International Journal of Food Microbiology
January 2017, Volume 241, Pages 69-77
http://dx.doi.org/10.1016/j.ijfoodmicro.2016.09.024
http://archimer.ifremer.fr/doc/00353/46412/

Improving simultaneously the quality and safety of cooked and peeled shrimp using a cocktail of bioprotective lactic acid bacteria

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Abstract :

Tropical shrimp is of considerable economic importance in the world but is highly perishable due to microbial and chemical degradation. Biopreservation is a food preservation technology based on the addition of “positive” bacteria able to kill or prevent the growth of undesirable microorganisms. Two strains of lactic acid bacteria (LAB) have previously been selected for a biopreservation strategy: *Lactococcus piscium* CNCM I-4031, for its ability to prevent the sensory deterioration of seafood and *Carnobacterium divergens* V41, which inhibits growth of *Listeria monocytogenes*. The objective was to test the association of the two strains to improve both the quality and safety of shrimp. In a first trial, the two LAB were inoculated alone or in a cocktail in cooked and peeled shrimp (CPS) *Penaeus vannamei* at 5×10^5 CFU/g. Chemical, sensory and microbiological analyses by culture-dependent and -independent methods were performed during storage under modified atmosphere packaging (MAP) at 8 °C. The results were compared to a non-inoculated batch. In a second trial, the same experiments were repeated in the presence of 10^2 CFU/g of *L. monocytogenes* RF191. The microbiota of CPS was composed of LAB, *Shewanella* spp. and *Enterobacteriaceae*. *Brochothrix thermosphacta* was not detected. *L. piscium* and *C. divergens* reached 10^8 and 10^9 CFU/g, respectively, in 7 days and did not inhibit each other when co-inoculated. *L. piscium* reduced *L. monocytogenes* by 1 Log (CFU/g) for 28 days. *C. divergens* had an immediate listericidal effect lasting 7 days. A regrowth of *L. monocytogenes* was then observed but the count was always 2 to 5 Log (CFU/g) lower than in the control. No additional or synergic effect between protective strains was observed and the cocktail had the same inhibitory effect as *C. divergens* alone. *C. divergens* was very effective at preventing the sensory deterioration of CPS. This may be related to the inhibition of *Shewanella* and *Enterobacteriaceae*. However, the panelists could detect the presence of *C.divergens* during the first 10 days of storage, with slight unpleasant odors and flavors. *L. piscium* improved the sensory quality of CPS for 14 days only. In co-culture, *L. piscium* eliminated the off-odors and flavors released by *C.*

divergens in the early stage of storage and the co-culture allowed maintaining a good quality of CPS throughout the storage. Therefore, the use of a cocktail of *L. piscium* CNCM I-4031 and *C. divergens* V41 is recommended in a strategy of biopreservation of shrimp.

Highlights

► A cocktail of *L. piscium* and *C. divergens* was tested to biopreserve cooked shrimp. ► *L. piscium* CNCM-I4031 and *C. divergens* V41 have no antagonist effect on each other. ► *L. piscium* inhibited *L. monocytogenes* by 1 Log and *C. divergens* by 2 to 5 Log CFU/g. ► The cocktail showed no additional or synergetic effect on inhibition of *Listeria*. ► The cocktail was more efficient to improve sensory quality than the strains alone.

Keywords : *Carnobacterium divergens* V41, *Lactococcus piscium* CNCM I-4031, *Penaeus vannamei*, *Listeria monocytogenes*, Biopreservation

1. Introduction

Shrimp has long been considered a rare, refined and expensive food. With the development of aquaculture, the shrimp market has increased rapidly (FAO, 2012). In 2014, the EU imported 557,700 t of shrimp between January and September (FAO-Globefish, 2015). The main suppliers of raw shrimp to Europe are Ecuador, Thailand and Greenland. Most of the imported tropical raw shrimp is processed in Europe to be sold as ready-to-eat (RTE) products, such as cooked or peeled/cooked shrimp with or without spices, and is packed under vacuum or modified atmosphere. These lightly-preserved shrimp are highly perishable due to microbiological and biochemical degradation (Dalgaard and Jørgensen, 2000). The microbial ecosystem of tropical shrimp is composed of Gram-positive bacteria (*Carnobacterium* sp., *Enterococcus* sp., *Vagococcus* sp., *B. thermosphacta*) and Gram-negative bacteria such as Enterobacteriaceae (*Serratia liquefaciens*) (Jaffrès et al., 2011, 2009; Laursen et al., 2006; Macé et al., 2014; Mejlholm et al., 2005). Some of them, called specific spoilage organisms, are responsible for unpleasant odors and flavors making the product inconsumable. Besides the associated economic losses, the presence of human pathogenic bacteria, such as *L. monocytogenes*, is a public health concern. *L. monocytogenes* is responsible for foodborne listeriosis, a rare but severe disease generally associated with a high mortality rate (20 – 30%). It infects all human population groups but those at greatest risk are pregnant women, newborn infants, elderly people and immune-compromised patients (diabetics, cancer sufferers, etc.) (Lecuit et al., 2015). Despite the development of the HACCP system, the number of cases has increased in the last decade from 3.1 cases/million people in 2001 to 5.3 in 2014 (Lecuit et al., 2015). This bacterium constitutes a major problem in refrigerated RTE marine food (Jami et al., 2014) due to its ability to grow in a wide range of temperatures (-1.5 to + 45°C), salt concentrations (up to 12%), water activity (> 0.92) and pH values (4.5 – 9.0) (Gandhi and Chikindas, 2007). The prevalence and growth *of L. monocytogenes* in shrimp have been documented (Matamoros et al., 2009a; Mejlholm et al., 2005;

Rutherford et al., 2007; Zarei et al., 2011) leading to the conclusion that, if present, *L. monocytogenes* can exceed the legal safety limit in EU Member States (100 CFU/g until the end of shelf-life).

Additives such as benzoic acid (E210) and sorbic acid (E200) or their derived salts, sodium benzoate (E211) and sodium sorbate (E201), are mostly used to prevent bacterial growth on shrimp. However, these products have an influence on taste and might also have negative consequences for human health due to *e.g.* possible pseudo-allergic reactions (Ortolani et al., 1999). Biopreservation is an alternative technology that involves natural and selected protective microorganisms to eliminate or reduce the growth of undesirable microorganisms. Many studies have reported promising results for seafood products (for reviews, see Calo-Mata et al., 2007 ; Ghanbari et al., 2013 ; Pilet and Leroi, 2011). Some protective cultures with a specific action on spoilage bacteria or pathogens have been commercialized (LLO by Biocéane and Lyoflora FP18 by Sacco S.r.l) and are currently used in cold-smoked salmon (CSS) and MAP shrimp. Currently, there is no European regulation on protective cultures in unfermented ready-to-eat food. In 2007, the European Food Safety Authority (EFSA) adopted guidelines for Qualified Presumption of Safety (QPS) that can be referred to as the European equivalent of the American GRAS status in terms of risk assessment (EFSA, 2007). In our laboratory (EM3B/Ifremer and Secalim/Oniris), one strain of *C. divergens* V41 isolated from trout viscera by Pilet et al. (1995) has been extensively studied for its anti-listeria activity. In sterile CSS, *C. divergens* V41, inoculated at 10^5 CFU/g, maintained *L. monocytogenes* at less than 50 (CFU/g) for 4 weeks of vacuum-storage at 4-8°C (Brillet et al., 2005). This inhibitory effect was due to the production of a class IIa bacteriocin, divercine V41 (Métivier et al., 1998). A second LAB, *L. piscium* CNCM I-4031 also referenced as EU2241, has been selected for its capacity to improve the sensory quality of CSS under VP and CPS under MAP (Fall et al., 2012; Matamoros et al., 2009a). This may be due to the inhibition of *B. thermosphacta*, a spoilage bacteria of refrigerated meat and seafood, which has been detected in artificially contaminated CPS and CSS (Fall et al., 2010a; Leroi et al., 2015). Therefore, a cocktail of *C. divergens* V41 and *L. piscium* CNCM I-4031 seems a promising

