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Seafood quality and safety improvement through hurdle technology, including biopreservation. Impact on microbial ecosystems.

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List of Abbreviations and Acronyms

3-HPA	3-hydroxypropionaldehyde (Reuterin)
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BIOHAZ	Biologicals Hazards scientific committee from EFSA
BHI	Brain Heart Infusion
bp	base pair
CFU	Colony-Forming Unit
CLSI	Clinical and Laboratory Standard Institute
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSS	Cold-Smoked Salmon
DGGE	Denaturing Gradient Gel Electrophoresis
DMA	Dimethylamine
DNA	Deoxyribonucleic Acid
EFFCA	European Food and Feed Cultures Association
EFSA	European Food Safety Authority
EM^3B	Laboratoire Ecosystèmes Microbiens et Molécules Marines pour les Biotechnologies
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization
FROGS	Find Rapidly OTUs With Galaxy Solution
GC	Gas Chromatography
GS/MS	Gas Chromatography - Mass Spectropmetry
GI	Gastro-Intestinal
HCA	Hierarchical Clustering Analysis
He	Helium
HPP	High Pressure Processing
IDF	International Dairy Federation
IFREMER	Institut Français de la Recherche pour l'Exploitation de la Mer

INRA	Institut National de la Recherche Agronomique
IMP	Inosine Monophosphate
ISO	International Organization for Standardization
kGy	Kilo Gray
LAB	Lactic Acid Bacteria
LH	Long and Hammer
LPFP	Lightly Preserved Fish Products
MAP	Modified Atmosphere Packaging
MDS	Multidimentional Scaling
MFC	Microbial Food Cultures
MIC	Minimal Inhibitory Concentration
MRS	de Man Rogosa & Sharpe
NADH	Nicotinamide Adenine Dinucleotide
NTNU	Norwegian University of Science and Technology
OTU	Operational Taxonomic Units
PAE	Phage Associated Lytic Enzymes
PC	Protective Cultures
PCA	Plate Count Agar
PCA	Principal Component Analysis
pH	Poentiel Hydrogène
pK_a	Acid Dissoctiation Constante
PTFE	Polytetrafluoroethylene
PV	Peroxide Value
q-PCR	Quantitative Polymerase Chain Reaction
QPS	Qualified Presumption of Safety
rRNA	Ribosomal Ribonucleic Acid
SECALIM	Securité des aliments et Microbiologie
SPME	Solid-Phase Microextraction
SSO	Specific Spoilage Organisms
STAA	Streptomycin Thallous Acetate Agar
TBARS	Thiobarbituric Acid Reactive Substances
TMA	Trimethylamine
TMAO	Trimethylamine Oxide

TTGE	Temporal Temperature Gradient Gel Electrophoresis
TVBN	Total Volatile Basic Nitrogen
TVC	Total Viable Count
UE	European Union
UMR	Unité Mixte de Recherche
USD	United State Dollars
VOC	Volatile Organic Compounds
VP	Vacuum Packed
VRBG	Violet Red Bile Glucose Agar

General introduction

General introduction

Benefiting from a healthy image, as a source of valuable nutrients (proteins, vitamins, minerals and omega-3 fatty acids), seafood and fishery products contribute to an important part of our diet. With an average world consumption value of 20.5 kg/per capita in 2017, these products represent a significant worldwide market estimated at 171 million tons for a trade value of USD 362 billion (FAO, 2018).

However, seafood are very fragile food commodities with a limited shelf-life not exceeding several days for fresh products up to 3-4 weeks for lightly processed high value-added products such as smoked fishes, fish gravlax, sea salads and marinated fishes. This short conservation period is mainly due to a high *post-mortem* pH and a large non-protein nitrogen and free amino acids fraction, which make these products an ideal growth environment for microorganisms (Gram and Dalgaard, 2002). Among these microorganisms, spoiling bacteria such as *Photobacterium phosphoreum*, *Brochothrix thermosphacta*, *Shewanella* spp., *Pseudomonas* spp., *Serratia* spp., *Hafnia* spp., *Psychrobacter* spp. and *Pseudoalteromonas* spp. are able through their metabolism to induce sensory degradation (off-odors and flavors, discoloration, etc…) (Gram and Dalgaard, 2002; Leroi, 2014; Boziaris and Parlapani, 2016). Seafood can also be an important vector of food poisoning and human infections. Indeed, 10-20% of foodborne illnesses are attributed to fish and shellfish consumption (Le Fur et al., 2013). In seafood industry, especially in lightly preserved or ready-to-eat products, *Listeria monocytogenes* currently represents the main pathogenic risk (Jami et al., 2014).

As they have been evaluated to be responsible for the loss of 20 to 25% of all post-harvesting food production, microbial food spoilage and pathogens growth control are currently representing a crucial challenge (Bondi et al., 2014; Bevilacqua et al., 2016). In addition to traditional preservatives technologies such as cooling, smoking, salting, drying, and to face the consumers' demand for minimally processed food with less chemical preservative, biopreservation represents a complementary way to extend food shelf-life and reduce microbial risks (Devlieghere et al., 2004; Ronholm et al., 2016; Odeyemi et al., 2018).

Biopreservation consists in adding microorganisms and/or their metabolites in food, in order to extend its shelf-life and enhance its microbial safety (Stiles, 1996). For this

purpose, lactic acid bacteria (LAB) are by far the most used and studied microorganisms. Indeed, they are naturally present in many foods and can produce a wide range of antimicrobial compounds such as bacteriocins, organic acids, hydrogen peroxide, diacetyl, etc... (Caplice and Fitzgerald, 1999; Singh, 2018). Moreover, due to their involvement in many ancestral fermentation processes (fermented dairy products, dry sausage, sauerkraut, etc...) most of them are recognized as safe for human consumption and benefit from a healthy and natural image from consumers (Rodgers, 2001; Ghanbari et al., 2013).

Within this context, the objective of the European project “SAFEFISHDISH“ (http://www.safefishdish.fr/) was to improve, through a combination of mild preservative technologies, cod, salmon and salmon based products (cold-smoked salmon and gravlax) microbial safety and quality, from capture to consumption. In order to address this objective, the project, supported by the COFASP ERA-NET program and funded in France by the French Agence Nationale de la Recherche (ANR-14-COFA-0001), involved 11 partners from France, Norway and Iceland:

- 5 universities or research institutes: NTNU (Trondheim, Norway), NOFIMA (Tromsø, Norway), Matís (Reykjavík, Iceland), Ifremer (Nantes, France) and Oniris (Nantes, France).
- 4 Industrial company or research platforms: Primex (Siglufjordur, Iceland), Arnarlax (Bíldudalur, Iceland), Samherji (Akureyri, Iceland), PFI Nouvelles Vagues (Boulogne-sur-Mer, France).
- 1 industrial consortium: CITPPM (Paris, France).

During the project, partners investigated the application of different preservation technologies such as superchilling, chitosan coating, biopreservation and modified atmosphere packaging, alone or in combination, at different steps of fish handling and transformation. The effect of these technologies was evaluated on microbial, physico-chemical and sensorial quality of whole fish and fish products. The work performed in during this thesis was included in the SAFEFISFDISH project and focused on the biopreservation technology applied to salmon gravlax.

Thesis outline

This thesis manuscript is structured in four sections. The first chapter introduces the subject with the state of the art regarding seafood place from a socio-economical and industrial point of view, the main spoiling and pathogenic bacteria encountered in these products and the preservative techniques used for their control. Finally a large focus was made especially on biopreservation: the main microorganisms used (LAB), their antimicrobial activity, protective cultures scientific and commercial applications in seafood, their regulation and safety status.

The second chapter concerns salmon dill gravlax characterization, on a microbiological (cultural method and metabarcoding approach), physico-chemical (biogenic amines, volatile organic compounds etc…) and sensorial point of view. This experiment was also extended to cold-smoked salmon stored under vacuum or modified atmosphere, in order to compare the manufacturing process effect on the microbial flora and products conservation.

In a third chapter, the selection of 6 LAB (*Carnobacterium maltaromaticum* SF1944, *Lactococcus piscium* EU2229, *Leuconostoc gelidum* EU2249, *Vagococcus fluvialis* CD264, *Carnobacterium inhibens* MIP2551 and *Aerococcus viridans* SF1044) as promising protective cultures for seafood, was performed from a collection of 35 LAB strains showing interesting antimicrobial profiles. The screening approach was based on technological criteria such as antimicrobial activity, spoilage potential, tolerance to freezing and chitosan, as well as safety criteria such as biogenic amines production and antibiotic resistance. These results were published in *Frontiers in Marine Science*.

The fourth chapter presents the application of these 6 LAB stains as protective cultures for salmon gravlax quality and safety improvement. Their impact on the product was measured by a polyphasic approach combining microbiological analyses (cultural method and metabarcoding), sensory analysis, biochemical analyses and volatilome.

Finally in a section untitled “General discussion”, main results are discussed in the light of the current scientific literature and possible perspectives for deepening the knowledge on seafood microbial ecosystems and on biopreservation are exposed.

Chapter 1

Bibliographic introduction

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1. World and European Fish and fishery products consumption and production

1.1. World fish market

Benefiting from a healthy image, as source of valuable nutrients (vitamins, minerals, polyunsaturated fatty acids etc.), seafood and fishery products contribute to an important part of our alimentation. Since 1961, when it was estimated at 9.0 kg, the average world fish consumption per capita quickly increased over the years to reach 17.0 kg in the 2000s, 18.9 kg in 2010 and 20.5 kg in 2017 according to preliminary estimates (FAO, 2016a, 2018c). In 2016, the global fishery and seafood production was estimated at 171 million tons (Figure 1.1), representing a trade value of USD 362 billion (FAO, 2018c).

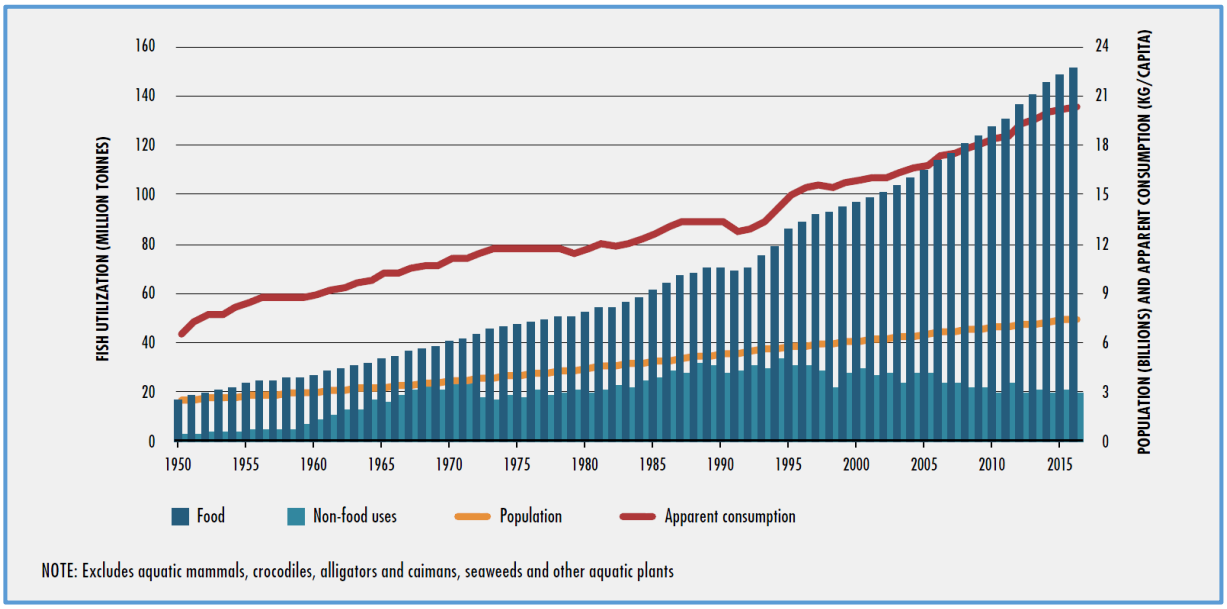

Figure 1.1: World Fish utilization and apparent consumption (FAO 2018).

Of this production, 151.2 million tons were destined for direct human consumption, while 19.7 million tons were intended to be processed for non-food utilization (FAO, 2018c). As the demand is increasing with the world population, according to some estimates, human consumption is expected to grow from 151.2 up to 181.1 million tons in 2022 (Lem et al., 2014; FAO, 2016a, 2018c).

To meet the growing demand for fish and seafood, aquaculture has grown considerably since the 1960s and nowadays accounts for nearly 50% of the world production. At the top of world aquaculture production, Asia is by far the largest producer with 89.4% of the volume produced in 2016, followed by Americas (4.2%), Europe (3.7%), Africa (2.5%) and Oceania (0.3%). As a reflection of its production volume, Asia comprises 5 of the world's largest aquaculture producers, which are China (61.5%), India (7.1%), Indonesia (6.2%), Vietnam (4.5%) and Bangladesh (2.8%). Among the most produced species, grass carp (*Ctenopharyngodon idellus*), silver carp (*Hypophthalmichtys malotrix*), common carp (*Cyprinus* carpio), whiteleg shrimp (*Penaeus vannamei*), cupped oyster nei (*Crassostrea* spp.) and Japanese carpet shell (*Ruditapes philippinarum*) ranked the top of the list (FAO, 2018c).

1.2. Salmon world production

Salmon production represents only a small share of the world fish and fishery production, with 3.1 million tons estimated in 2014 for both wild and farmed (FAO, 2018a). This market mainly concerns 5 salmon species: atlantic salmon (*Salmo salar*), pink salmon (*Oncorhynchus gorbuscha*), chum salmon (*Oncorhynchus keta*), coho salmon (*Oncorhynchus kisutch*) and sockeye salmon (*Oncorhynchus nerka*) (Globefish, 2018). Among these salmon species, *Salmo salar* is the most produced with 66% of total salmon production (Figure 1.2).

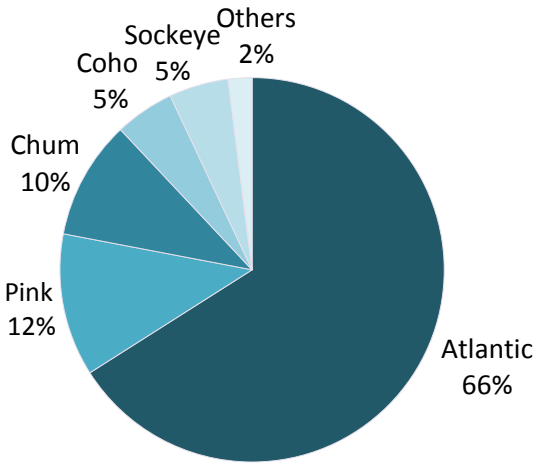

Figure 1.2: Salmon production by species, both wild and farmed in 2015 (Globefish, 2018).

