ($CFU.g^{-1}$)) are required to produce off-odours in seafood (Dalgaard, 1995). In the present study, the same bacterial level was observed in samples spoiled by *S. baltica.*

Some *C. maltaromaticum* strains present spoilage ability on several seafood products, notably cooked shrimp (Laursen *et al.*, 2005; Mejlholm *et al.*, 2005; Laursen *et al.*, 2006; Leisner *et al.*, 2007; Jaffrès *et al.*, 2011). Sour, feet/cheese, milky, and/or butter odour were noticed in this study for *C. maltaromaticum*-inoculated spoiled samples but these off-odours were also associated with this species during characterization of cooked and peeled tropical shrimp, raw salmon and cold-smoked salmon spoilage (Joffraud *et al.*, 2001; Jaffrès *et al.*, 2011; Macé *et al.*, 2013). Laursen *et al.*(2006) linked *C. maltaromaticum* off-odours to malty, nutty, nauseous sweet odours but also to sour odour on an inoculated cooked shrimp sample.

S. putrefaciens and *S. putrefaciens*-like bacteria, such as *S. baltica*, play an important role in seafood spoilage and produce spoilage compounds like TMA and sulphur compounds like H_2S which lead to fishy and sulphur odours. They are able to develop on ice-stored products like seafood (Papadopoulos *et al.*, 2003; Vogel *et al.*, 2005; Dalgaard, 2006) and have also been identified as part of the spoilage microbiota of MAP seafood (Hovda *et al.*, 2007; Tryfinopoulou *et al.*, 2007). The production of sulphur compounds could be linked to the typical cabbage/sulphur odours of *S. baltica* spoiled samples.

The spoilage potential of these two bacterial isolate groups was thus studied singly or in combination using a multi-parameter approach to investigate any interaction between the species in terms of growth, chemical changes and sensory evolution. This type of approach has been used in several works on raw and cold-smoked salmon (Jorgensen *et al.*, 2000; Joffraud *et al.*, 2006; Macé *et al.*, 2013) and on shrimp (Mejlholm *et al.*, 2005; Laursen *et al.*, 2006).

Few growth differences were found between bacteria in co-culture and alone in the matrix. In the presence of *S. baltica*, *C. maltaromaticum* was not inhibited and still grew quite quickly on cooked tropical shrimp, reaching about 9 log ($CFU.g^{-1}$) in 8 days. In other cooked shrimp challenge tests, it has already been observed that *C. maltaromaticum* grows to high concentrations after about 10 days of storage, alone or in co-culture with *Brochothrix thermosphacta* (Mejlholm *et al.*, 2005; Laursen *et al.*, 2006; Jaffrès *et al.*, 2011). TTGE analyses enabled the dominant group evolution to be visualized during storage. Fingerprints of co-inoculated samples displayed the dominance of the *C. maltaromaticum* isolate group and its faster implantation in the matrix.

S. baltica growth seemed to be accelerated by the presence of *C. maltaromaticum* with a bacterial concentration of about 7.4 log ($CFU.g^{-1}$) in co-culture compared to 5.6 log ($CFU.g^{-1}$) in mono-culture after 8 days. This could be due to metabiosis behaviour between the two species favouring *S. baltica* growth (Gram *et al.*, 2002).

The spoilage kinetics of co-inoculated samples corresponded to *C. maltaromaticum* samples behaviour whereas their off-odours were closer to those of *S. baltica* samples with a stronger pyrrolidine odour. The increase in intensity of this odour was perhaps due to an interaction between metabolites produced by the two bacterial groups. The same kind of observation appeared for *C. maltaromaticum*- and *B. thermosphacta*-inoculated shrimp samples, where a particular odour was formed only when they were co-cultured (Mejlholm *et al.*, 2005; Laursen *et al.*, 2006). The modification of bacterial metabolism in co-cultures (metabiosis) is frequently involved in spoilage mechanisms (Jorgensen *et al.*, 2000; Gram *et al.*, 2002). For example, Jorgensen *et al.*, (2000) demonstrated that the spoilage activity and biogenic amine production of *H. alvei* was enhanced in the presence of LAB.