solution to prevent simultaneously the sensory deterioration and growth of *L. monocytogenes*. However, the two strains must not inhibit each other and not develop off-odors when co-cultured. In fact, two bacteria that do not spoil CSS when cultivated alone were shown to release strong unpleasant odors when tested in combination, probably due to metabiosis phenomena (Joffraud et al., 2006).

In this study, challenge tests were performed to evaluate the effect of a cocktail of *C. divergens* V41 and *L. piscium* CNCM I-4031 on the quality and safety of CPS stored under MAP at 8°C for 28 days. The results were compared to a control (not inoculated with protective LAB) and to CPS inoculated with *C. divergens* and *L. piscium* alone. The effect of this cocktail on the quality of shrimp and on the endogenous microorganisms was evaluated by sensory evaluation of off-odors, flavors and texture and by cultural and non-cultural microbial analysis. The role of the protective LAB in *L. monocytogenes* was studied on CPS (with its natural endogenous ecosystem) artificially inoculated with the pathogen.

2. Materials and methods

2.1. Bacterial strains

The bioprotective strains *C. divergens* V41 and *L. piscium* CNCM I-4031 were isolated from trout and from fresh MAP salmon steak, respectively (Pilet et al., 1995; Matamoros et al., 2009a). The target strain *L. monocytogenes* RF191 was isolated from tropical CPS (PFI Nouvelles Vagues, Boulogne-sur-Mer, France). All the strains were stored at -80°C in their culture media with 20% glycerol (Panreac, Barcelona, Spain). For all experiments, *C. divergens* and *L. piscium* were sub-cultured individually in Elliker broth (Biokar Diagnostic, Beauvais, France) and *L. monocytogenes* in Brain Heart Infusion (BHI) (Biokar Diagnostic) for 24 h at 26°C. After 24 h of culture, the strains reached their maximal concentrations, 10^9 CFU ml^{-1} for *C. divergens* V41 and *L. monocytogenes* and 10^8 CFU ml^{-1} for *L. piscium* CNCM I-4031. The cultures were diluted in physiological saline solution (0.85% NaCl, 0.1% tryptone) (Biokar Diagnostic) to achieve an appropriate inoculation solution.

2.2. Shrimp and challenge tests

About 12 kg of tropical peeled shrimp *Penaeus vannamei* from Thailand (size 71/90) was cooked by the manufacturer at 71°C for 2 min (Miti, Nantes, France) and directly transported to the laboratory under refrigerated conditions. In the first trial, the bioprotective effect of the strains on the quality of tropical CPS was evaluated. Four batches were prepared: (1) untreated shrimp (control); (2) inoculation with *L. piscium* CNCM I-4031 alone; (3) inoculation with *C. divergens* V41 alone and (4) inoculation with a cocktail of *C. divergens* V41 and *L. piscium* CNCM I-4031. Shrimp were inoculated at 2% (v/w) by spraying diluted cultures to obtain an estimated final level of 5×10^5 CFU/g for *C. divergens* V41 and *L. piscium* CNCM I-4031. No physiological saline solution was added to the control to achieve the same condition as in industry. The spray method was chosen because this method is already used by a shrimp industry based in Nantes (France).

After inoculation, each batch was divided into 18 punnets, each containing a 120-g portion of shrimp, and packed under MAP (50% CO_2 and 50% N_2) as in the French industry (Multivac T250, Wolfertschwenden, Germany). All punnets were stored at 8°C for 28 days. Sensory, chemical and microbial analyses were carried out after 0, 7, 10, 14, 21 and 28 days in triplicate (3 punnets).

In a second trial one month later, with a new batch of 12 kg of shrimp, the bioprotective effect of the strains on *L. monocytogenes* was evaluated. Four batches were prepared: (5) CPS inoculated with *L. monocytogenes* RF191 at a final concentration of 10^2 CFU/g; (6) inoculation with *L. monocytogenes* RF191 and *L. piscium* CNCM I-4031 (10^2 and 5×10^5 CFU/g, respectively); (7) inoculation with *L. monocytogenes* RF191 and *C. divergens* V41 (10^2 and 5×10^5 CFU/g, respectively) and (8) inoculation with *L. monocytogenes* RF191, *C. divergens* V41 and *L. piscium* CNCM I-4031 (10^2, 5×10^5 and 5×10^5 CFU/g, respectively). After inoculation, each batch was divided into 21 punnets of 120-g portions and packed under MAP (50% CO_2 and 50% N_2). All punnets were stored at 8°C for 28 days. Chemical (pH) and

microbial analyses were carried out after 0, 3, 7, 10, 15, 21 and 28 days. All the analyses were done in triplicate and the results presented as the mean value ± a confidence interval of 95%.

2.3. Microbial enumeration

For each day of analysis, 3 punnets per batch were analyzed. 20-g portions (first trial) or 30-g portions (second trial) of shrimp were stomached (Stomacher 400 circulator, Seward Medical, London, UK) for 2 min with 80 and 120 ml, respectively, of chilled sterile physiological saline solution with 1% Tween 80 (Grosseron, Saint-Herblain, France). 0.1 ml of appropriate 10-fold dilutions was spread on different selective agar plates. For Enterobacteriaceae, 1 ml was pour plated. The microorganisms, culture medium and incubation conditions are listed in Table 1. Anaerobic conditions were performed in anaerobic jars with Anaerocult A and Microbiology Anaerotest (Merck, Darmstadt, Germany). In the cocktail of protective bacteria, *C. divergens* was enumerated on Elliker agar at 30°C as *L. piscium* cannot grow at this temperature (Matamoros et al., 2009a). *L. piscium* was enumerated on Elliker agar at 8°C where *C. divergens* developed less rapidly than *L. piscium* and the morphology of the colonies was different. However, the endogenous LAB could not be distinguished from the inoculated bioprotective strains, except that during the first days of the experiment, their concentration was less than inoculated LAB. The detection threshold was 0.7 and 1.7 Log (CFU/g) for Enterobacteriaceae and the other counts, respectively.