Salmonid farming, which accounts for more than 2/3 of the salmon production, is one of the best examples of intensive aquaculture success and has grown particularly strongly between the 1980s and 2000s (Asche et al., 2013; FAO, 2018a). Atlantic salmon, which is the most world wild consumed species, represents nowadays 90% of the farmed salmon market. Its production was nearly inexistent in 1980 with 5 288 tons and rapidly grow to reach a volume of 2 247 759 tons in 2016, for a trade value of USD 14.4 billion (Figure 1.3) (FAO, 2018b).

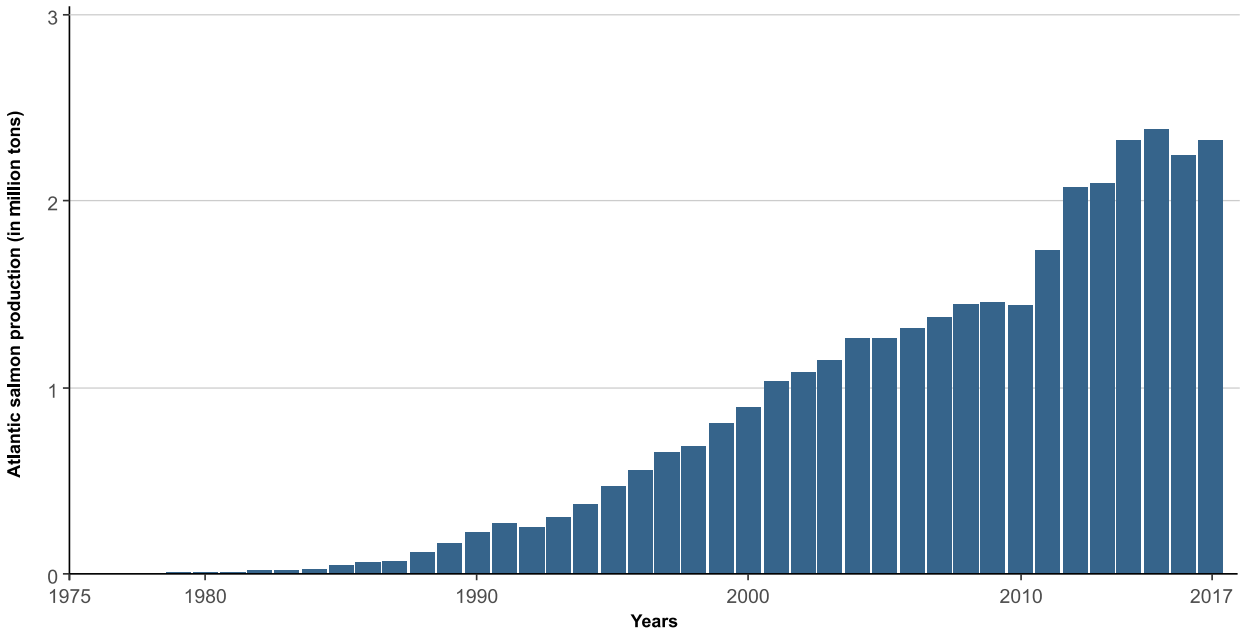

Figure 1.3: Evolution of atlantic salmon (*Salmo salar*) production from 1975 to 2017. Data extracted from FAO (2018a) and completed with estimated data from Globefish (2018) and FAO (2018b).

Norway, Chile and United-kingdom are the 3 mains farmed atlantic salmon producers. In 2013 they accounted for 90.3% of world atlantic salmon production (EUMOFA, 2016; Globefish, 2018). Despite a rapid growth in salmon production in Chile this last decade, Norway remains the undisputed leader with almost 60% of the world production (EUMOFA, 2016; FAO, 2018a; Globefish, 2018).

World wild demand for atlantic salmon is continually increasing over the years, boosted by the development of the sushi market notably. Thus Japan, European Union (EU) and North America represent the main markets (EUMOFA, 2016; FAO, 2018a). In EU, salmon products market supply is mainly driven by fresh salmon, as fillets (47%) or as whole piece (12%). However, lightly processed salmon such as smoked salmon and others value-added products still have a prominent place with 28 and 13% of the market share (EUMOFA, 2016).

Currently, high value-added products, including smoked fishes, fish gravlax and sea salads, are gaining popularity in Europe. Cold-smoked salmon figures among the best-selling delicatessen products (FranceAgriMer, 2018). Its market in EU was estimated at 155 000 tons in 2013, an increase by 29% compared to 2010. Germany is the largest market (28%), followed by France (20.9%) which was the first market until 2010. UK (9.1%), Italy (8.6%), Spain (6.9%) and Poland (6.8%) also represent important market share in EU (EUMOFA, 2016).

In France, the smoking industry covers around 70% of the domestic demand, the rest being imported from other EU countries. To ensure its production, France imports fresh salmon mainly from Norway (66%), UK (Scotland) (25%) and Ireland (4%). In 2014, 28 465 tons of smoked salmon were produced, of which 12% were exported. Currently, on the French market, smoked salmon comes equally from wild and farmed fish (EUMOFA, 2016).

1.3. Food losses in fishery production chain

All food commodities combined, it is estimated that approximatively one third of word global food production for human consumption is lost or wasted every year. These food losses represent a volume of 1.3 billion tons for an equivalent market value of USD 990 billion (FAO, 2011, 2012).

The fishing industry is one of the most affected agri-food chain with 35% of its total production lost (Figure 1.4) (FAO, 2012).The most part of the primary production losses took place at the harvest level, where 6-8% of the marine catches are discard back to the sea. Another important part of the losses (almost 50%) is distributed between the distribution and consumption channels (FAO, 2012).

At this stage of the food supply chain, products are mainly declassed for inadequate shape, appearance or organoleptic properties for consumption, resulting from microorganisms spoilage activity (The Microbiological Quality of Food, 2017). Globally, all food commodities considered, it has been estimated that about 20 to 25% of all food produced is lost due to microbial spoilage (Gram and Dalgaard, 2002; Bondi et al., 2014; Bevilacqua et al., 2016).

Figure 1.4: World global fish and seafood losses (FAO, 2012).

2. Fish microbiota

Although mostly composed of bacteria, the microbiome of living fish can be very diverse, including fungi, yeasts, viruses and archaea (Merrifield and Rodiles, 2015; Egerton et al., 2018). The microbiota acquisition begins from the animal egg and hatching, and will continue throughout its whole life (Hansen and Olafsen, 1999; Ringø and Birkbeck, 1999; Austin, 2006; Wang et al., 2018). Many environmental factors shape the fish microbiome diversity and composition, such as the life stage, trophic level, diet, season, habitat, including surrounding water quality (biological and chemical composition), captive-state, sex and phylogeny (Hansen and Olafsen, 1999; Ringø and Birkbeck, 1999; Austin, 2006; Sullam et al., 2012; Llewellyn et al., 2016; Ringø et al., 2016; Egerton et al., 2018; Wang et al., 2018). Concomitantly to the aquaculture industry expansion, research on fish microbiome, in particular on gastrointestinal microbiota, increased over the 3 last decades. Consequently this field of research was

reviewed many times (Cahill, 1990; Ringø et al., 1995; Hansen and Olafsen, 1999; Ringø and Birkbeck, 1999; Austin, 2002, 2006; Merrifield and Rodiles, 2015; Ringø et al., 2016; Tarnecki et al., 2017; Egerton et al., 2018).

2.1. Fish early stages of life microbiota

Microbial colonization of fish larvae originates from eggs, surrounding water and the first feed. The microbiota acquired during the first stage of fish life is likely to influence the microbiome composition in later stage (Austin, 2006; Egerton et al., 2018). Bacterial colonization of eggs surface rapidly occurs within few hours after fertilization (Hansen and Olafsen, 1999; Austin, 2006). The adherent microbiota of cod, halibut and turbo eggs were found to be mainly constituted by Gram-negative bacteria such as pseudomonads (*P. aeruginosa*, *P. fluorescens*), *Alteromonas* sp., *Aeromonas* sp., *Flavobacterium* sp. and *Moraxella* sp. (Hansen and Olafsen, 1989, 1999). The intraovum part of the fish egg can also contains a high number of bacteria ranging from 10^2 up to 10^6 CFU/g (Hansen and Olafsen, 1999; Austin, 2006). Various bacterial genera and species were reported inside fresh- and seawater fish eggs such as *Renibacterium salmoninarum*, *Lactobacillus* spp., *Aeromonas* spp., including *A. hydrophila*, *Pseudomonas* spp. including *P. fluorescens*, *Vibrio* spp. (*V. fluvialis, V. parahaemolyticus*), *Enterobacter* sp., *Hafnia alvei*, *Serratia liquefaciens*, *Listeria* sp., *Staphylococcus aureus*, *Corynebacterium hoffmannii*, *Bacillus* sp., *Aerococcus viridans, Cytophaga* sp. and *Flavobacterium psychrophilum* (Bell et al., 1971; Cone, 1982; Evelyn et al., 1984; Sauter et al., 1987; Barker et al., 1989; Brown et al., 1997; Hänninen et al., 1997; Hansen and Olafsen, 1999; Austin, 2006). However, the presence of bacteria in the intraovum content is not something usual and may lead to egg mortality, embryo abnormal development, hatching delay and spawnlings disease. For instance, *Flavobacterium psychrophilum*, the causal agent of bacterial coldwater disease, have been demonstrated to be vertically transmitted to rainbow trout (*Oncorhynchus mykiss*) through their eggs (Hansen and Olafsen, 1999).

2.2. Skin Microbiota

The surface (skin and mucus) microbial population has been estimated to 10^2 to 10^4 bacteria/cm^2 (Horsley, 1973; Gillespie and Macrae, 1975; Austin, 2006). However, some variations can be observed in microbial concentration, according to the quality of the surrounding water (Austin, 2006). Diler et al. (2000) described concentrations

that can reach 10^7 bacteria/cm^2 on rainbow trout (*O. mykiss*) surface, when ponds water were heavily polluted.

Sea and freshwater fish skin microbiota are both mainly dominated by Gram-negative bacteria, but slightly differ from one to the other due to water biochemical composition (Austin, 2006). Freshwater fish microbial community includes bacteria such as, *Acinetobacter johnsonii*, aeromonads (*A. hydrophila, A. bestiarum, A. caviae, A. jandaei, A. schubertii and A. veronii*), *Alcaligenes piechaudii*, *Enterobacter aerogenes*, *Escherichia coli*, *Flavobacterium* sp., *Flexibacter* spp., *Moraxella* spp., *Pseudomonas fluorescens*, *Vibrio fluvialis*, *Psychrobacter* spp. and *Micrococcus luteus* (Allen et al., 1983; Diler et al., 2000; González et al., 2000, 2001; Zmyslowska et al., 2001; Austin, 2006). Seawater fish skin microbiota includes bacteria such as *Acinetobacter calcoaceticus*, *Alcaligenes faecalis*, *Bacillus* (*B. cereus, B. firmus*), *Caulobacter*, coryneforms, *Cytophaga* sp., *Flexibacter* sp., *E. coli*, *Hyphomicrobium vulgare*, *Photobacterium* (*P. angustum* and *P. damselae*), *Prosthecomicrobium* sp., *Pseudomonas* (*P. fluorescens*, *P. marina*), *Aeromonas spp.*, *Alteromonas spp.*, *Plesiomonas shigelloides*, *Moraxella* spp., *Neisseria* spp. and many species belonging to *Vibrio* genera (*V. harveyi, V. alginolyticus, V. albensis, V. anguillarum, V. splendidus, V. fischeri, V. ordalii* and *V. scophthalmi*) (Allen et al., 1983; Grimes et al., 1993; Montes et al., 1999; Austin, 2006).

Several recent studies, using metabarcoding approach, described the genera *Burkholderia*, *Lysobacter*, *Methylobacterium*, *Rhalstonia*, *Pseudomonas*, *Aeribacillus*, *Janthinobacterium*, *Delftia*, *Microbacterium*, *Acinetobacter* and *Flavobacterium* as the main dominant genera found in fish skin microbiota (Larsen et al., 2013; Boutin et al., 2014; Minniti et al., 2017).

2.3. Gills Microbiota

Microbial population in fish gills can reach concentration values ranging from 10^4 (Ringø and Holzapfel, 2000) to 10^6 bacteria/g (Trust, 1975). Gills from freshwater fish were found to be mainly dominated by Gram-negative bacteria such as *Cytophaga spp.*, aeromonads, coryneforms, enterobacteria, pseudomonads and vibrios (Trust, 1975; Nieto et al., 1984). In comparison, in gills from marine fish, genera such as *Achromobacter*, *Alcaligenes*, *Bacillus*, *Flavobacterium*, *Micrococcus*, *Chryseobacterium*, *Flexibacter* and *Cytophaga* were mainly isolated (Mudarris and

Austin, 1988; Austin, 2006). *Carnobacterium maltaromaticum* (formerly *C. piscicola*) were also found to be the main Gram-positive bacteria isolated from atlantic salmon gills (Ringø et al., 2000). By metabarcoding approach recent studies showed that the main bacterial genera found in many reef fish and yellowtail king fish gills were *Vibrio*, *Salinivibrio*, *Shewanella*, *Photobacterium*, *Clostridium*, *Pseudoalteromonas*, *Burkholderia*, *Rhalstonia*, *Propionibacterium*, *Paracoccus, Neorickettsia, Exiguobacterium, Endozoicomonas, Delftia, Corynebacterium, Akkermansia, Acinetobacter, Roseovarius, Ferrovum, Alteromonas, Glaciecola, Idiomarina, Marinobacter* and *Halomonas* (Reverter et al., 2017; Pratte et al., 2018; Legrand et al., 2018).

2.4. Gut Microbiota

The interest in aquaculture industry for microbiome modulation through dietary and/or probiotic to enhance both animal performance and health is growing in aquaculture industry. Consequently, the gastrointestinal (GI) microbiota of fish has been extensively studied in the recent years (Ringø et al., 2016; Tarnecki et al., 2017; Egerton et al., 2018). The bacterial density in fish GI is ranging from 10^4 up to 10^9 CFU/g (Austin, 2006; Egerton et al., 2018), constituted of 10^{8-9} CFU /g of heterotrophs (Trust, 1975; Yoshimizu and Kimura, 1976; Campbell and Buswell, 1983; Yano et al., 1995; Savaş et al., 2005; Austin, 2006) and 10^5 CFU/g of anaerobes (Trust, 1975; Yoshimizu and Kimura, 1976; Kamei et al., 1985; Austin, 2006). Sakata et al. (1978) were the first ones to describe an increase in bacterial population throughout the gastrointestinal tract (GIT). They found in yellowtail GIT a bacterial concentration of 2.0 to 6.5 x 10^4 CFU/g in the pyloric caeca, 2.3 to 2.6 x 10^5 CFU/g in the stomach part and 6.6 x 10^5 to 5.9 10^6 CFU/g in the intestine. This finding was also observed later in several other studies (Fidopiastis et al., 2006; Ringø et al., 2006; Hovda et al., 2007a; Silva et al., 2011).

Marine fish gut microbiota can be extremely diverse depending on the fish species considered and its trophic level (Table 1.1). However, among the most dominant bacterial genera described in the literature, *Vibrio*, *Photobacterium*, *Clostridium* and *Pseudomonas* are the most reported (Egerton et al., 2018).