During the last few years, several studies have shown a correlation between the release of spoilage volatile compounds and the development of specific microbial species during

storage of seafood products (Jorgensen *et al*., 2001; Joffraud *et al.*, 2001; Wierda *et al.*, 2006) and notably of shrimp (Laursen *et al.*, 2006; Jaffrès *et al.*, 2011; Noseda *et al*., 2012). Volatile compounds produced by one of the dominant spoilers, *S. baltica*, were investigated in this study. We chose to focus on isolates of this species because volatile compound production by *C. maltaromaticum* isolates on cooked tropical shrimp product has already been studied recently (Jaffrès *et al.*, 2011). As several origins are possible for these volatile metabolites, it is difficult to attribute them to a specific pathway but some hypotheses can be made. 3-hydroxybutanone is an interesting compound resulting from microorganism glycogen catabolism (Joffraud *et al.*, 2001; Laursen *et al.*, 2006). Acetic acid production during spoilage of shrimp has already been related to microbial growth (Noseda *et al.*, 2012) and it can be produced in different metabolic pathways (Joffraud *et al.*, 2001). *Shewanella* has been assumed to be involved in acetone production on spoiled shrimp (Noseda *et al.*, 2012). Sulphur components, such as methyl mercapthan, play an important role in shrimp spoilage (Noseda *et al.*, 2012). Moreover, methyl mercaptan is produced by *Shewanella* species and results from the breakdown of methionine but it can also be an intermediate product of the metabiosis of dimethylsulphide and dimethyldisulphide (Noseda *et al.*, 2012). In this study, methyl mercaptan production is described in *S. baltica* spoiled sample and it is the only compound which can be linked to the sulphur/cabbage odour of this sample (Fenaroli, 2001). Methyl mercaptan could be an interesting spoilage marker for MAP cooked whole tropical shrimp, even though the sensory profile of seafood products depends on a combination of different compounds rather than a single one (Laursen *et al.*, 2006; Jaffrès *et al.*, 2011).

Conclusion

The results of these challenge-test studies show that two isolate groups from five bacterial groups associated with cooked whole tropical shrimp stored under MAP were

447 determined as fast and strong spoilers: *C. maltaromaticum* and *S. baltica.* Their assessment
448 alone or in co-culture, using a multi-parametric approach, demonstrated that *C.*
449 *maltaromaticum* is one of the SSO of cooked whole tropical shrimp. In fact, this bacterial
450 spoiler was able to cause rapid and strong spoilage. Therefore, this work has also contributed
451 to characterizing the spoilage potential of bacterial species isolates in association and to
452 highlight that interaction between *C. maltaromaticum* and *S. baltica* can enhance the intensity
453 of the pyrrolidine odour of a cooked shrimp sample. Moreover, in co-inoculated samples,
454 each species group imposes its characteristics: the spoilage kinetics of *C. maltaromaticum* and
455 the odours of *S. baltica*. In order to understand the mechanism involved in these interactions,
456 more detailed investigations are required. However, this work is a first step towards a better
457 comprehension of the natural spoilage of cooked whole tropical shrimp.
458

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Figure 1: Growth of five bacterial groups in MAP cooked tropical shrimp during storage at 8°C.

Figure 2: Spoilage scores of cooked tropical shrimp inoculated with 5 bacterial groups stored under MAP at 8°C for 32 days determined by the sensory panel (9 panellists). For each day of storage, analysis of variance with samples and panellists as independent factors, and Duncan's multiple comparison test ($p < 0.05$) were performed. Different letters indicate significant differences between samples.

Figure 3: Growth of each bacterial group in cooked tropical shrimp during MAP storage at 8°C for 18 days: enumeration of *C. maltaromaticum* and *S. baltica* group alone or in co-culture (*C. maltaromaticum* in the presence of *S. baltica* or *S. baltica* in the presence of *C. maltaromaticum*)

Figure 4: Spoilage level mean scores as determined by the sensory panel (12 panellists) for each of the inoculated cooked shrimp samples (continuous scale from 0 to 10) during storage at 8°C for 18 days. For each day of storage, analysis of variance, with samples and panellists as independent factors, and Duncan's multiple comparison test ($p < 0.05$) were performed. Different letters indicate significant differences between samples.

Figure 5: Simultaneous representation of inoculated cooked shrimp samples and odour descriptors on planes 1-2 of principal component analysis. Sample nomenclature: C, control samples (non-inoculated); carno, *Carnobacterium maltaromaticum*; shew, *Shewanella baltica*; msc, co-culture of *S. baltica* and *C. maltaromaticum*. Numbers in labels of samples represent time of storage (in days).

Figure 6: Development of total volatile basic nitrogen (TVBN, mg-N 100 g^{-1}) in cooked tropical shrimps inoculated with different bacterial groups during MAP storage for 18 days at 8°C.

Figure 7: Fingerprints and dynamics of co-cultures inoculated on cooked tropical shrimp during storage at 8°C for 18 days. Digitized TTGE profiles of 16S rRNA gene V3 regions obtained by PCR amplification from bacterial DNA of 6 samples inoculated with one mixed culture: *C. maltaromaticum* with *S. baltica* stored at 8°C for 18 days. Lanes D1 to D18 corresponded to samples: day 1, day 4, day 8, day 11, day 15, day 18. Bands “c” were assigned as *C. maltaromaticum* and bands “s1-s3” as *S. baltica* by comparison with pure isolate profiles. Bands * are assumed to be endogenous bacteria.