2.4. Physicochemical parameters

The pH was measured in the stomached flesh immediately after microbiological analysis by immersing the electrode of the pH-meter (Mettler Toledo AG, Schwerzenbach, Switzerland). Total Volatile Basic Nitrogen (TVBN) was quantified by the Conway micro-diffusion method (Conway and Byrne, 1933)

2.5. Sensory analysis

A quantitative descriptive analytical method was used, with a conventional profiling performed by an internal, regularly trained panel of Ifremer, as described by Macé et al. (2014). Panelists (12 people)

experienced in seafood evaluation were specifically trained on tropical CPS (Fall et al., 2012; Jaffrès et al., 2009; Macé et al., 2014). This analysis was carried out on batches 1, 2, 3, and 4, without *L. monocytogenes*. The panelists first evaluated the overall spoilage level on a continuous scale from 0 to 10, just after opening the punnet and then 30 s later (0 corresponded to not spoiled and 10 to strongly spoiled products). They then scored the following odor descriptors: rice/crustacean, diacetyl/butter, pyrrolidine, floor cloth, sour, acid, fermented, and sulfur. For acceptable samples (global odor score < 5-6), the panelists also scored the texture (crunchy, pasty, humidity) and flavor (rice/crustacean, floor cloth, sour, acid, fermented and bitter).

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

2.6. Temporal Temperature Gradient Gel Electrophoresis (TTGE) analysis

The suspension prepared for bacteriological analysis was used to obtain molecular fingerprints from the control and shrimp matrix inoculated with protective cultures. The DNA was then extracted using the method described previously by Macé et al. (2012).

Bacterial DNA from the inoculated shrimp matrix was analyzed by PCR-TTGE. The V3 region of the 16S rRNA gene (194 bp) was PCR-amplified as described by Jaffrès et al. (2009).

The PCR products obtained were subjected to TTGE gel analysis as described previously by Macé et al. (2012). Standardization, analysis and comparison of TTGE fingerprints were monitored using BioNumerics Software, version 7.1 (Applied Maths NV, Sint-Martens-Latem, Belgium) as described by Macé et al. (2012). Experimental fingerprint data were compared to an internal fingerprint database established from bacterial species isolated from marine products.

3. Results

3.1. Microbiota of naturally contaminated shrimp

Fig. 1 represents the kinetics of total mesophilic microflora, mesophilic and psychrotrophic LAB, Enterobacteriaceae and *B. thermosphacta* of naturally contaminated PCS (control) during MAP storage at 8°C. The initial contamination level was low (1.8 ± 0.1 Log (CFU/g) for the total mesophilic count and less than the detection level for the other counts. All the microbial groups reached their maximum count in 14 days, except *B. thermosphacta* which was never detected. The highest growth rates and maximum counts were observed for psychrotrophic LAB, whereas mesophilic LAB grew more slowly but reached the same final count after 14 days (8-9 Log (CFU/g)). The colonies in ELK at 8°C were small mucoid and opaque and composed of Gram-positive, catalase- and oxidase-negative bacteria, probably LAB producing a polysaccharide. The Enterobacteriaceae count was always 1-2 Log (CFU/g) lower (statistical difference, $P < 0.05$). TTGE fingerprints of the V3 region of 16S rRNA genes were performed to evaluate the evolution of the dominant bacterial groups. The results are shown in Fig. 2. At day 0, 5 bands with a very weak intensity were observed. One band (c) was assigned to *Carnobacterium*/*Psychrobacter* and 4 bands (n1 to n4) were not assigned. After 7 days, the fingerprints of n1, n2, n3 and n4 disappeared whereas fingerprints of *Carnobacterium*/*Psychrobacter* intensified until the end of storage. Three fingerprints (sb and sp assigned to *Shewanella* sp., respectively, and unassigned band n5) appeared at day 7 and persisted until the end of storage. Additionally an unassigned band (n6) appeared at days 21 and 28.

3.2. Implantation of the bioprotective LAB strains in shrimp

In batch 2, *L. piscium* was inoculated at 5.9 ± 0.2 Log (CFU/g), very close to the desired level (data not shown). The concentration on ELK at 8°C reached 8.7 ± 0.0 Log (CFU/g) in 14 days and then remained constant. The colony had the same morphology as *L. piscium* in pure culture and no mucoid colony was present. Moreover, the TTGE results showed intense bands assigned to *L. piscium*. This result confirmed

that *L. piscium* was the dominating psychrotrophic LAB throughout storage (band lp, Fig. 2). In batch 3, the inoculation level of *C. divergens* was 5.4 ± 0.1 Log (CFU/g) and the count on ELK at 30°C reached 9.5 ± 0.0 Log (CFU/g) in 7 days and until the end of storage (data not shown). This count was always 1 Log (CFU/g) higher than the count on ELK at 30°C in batch 1. The morphology of the colonies looked like *C. divergens* in pure culture. These results indicated that the bioprotective strain dominated the total LAB count in batch 3. This was confirmed by the TTGE profiles (band cd, assigned to *C. divergens* bands, Fig. 2). In batch 4, the initial *L. piscium* and *C. divergens* counts were 5.8 ± 0.2 Log (CFU/g) and 5.3 ± 0.1 Log (CFU/g), respectively. *C. divergens* was able to grow on ELK at 8°C but the colonies could easily be distinguished (white colonies of 1-2 mm and 0.5 mm in diameter for *L. piscium* and *C. divergens*, respectively). Throughout storage, the counts of presumptive *L. piscium* and *C. divergens* were exactly similar to the counts of pure culture in batches 2 and 3 (data not shown). Therefore, we could conclude that there was no competition between the strains. *C. divergens* was 1 Log (CFU/g) higher than *L. piscium* after 7 days and until 28 days. This was confirmed by the TTGE fingerprints (Fig. 2) in which the bands assigned to both protective cultures were present.

3.3. Effect of the protective cultures on the microbial ecosystem of shrimp

B. thermosphacta was never detected in the control and biopreserved CPS. Fig. 3 represents the growth kinetics of the Enterobacteriaceae in the different batches of CPS. Enterobacteriaceae grew from 0.7 ± 0.2 to 6.0 ± 0.3 at day 7 and 7.1 ± 0.8 Log (CFU/g) at day 14 and then remained constant. In the presence of *L. piscium* (batch 2), no inhibition of the Enterobacteriaceae count was noticed, except at day 28 when the count was 1.0 Log (CFU/g) lower (statistical difference $P< 0.05$). With *C. divergens* (batch 3), a significant inhibition of Enterobacteriaceae of 2-3 Log (CFU/g) was observed, which began at the start of storage and persisted until day 28. The results were similar in batch 4 so it was concluded that the presence of *L. piscium* did not modify the inhibitory effect of *C. divergens*.