Table 1.1: Dominant bacterial genera and species isolated from various marine fishes intestinal tract at different trophic levels. Extracted from Egerton et al. (2018) and completed with a special focus given on atlantic salmon GI microbiota.

Trophic level	Fish species	Dominant bacteria	Reference
Herbivores			
	Butterfish	*Clostridium*	Clements et al., 2007
	Marble fish	*Clostridrium, Eubacterium desmolans, Papillibacter cinnaminovorans*	Clements et al., 2007
	Parrotfish	*Vibrio*, *Photobacterium*	Smriga et al., 2010
	Silver drummer	*Clostridium*	Moran et al., 2005
	Surgeonfish	*Bacteroidetes*, non-vibrio *Proteobacteria*, Firmicutes	Smriga et al., 2010
		Epulopiscium	Miyake et al., 2015
	Zebraperch	*Enterovibrio*, Bacteroides, *Faecalibacterium*, *Desulfovibrio*	Fidopiastis et al., 2006
Omnivores			
	Pinfish	*Clostridium*, *Mycoplasma*	Ransom, 2008
		Photobacterium, *Propionibacterium*, *Staphylococcus*, *Pseudomonas*, *Corynebacterium*	Givens et al., 2015
	Long-jawed mudsucker	*Mycoplasma*	Bano et al., 2007
Carnivores			
	Atlantic cod	*Clostridium perfringens*	Aschfalk and Müller, 2002
		Vibrio	Star et al., 2013
	Atlantic halibut	*Vibrionaceae* (juveniles), *P. phosphoreum* (adults)	Verner-Jeffreys et al., 2003
	Blackfin icefish	*Photobacterium*	Ward et al., 2009
	Black rockcod	*Photobacterium*, *Vibrio*	Ward et al., 2009
	Bluefish	*Vibrio*, *Pseudomonas*, *Enterobacteriaceae*	Newman et al., 1972
	Gilthead sea bream	*Pseudomonas*	Floris et al., 2013
	Grass puffer	*Vibrio*, *Pseudomonas*, *Flavobacterium*	Sugita et al., 1989
	Grouper	*Bacillus*, *Vibrio*, *Delftia*, *Psychrobacter*, *Acinetobacter*, *Pseudomonas*	Sun et al., 2013
	Red drum	*Mycoplasmataceae*	Ransom, 2008
	Sea trout	*Aeromonas sobria*, *Pseudomonas*	Skrodenytė-Arbačiauskienė et al., 2008
	Siberian sturgeon	*Cetobacterium somerae*	Geraylou et al., 2013
	Red Snapper	*Vibrio*, *Photobacterium*	Smriga et al., 2010
		Pseudoalteromonas, Photobacterium	Tarnecki et al., 2016

Trophic level	Fish species	Dominant bacteria	Reference
	Southern flounder	*Clostridium*	Ramirez and Dixon, 2003
		Clostridium	Ransom, 2008
		Photobacterium, *Clostridiaceae*, *Clostridium*	Givens et al., 2015
	Speckled trout	*Escherichia coli*	Ransom, 2008
	Striped bass	*Aeromonas*, *Pseudomonas*, *Vibrio*	MacFariane et al., 1986
	Atlantic salmon	*Carnobacterium*	Ringø et al., 2000
		Acinetobacter junii, *Mycoplasma*	Holben et al., 2002
		Lactobacillus, *P. phosphoreum*, *Lactococcus*, *Bacillus*	Hovda et al., 2007
		Plesiomonas, *Enterobacteriaceae*, *Carnobacterium*, *Aeromonas*, *Lactococcus*	Skrodenytė-Arbačiauskienė et al., 2008
		Pseudomonas, Shewanella	Navarrete et al., 2009
		Vibrio, *Photobacterium*, *Lactococcus*, *Weissella*, *Leuconostoc*	Zarkasi et al., 2014
		Photobacterium, *Delftia*, *Weissella*, *Leuconostoc*, *Janthinobacterium*	Gajardo et al., 2016
		Aliivibrio, *Mycoplasma*, *Photobacterium* (adults)	Llewellyn et al., 2016
		Ruminococcaceae, *Mycoplasmataceae*, *Pseudomonas*	Dehler et al., 2017
		Sphingomonas, *Streptomyces*, *Lactococcus*	Wang et al., 2018
Zooplanktivores			
	Cardinalfish	*Vibrionacceae*, *Pasteurellaceae*, *Vibrio harveyi*, *Shewanella*, *Endozoicomonas*	Parris et al., 2016
	Damselfish		
	Herring	*Pseudomonas*, *Alteromonas*	Hansen et al., 1992
		Pseudomonas, *Psychrobacter*	Curson et al., 2010
	Sardines	*Achromobacter*, *Vibrio*, *Pseudomonas*	Karthiayani and Mahadeva Iyer, 1967
	Atlantic mackerel	*Psychrobacter*, *Vibrio*, *Shewanella*	Svanevik and Lunestad, 2011

In contrast, the GI microbiota of freshwater fish is mainly dominated by *Aeromonas*, *Pseudomonas*, *Bacteroides*, *Plesiomonas*, *Enterobacteriaceae*, *Micrococcus*, *Acinetobacter*, *Clostridium*, *Cetobacterium*, *Bacillus*, *Veillonella* and *Rothia*. LAB genera such as *Weissella*, *Streptococcus*, *Leuconostoc*, *Lactobacillus* and *Enterococcus* were also reported as major members of the intestinal microbiota (Austin, 2006; Tarnecki et al., 2017; Wang et al., 2018).

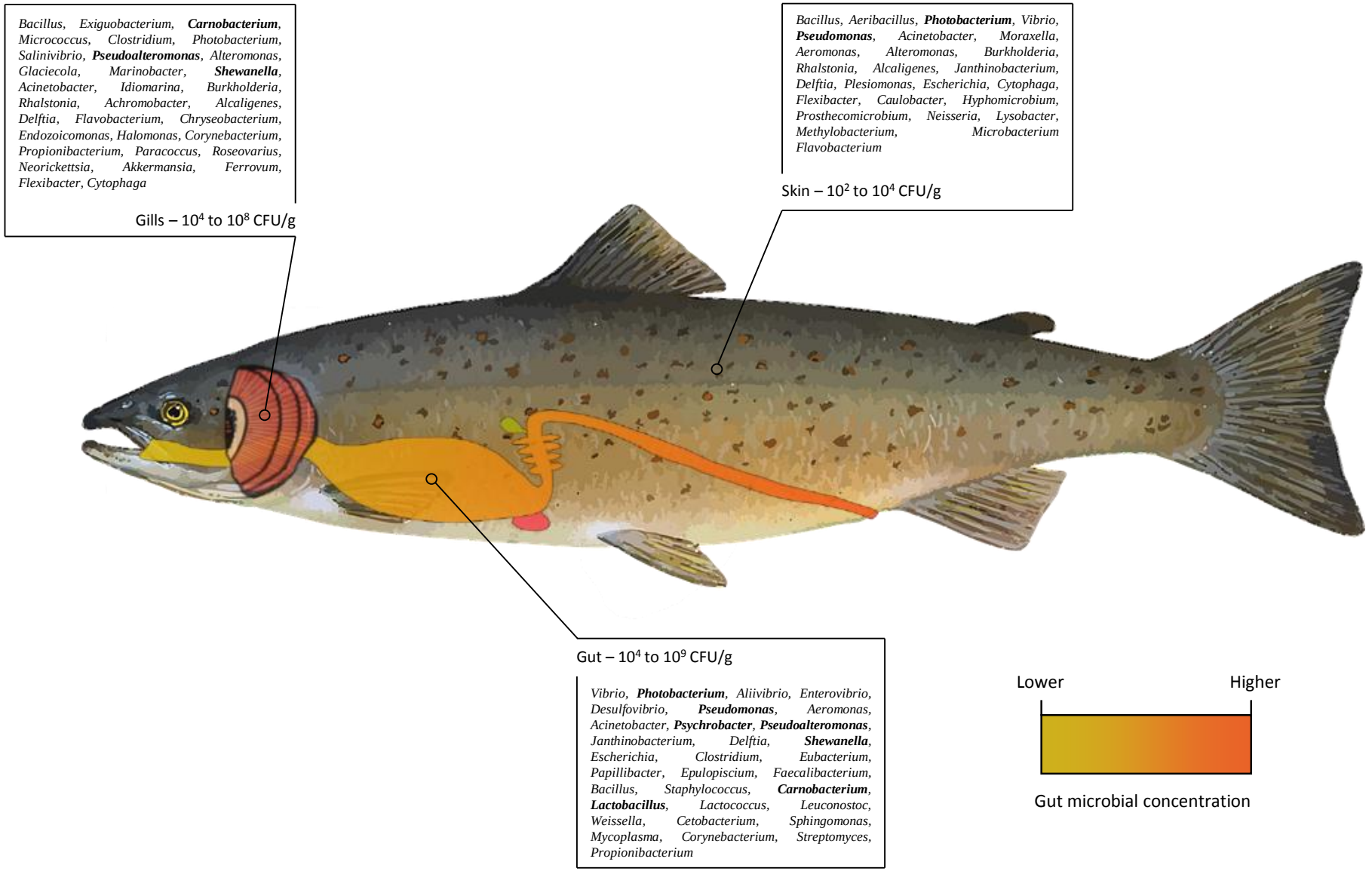

Figure 1.5: Illustration of marine fishes microbiota. Bacterial genera in bold are commonly associated with seafood and fishery products spoilage.

3. Seafood spoilage microorganisms

The living fish microbiome is likely to influence the microbial ecosystem present on processed seafood as illustrated in Figure 1.5. Transformation steps such as gutting, scaling and filleting, lead to the flesh contamination with fish endogenous bacteria. Furthermore, each processing steps like salting, smoking, marinating, slicing and packaging, represents as many additional sources of post-contamination by exogenous bacteria from human, environmental and material origin (Leroi, 2014; Boziaris and Parlapani, 2016).

3.1. Seafood specific spoilage microorganisms (SSOs) concept

After fish death, the immune system collapses, allowing bacteria to proliferate freely. This, combined to a high content in non-protein low molecular weight nitrogenous compounds such as free amino acids, creatine, taurine, nucleotides, urea and TMAO and a high *post mortem* pH superior to 6.0, makes seafood an ideal growth environment for psychrophilic and psychrotrophic spoilage bacteria (Gram and Huss, 1996; Gram and Dalgaard, 2002; Leroi, 2014; Boziaris and Parlapani, 2016). In seafood and fishery products, sensory degradation and freshness are most of the time not correlated with the number of microorganisms. The sensory rejection of a product can be reached several days after the total bacterial count reaches its maximum concentration (Gram and Huss, 1996; Huss, 1999).

Among the microorganisms that dominate the spoilage microbiota, only those possessing the ability to produce metabolites responsible for off-flavors and odors (spoilage potential), in sufficient quantities (spoilage activity) to induce organoleptic rejection and spoilage are considered as specific spoilage microorganisms (SSOs) (Dalgaard, 1995; Gram and Huss, 1996; Gram and Dalgaard, 2002; Boziaris and Parlapani, 2016). SSOs often represent only a small portion of the product initial microbial flora (Figure 1.6).

In refrigerated conditions, these bacteria rapidly grow to become a dominant part of the final spoilage microbiota, producing metabolites leading to the sensory rejection (Gram and Huss, 1996; Gram and Dalgaard, 2002; Boziaris and Parlapani, 2016). In consequence, monitoring SSOs concentration and their metabolites can be adequate for seafood spoilage assessment (see 5.4).

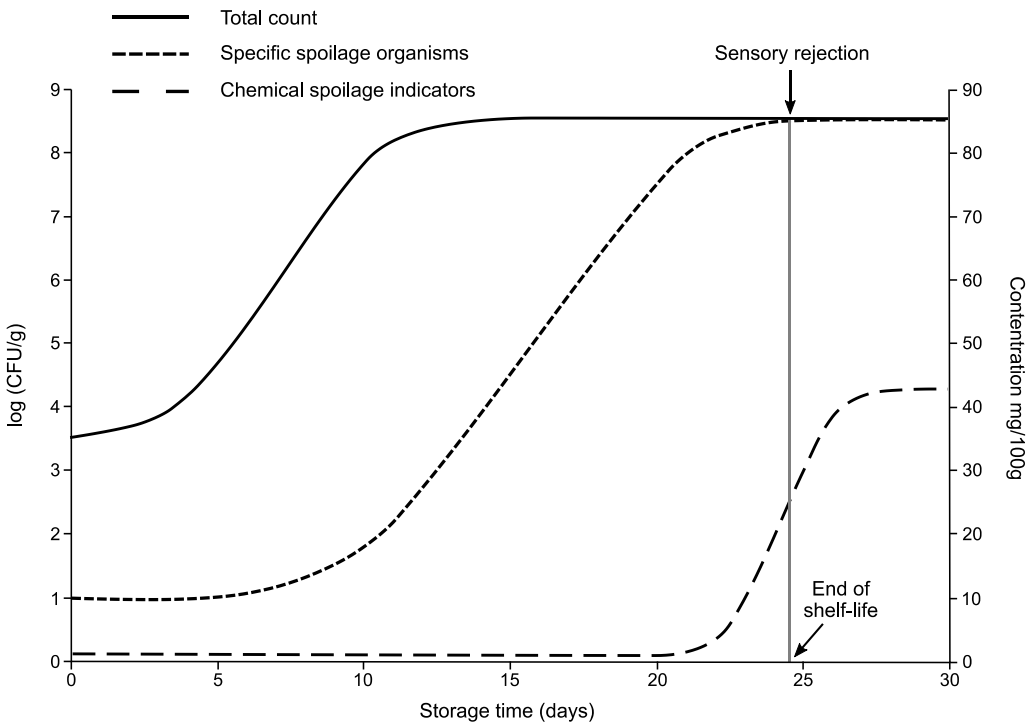

Figure 1.6: Microbial spoilage and SSO concept. Modified from Gram and Huss (1996) and Boziaris and Parlapani (2016).

Identifying SSOs from the rest of the seafood microbial ecosystem and investigating their spoilage potential can represent a challenging task. For a long time, seafood products microbiota at spoilage was enumerated and studied via the use of non-specific culture media such as the Plate Count Agar (PCA) often incubated at 30 °C (Huss et al., 1995; Boziaris and Parlapani, 2016). However this culture medium was inadequate due to the lack of salts and the incubation temperature was too high for the growth of psychrotrophic microorganisms. Bacteria, now well known to be major seafood spoiling microorganisms, such as *Photobacterium phosphoreum*, *Shewanella baltica* or *Pseudomonas fluorescens* are indeed unable to grow on PCA medium (Broekaert et al., 2011). More adequate media like Long and Hammer (LH) or Marine Agar and incubation temperatures around 15-20 °C are now commonly used. In any case, bacteria were isolated in pure culture from these non-specific media, and were laboriously identified using phenotypic tests such as Gram-reaction, oxidase test, Hugh and Leifson reaction, production of acid and/or gas from glucose, mobility (etc…), and later via conserved genes sequencing (16S rRNA, *gyrB*, *rpoB*, etc…) (Boziaris and Parlapani, 2016).