Table 1. List of bacterial isolates inoculated on ionized cooked tropical shrimp during challenge tests

Bacterial Identity/Group	Ifremer/Oniris Strain Library Code	Species
Shewanella baltica	MIP 2649	*S .baltica*
	MIP 2641	*S. baltica*
	MIP 2666	*S. baltica*
	MIP 2520	*S. baltica*
Carnobacterium maltaromaticum	MIP 2505	*C. maltaromaticum*
	MIP 2636	*C. maltaromaticum*
	MIP 2654	*C. maltaromaticum*
	MIP 2504	*C. maltaromaticum*
	MIP 2526	*C. maltaromaticum*
	MIP 2631	*C. maltaromaticum*
Aeromonas salmonicida	MIP 2644	*A. salmonicida*
	MIP 2646	*A. salmonicida*
Vibrio sp.	MIP 2657	*Vibrio sp.*
	MIP 2648	*Vibrio sp.*
"Other Gamma-Proteobacteria"	MIP 2626	*Pseudoalteromonas* sp.
	MIP 2537	*Psychrobacter* sp.

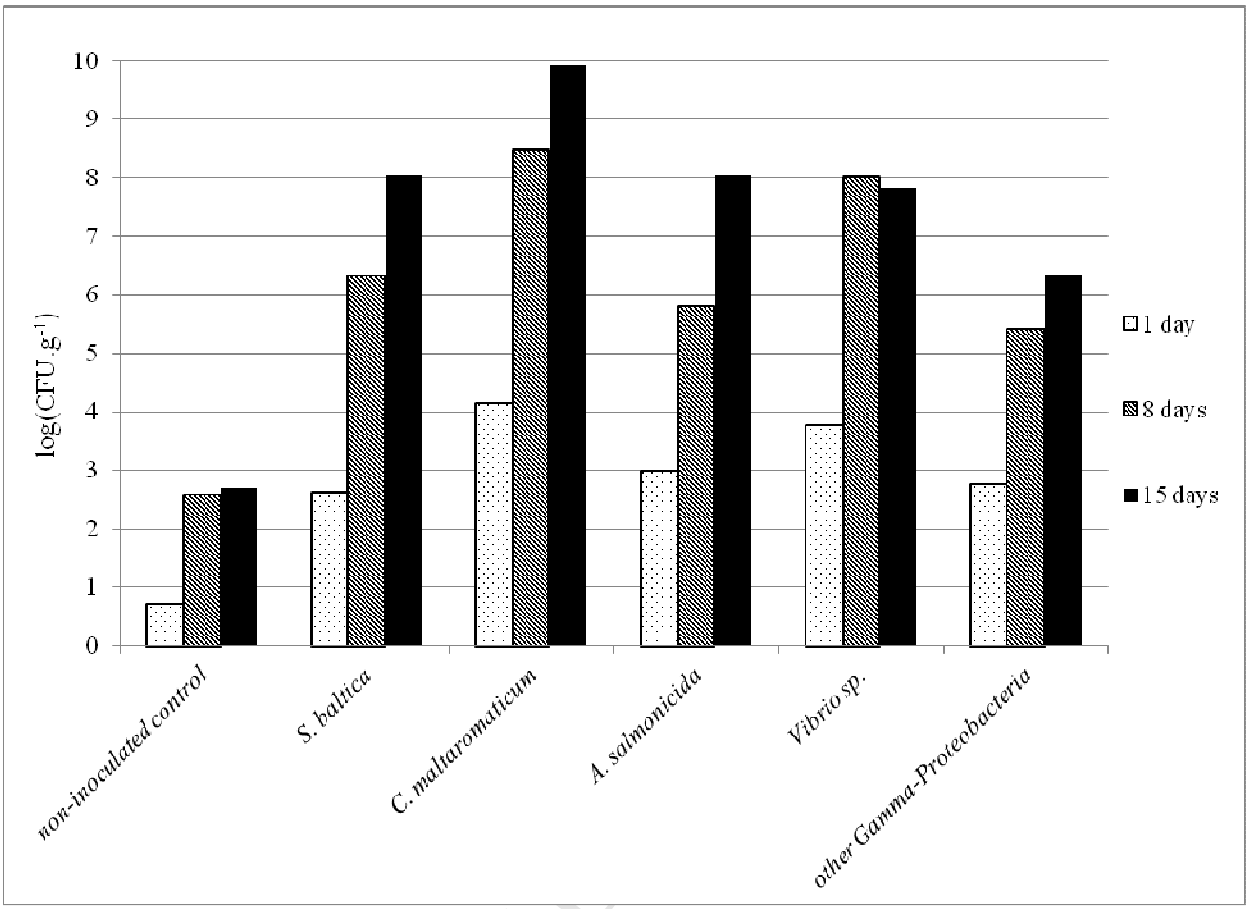
Table 2: Main odours in cooked tropical shrimp inoculated with 5 bacterial groups followed by storage under MAP at 8°C for 32 days. Percentage of panellists (n=9) noting specific odour characteristics is indicated in brackets. Main odours presented were noted by at least 3 panellists.