The effect of the protective strains was also determined with the TTGE fingerprints. No strong differences were observed between the fingerprints of the control and CPS inoculated with *L. piscium* (batch 2), except at day 7 when most of the bands observed in batch 2 were not detected. At day 0, 3 bands were observed (lp assigned to *L. piscium* as well as the unassigned bands n7 and n8). The increase in intensity of the band assigned to *L. piscium* showed the good implantation of this strain in the shrimp matrix. The unassigned bands n7 and n8 totally disappeared after day 7. After 10 days, the same group of bacteria was found in batches 1 and 2 (sp, sb, c, n5 and n6) throughout storage but with a slightly weaker intensity in batch 2. It is difficult to know if this was due to a weaker concentration of these species in batch 2 compared to the control. In fact, in batch 2, *L. piscium* was present in high number and species representing less than 1% of the total community are not visible in TTGE profiles. In batch 3, at day 0, the cd band assigned to *C. divergens* was present and increased immediately after 7 days. Except for the band sb corresponding to *Shewanella* sp. visible at day 28 but with a very weak intensity, no other band could be detected, proving that *C. divergens* inhibited many of the endogenous bacteria naturally present in CPS. The fingerprints of batches 3 and 4 were similar, except that the band corresponding to the inoculated *L. piscium* was present. The intensity of the band assigned to *C. divergens* fingerprint was higher than that of *L. piscium* confirming the results obtained by cultural analysis. To conclude, *L. piscium* had a small effect on the microbial diversity of CPS except at day 7 while *C. divergens* reduced this biodiversity. The co-culture of *C. divergens/L. piscium* had the same inhibitory capacity as *C. divergens* alone.

In the second trial, in which samples were inoculated with *L. monocytogenes*, the total ecosystem was once again analyzed and all the results obtained in the first trial were confirmed. Inhibition of Enterobacteriaceae was observed in batches 7 and 8, although it was a little weaker (1 to 2 Log (CFU/g), data not shown). The effect on the microbial diversity of CPS studied by TTGE fingerprints was very weak

with *L. piscium* whereas *C. divergens* alone or in co-culture inhibited most of the dominating bacteria naturally present in CPS (data not shown).

3.4. Chemical analysis

The initial pH value of CPS was 6.65 ± 0.05. In **batches** 1, 3 and 4, the pH remained stable throughout storage (data not shown) and no significant difference was observed. In batch 2 with *L. piscium* alone, acidification to 6.22 ± 0.05 was observed in the first 7 days, but after 14 days the pH increased and reached the same level as in the other batches (6.72 ± 0.01 at day 28) (data not shown). TVBN in CPS (batch 1) was very low until day 7 (<10 mg-N/100 g) but reached high concentrations at days 10 and 14 (40 and 60 mg-N/100 g, respectively, data not shown). *L. piscium* totally prevented this formation for 10 days. In the presence of *C. divergens*, alone or in co-culture, TVBN production (50 mg-N/100 g) was observed from 7 days, leading to a much higher concentration than in batches 1 and 2. At day 14 and until the end of storage, the concentrations were similar in all batches.

3.5. Effect of the protective cultures on the sensory characteristics of shrimp

At day 0, all the samples were considered of very high quality by the panelists, based on the odor, flavor, texture and color evaluation. The presence of the inoculated protective bacteria was not detected.

3.5.1. Odor characteristics

Fig. 4A represents the global spoilage scores of CPS based on odors during MAP storage at 8°C. After 7 days, the control was considered not-spoiled with a score of 1.2 on a 10-point scale. However, deterioration occurred rapidly. After 10 and 14 days, the score ranged between 4 and 5 (still acceptable) but after 21 days samples were rejected by the panelists with a score higher than 7. In the presence of *L. piscium* (batch 2), a significant improvement in quality was noticed until day 10 with a score of 2.7, and samples were also acceptable until fourteen days. Then, the global spoilage score was similar to the control and samples were considered unacceptable for consumption. The samples inoculated with *C. divergens* alone (batch 3) or in co-culture with *L. piscium* (batch 4) had a score similar to the control until

day 14. Then, *C. divergens* alone or in combination with *L. piscium* prevented off-odor formation until day 28 and the final scores were 4.8 and 6.0 in batches 3 and 4, respectively (no statistical difference between batches 3 and 4).

The principal component analysis (PCA) represented in Fig. 5A provides more information on the odor characteristics. Samples and their odor descriptors were projected on the first plane of the PCA, which restored 62.4% of the total information. The first axis (42.7% of information) enabled the separation of the non-spoiled (right part of Fig. 5A) and lightly/strongly spoiled (left part) samples. Lightly spoiled (upper part) and strongly spoiled (lower part) samples were discriminated on the second axis (19.7% of information). The control sample at 7 days was considered non-spoiled with a rice odor, typical of freshly cooked tropical shrimp. At 10 and 14 days, the control was lightly spoiled with pyrrolidine, acid, floor cloth and sour odors but still with a rice odor, with a score of around 2 for each descriptor. After 21 days, the control was highly spoiled with strong sulfur, sour and fermented odors (scores from 3 to 5 for these attributes, data not shown). In the presence of *L. piscium*, the samples were considered non-spoiled at 7 and 10 days with a characteristic rice odor (intensity of 3.7 and 3.3, respectively), showing a prevention of the sensory deterioration of CPS during 10 days. After 14 days, the samples were considered lightly spoiled like the control but the odors were different and less unpleasant (rice and diacetyl/butter odor with an intensity of 2.1 and 1.4, respectively). At days 20 and 28, the off-odor characteristics were similar in batches 1 and 2. The results in Fig. 5A clearly show that batches 3 and 4 inoculated with *C. divergens* alone or in co-culture remained in the upper-left part of the figure and never became highly spoiled. The descriptors were pyrrolidine, floor cloth, sour and fermented for batch 3 and diacetyl/butter for batch 4. It is important to note that until 7 days, the presence of *C. divergens* was responsible for slight off-odor release whereas in association with *L. piscium* (batch 4) these odors were not detected and the sample was very similar to the control (i.e. good quality).

3.5.2. Flavor characteristics

Fig. 4B represents the global spoilage scores based on flavor and texture evaluation of CPS until 14 days of storage. After 21 days, the panelists did not consume the products because of strong off-odors in the control. After 7 days, the control was of very high quality with a score of 0.6. Then it increased to reach 3.1 after 14 days. *L. piscium* significantly prevented the spoilage of CPS. The global flavor/texture scores were 1.2 and 1.6 points lower than the control after 10 and 14 days, respectively, and never exceeded the score of 1.3. After 7 days, and until 10 days to a lesser extent, the presence of *C. divergens* was detected by the panelists and the spoilage score was higher than in the control (3.3 vs. 0.6 at day 7). After 14 days (and probably more if the products had been eaten), the beneficial effect of *C. divergens* against the spoilage of CPS (batch 1) was evidenced. Interestingly, the presence of *L. piscium* significantly prevented the off-flavors released by *C. divergens*. In fact, in co-culture, the spoilage score was 1.7 and 1.5 after 7 and 10 days, respectively, and 3.3 and 2.4 with *C. divergens* alone. At day 14, the effect of *C. divergens* alone or in co-culture was similar, as for the odors. The PCA summarizing the main flavor and texture characteristics is represented in Fig. 5B. The first axis restored 58.8% of the total information and enabled the separation of the non-spoiled (left part of Fig. 5B) and lightly spoiled (right part) samples. The second axis (18.6% of information) discriminated samples with sour and fermented flavors (upper-right part of the plan) from those with a pasty texture and floor cloth and bitter taste (lower-right part). The non-spoiled samples were characterized by a rice/crustacean flavor and crispy texture as were the control at day 7, all samples of batch 2 (in the presence of *L. piscium*) and samples inoculated with *C. divergens* and *L. piscium* at day 7. All the other samples were lightly spoiled, mainly with a pasty texture and floor cloth and bitter tastes. Samples of batch 3 inoculated with *C. divergens* had a specific signature of a sour and fermented taste at the beginning of the experiment. In conclusion, as for the odors until 14 days, *L. piscium* had the main positive effect on the quality of CPS. The unpleasant taste of *C. divergens* until 10 days of storage disappeared in the presence of *L. piscium*.