During the last decade, the introduction of new sequencing technology platforms such as 454-pyrosequencing, Ion Torrent and Illumina changed and revolutionized the way

of exploring microbial communities in food. Through metabarcoding or metagenomic approaches it is now possible to describe microbial dynamics within an ecosystem and to get a clear picture of the microbial composition. Some examples illustrating the use of Next Generation Sequencing (NGS) technologies to study microbial communities at spoilage time in seafood are given in Table 1.2.

Table 1.2: Examples of scientific works studying the microbial ecosystem at spoilage time in seafood through the use of metabarcoding approach.

Sequenc. technol. **Target gene region**	**Matrix and storage cond.**	**Main bacteria identified, ordered by sequence number**	**Reference**
454-pyrosequencing			
V1-V3 (16S rRNA)	Cold-smoked salmon (VP)	*C. divergens, B. thermosphacta, P. phosphoreum, S. equorum, C. maltaromaticum, L. lactis, L. curvatus*	Chaillou et al., 2015
	Cod fillets (MAP)	*Fusobacteriaceae (unknown), B. thermosphacta, P. phosphoreum, V. fluvialis, C. maltaromaticum*	
	Cooked shrimp (MAP)	*L. gasicomitatum, S. parauberis, V. ordalii, W. viridescens, C. divergens, C. malta, L. mesenteroides, C. inhibens, A. viridans*	
	Salmon fillets (MAP)	*C. maltaromaticum, S. sakei, P. phosphoreum, C. divergens, L. piscium, L. curvatus*	
Illumina MiSeq			
V3 (16S rRNA)	Yellowfin tuna (air)	*Brochothrix, Pseudomonas*	Silbande et al., 2016
V3-V4 (16S rRNA)	Red drum (air)	*Brochothrix, Carnobacterium, Lactococcus Vagococcus, Leuconostoc*	Silbande et al., 2018a
	Sea bream (air)	*Pseudomonas, Psychrobacter, Carnobacterium*	Parlapani et al., 2018

VP: vacuum packed, MAP: Modified atmosphere packaging

However, although the microorganisms mainly present at the spoilage time are characterized through culture-dependent or -independent methods, it is imperative to assess their real involvement in sensory degradation. Some bacteria belonging to genera such as *Photobacterium*, *Shewanella*, *Pseudomonas*, *Brochothrix*, *Serratia*, *Hafnia*, *Pseudoalteromonas* and *Psychrobacter* were identified as the main seafood spoiling bacteria, notably via use of organoleptic analyses performed on inoculated

sterile matrix or model substrate (fish juice) (Dalgaard, 1995; Chinivasagam et al., 1998; Leroi et al., 1998; Truelstrup Hansen and Huss, 1998; Gennari et al., 1999; Stohr et al., 2001; Joffraud et al., 2001, 2006; Jaffrès et al., 2011; Broekaert et al., 2013b; Macé et al., 2013a, 2014; Parlapani et al., 2017; Silbande et al., 2018b).

3.2. *Photobacterium phosphoreum*

The *Photobacterium* genus is a marine motile, psychrotrophic, facultative aerobic and Gram-negative bacteria belonging the *Vibrionaceae* family (Urbanczyk et al., 2011; Jérôme et al., 2016). Depending on the strain and species, some *Photobacterium* isolates possess the ability to be bioluminescent. They are usually found in association with various marine animals (see 2.) and are largely present in seawater (Urbanczyk et al., 2011). Twenty one species currently constitute the *Photobacterium* genus (Urbanczyk et al., 2011), however, *P. phosphoreum P. illiopiscarium* and *P. kishitani* are the most commonly found and isolated *Photobacterium* species in seafood (Chaillou et al., 2015; Jérôme et al., 2016).

P. phosphoreum were reported to dominate the spoilage microbiota of cod, fresh and smoked salmon, coalfish and halibut (Paludan-Müller et al., 1998; Truelstrup Hansen and Huss, 1998; Emborg et al., 2002; Rudi et al., 2004; Hovda et al., 2007b, 2007c; Olofsson et al., 2007; Hansen et al., 2009; Reynisson et al., 2009; Macé et al., 2012). This species has been identified as the main SSO responsible for the spoilage of several seafood products stored under MAP and VP, such as cod, salmon and lobster (Dalgaard et al., 1993; Dalgaard, 1995; Leroi et al., 1998; Emborg et al., 2002; Gornik et al., 2011; Macé et al., 2013a). Seafood spoilage by *P. phosphoreum* is mainly characterized by off-odor production such as sour, amine, ammonia-like (Dalgaard, 1995; Leroi et al., 1998; Emborg et al., 2002; Macé et al., 2013a), which can be related notably to the important production of TVBN compounds (TMA, DMA etc…) (Joffraud et al., 2001; Stohr et al., 2001). *P. phosphoreum* is indeed capable of using TMAO as final electron acceptor and reduces it into TMA (Gram and Huss, 1996). *P. phosphoreum* were also found to produce in sterile raw salmon various volatile organic compounds, mainly alcohols and aldehydes, which are summarized in Table 1.3.

P. phosphoreum and more broadly the *Photobacterium* genus, can be a strong biogenic amine producer in seafood, especially tyramine, histamine, putrescine and cadaverine (Jørgensen et al., 2000b; Visciano et al., 2012; Biji et al., 2016). This

species was also implicated in cases of histamine poisoning in sardines and tuna (Kanki et al., 2004; Emborg et al., 2005).

3.3. *Shewanella*

Shewanella species are Gram-negative, oxidase and catalase positive, H_2S-producing rod bacteria. To date, 47 species, mostly found in aquatic environment, are described (Vogel et al., 2005; Zhu et al., 2015; Odeyemi et al., 2018). The ability to reduce TMAO into TMA and to produce volatiles sulfides compounds from sulfur-containing amino acids, makes some species, especially *S. putrefaciens* and *S. baltica*, important fish SSOs (Gram and Dalgaard, 2002; Vogel et al., 2005). These two species were found to be predominant in many seafood spoilage microbiota such as sea bass, fresh salmon, tropical and brown shrimps, cooked and peeled shrimps, European bogue, and yellow croaker (Chinivasagam et al., 1998; Koutsoumanis and Nychas, 1999; Papadopoulos et al., 2003; Taliadourou et al., 2003; Jaffrès et al., 2009; Macé et al., 2012; Zhu et al., 2015; Calliauw et al., 2016).

S. putrefaciens and *S. baltica* spoilage activity is characterized by cabbage, putrid H_2S related odors and in a lesser extent by amine, acid, sour, feet/cheese (Dalgaard, 1995; Chinivasagam et al., 1998; Leroi et al., 1998; Gennari et al., 1999; Macé et al., 2013a, 2014a). In prawn sterile extract, *S. putrefaciens* produced many sulfur related compounds (Table 1.3) (Chinivasagam et al., 1998). In cooked and peeled sterile shrimp, *S. baltica* produced spoilage related volatile compounds which are summarized in Table 1.3 (Macé et al., 2014).

3.4. *Brochothrix thermosphacta*

B. thermosphacta is a psychrotrophic, facultative aerobic, non-motile, non-sporulating, Gram and catalase positive rod shape bacteria (Mamlouk et al., 2012; Odeyemi et al., 2018; Illikoud et al., 2018). It is part of the dominant microbiota of gilt-head sea bream, fresh and smoked salmon and cooked and peeled shrimp (Drosinos and Nychas, 1996; Leroi et al., 1998; Hoz et al., 2000; Taliadourou et al., 2003; Rudi et al., 2004; Mejlholm et al., 2005; Jaffrès et al., 2009; Fall et al., 2010a). In seafood, *B. thermosphacta* has a strong spoilage potential and can produce off-odors such as sour, amine, feet/cheese, sour/fermented, butyric acid, rancid, caramel and butter-like (Leroi et al., 1998; Stohr et al., 2001; Laursen et al., 2006; Jaffrès et al., 2011; Fall et al., 2012; Macé et al., 2013; Silbande et al., 2018b). This caramel/butter smell, characteristic of

B. thermosphacta spoilage, is related to the production of 2,3-butanedione (diacetyl) and 3-hydroxybutanone (acetoin) (Laursen et al., 2006; Macé et al., 2013a). In addition to these volatile organic compounds, *B. thermosphacta* produced in cooked and peeled shrimp various alcohols, aldehydes, ketones summarized in Table 1.3 (Laursen et al., 2006; Jaffrès et al., 2011; Fall et al., 2012).

Table 1.3: List of organic volatile compounds produced by the main seafood spoilage microorganisms (SSOs).

	SSOs / Microorganisms								
Volatile Compounds	**P. phot**	**S. putr**	**S. balt**	**B. ther**	**Pseud**	**S. lique**	**Psychr**	**Pseudalt**	**C. malt**
Alcohols									
Ethanol	x			x	x				
1-propanol				x		x			
2-propanol							x	x	
2-methylpropanol	x			x	x			x	
2-methyl-2-propanol							x		
2-butanol						x			
2-methylbutanol				x		x			x
3-methylbutanol				x					x
3-methyl-2-butanol	x								
Butanediol				x					x
1,2-butanediol								x	
1-pentanol			x						
2-pentanol				x					
1-penten-3-ol			x						x
Cyclopentanol									x
Hexanol			x						
2-hexanol				x					
2-ethyl-1-hexanol					x				
1-octen-3-ol					x				
1-nonanol					x				
Aldehydes									
Acetaldehyde				x		x			x
2-methylpropanal	x			x			x		x
2-methylbutanal	x			x					x
3-methylbutanal	x			x				x	x
Pentanal	x					x			
Hexanal				x					
2-hexenal					x				
Benzaldehyde			x						
Heptanal					x				
Benzenacetaldehyde	x								
Nonanal					x				
Ketones									
Acetone							x	x	
2-propanone			x						x
2-butanone							x		
2,3-butanedione (diacetyl)				x		x			x
3-hydroxybutanone (acetoin)	x		x	x					
2-pentanone				x				x	
2,3-pentanedione									x
2-heptanone			x		x				
2,3-heptanedione				x					
2-octanone					x				
2-nonanone			x		x				
2-decanone			x						
2-undecanone					x				
Decanal					x				
Acids									
Acetic acid	x								
Isobutyric acid			x						

Volatile Compounds	SSOs / Microorganisms								
	P. phot	S. putr	S. balt	B. ther	Pseud	S. lique	Psychr	Pseudalt	C. malt
Isovaleric acid			x						
Amines									
Trimethylamine						x	x	x	
2-formylhistamine							x	x	
Sulfur compounds									
Methanethial		x							
Methanethiol		x				x		x	
Dimethyl sulfide		x						x	
Dimethyl trisulfide		x							
Fluoromethyl methyl disulfide		x							
2-propanethiol		x							
Bis (1-methylethyl) disulfide		x							
Esters									
Ethyl acetate	x			x		x			
Ethyl-2-methylbutyrate					x				
Ethyl isovalerate					x				
Ethyl tiglate					x				
Ethyl octanoate					x				
Aromatic compounds									
Benzene				x					
Dimethylbenzene				x					
Ethers									
Ethylene oxide							x	x	
2,3-dimethyl-oxirane							x	x	
2-ethoxy-propane									
1-methoxy-butane								x	
Furanic compound									
2-methylfuran								x	
References	1	2	3	4, 5, 6	7	5	8	8	4, 5

Microorganisms' abbreviations: P. phot (*P. phospreum*), S. putr (*S. putrefaciens*), S. balt (*S. baltica*), B. ther (*B. thermosphacta*), Pseud (*Pseudomonas*), S. lique (*S. liquefaciens*), Psychr (*Psychrobacter*), Pseudalt (*Pseudoalteromonas*) and C. malt (*C. maltaromaticum*).

References: 1 (Macé et al., 2013b), 2 (Chinivasagam et al., 1998), 3 (Macé et al., 2014), 4 (Laursen et al., 2006), 5 (Jaffrès et al., 2011), 6 (Fall et al., 2012), 7 (Parlapani et al., 2017), 8 (Broekaert et al., 2013b).

3.5. *Pseudomonas*

Pseudomonas is a genus comprising psychrotrophic, aerobic, non-fermentative, oxidase positive, motile Gram-negative bacteria (Odeyemi et al., 2018). *Pseudomonas* species are commonly detected and found dominant in various seafood stored under aerobic condition or MAP containing high level of O_2, such as halibut, lobster, sea bass, sardines, European bogue and sea bream (Gennari et al., 1999; Koutsoumanis and Nychas, 1999; Papadopoulos et al., 2003; Taliadourou et al., 2003; Hovda et al., 2007c; Boziaris et al., 2011; Carrascosa et al., 2014; Parlapani et al., 2014; Bekaert et al., 2015; Boziaris and Parlapani, 2016). As aerobic microorganisms, *Pseudomonas* are often found dominant at the onset of storage, but quickly collapse in favor of other bacteria when storage conditions includes packaging under modified atmosphere with high level of CO_2 or vacuum (Macé et al., 2012; Leroi, 2014; Odeyemi et al., 2018).

Among *Pseudomonas*, *P. fragi*, *P. fluorescens* and *P. putida* are the main spoilage species in seafood (Leroi, 2014; Boziaris and Parlapani, 2016), able to induce characteristic fruity, rotten, putrid and ammoniacal off-odors (Chinivasagam et al., 1998; Gennari et al., 1999; Leroi, 2014; Silbande et al., 2018b). *Pseudomonas* are not capable to produce TMA and H_2S, thus these odors are mainly resulting from the high production of various volatile compounds in presence of O_2, such as ammonia, aldehydes, alcohols, ketones and esters (Table 1.3) (Leroi, 2014). In sterile sea bream juice agar medium stored under air and modified atmosphere, *Pseudomonas*, including *P. fluorescens*, *P. veronii* and *P. fragi*, was found to produce lots of VOCs showed in Table 1.3 (Parlapani et al., 2017).

3.6. *Enterobacteriaceae*

Among the *Enterobacteriaceae* family, *Serratia* and *Hafnia* are the two main genera associated with seafood spoilage (Macé, 2013; Leroi, 2014). As all *Enterobacteriaceae*, these two genera comprise Gram-negative, oxidase negative, non-sporulating, facultative aerobic and motile bacteria.

Different species of *Serratia*, like *S. liquefaciens* and *S. proteamaculans*, were found in the dominant spoilage microbiota of salmon fillets, smoked salmon and trout and cooked and peeled shrimp (Paludan-Müller et al., 1998; Truelstrup Hansen and Huss, 1998; Jørgensen et al., 2000b; Stohr et al., 2001; González-Rodríguez et al., 2002; Joffraud et al., 2006; Jaffrès et al., 2009; Macé et al., 2013a). These two species demonstrated the ability to induce strong amine/urine, pyrrolidine, sulphur, cabbage/garlic/gas off-odors when inoculated in sterile cooked and peeled shrimp, fresh and cold-smoked salmon and tuna (Joffraud et al., 2006; Jaffrès et al., 2011; Macé et al., 2013a; Silbande et al., 2018b). Still in cooked and peeled shrimp *S. liquefaciens* also produce COVs related to spoilage such as 2-methyl-1-butanol, 1-propanol, 2-butanol, acetaldehyde, pentanal, 2,3-butanedione (diacetyl), trimethylamine, methanethiol and ethyl acetate (Table 1.3) (Jaffrès et al., 2011).