	Non-inoculated control	*Shewanella baltica*	*Carnobacterium maltaromaticum*	*Aeromonas salmonicida*	*Vibrio* sp.	"Other Gamma-Proteobacteria"
Day 8	Cabbage/sulphur (44.4) Crustacean (33.3)	Sour (44.4) Cabbage/sulphur (33.3) Pyrrolidine (33.3)	Sour (66.7) Butter (33.3) Milky (33.3)	Butter (77.8) Pyrrolidine (44.4)	Crustacean (55.6) Sour (44.4) Cabbage/sulphur (33.3)	Crustacean (33.3) Sour (33.3) Feet/cheese (33.3)
Day 15	Crustacean (55.6) Rice (33.3)	Cabbage/sulphur (66.7) Sour (44.4) Amine (33.3)	Sour (66.7) Feet/Cheese (33.3) Butter (33.3)	Pyrrolidine (44.4) Amine (33.3)	Crustacean (44.4) Floorcloth (33.3)	Feet/cheese (55.6) Butter (33.3)
Day 26	Crustacean (33.3) Rice (33.3) Sour/fermented (33.3) Acid (33.3)	Cabbage/sulphur (66.7) Sour (44.4)	Sour (55.6) Feet/cheese (44.4) Acid (33.3)	Sour (44.4) Cabbage/sulphur (33.3) Butter (33.3) Feet/Cheese (33.3)	Sour (33.3) Acid (33.3)	Sour (66.7) Feet/cheese (55.6)
Day 32	Nothing (33.3) Feet/cheese (33.3) Acid (33.3)	Sour (66.7) Cabbage/sulphur (55.5)	Sour (77.8) Feet/Cheese (44.4) Acid (44.4)	Sour (44.4) Cabbage/sulphur (44.4) Amine (33.3)	Sour (44.4) Cabbage/sulphur(33.3) Crustacean (33.3)	Sour (44.4) Nothing (33.3)

Table 3: Specific volatile compounds identified in spoiled *S. baltica*-inoculated cooked tropical shrimp after 18 days of storage at 8°C

Volatile compound	Non-inoculated (control) Day 1	Non-inoculated (control) Day 18	*S. baltica* Day 1	*S. baltica* Day 18
Alcohols				
Butanol	ND	ND	ND	4.99 ±0.09
1-pentanol (amyl alcohol)	5.48 ±0.16	5.73± 0.01	5.77 ±0.17	6.37 ±0.04
Isoamyl alcohol	ND	ND	ND	5.54 ±0.11
Ethanol	7.38 ±0.01	7.44 ± 0.01	7.28 ±0.03	6.86 ±0.23
1-penten-3-ol	5.39 ±0.02	6.00 ±0.02	5.36 ±0.03	6.14 ±0.14
1-hexanol	ND	ND	ND	6.14 ±0.15
Aldehydes				
2-hexenal	ND	ND	ND	5.52 ±0.03
Acid				
Acetic acid	ND	ND	ND	6.27 ±0.15
Isovaleric acid	ND	ND	ND	4.53 ±1.41
Isobutyric acid	ND	ND	ND	4.39*
Ketones				
2-propanone (acetone)	ND	ND	ND	6.81 ±0.15
2-heptanone	ND	ND	ND	5.34 ±0.13
3-hydroxybutanone (acetoin)	ND	ND	ND	5.28*
2-nonanone	ND	ND	ND	5.52 ±0.24
2-decanone	ND	ND	ND	4.72*
Other				
Methyl mercaptan	5.24 ±0.37	ND	5.04 ±0.18	6.34 ±0.10
Methyl-P-tert-butyl phenyl acetate	ND	ND	ND	4.57 ±0.10
p-Menthane	ND	ND	ND	5.32 ±0.19

ND: Not detected
Values are averages ± standard deviation of log (peak area/g). n=3
* n=1


log(CFU.g-1)
10
9
8
7
6
5
4
3
2
1
0
1 day
8 days
15 days
non-inoculated control
S. baltica
C. maltaromaticum
A. salmonicida
Vibrio sp.
other Gamma-Proteobacteria