3.6. Effect of the protective cultures on *L. monocytogenes*

Fig. 6 shows the growth kinetics of *L. monocytogenes* alone in CPS (control) and in the presence of *L. piscium*, *C. divergens* and the two strains simultaneously. *L. monocytogenes* increased rapidly from an initial level of 2.3 ± 0.1 to 7.5 ± 0.2 Log (CFU/g) at day 10, then decelerated to 8.1 ± 0.1 Log (CFU/g) at 14 days and stabilized until the end of storage. In the presence of *L. piscium* (batch 6), a statistical inhibition of 1-2 Log (CFU/g) was observed throughout storage. In the presence of *C. divergens* (batch 7) and a mixture of the two protective LAB strains (batch 8), the growth of *L. monocytogenes* was totally prevented for 10 days (at day 7, the *L. monocytogenes* count was even lower than the detection threshold). Then *L. monocytogenes* grew at the same rate as when alone in batch 6 and reached 6.0 ± 0.5 and 6.6 ± 0.2 Log (CFU/g) in batches 7 and 8, respectively, after 28 days, corresponding to a 2 Log (CFU/g) diminution. No statistical difference was observed between the *L. monocytogenes* counts in batches 7 and 8. No additional or synergetic effect was noticed between *L. piscium* and *C. divergens*

4. Discussion

Our results confirmed that the microbial ecosystem of tropical CPS is complex and can reach 8-9 Log (CFU/g) in a few days at 8°C under MAP. The heat treatment of shrimp reduces the initial contamination but does not eliminate all the microorganisms, probably because shrimp are not gutted. In addition, the various processing and handling steps and environmental conditions in the industry play a role in the recontamination. The different microbial counts revealed that psychrotrophic and mesophilic LAB dominated the ecosystem, with the presence of *C. divergens* revealed by TTGE fingerprints. Many authors, using culture and culture-independent techniques, have reported the importance of LAB such as *Carnobacterium* (*divergens, maltaromaticum, jeotgali, inhibens, funditum, alterfunditum, mobile*), *Enterococcus* (*faecalis, faecium*), *Vagococcus* (*penaei, fluvialis*), *Leuconostoc* (*gasicomitatum, mesenteroides*), *Weissella viridescens* and *Streptococcus parauberis* in cooked and peeled *P. vannamei*

stored under CO_2/N_2 (50/50) (Chaillou et al., 2015; Jaffrès et al., 2009, 2010). The predominance of LAB, frequently including *Carnobacterium,* has also been reported in other species of arctic or tropical shrimp under various storage conditions (Calliauw et al., 2016; Dabade, 2015; Jaffrès et al., 2009; Mejlholm et al., 2005). The role of *Carnobacterium* in spoilage depends on the species and strains but some *C. divergens* and *C. maltaromaticum* have produced chlorine, cheese/feet, sour and butter off-odors in peeled *Pandalus borealis* and *P. vannamei* (Jaffrès et al., 2011; Laursen et al., 2006; Mejlholm et al., 2005). Although the count of Enterobacteriaceae was high (7 Log (CFU/g)), it is important to note that the most frequently found species *Serratia liquefaciens/proteamaculans* (Jaffrès et al., 2009) was not evidenced in this study by TTGE profiling. It is possible that their count was too low to be detected by TTGE. Moreover, the selectivity of VRBG is questionable. In a study performed on tuna loins, herring fillets and hot-smoked mackerel by Podeur et al. (2015), all the colonies isolated from VRBG were *Pseudomonas/Shewanella*-like. In this study, the DNA fingerprints of *Shewanella* spp. were present in the control CPS with a high intensity of the bands. These species are known as strong spoilers of some vacuum-packed fish (Gram and Huss, 1996; Vogel et al., 2005) and for their production of trimethylamine and H_2S components. *Shewanella* spp. have been isolated from CPS in our laboratory in the framework of a national project MIPROMER (unpublished data) and are part of the dominant microbiota of *Crangon crangon* (Calliauw et al., 2016). *S. baltica*, inoculated at high level in CPS, has clearly been pointed out as a major spoiler, with sour and cabbage/sulfur odors (Macé et al., 2014). The TVBN concentration in CPS was very high. Different authors have reported the production of up to 90 mg-N/100 g by *C. maltaromaticum, C. divergens* and *V. penaei* (challenge tests in artificially inoculated shrimp, 3 weeks at 8°C under MAP) as well as 60, 50 and 40 mg-N/100 g for *S. baltica, S. liquefaciens* and *B. thermosphacta*, respectively (Jaffrès et al., 2011; Macé et al., 2014).

L. piscium CNCM I-4031 inoculated in shrimp grew very well, as already shown by Fall et al. (2010a, 2010b; 2012,). Its presence prevented the sensory deterioration of CPS for 14 days compared to the

control, but no beneficial effect was observed after 21 days. These results were correlated with the TVBN content, which was lower in batch 2 for 10 days. In the study of Matamoros et al. (2009b), the bioprotective effect of *L. piscium* was more pronounced, with a shelf-life extended to 28 days. The hypothesis was that *L. piscium* had inhibited the growth of *B. thermosphacta*, which is a well-known spoilage bacteria of shrimp (Jaffrès et al., 2011; Laursen et al., 2006). In fact, *B. thermosphacta* inoculated in sterile shrimp spoiled the products after 11 days at 8°C under MAP, whereas in the presence of *L. piscium*, the samples were still acceptable after 38 days (Fall et al., 2012). In the present study, *B. thermosphacta* was absent in the control. The improvement in the sensory quality may be due to an inhibition of *Carnobacterium* or *Shewanella* spp., for which the intensity of the assigned bands was weaker in batch 2 than in the control. However, the antimicrobial effect of another protective strain of *L. piscium* (EU2229) against a mixture of *S. baltica*, *B. thermosphacta* and *C. maltaromaticum* in CPS confirmed the high sensitivity of *B. thermosphacta* whereas *C. maltaromaticum* was slightly inhibited (1 Log (CFU/g) and *S. baltica* was not (unpublished data from our laboratory).