Although less detected than *Serratia*, *Hafnia*, especially *H. alvei* and *H. paralvei* can be found in spoiled fresh salmon and trout flesh and cold-smoked salmon (Truelstrup Hansen and Huss, 1998; Jørgensen et al., 2000b; González-Rodríguez et al., 2001; Macé et al., 2012). This bacterium was able to produced sulfur/cabbage, fecal,

pyrrolidine and sour off-odors in sterile fresh salmon and tuna fillets (Macé et al., 2013a; Silbande et al., 2018b).

In addition, *S. liquefaciens* and *H. alvei* were found to be strong biogenic amines producers in cold-smoked salmon, in particular cadaverine (Jørgensen et al., 2000b). These two species are also more globally focused in food industry, since they may be responsible for meat, dairy and brewery products spoilage (Sofos, 2014; Paradh, 2015; Mounier et al., 2017).

3.7. *Psychrobacter*

Members of the genus *Psychrobacter* are Gram-negative, psychrotrophic, non-motile, oxidase positive, aerobic, osmotolerant bacteria belonging the *Moraxellaceae* family (Odeyemi et al., 2018). Several species like *P. immobilis*, *P. cibarius, P. maritimus* and *P. fozii* were isolated from cured-salted cod, mackerel, angler fish, cooked and peeled shrimps and oysters (Bjørkevoll et al., 2003; Mejlholm et al., 2005; Jaffrès et al., 2009; Prapaiwong et al., 2009; Broekaert et al., 2011). *Psychrobacter* was also found to be the main dominant spoilage flora with *Pseudoalteromonas* in lobster and brown shrimp (Broekaert et al., 2013a; Bekaert et al., 2015). *Psychrobacter* spoilage activity seems to be species strain and matrix dependent. Indeed, *P. cibarius* had only a weak spoilage potential when inoculated on sterile peeled brown shrimp (Broekaert et al., 2013b). In the same way, *P. immobilis* also showed a weak spoilage activity in oysters and cooked and peeled shrimp with weak musty and fishy off-odors (Mejlholm et al., 2005; Prapaiwong et al., 2009). In contrast, this species was inoculated in sterile cured-salted cod, it induced strong musty and fishy off-odors, leading to the sensory rejection of the product (Bjørkevoll et al., 2003). A mix of *Psychrobacter* species, including strains belonging to *P. cibarius* and *P. maritimus* produced in sterile brown shrimp few volatile compounds, some of which are related to seafood spoilage, such as 2,3-dimethyl-oxirane, 2-butanone, 2-formylhistamine, 2-methyl-2-propanol, acetaldehyde, acetone, ethylene oxide, isopropylalcohol and trimethylamine (Table 1.3) (Broekaert et al., 2013b).

3.8. *Pseudoalteromonas*

Pseudoalteromonas are aerobic, heterotrophic, halophilic, oxidase and catalase positive, non-glucose fermentative, heat labile, Gram-negative rod shape bacteria (Odeyemi et al., 2018). Members of this genus, especially *P. nigrifaciens*, *P. elyakovii*

and *P. paragorgicola* were detected and isolated from various fishes and seafood such as cod, plaice, common sole, sea bass, gilthead sea bream, atlantic salmon, whiting, mackerel, pangasius, ray, angler fish, blunt snout bream, and brown shrimp (Reynisson et al., 2009; Broekaert et al., 2011, 2013a; Li et al., 2018). This genus was also found as the main dominant microbial group in oysters and lobster (Romero et al., 2002; Bekaert et al., 2015). The spoilage potential of *Pseudoalteromonas* species is not well described. However, according to Broekaert et al. (2013b), *P. nigrifaciens* and *P. elyakovii* can actively participate to seafood spoilage due to their capacity to produce many volatile compounds in large amounts such as sulfur compounds, alcohols and ketones (Table 1.3). Moreover, these two species and more broadly the *Pseudoalteromanas* genus, possess numerous enzymes such as lipases, chitinase, agarase, amylase and protease which can be involved in spoilage phenomena (Broekaert et al., 2013b).

3.9. Lactic acid bacteria (LAB)

Lactic acid bacteria are also commonly detected and isolated from seafood products (Leroi, 2010; Ghanbari et al., 2013). They usually constitute the dominant microbial group of seafood that are processed by salting, smoking, curing, marinating and/or packed under vacuum or athmosphere enriched with CO_2. Among the main genera, *Carnobacterium* (*C. maltaromaticum* and *C. divergens*), *Lactobacillus* (*L. sakei, L. curvatus*), *Leuconostoc* and *Lactococcus* (*L. piscium*) are the most frequently isolated LAB (Matamoros, 2008; Leroi, 2010; Ghanbari et al., 2013; Leroi, 2014). Although they can be found in significant concentration ($10^{7\text{-}8}$ CFU/g) especially in seafood stored under MAP and VP, the lactic acid spoiler status remains ambiguous and seems to be strain and matrix dependent (Leroi, 2010).

3.9.1. Carnobacterium

Carnobacterium are ubiquitous, psychrotrophic, rod shape lactic acid bacteria. This genus comprises 12 species (*C. alterfunditum*, *C. antarticum*, *C. divergens*, *C. funditum*, *C. gallinarum*, *C. iners*, *C. inhibens*, *C. jeotgali, C. maltaromaticum*, *C. mobile*, *C. pleistocenium*, *C. viridans*,) isolated from various cold and temperate environments such as living fish, polar lakes, deep sea sediments, permafrost, soil, compost and refrigerated food commodities (milk and dairy products, seafood and meat products) (Leisner et al., 2007; Afzal et al., 2010; Snauwaert et al., 2013; Zhu et al., 2018). However, *C. maltaromaticum* (formerly *C. piscicola*) and *C. divergens* are

the most frequently isolated species in seafood (Leisner et al., 2007). *Carnobacterium* spp., and in particular *C. maltaromaticum*, is often found among the dominant microbial groups of many products such as cold-smoked salmon (Leroi et al., 1998; Paludan-Müller et al., 1998; González-Rodríguez et al., 2002), cold-smoked trout (González-Rodríguez et al., 2002), raw atlantic salmon (Rudi et al., 2004; Powell and Tamplin, 2012), red drum (Silbande et al., 2018a), cooked and peeled shrimp (Mejlholm et al., 2005; Jaffrès et al., 2009), brown shrimp (Calliauw et al., 2016), salmon gravlax (Leisner et al., 1994), atlantic horse mackerel (Alfaro and Hernandez, 2013) and sea bream (Parlapani et al., 2015b).

Carnobacterium spoiler status is still currently discussed in the literature. *C. maltaromaticum* and *C. divergens* were found to be responsible for the spoilage of both sterile and naturally contaminated cooked and peeled shrimp (Mejlholm et al., 2005; Laursen et al., 2006; Jaffrès et al., 2011; Macé et al., 2014) and sterile raw salmon (Macé et al., 2013a), with strong feet, butter, nutty/malty, sweet/nauseous sour, acid and chlorine smells. On the other hand, some authors did not find any spoilage evidence or only weak organoleptic changes when *C. maltaromaticum* and *C. divergens* were inoculated on cold-smoked salmon, sterile tuna or various fish juices (Leroi et al., 1996, 1998; Paludan-Müller et al., 1998; Duffes et al., 1999a; Nilsson et al., 1999; Stohr et al., 2001; Joffraud et al., 2006; Vescovo et al., 2006; Brillet et al., 2005; Matamoros et al., 2009a; Tahiri et al., 2009; Wiernasz et al., 2017; Silbande et al., 2018b). However, even with weak spoilage potential, these studies almost all reported the presence of malty, rhubarb or feet/banana odors. These sensory notes, typical of *C. maltaromaticum*, are related to the ability of this species to produce 2-methylbutanal and 3-methybutanal from leucine and isoleucine catabolism (Leisner et al., 2007; Afzal et al., 2010). In addition, *C. maltaromaticum* can produce volatile compounds such as 2- /3-methylbutanol, 1-penten-3-ol, cyclopentanol, acetaldehyde, 2-methyl-1-propanal, 2,3-butanedione (diacetyl), 2,3-pentanedione, 2-propanone (Table 1.3) (Joffraud et al., 2001; Afzal et al., 2010; Jaffrès et al., 2011).

Some *Carnobacterium* species, especially *C. divergens*, *C. maltaromaticum* are known to be strong tyramine producers (Leisner et al., 1994; Bover-Cid and Holzapfel, 1999; Masson et al., 1996; Leisner et al., 2007). Both species were able to produce up to 120-260 mg/kg of tyramine in cold-smoked salmon (Duffes et al., 1999a; Brillet et al., 2005). Although the risk is low and concerns especially sensitive individuals,

concentrations superior to 100-800 mg/kg may be the cause of headaches and hypertensive effects (ten Brink et al., 1990; Halász et al., 1994; Visciano et al., 2012).

3.9.2. Lactobacillus sakei

Firstly isolated from sake in 1934, *L. sakei* is commonly found in various fermented and non-fermented food commodities such as sauerkraut, sausages, fresh meat products and seafood (Champomier-Vergès et al., 2001). This results from its metabolic activities and phenotypic traits, including its psychrotrophic nature, that are particularly well adapted to the growth and survival under refrigerated temperatures and modified atmosphere or vacuum packaging (Zagorec and Champomier-Vergès, 2017b).

L. sakei was isolated from the spoilage microbiota of cold-smoked salmon (Leroi et al., 1998; Truelstrup Hansen and Huss, 1998; González-Rodríguez et al., 2002; Olofsson et al., 2007), trout gravlax (González-Rodríguez et al., 2002; Lyhs et al., 2002) and maatjes herring (Lyhs and Björkroth, 2008). As for *Carnobacterium*, its spoilage potential seems to be strain dependent. In fresh and cold-smoked salmon, this species induced strong amine and sulfur/cabbage off-odors (Truelstrup Hansen, 1995; Nilsson et al., 1999; Stohr et al., 2001; Joffraud et al., 2006; Leroi et al., 2015). However, in the same matrix, Weiss and Hammes (2006) did not find spoilage evidence. In the same way, when inoculated into cooked tuna, rainbow trout fillets and smoked salmon juice, *L. sakei* did not induce any sensory degradation (Leroi et al., 1998; Truelstrup Hansen and Huss, 1998; Aras et al., 2005; Katikou et al., 2007; Podeur, 2014).

Furthermore, the ambiguous spoiler status of *C. maltaromaticum* and *L. sakei*, but also more broadly of the LAB, is reinforced by the fact that these microorganisms are frequently involved in organoleptic defects in other foodstuff such as meat based products (Zagorec and Champomier-Vergès, 2017a).

4. Seafood pathogenic organisms

Seafood can also be an important vector of foodborne diseases. It was estimated that 10 to 20% of world foodborne diseases is due to fish and seafood consumption. This number may vary according to the health surveillance system quality implemented in countries, but also according to seafood consumption level and consumers' habits (fish and shellfish eaten raw represent a higher risk) (Pilet and Leroi, 2011; Le Fur et al., 2013). A country with high per capita consumption, such as Malaysia (>50 kg/year/capita), will inevitably have a higher foodborne incidence than a country with a low consumption, as for example Bolivia (<5 kg/year/capita) (values from FAO, 2018c). Among seafood, fish is responsible for more outbreaks than shellfish. However, for the latter, the number of cases per outbreak is often much higher (Huss et al., 2000). In finfish, outbreaks are more likely from direct or indirect bacterial origin, the main cause of which is due to histamine, a biogenic amine, which is responsible for 30 to 40% of fish poisoning. In bivalve filter feeding molluscs, viruses are accounting for more than 50% of outbreak cases (Huss et al., 2000; Pilet and Leroi, 2011; Le Fur et al., 2013). Microalgae toxins (saxitoxin, brevetoxin, domoic acid and okadaic acid) and some *Vibrio* species (*V. cholerae* and *V. vulnificus*) can also be responsible for outbreak from shellfish consumption (Huss et al., 2000).

Bacterial seafood pathogens can be distinguished according to their origin. Some bacteria can be naturally present in the marine environment or be a part of the animal indigenous microbiota, such as *V. vulnificus*, *V. parahaemolyticus*, *V. cholerae*, *L. monocytogenes*, *Clostridium botulinum* and *Aeromonas hydrophila*, but also histamine producing bacteria such as *H. alvei*, *P. phosphoreum*, *Morganella morganii*, *Morganella psychrotolerans* and *Raoultella planticola*. As for all food commodities, seafood products can also be contaminated by exogenous pathogens during processing. Among these bacteria, we can found *Staphylococcus aureus*, *Salmonella* spp., *Shigella* spp., *Clostridium perfringens*, *Bacillus cereus*, *Yersinia enterocolitica* and enterohaemorrhagic *E. coli*. Some of these microorganisms, notably salmonellas, can also be present in coastal and estuarine polluted marine water (Huss, 1997; Huss et al., 2000; Pilet and Leroi, 2011; Le Fur et al., 2013).

In lightly processed and preserved seafood, such as smoked products, fish gravlax, marinated fish, sea salads and insufficiently cooked products, *L. monocytogenes* represent the main pathogenic risk (Pilet and Leroi, 2011; Jami et al., 2014; Løvdal,

2015). *L. monocytogenes* is a psychrotrophic, non-sporeforming, Gram-positive bacterium with a high tolerance to NaCl concentration (up to 28% w/v), resistant to freezing temperatures and able to grow in a pH range of 4.3 to 9.6. *L. monocytogenes* infection represent an important public health concern due to the high mortality rate (20 to 30%) in immunocompromised individuals, children and pregnant women. Listeriosis can be associated to meningitis, septicemia, pneumonia, gastroenteritis and abortion (Jami et al., 2014; Løvdal, 2015). By being able to survive to smoking and salting process, to grow well both aerobically and anaerobically at refrigerated temperatures, *L. monocytogenes* prevalence in lightly processed fish products can be very high. *L. monocytogenes* prevalence in smoked fishes was estimated to be comprised between 0 up to 80.3% and between 0 to 38.4% in slightly preserved seafood products (including gravlax, seafood salad and fish roe) (Jami et al., 2014). Through a meta-analysis, Løvdal, (2015) estimated at 9.8% the prevalence of *L. monocytogenes* in cold-smoked salmon retail. *L. monocytogenes* concentration in ready-to-eat food commodities is currently under regulation (Regulation (EC) n°2073/2005, 2005) and must not exceed the threshold value of 100 CFU/g.