C. divergens V41 grew rapidly in CPS and reached a final concentration 1 Log (CFU/g) higher than *L. piscium* CNCM I-4031. In contrast with *L. piscium,* this strain was detected by the panelists in the first 10 days of storage, with pyrrolidine, floor cloth, sour and fermented odors and taste, when the control (batch 1) was still of good quality. Slight off-odors or a bitter flavor have already been detected in sterile blocks of CSS inoculated with *C. divergens* V41 (Brillet et al., 2005). Very interestingly, the presence of *L. piscium* (batch 4) reduced this negative perception at the early stage of storage. However, the concentration of TVBN with the cocktail was the same as with *C. divergens* alone. This indicates that compounds other than nitrogen-containing molecules are involved in spoilage, as demonstrated in naturally contaminated cooked shrimp by Dalgaard and Jørgensen (2000) and Mejlholm et al. (2005) and for raw peeled royal shrimp *Penaeus kerathurus* (Sadok et al., 2004). From day 14 and until the end of storage, *C. divergens* prevented the off-odor formation observed in the control. The presence of *L.*

piscium did not reduce this protective effect. To our knowledge, this is the first time that an activity of *Carnobacterium* against seafood spoilage has been reported. In CSS for example, *C. divergens* V41 did not extend the sensory shelf-life (Brillet et al., 2005). The beneficial effect may be due to the inhibition of bacteria enumerated in VRBG (2 to 3 Log (CFU/g)). Furthermore, TTGE analysis clearly showed the extinction of many bands at 7 days and after.

Concerning *L. monocytogenes*, an inhibition of 1 Log (CFU/g) was observed after day 7 in the presence of *L. piscium.* This is lower than the 3-4 Log (CFU/g) observed by Fall et al. (2010b). In the latter study, the strains were inoculated in shrimp sterilized by ionization whereas in the former, CPS contained its own endogenous microbiota. As the mechanism involved in this inhibition is cell-to-cell contact-dependent (Saraoui et al., 2016), the presence of endogenous bacteria in CPS at high concentration may prevent physical contact between *L. piscium* and *L. monocytogenes.* However, Matamoros et al. (2009b) obtained a 2 Log (CFU/g) reduction by challenge tests in naturally contaminated shrimp. *C. divergens* had a listericidal effect that lasted 7-10 days. Then *L. monocytogenes* grew at approximately the same rate as in the control but the count was always 2 to 5 (CFU/g) lower. *C. divergens* V41 produces a very active class IIa bacteriocin, divercin V41 (Metivier et al., 1998). In sterile CSS blocks, *C. divergens* V41 totally prevented the growth of *L. monocytogenes* for 28 days under vacuum packaging at 4-8°C (Brillet et al., 2004), and it was proved, using a divercin- mutant, that the inhibition of *L. monocytogenes* was specifically due to this bacteriocin (Richard et al., 2003)**.** This difference may be explained by the fact that the target *L. monocytogenes* strain, the matrix and the packaging conditions were different as well as by the presence of naturally contaminating bacteria, which can also produce proteases active against bacteriocin. Some spoilage LAB may be sensitive to divercin, which may explain the improvement in the sensory quality of CPS with *C. divergens*. In the co-culture of *L. piscium* and *C. divergens*, the inhibition of *L. monocytogenes* was similar to that with *C. divergens* alone. There was no additional or synergic effect.

In conclusion, we have shown that the two protective bacteria do not inhibit each other when co-inoculated in CPS. The inhibitory effect of *C. divergens* on *L. monocytogenes* in naturally contaminated shrimp has been demonstrated for the first time. *L. piscium* also inhibits *L. monocytogenes* but there is no additional or synergic effect between the two LAB. *C. divergens* V41 is very effective at preventing the sensory deterioration of CPS stored under MAP at 8°C. However, the panelists could detect the presence of *C. divergens* during the first 10 days of storage. *L. piscium* improves the sensory quality of CPS for 14 days only. In co-culture, *L. piscium* is very effective at eliminating the off-odors and flavors released by *C. divergens* in the early stage of storage. Therefore, the use of a cocktail of *L. piscium* CNCM I-4031 and *C. divergens* V41 is recommended in a strategy of biopreservation of seafood.

Acknowledgements

Taous Saraoui was the recipient of a Ph.D. fellowship from the French Ministry of Higher Education and Research. This study was supported by a public grant from the Région Pays de la Loire (France) (project COM-BACT). The authors thank Mireille Cardinal for assistance with sensory analysis and for her advice, Justine Greyo for her help in the experimental tests and Carol Robins for the English language editing of the manuscript.

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Figure 1: Growth of the endogenous microflora of cooked and peeled shrimp packaged under modified atmosphere and stored at 8°C for 28 days. (**x**) total mesophilic bacteria (◇) mesophilic lactic acid bacteria; (○) psychrotrophic lactic acid bacteria; (Δ) *Brochothrix thermosphacta;* (□) Enterobacteriaceae

Figure 2: Digitized TTGE profiles of the 16S rRNA gene of the V3 region obtained by PCR amplification of bacterial DNA extracted from shrimp. Batch 1 (control): non-inoculated shrimp; batch 2 (Lp): inoculated with *Lactococcus piscium* CNCM I-4031; batch 3 (Cd): inoculated with *Carnobacterium divergens* V41; batch 4 (Lp+Cd): inoculated with a co-culture of *Carnobacterium divergens* V41 and *Lactococcus piscium* CNCM I-4031. Lanes D0-D28 correspond to samples at days 0, 7, 10, 14, 21, and 28. Bands: "c" represents *Carnobacterium divergens*, *jeotgali* or *Psychrobacter glacincola*, "cd" is *C. divergens*, "lp" is *L. piscium*, "sp" is *Shewanella putrefaciens*, "sb" is *Shewanella baltica* and "n1 to n8" are bands not assigned to any bacteria in our database. Encircled bands represent the inoculated lactic acid bacteria.

Figure 3: Growth of Enterobacteriaceae in cooked and peeled shrimp packed under modified atmosphere and stored at 8°C for 28 days. (♦) control; (■) batch 2 inoculated with *Lactococcus piscium* CNCM I-4031; (▲) batch 3 inoculated with *Carnobacterium divergens* V41 (●) batch 4 inoculated with *Lactococcus piscium* CNCM I-4031 and *Carnobacterium divergens* V41.

Figure 4: Sensory score of cooked and peeled shrimp packed under modified atmosphere and stored at 8°C based on (A) odors and (B) flavors and texture, determined by 12 trained panelists. Continuous scale from 0 to 10, with 0 corresponding to unspoiled and 10 to highly spoiled samples. Different letters indicate significant differences between samples. (♦) control; (■) batch 2 inoculated with *Lactococcus piscium* CNCM I-4031; (▲) batch 3 inoculated with *Carnobacterium divergens* V41 (●) batch 4 inoculated with *Lactococcus piscium* CNCM I-4031 and *Carnobacterium divergens* V41.

Figure 5: Simultaneous representation of samples of cooked and peeled shrimp packed under modified atmosphere and stored at 8°C and (A) odor descriptors and (B) flavor and texture descriptors on plane 1-2 of principal component analysis. Sample nomenclature: 1: control; 2: *Lactococcus piscium* CNCM I-

4031; 3: *Carnobacterium divergens* V41; 4: co-culture of *Carnobacterium divergens* V41 and *Lactococcus piscium* CNCM I-4031, D7-D28: days of storage (7, 10, 14, 21, and 28).