Often omitted or underestimated, seafood, especially fish products can be vectors of parasitic zoonotic diseases. In the recent years, these fishborne parasitoses had gained in incidence due to diagnosis and surveillance system improvement, but also due to the world-wide increased consumption of minimally processed fish, eaten raw such as sushis, sashimis, ceviches and carpaccios, or insufficiently cooked products (Lima dos Santos and Howgate, 2011, Løvdal, 2015). Although there is a large number of parasites able to infect fish, only few are responsible for human illnesses. Among these parasites, species belonging to the genera *Clonorchis* and *Opisthorchis* (Opisthorchiidae family, liver flukes, trematodes), *Diphyllobothrium* (especially *D. latum*, a cestode) and *Anisakis* (especially A. simplex, a nematode) are the main causal agents of fishborne parasitoses. Although there are some rare cases of anikiasis and cestodiasis infections described in salmon, trout and cod aquaculture, the main risk comes from wild fish consumption (Lima dos Santos and Howgate, 2011). Moreover, these species of parasites have until now, only been very sporadically associated with lightly processed and preserved seafood like cold-smoked fishes. However, a freezing step of 24h up to 7 days is sufficient to rule out the parasitic health hazard from a fish product (Løvdal, (2015).

5. Fish freshness assessment: quality indexes

There is a significant number of methods to assess seafood products freshness based on organoleptic, biochemical, physical and microbiological measures (Olafsdóttir et al., 1997; Huss, 1999; Leduc, 2011; Macé, 2013; Dehaut, 2014). However, no reliable or universal fish spoilage markers currently exist. Thus, a multiphasic approach, based on a combination of several markers, is often required to assess fish quality.

5.1. Organoleptic analyses

Sensory analyses are the most used and instinctive methods when it comes to assess food freshness. The state of freshness is evaluated through several organoleptic criteria such as product aspect, odors, texture and flavor. For fishery and seafood products, three scoring schemes are mainly used: the Quality Index Method or QIM, the Torry Sensory Assessment scheme and the European E-A-B scheme (Olafsdóttir et al., 1997; Huss, 1999).

5.1.1. European E-A-B scheme

Originally created in 1970 (Howgate et al., 1992), the European E-A-B scheme or Council Regulation N° 2046/96 (1996) is still today the most used method for seafood freshness assessment in Europe. The product state of freshness is classified according to 4 categories (E, A, B and C) corresponding to the different spoilage stage. This scoring system is based on visual and olfactory description of certain criteria such as the aspect of the skin, skin mucus, eyes, gills, peritoneum (for gutted fishes) or the smell of abdominal cavity. An E (for Extra) grade corresponds to the highest level of quality, while a product with a C grade is considered as non-consumable.

An example of the E-A-B scheme applied to whitefish figures in Table 1.4. Thus far, this notation scheme only concerned a small number of fishes (38), belonging to the group of whitefish, bluefish and *Selachii*, as well as a small number of crustaceans and cephalopods. For instance, no salmon species are among the list. In addition, this assessment method does not make any distinctions between species from a same fish category. Moreover, the freshness category attribution to a seafood product can also be relatively tricky when several criteria belong to different classes (Huss, 1999).

Table 1.4: Example of E-A-B scheme applied to whitefish (Council Regulation (CE) n° 2406/96).

	Criteria			
	Freshness category			
	Extra	**A**	**B**	**Not admitted**
Skin	Bright, iridescent pigment (save for redfish) or opalescent; no discolouration	Pigmentation bright but not lustrous	Pigmentation in the process of becoming discoloured and dull	Dull pigmentation
Skin mucus	Aqueous, transparent	Slightly cloudy	Milky	Yellowish grey, opaque mucus
Eye	Convex (bulging; black, bright pupil; transparent cornea	Convex and slightly sunken; black dull pupil; slightly opalescent cornea	Flat; opalescent cornea; opaque pupil	Concave in the centre; grey pupil; milky cornea
Gills	Bright colour; no mucus	Less coloured; transparent mucus	Brown/grey becoming discoloured; thick, opaque mucus	Yellowish; milky mucus
Peritoneum (in gutted fish)	Smooth; Bright; difficult to detach from flesh	Slightly dull; can be detached from flesh	Speckled; comes away easily from flesh	Does not stick
Smell of gills and abdominal cavity – except plaice	Seaweedy	No smell of seaweed; neutral smell	Fermented; slightly sour	Sour
– Plaice	Fresh oily; peppery; earthy smell	Oily; seaweedy or slightly sweetish	Oily; fermented; stale, slightly rancid	Sour
Flesh	Firm and elastic; smooth surface	Less elastic	Slightly soft (flaccid), less elastic; waxy (velvety) and dull surface	Soft (flaccid); scales easily detached from skin, surface rather wrinkled

5.1.2. Quality Index Method

Developed in Australia by Bremner (1985), the QIM method is a raw fish defect scoring system (Huss, 1999). Each quality parameter is associated with a score according to its spoilage level. These scores can range from 0 to 1, 0 to 2 or 0 to 3 depending on their importance in fish spoilage. The more a parameter is altered, the higher its score will be. When all parameters scores are summed, a global sensory score or QI (Quality Index) is obtained. The higher the QI is, the less fresh the fish is. The QIM scheme sheet for farmed salmon is shown in Table 1.5. The QIM method has been developed for many commonly consumed seafood species in Europe, whose salmon, cod, sardines, mackerel or sea bream are just few examples (Sveinsdóttir et al., 2002; Martinsdóttir et al., 2003; Barbosa and Vaz-Pires, 2004; Sant'Ana et al., 2011). For each seafood product, through calibration curves, a linear mathematical relation has been defined between QI and the storage time. Thus, it is possible to estimate the product remaining shelf-life depending on its QI score (Huss, 1999; Martinsdóttir et al., 2003). An example for salmon and plaice is shown in Figure 1.7.

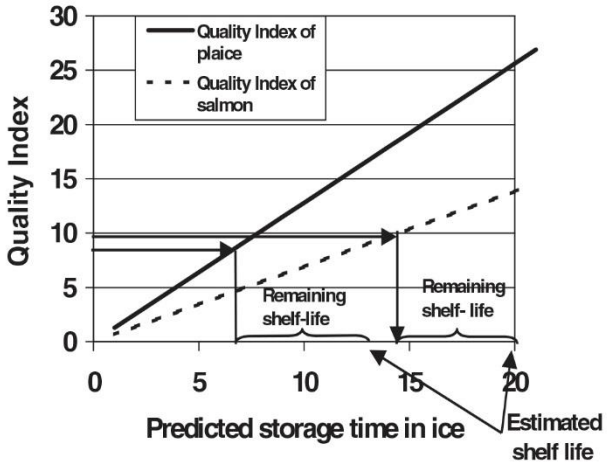

Figure 1.7: Illustration of the linear relation between QI and storage time, applied to salmon and plaice (Martinsdóttir et al., 2003).

However, this relationship is relatively limited since it was established only for products stored under ice between 0 to 4 °C. At the initiative of the project QIM Eurofish, a smartphone application "How Fresh is Your Fish?" is available since 2011. This app, aimed for general public (from retailers to consumers), regroups a QIM scheme for 14 of the most consumed seafood products in Europe.

Table 1.5: QIM scheme for farmed salmon (Sveinsdóttir et al., 2002).

Quality parameters		Description	Points
Skin:	Color/appearance	Pearly-shiny all over the skin	0
		The skin is less pearl-shiny	1
		The fish is yellowish, mainly near the abdomen	2
	Mucus	Clear, not clotted	0
		Milky, clotted	1
		Yellow and clotted	2
	Odor	Fresh seaweed, neutral	0
		Cucumber, metal, hay	1
		Sour, dish cloth	2
		Rotten	3
	Texture	In rigor	0
		Finger mark disappears rapidly	1
		Finger leaves mark over 3 s	2
Eyes:	Pupils	Clear and black, metal shiny	0
		Dark gray	1
		Mat, gray	2
	Form	Convex	0
		Flat	1
		Sunken	2
Gills:	Color/appearance	Red/dark brown	0
		Pale red, pink/light brown	1
		Grey-brown, brown, gray, green	2
	Mucus	Transparent	0
		Milky, clotted	1
		Brown, clotted	2
	Odor	Fresh, seaweed	0
		Metal, cucumber	1
		Sour, moldy	2
		Rotten	3
Abdomen:	Blood in abdomen	Blood red/not present	0
		Blood more brown, yellowish	1
	Odor	Neutral	0
		Cucumber, melon	1
		Sour, fermenting	2
		Rotten/rotten cabbage	3
Maximum sum (Quality index):			24

5.1.3. Torry sensory assessment scheme

Originally developed in UK and completed over the years, the Torry sensory scheme is a freshness scoring system based on cooked fish (Shewan et al., 1953). Both smell and flavor are assessed and scored on a scale from 3 to 10. A high score is related to a high state of freshness. A product with a mean score of 5.5 is almost considered as not fit for human consumption (Ifremer, 2009). An example of the Torry scoring scheme applied to cod figures in Table 1.6.

Table 1.6: Torry scoring sheet for cod freshness assessment (Archer, 2010).

Score	Odour	Flavour	Texture, mouth feel and appearance
10	Initially weak odour of sweet, boiled milk, starchy followed by strengthening of these odours	Watery, metallic, starchy. Initially no sweetness but meaty flavours with sligh sweetness may develop	Dry, crumbly with short tough fibres
9	Shellfish, seaweed, boiled meat, raw green plant	Sweet, meaty, creamy, green plant	Succulent, fibrous. Initially firm going softer with storage.
8	Loss of odour, neutral	Sweet and characteristic flavours but reduced in intensity	Appearance originally white and opaque going yellowish and waxy on storage
7	Woodshavings, woodsap, vanillin	Neutral	
6	Condensed milk, caramel, toffee-like	Insipid	
5	Milk jug odours, boiled potato, boiled clothes like	Slight sourness, trace of off flavours	
4	Lactic acid, sour milk, “byre-like”	Slight bitterness, sour off flavours	
3	Lower fatty acids (e.g. acetic or butyric acids), composted grass, soapy, turnip, tallowy		

5.1.4. Sensory evaluation with panelist

Although the QIM and European E-A-B schemes are standardized methods commonly used at the fishmonger level, they are not applicable to all seafood products. Indeed, these two methods are only used for whole fresh fish. However, at the consumer level, products are mainly presented as fillets or as already lightly processed ready-to-eat products. In these specific cases, other sensory analyses methods, such as profile or triangular tests can be used to assess seafood quality (Huss, 1999).

The sensory profile test, which is commonly used at the Ifremer laboratory EM^{3}B ("Ecosystème Microbien et Molécules Marines pour les Biotechnologies") (Stohr et al., 2001; Joffraud et al., 2006; Jaffrès et al., 2011; Fall et al., 2010a; Macé et al., 2013a, 2014; Leroi et al., 2015; Saraoui et al., 2017; Wiernasz et al., 2017), allows to describe exhaustively a product according to a list of sensory descriptors specific to spoilage and/or freshness. For instance, in the study conducted by Macé et al. (2013a), the spoilage of raw sterile salmon inoculated with bacteria was assessed by an intern panel of trained judges. All sensory sessions were performed in individual partitioned booths, according to the procedure NF V-09-105 (ISO, 2010), equipped with a computerized system for data acquisition. Panelists first had to assess the overall spoilage level and then to characterized it with appropriate odor descriptors such as fatty fish, butter/caramel, acid/vinegar, sour/fermented, amine, pyrrolidine, feet/cheese etc. Both spoilage level and descriptors were scored on a continuous scale from 0 to 10 according to the intensity: 0 meaning low intensity, while 10 was for a high intensity. In addition to the smell, for the sensory characterization of cold-smoked salmon products from various European retailers, Cardinal et al. (2004) also scored the appearance (fatty aspect, drip loss, color of the product), the texture (firmness, crunchy texture, melting texture, fatty film, pasty texture) and the flavor (smoke, cold ashes, bacon, raw fish, rancid, salty, sour, amine, rubber).

Despite their completeness for seafood quality assessment, sensory methods possess several major disadvantages. They require the use of expert and regularly trained panel of judges, are relatively time and cost consuming and difficult to perform routinely (Dehaut, 2014).

5.2. Chemical and Biochemical indexes

Some compounds resulting from fish and seafood tissues degradation, such as volatile organic compounds (VOCs), nucleotides catabolic derivatives, biogenic amines and products of lipids oxidation can be good spoilage indicators. Moreover, by being less subjective than sensory analyses, chemical and biochemical methods to assess seafood quality can allow to establish quantitative norms and threshold of acceptance (Huss, 1999).

5.2.1. Total volatile basic nitrogen (TVBN)

Total volatile basic nitrogen content (TVBN) is a criterion often used for seafood spoilage assessment. TVBN is mainly composed by ammonia (NH_3), dimethylamine (DMA), trimethylamine (TMA), as well as other volatile amines with low molecular weight (Huss, 1999; Ifremer, 2008a). TMA and DMA are derived from the degradation of trimethylamine-N-oxide (TMAO), a molecule naturally present in most sea fish (Timm and Jørgensen, 2002) and seafood (Ifremer, 2008a). TMAO is reduced to TMA by spoilage bacteria such as *P. phosphoreum*, *S. putrefaciens*, *S. liquefaciens* and some *Vibrio* species through the action of the TMAO reductase (Gram and Huss, 1996; Huss, 1999; Stohr et al., 2001). DMA is produced by autolysis during refrigerated or frozen storage via the TMAO demethylase, an enzyme present in the fish flesh. Ammonia is formed by proteins, peptides and amino acids deamination through the action of fish flesh enzymes or bacterial degradations. As mainly resulting from the degradation of proteins and nitrogenous compounds by bacteria or seafood endogenous enzymes, TVBN can be a good index for spoilage assessment. In addition, these volatile compounds are associated with characteristic amine-like unpleasant smell (Huss, 1999; Ifremer, 2008a).

Furthermore, the Council Regulation (CE) n° 2074/2005 (2005) provides a regulation for the TVBN content for certain fish species, only if any doubts were raised concerning the freshness of the product after organoleptic assessment. For instance, the regulatory limit for fresh *Salmo salar* is set at 35 mg of nitrogen/100 g of fish flesh.

However, TVBN content as spoilage indicator has some disadvantages. It is not suitable for all fish species, including fishes belonging to the *Scombroidei* order (e.g. tuna, swordfish and bonito) and for the sea bass (Castro et al., 2006), but also for canned, cooked, or processed products (Ifremer, 2008b). The TVBN measurement is also bad indicator for whole fish or fillet stored on ice or liquid ice, due to the water leaching activity (Ifremer, 2008b). TMA and DMA are sometimes measured separately from TVBN as additional spoilage indicator (Timm and Jørgensen, 2002). However, they possess the same disadvantages than TVBN. Moreover, TMA and DMA content are not a reliable measures for some fatty fish, such as *Salmonidae*, because of their low endogenous TMAO content (Macé, 2013). In many cases, it is not advisable to use TVBN content as the only index for seafood spoilage interpretation.

5.2.2. Nucleotides derivatives

Following death, adenosine triphosphate (ATP) is degraded by fish endogenous and microbial exogenous enzymes into different successive catabolites: adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine and hypoxanthine as described in Figure 1.8 (Olafsdóttir et al., 1997; Huss, 1999; Howgate, 2006).