Figure 6: Growth of *Listeria monocytogenes* RF191 in cooked and peeled shrimp packed under modified atmosphere and stored at 8°C for 28 days. (♦) control; (■) batch 2 inoculated with *Lactococcus piscium* CNCM I-4031; (▲) batch 3 inoculated with *Carnobacterium divergens* V41; (●) batch 4 inoculated with *Lactococcus piscium* CNCM I-4031 and *Carnobacterium divergens* V41.

Table 1: Culture media and conditions used to enumerate the inoculated bacteria and endogenous microflora of cooked and peeled shrimp packaged under modified atmosphere and stored at 8°C for 28 days

Culture medium	Growth conditions	Target strains
Elliker	48 h at 30°C, anaerobic	*Carnobacterium divergens* and other mesophilic lactic acid bacteria
Elliker	5 days at 8°C, anaerobic	*Lactococcus piscium*, *Carnobacterium divergens* and other psychrotrophic lactic acid bacteria
VRBG	72 h at 30°C, aerobic	Enterobacteriaceae
STAA	48-72 h at 20°C, aerobic	*Brochothrix thermosphacta*
PCA	48 h at 30°C, aerobic	Total mesophilic bacteria
PALCAM	48 h at 37°C, aerobic	*Listeria monocytogenes*

Figure 1

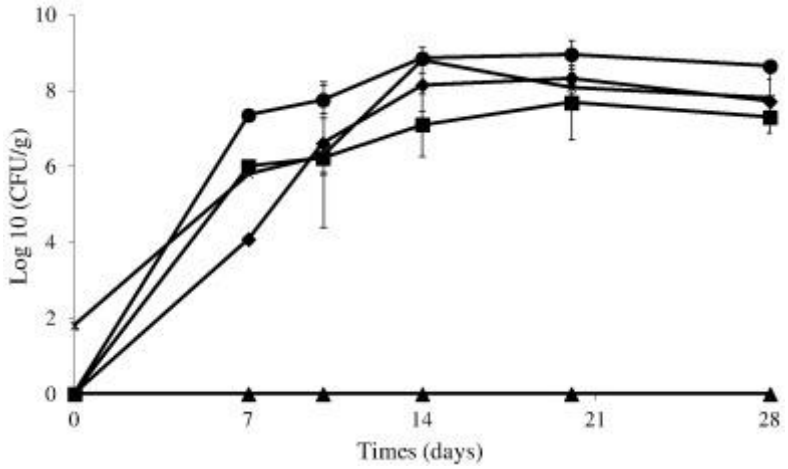

Figure 2

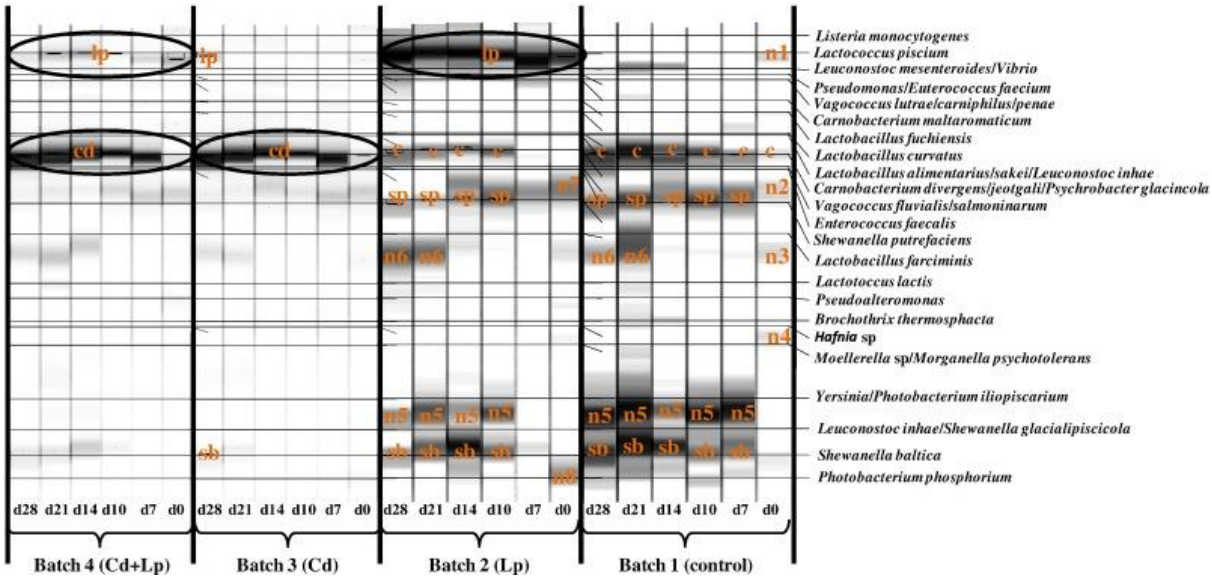

Figure 3

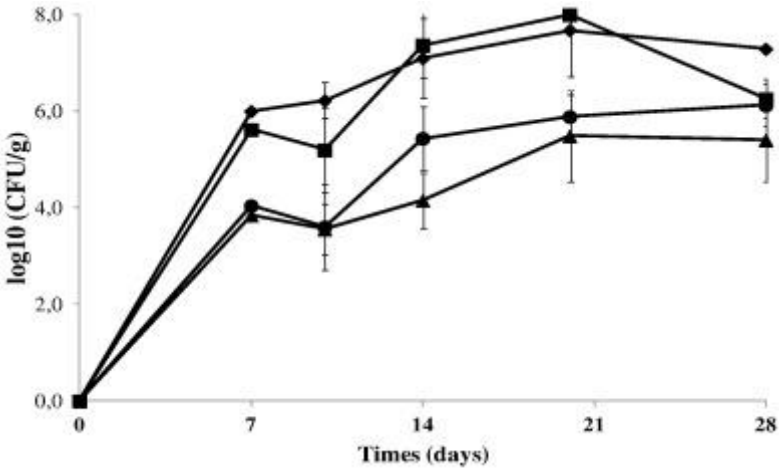

Figure 4

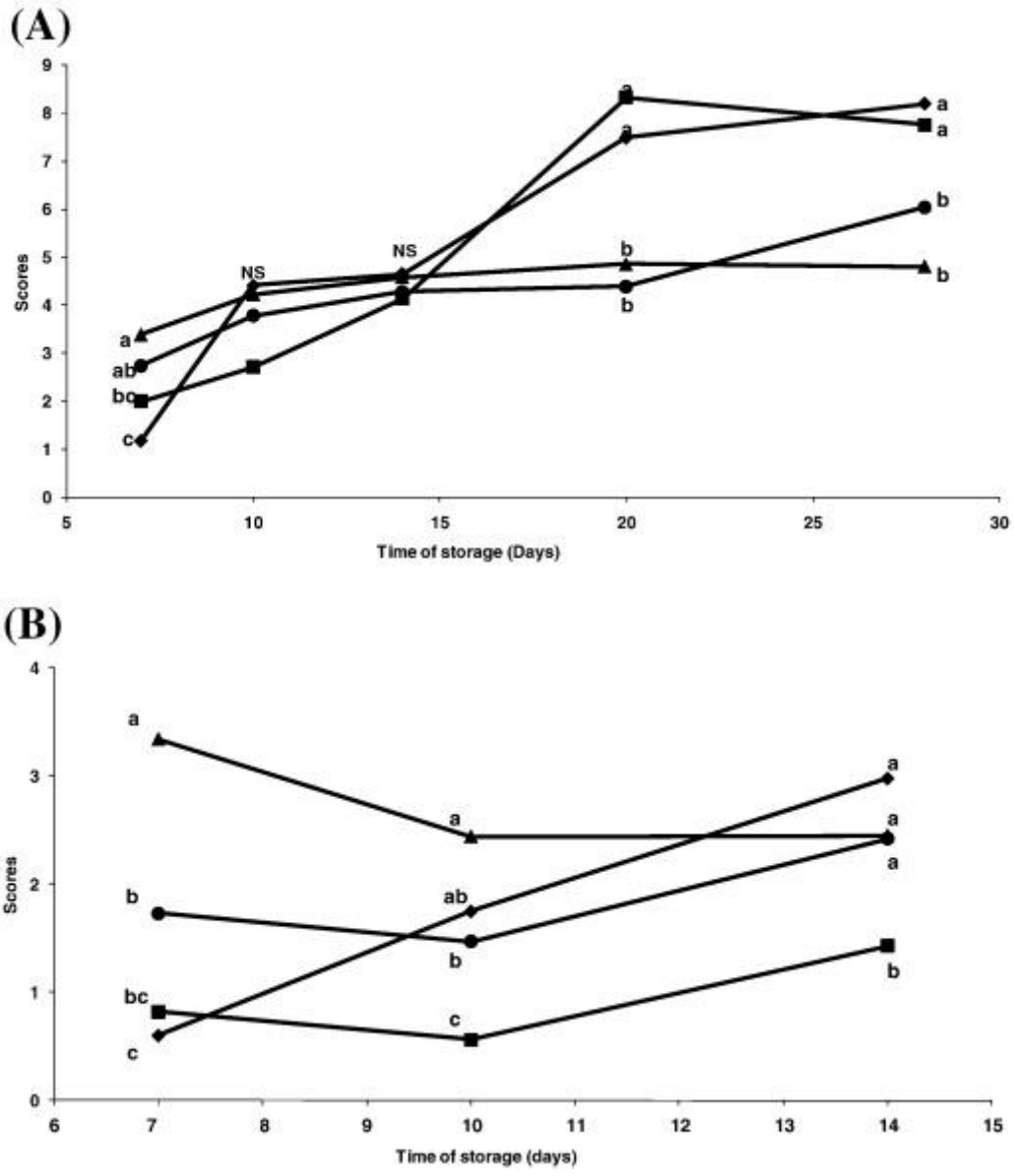

Figure 5

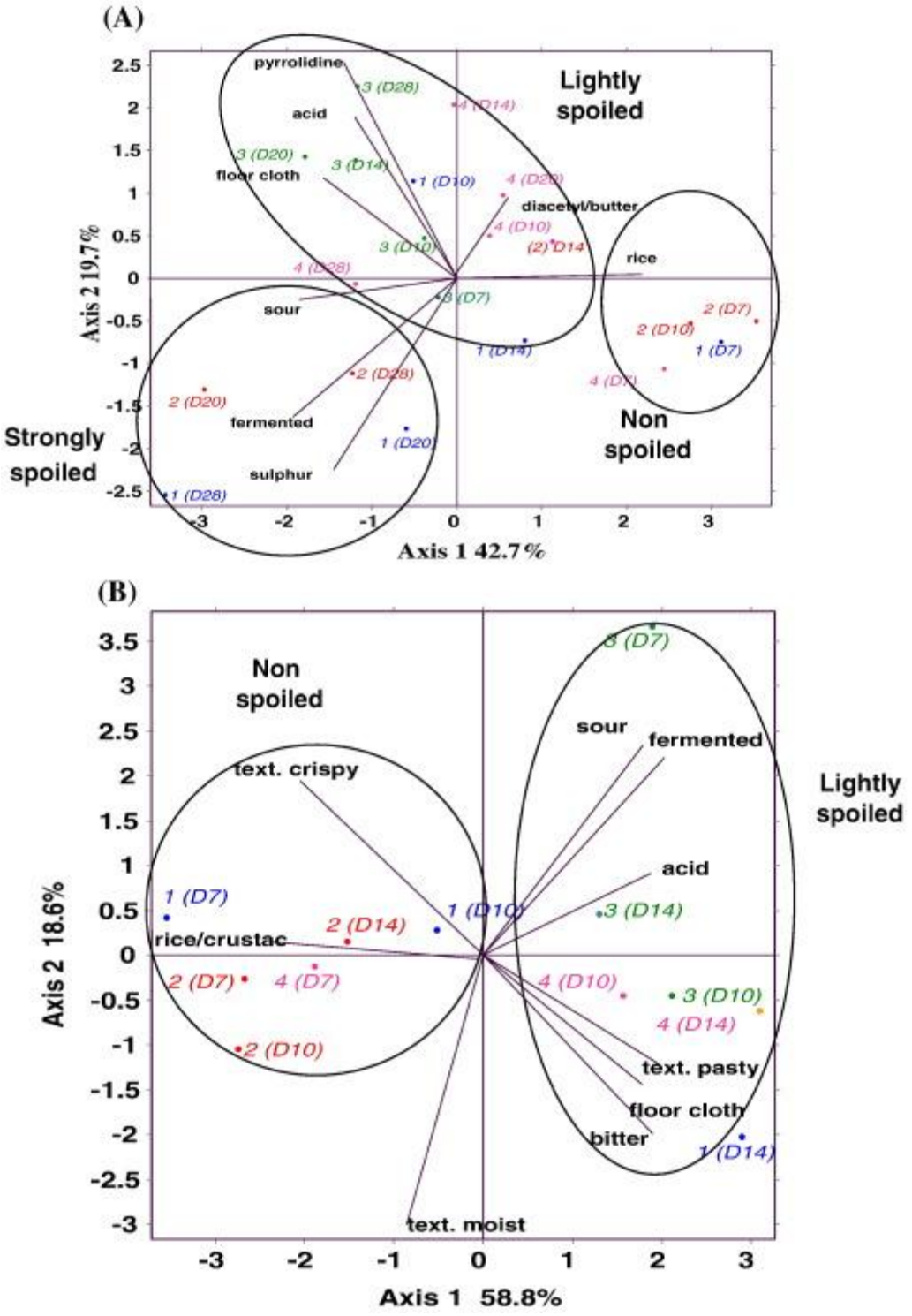
(A)
pyrrolidine
acid
floor cloth
diacetyl/butter
rice
sour
fermented
sulphur
Lightly spoiled
Strongly spoiled
Non spoiled
Axis 2 19.7%
Axis 1 42.7%
(B)
Non spoiled
text. crispy
rice/crustac
sour
fermented
acid
text. pasty
floor cloth
bitter
text. moist
Lightly spoiled
Axis 2 18.6%
Axis 1 58.8%

Figure 6