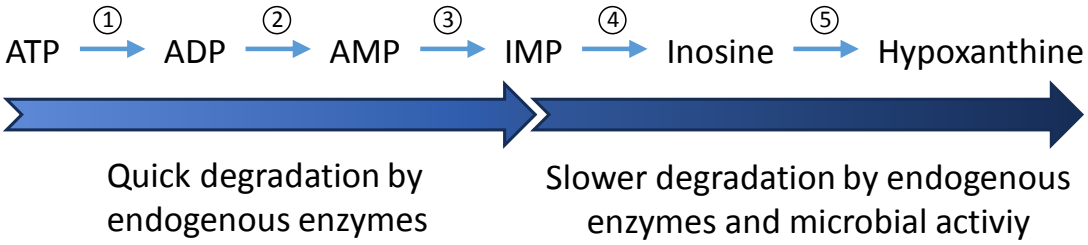

Figure 1.8: ATP degradation and successive transformation into hypoxanthine. 1: ATPase, 2: Myokinase, 3: AMP deaminase, 4: IMP phosphohydrolase, 5: Inosine nucleosidase. Adapted from Ifremer, 2009 and completed from Huss (1999) and Howgate (2006).

These nucleotides derivatives are excellent spoilage markers for fresh fish, thus Saito et al. (1959) developed the K value to estimate the freshness. K value is calculated as following, as the ratio between ATP nucleotide derivatives concentration:

$$K(\%) = \frac{[\text{Inosine}]+[\text{Hypoxanthine}]}{[\text{ATP}]+[\text{ADP}]+[\text{AMP}]+[\text{IMP}]+[\text{Inosine}]+[\text{Hypoxanthine}]} \times 100$$

However, as ATP, ADP and AMP are almost completely degraded into IMP in the first 24 hours, it was suggested to simplify this ratio by another (K') (Howgate, 2006):

$$K'(\%) = \frac{[\text{Inosine}]+[\text{Hypoxanthine}]}{[\text{IMP}]+[\text{Inosine}]+[\text{Hypoxanthine}]} \times 100$$

These two ratios evolve almost linearly during storage time and the higher their value is, the more the product is spoiled. However, although accurate, the method to determine K and K' values are both time and cost consuming. In addition, these two indexes varies according to the fish species and can be influenced by the fishing method, the fish state of fatigue and stress and by the slaughter and storage conditions. As for the TVBN content, a leaching activity can be observed when fishes are stored on ice (Howgate, 2006; Ifremer, 2009).

5.2.3. Lipid oxidation index

Polyunsaturated lipids, which are found in significant amounts in fish, are very sensitive to oxidation. Lipids oxidation starts rapidly in fish tissues after its capture and slaughter, resulting in the formation of primary and then secondary compounds. Primary compounds correspond to hydroperoxide lipids, which can be measured by titric or spectrophotometric methods, giving the peroxide value (PV). Primary oxidation products being flavor- and odorless, the PV is not directly related to product spoilage. However, this value indicates the potential formation of secondary oxidation compounds, which are strongly implicated in spoilage. These secondary products include aldehydes, ketones and short chain fatty acids, among many of which has unpleasant rancid and flowery smell and flavor of oxidized lipids. Due to their volatile properties, secondary products can be measured by gas chromatography (GC). Aldehydes react readily with *p*-anisidine and thiobarbituric acid, forming colored products. By spectrophotometrically measuring these products, the *p*-anisidine and TBARS (Thiobarbituric Acid Reactive substances) values can be obtained (Olafsdóttir et al., 1997; Huss, 1999).

5.2.4. Biogenic amines

Biogenic amines are nitrogenous compounds with low molecular weight, possessing biological activity. In food, they are essentially produced by free amino acids microbial decarboxylation (ten Brink et al., 1990; Halász et al., 1994; Santos, 1996). In seafood, the most commonly found biogenic amines are histamine, tyramine, putrescine, cadaverine, tryptamine, 2-phenylethylamine, spermine, spermidine and agmatine (Visciano et al., 2012). Biogenic amines may be of interest as quality deterioration markers, since they reflect the spoilage microbiota activity in food. In addition, tyramine and histamine may be vectors of human health issues, if ingested in high quantity (ten Brink et al., 1990; Halász et al., 1994; Visciano et al., 2012). In food, concentrations superior to 100-800 mg/kg of tyramine may cause headaches and hypertensive effects in individual deficient in monoamine oxidase (ten Brink et al., 1990; Halász et al., 1994). More serious, histamine poisoning, also referred as scombroid poisoning, can occur at concentrations from 100 mg/kg and may rapidly cause a wide range of symptoms such as headache, oral numbness, dizziness, palpitations, drop in blood pressure, difficulty in swallowing, rash, flushing, swelling nausea, vomiting and diarrhea (ten Brink et al., 1990; Halász et al., 1994; Visciano et al., 2012). Therefore, their quantification also

allows ensuring food commodities safety. In addition, histamine content is regulated, in particular for fish species with high histidine content (species belonging to *Scombridae*, *Clupeidae*, *Coryphaenidae*, *Pomatomidae* and *Scomberesocidae* families) (Regulation (EC) n°2073/2005, 2005).

Several authors developed seafood degradation indexes based on biogenic amines concentration. Mietz and Karmas (1977) proposed a quality index, which has been applied to canned tuna and calculated as follow:

$$\text{Quality index} = \frac{[\text{Histamine}] + [\text{Putrescine}] + [\text{Cadaverine}]}{1 + [\text{Spermidine}] + [\text{Spermine}]}$$

Duflos et al. (1999) also proposed a quality index (AI') which they applied on plaice (*Pleuronectes platessa*) and whiting (*Merlangus merlangus*) for spoilage assessment:

$$\text{AI}'(\%) = \frac{[\text{Hist}]+[\text{Put}]+[\text{Cad}]}{[\text{Hist}]+[\text{Put}]+[\text{Cad}]+[\text{Spd}]+[\text{Spm}]+[\text{Tyra}]+[\text{Tryp}]+[\text{Meth}]} \times 100$$

With: histamine (Hist), putrescine (Put), cadaverine (Cad), spermidine (Spd), spermine (Spm), tyramine (Tyra), tryptamine (Tryp), methylamine (Meth).

Although some authors found a good correlation between biogenic amines content and spoilage in seafood (Mietz and Karmas, 1977; Duflos et al., 1999; Jørgensen et al., 2000a; Dondero et al., 2004), caution must be taken. A product with low biogenic amines content cannot be necessarily considered as fresh. Hoz et al. (2000), Emborg et al. (2002) and Brillet et al. (2005) found very low biogenic amines content after 4 weeks of storage in spoiled salmon fillets and cold-smoked salmon. Biogenic amines formation in food relies indeed on the microbiota present in the product. For instance in seafood, histamine is mainly produced by Gram-negative bacteria such as *H. alvei*, *Photobacterium* (*P. phosphoreum, P. damselae*, and *P. leognathi*), *Morganella morganii*, *Morganella psychrotolerans*, *Klebsiella* (*K. oxytoca*, *K. cloacae*), *Enterobacter* (*E. freundii*, *E. aerogenes*), *Acinetobacter baumanii*, *Proteus* (*P. mirabilis*, *P. penneri* and *P. vulgaris*), *Pseudomonas fluorescens*, *Raoultella* (*R. ornithinolytica*, *R. planticola*), *Serratia* (*S. fonticola*, *S. marcescens*) and *Vibrio* (*V. fischeri* and *V. harveyi*) (Visciano et al., 2012; Podeur, 2014). Another limiting factor regarding the use of biogenic amines as spoilage indicator in seafood is that the free amino acids composition can vary a lot from a species to another (Leduc, 2011; Dehaut, 2014).

5.2.5. Volatile organic compounds (VOCs) quantification

Newly caught marine fish contains low levels of volatile compounds and is nearly odorless (Olafsdóttir and Jónsdóttir, 2009). Resulting from microbial metabolism (SSO) and oxidation, but also from fish flesh endogenous enzyme activity, volatiles compounds such as aldehydes, ketones, alcohols, organic acids, esters and sulfur compounds can be good spoilage markers (Jørgensen et al., 2001; Olafsdóttir et al., 2005; Varlet et al., 2006, 2007; Jónsdóttir et al., 2008; Olafsdóttir and Jónsdóttir, 2009). Their concentration increases during storage and for some, are related to fish specific spoiling off-odors (Olafsdóttir and Jónsdóttir, 2009). For instance, aldehydes, compounds mainly deriving from lipids oxidation by microorganisms, actively participate in the characteristic rancid, cooked/boiled potatoes, fatty, floral, grassy odors of spoiled fish (Jørgensen et al., 2001; Varlet et al., 2007; Jónsdóttir et al., 2008; Olafsdóttir and Jónsdóttir, 2009). For examples of VOCs production associated with spoiling bacteria see Table 1.3 (Part 3.).

Despite many studies on fish and seafood volatilome, identify volatile compounds as universal spoilage markers remains a difficult task. Their presence can vary according to the fish species, the storage condition, the extraction method and the microbial population present in the product (Olafsdóttir and Jónsdóttir, 2009; Leduc, 2011). In addition, their quantification with methods such as HS-SPME-GC/MS (Headspace-solid-phase microextraction coupled with gas chromatography/mass spectrometry) is time and cost consuming and difficult to perform routinely.

5.3. Physical indexes

These methods for seafood freshness assessment are based on fish physical properties modifications after slaughtering. They are fast and routinely useable. However, they are applied mainly to whole fish or fillets. Moreover, an additional difficulty lies in the fact that in a same fish or fillet, the physical properties can be heterogeneous.

5.3.1. Texture measurement

During spoilage, the fish flesh relaxes and becomes soft. Several methods, involving texture meters, have been developed to measure products rheological changes during spoilage (Leduc, 2011; Dehaut, 2014; Hassoun and Karoui, 2017). Different tests exist when it comes to assess food texture, as for example the Kramer or Warner-Bratzler tests, based on compression and shearing force measurement when the product is crossed by two metallic blades; the penetration puncture test (product is pierced with a penetrometer), etc… (Hassoun and Karoui, 2017). Nevertheless, many parameters influence the fish muscle texture, such as the species, the animal biological and physiological status, as well as the capture, slaughtering and storage condition (Dehaut, 2014).

5.3.2. Electrical conductivity measurement

Dielectric properties of fish skin and muscle change during tissues degradation. During spoilage, the cells are lysed and release their cytoplasmic content, rich in electrolytes, inducing a modification of the muscle and skin electrical property (Oehlenschläger, 2003). Thus, the electric conductivity of tissues increases when the freshness decreases. Several measuring instruments have been developed such as the Torry meter, the Fischtester VI and the RT-Freshtester (Oehlenschläger, 2003; Leduc, 2011; Dehaut, 2014; Hassoun and Karoui, 2017). However, freshness assessment using electrical conductivity measurement are not suitable for fatty fish, fish washed with sea water or frozen-thawed products (Duflos et al., 2002; Leduc, 2011; Dehaut, 2014; Hassoun and Karoui, 2017).

5.4. Microbiological indexes

As we saw, the microbial activity is the main limiting factor for the shelf-life of fish and seafood with all consequences described above. For a long time the total flora (Total viable count or TVC), often referring to aerobic mesophilic microorganisms, was used as quality index for seafood acceptability in many standards and guidelines (Olafsdóttir et al., 1997; Huss, 1999). Total flora were usually enumerated on Plate Count Agar medium (PCA) and incubated several days at 30° C in aerobic condition. However, most of the time, total flora is not a good index to monitor seafood freshness. Indeed, total flora can be in very high number (10^{8-9} CFU/g) several days before the sensory rejection (Gram and Huss, 1996; Huss, 1999). Thus, the total flora count, as

acceptability index for fish products was removed with the publication of the Commission Regulation (EC) n°2073/2005 (Ifremer, 2009).

More than the total flora, it is then necessary to target more specifically seafood specific spoilage organisms (SSOs), such as *P. phosphoreum*, *S. putrefaciens*, *Pseudomonas* spp., *H. alvei*, *S. liquefaciens* and *B. thermosphacta* (Gram and Huss, 1996; Gram and Dalgaard, 2002). For instance, models to predict the shelf-life of seabream and cod respectively stored under aerobic condition and MAP, has been developed by monitoring *B. thermosphacta*, *Pseudomonas* spp., *S. putrefaciens* and *P. phosphoreum* growth (Dalgaard et al., 1997; Koutsoumanis and Nychas, 2000; Koutsoumanis, 2001; Gram and Dalgaard, 2002). Nevertheless few SSOs, are specifically enumerable by cultural methods: *B. thermosphacta* with Streptomycin-Thallous Acetate-Actidione medium (STAA) (Gardner, 1985) and *P. phosphoreum* with a Malthus conductance method developed by Dalgaard et al. (1996). H_2S-producing bacteria (including *S. putrefaciens*) can be enumerated on Iron agar medium (Huss, 1999).

Nowadays, the development of culture-independent tools such as q-PCR has considerably enhanced and shortened the time response for SSOs enumeration. From one to several day with classical cultural methods, *P. phosphoreum* (Macé et al., 2013b), *Pseudomonas* spp. (Reynisson et al., 2008) and *B. thermosphacta* (Mamlouk et al., 2012) are currently detectable and quantifiable in few hours in seafood. This technique also allows to target other spoilage markers such as the gene *torA* to assess seafood quality. Indeed, found in many SSOs‘ genome (*Vibrio*, *Shewanella* spp., *P. phosphoreum*), this gene is coding for the TMAO reductase, the microbial enzyme responsible for TMAO reduction into TMA (Duflos et al., 2010; Dehaut et al., 2016).

6. Seafood safety and quality control through hurdle technologies

As mentioned before, seafood are very fragile food commodities with a short shelf-life not exceeding 1-2 weeks for fresh products and 3-4 weeks for lightly processed and preserved ones. Their quick sensory degradation is mainly due to SSOs and they can also be vectors for food-borne diseases through the presence of pathogenic microorganisms. Consequently, with seafood increased popularity and consumption, the development of innovative preservative techniques, to reduce pathogenic risk and to limit the growth of spoilage organisms, thus participating to minimize food waste, represents a crucial challenge for the food industry.

Traditional preservation methods usually combined different technologies such as cooling, salting, drying, smoking and cooking. This concept, described as hurdle technologies, includes new promising techniques that have emerged in the last 3 decades in seafood industry. Among these techniques, we can mention the use of modified atmosphere and vacuum packaging, superchilling, chitosans, phages and biopreservation. Moreover non-thermal inactivation technologies such as high pressure processing, irradiation and ozone have also been investigate to improve seafood quality and safety (Devlieghere et al., 2004; Cortesi et al., 2009; Sampels, 2015; Ronholm et al., 2016; Odeyemi et al., 2018; Olatunde and Benjakul, 2018). Action mechanisms of some techniques cited above on bacterial cells are illustrated in Figure 1.9.

6.1. Salting, smoking, drying and fermentation processes

Used for centuries, even for millennium in the case of fermentation and drying process, these methods are the most ancient and traditional practices to ensure food preservation and availability in time. The limitation of microorganisms growth by salting, drying and smoking processes is essentially resulting from the reduction of the water activity and content in food. In addition, during the smoking process, phenolic compounds, issued from wood combustion and known to possess antimicrobial activity (Maqsood et al., 2013), add another hurdle to the microbial growth (Leroi et al., 2000; Giménez and Dalgaard, 2004; Porsby et al., 2008; Hwang, 2009). In the case of fermentation, the quick pH drop due to the production of organic acids mainly ensures the foodstuff microbiological stability over time (Ross et al., 2002).

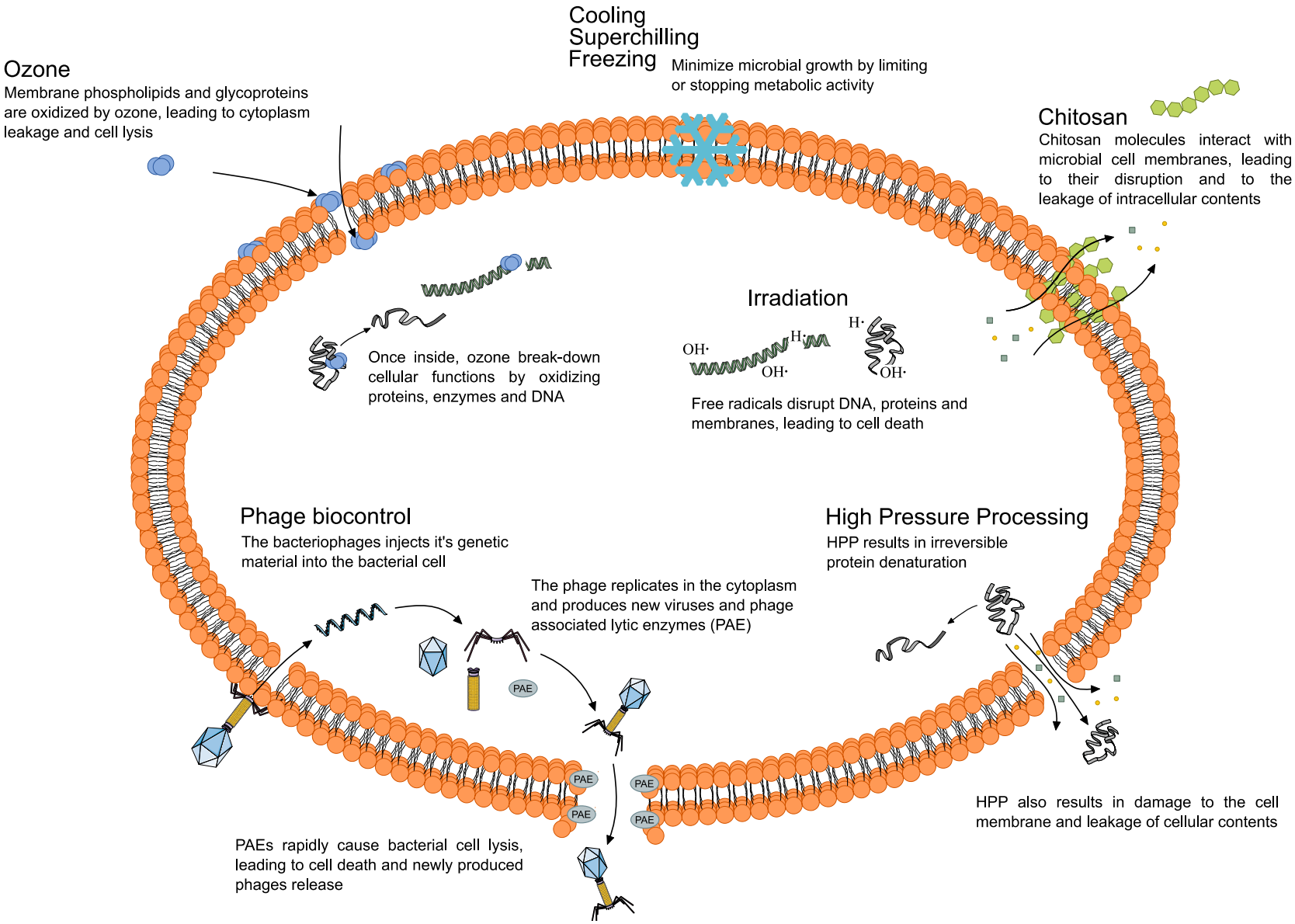

Figure 1.9: Mechanisms of action of technologies used for food safety and quality control. Inspired from Ronholm et al. (2016).

However, in any case, these preservative methods completely and irreversebly modify food organoleptic properties (odors, flavors and texture). Currently smoking and fermentation are more applied in the food industry for their sensory impact than for food preservation itself.

6.2. Cooling

Among the preservative methods, cooling or refrigeration is the first technique that comes to mind when it comes to extend sanitary, nutritionnal and organoleptic properties of food. However, it does not eliminate microorganisms or inactivate enzymatic activity. Most of the biochemical spoilage processes are just slowed down at lower temperature (Figure 1.9). Psychrotrophic bacteria present in seafood products are indeed able to grow and induce spoilage relatively fast under chilled conditions (Sampels, 2015).

6.3. Freezing

Freezing is an effective way to preserve fish and seafood over a long term period, which can last from one month up to more than one year (Burgaard, 2010; Sampels, 2015). It minimizes the microbial growth, enzymatic activity and preserves the product nutritional qualities much more effectively than storage under chilled temperatures (Figure 1.9) (Sampels, 2015). In addition, *P. phosphoreum*, a major spoiling bacteria in seafood, is sensitive to very low temperature and can be eliminated after several weeks at -20 °C (Emborg et al., 2002).

However, freezing can act negatively on seafood organoleptic properties. A slow freezing rate induces the formation of large ice crystals in tissues, leading to cells membrane disruption. This results in an increase in free fatty acid content, drip loss and solutes concentration in the unfrozen water fraction (e. g. salts), leading to muscle texture degradation and lipid oxidation (Burgaard, 2010; Sampels, 2015). The fish species also plays an important role in organoleptic qualities preservation over time. A fatty fish species, such as trout or salmon, will be more susceptible to lipid oxidation during freezing storage than a lean fish, leading to the appearance of rancid taste and smell. A quick freezing rate with no temperature fluctuations during storage and in absence of oxygen (VP and MAP) can limit seafood quality deterioration (Burgaard, 2010).

6.4. High Pressure Processing

High pressure processing (HPP), also referred as high hydrostatic pressure, is a potential non-thermal processing method which consists in applying a high pressure to packaged food products (often under VP) through the use of compressed liquids (Devlieghere et al., 2004; Considine et al., 2008; Campus, 2010; Sampels, 2015; Ronholm et al., 2016). In industry, pressure level applied to food commodities is usually ranging from 200 up to 600 MPa (Rastogi et al., 2007; Considine et al., 2008; Campus, 2010). HPP applications can be found for many types of fresh or processed food such as dairy products (milk and cheese), fruits and vegetables, meat and meat based products, seafood and fishery products (Devlieghere et al., 2004; Rastogi et al., 2007; Campus, 2010).

The use of high pressure in food can inactivate and reduce loads of pathogenic organisms or spoilage bacteria, but also more broadly enzymes that are involved in deterioration mechanisms (e. g. lipases, proteases) (Lakshmanan et al., 2003; Rastogi et al., 2007; Considine et al., 2008; Campus, 2010). Indeed, effects of high pressure on microorganisms are quite similar to those obtained by high temperatures equivalent to pasteurization. Microorganism's inactivation by HPP is the result of combined factors including cell membrane deformation, cell wall damage and breakage, intracellular content leakage and proteins and enzymes denaturation (Figure 1.9) (Campus, 2010; Sampels, 2015; Ronholm et al., 2016).

HPP showed promising results in seafood, by inactivating *L. monocytogenes* in cold-smoked salmon when combined with frozen storage at -18 °C (Ritz et al., 2008), but also by significantly reducing *V. parahaemolyticus* and *V. vulnificus* microbial loads in oysters (Ronholm et al., 2016). Some authors have also demonstrated a shelf-life extension for seafood products treated by high pressure, such as trout (Matějková et al., 2013), red mullet (Erkan et al., 2010), prawn (López-Caballero et al., 2000) and oyster (He et al., 2002). In fishery industry, besides its utility to preserve food quality, HPP is also used for technological purposes. It facilitates shellfish opening, lobster and crab meat shucking from shells and favors the formation of gel structures in fish meat mince preparations like surimi (Campus, 2010).

However, HPP may have adverse effects on seafood organoleptic properties. A treatment with pressure superior to 300 MPa can induce irreversible changes on

proteins and enzymes structures, leading to flesh tenderization, unattractive coloration, cooked appearance and an increase of lipid degradation (Lakshmanan et al., 2003; Rastogi et al., 2007; Considine et al., 2008; Campus, 2010).

The use of HPP to minimize pathogenic risk or to extend shelf-life of food is relatively recent in food industry. Thus, depending on the process, foodstuff treated by high pressures might be considered as a “novel food” and therefore fall under the Novel Foods Regulation (European Commission, Regulation (EC) No 258/97). Food processors, will then need to provide to the regulation authorities the proof that their process complies with all safety requirements regarding chemical, microbiological, nutritional and toxicological aspects, but also its added value in comparison with existing methods (Kurowska et al., 2016). This, in addition to the important investment cost, might act as a brake for its use in food industry.

6.5. Irradiation

Irradiation is one of the most effective techniques for food microbial safety and quality preservation. Also known as “cold pasteurization” and extensively studied since the 90s’, this preservative method involves irradiating food commodities with gamma radiation from radioisotope source (e. g. cobalt-60), or more recently with electrons or X-ray (Arvanitoyannis and Tserkezou, 2013; Olatunde and Benjakul, 2018). Irradiation effects in food preservation depend on the delivered radiation dose. A low dose of 1 kGy is enough to eliminate insects and parasites, while radiation doses comprise between 1 up to 10 kGy can reduce spoilage and pathogenic microorganisms in many foods, leading to shelf-life extension. Beyond 10 kGy, doses are sufficient to sterilize food commodities, but can however induce detrimental sensory effects (Arvanitoyannis and Tserkezou, 2013). In cells, ionizing radiations causes water radiolysis, resulting in free radicals production such as hydroxyl and hydrogen radicals. These highly reactive species can disrupt DNA, proteins and membranes, leading to cell death (Figure 1.9) (Sampels, 2015; Ronholm et al., 2016; Olatunde and Benjakul, 2018).

The use of irradiation applied to fishery products and seafood was exhaustively reviewed by Arvanitoyannis and Tserkezou (2013) and many studies demonstrated a significant shelf-life extension for various fish, crustacean and shellfish species such as mackerel, tilapia, anchovy, rainbow trout, sea bass, sole, bombay duck, haddock, cod, sardine, herring, pacific shrimp, brown shrimp, lobsters, oysters, clams, scallops,

but also lightly processed and preserved products such as cold-smoked salmon, salted trout and smoked mullet. Furthermore, irradiation doses of 3 up to 10 kGy successfully sanitized oysters from pathogenic *Vibrio* species (Ronholm et al., 2016; Olatunde and Benjakul, 2018).

However, high-dose irradiation can induce organoleptic defects in seafood. An important generation of free radicals can increase lipids oxidation and free amino acids cleavage leading to flavor and aroma modification (Arvanitoyannis and Tserkezou, 2013; Sampels, 2015; Olatunde and Benjakul, 2018). In addition, there is a major drawback to its routine commercial use as preservative method: food irradiation suffers from a bad and unsafe image from consumers (Arvanitoyannis and Tserkezou, 2013).

6.6. Ozonification

Ozone treatment, either by gaseous or dissolved forms, is among one of the most powerful oxidizing and food contact sanitizing treatments allowed in food industry (Gonçalves, 2009; Ronholm et al., 2016; Olatunde and Benjakul, 2018). Usually employed in industry as disinfectant to sanitize equipment and surfaces, water, freezing chambers and warehouses atmosphere, ozone gained in interest in recent years for food preservation (Crapo et al., 2004; Gonçalves, 2009; Olatunde and Benjakul, 2018). Due to its extremely high oxidizing activity, ozone attacks glycolipids, glycoproteins on cells surface, leading to bacterial membrane disruption, increase in permeability and lysis. If inside the cell, ozone can also break-down cellular functions by oxidizing proteins, enzymes and DNA (Figure 1.9) (Ronholm et al., 2016; Olatunde and Benjakul, 2018).

Several studies reported strong bactericidal effects and shelf-life extension for seafood and fishery products treated with ozonized water or stored under ozone slurry ice, as for example for tilapia (Gelman et al., 2005), shrimp (Okpala, 2015), trout (Dehkordi and Zokaie, 2010), mussels (Manousaridis et al., 2005), sardines (Campos et al., 2005), oysters (Chen et al., 2014) and japanese sea bass (Lu et al., 2012). Although ozonification is an effective method for food preservation, it has nevertheless one major limitation. If applied for too long and in too high concentrations, ozone can induce a quick lipids and proteins oxidation, leading to rancid off-flavor and smell.

6.7. Modified atmosphere and vacuum packaging

Modified atmosphere packaging (MAP) and vacuum-packaging (VP) have become popular these last 2 decades and are now common packaging methods used in seafood industry (Sampels, 2015; Odeyemi et al., 2018). They consist in replacing or reducing oxygen contained in a package with a defined gas mixture, mainly composed of carbon dioxide (CO_2) and nitrogen (N_2); or simply to remove any atmosphere in contact with the product (VP) (Sivertsvik et al., 2002; Devlieghere et al., 2004; Sampels, 2015). Besides CO_2 antimicrobial (e. g. against *Shewanella* sp.) and antifungal activity, the absence or low O_2 content limits oxidative reaction and prevent the growth of aerobic spoiling bacteria such as *Pseudomonas* sp., *Acinetobacter* sp. and *Pseudoalteromonas* sp. (Sivertsvik et al., 2002; Leroi, 2014; Sampels, 2015). However, to address safety concerns regarding the growth of strictly anaerobic pathogens bacteria like *Clostridium botulinum* type E, a small portion of oxygen is often added in modified atmosphere (Sivertsvik et al., 2002).

In seafood industry, the recommended gas mixtures are: 35-45% CO_2/25-35% O_2/25-35% N_2 for white lean fish and shellfish and 35-45% CO_2/55-65% N_2 for fatty fish (Sampels, 2015). Many authors succeed to increase the shelf-life of various fishery products (Table 1.7), in comparison to storage under air conditions (Masniyom, 2011; Odeyemi et al., 2018).

The use of enriched CO_2 atmosphere and vacuum has however some limitations. Seafood spoiling bacteria such as *B. thermosphacta*, *P. phosphoreum*, *H. alvei*, *Serratia* and lactic acid bacteria are CO_2 resistant and anaerobic and can grow under MAP and VP, leading to quick sensory degradations (Sivertsvik et al., 2002; Leroi, 2014). In addition, although the use of high concentration in CO_2 (80-100%) showed good results for microbial growth delay, it can cause collapsing of the package (Devlieghere et al., 2004) and lead to organoleptic defects (Fletcher et al., 2005). This is due to the high CO_2 solubility in water and fat, which is rapidly dissolved in water and fatty phase of the product, leading to a vacuum effect, high drip loss, acidic taste and poor texture (Devlieghere et al., 2004; Fletcher et al., 2005).

Table 1.7: Examples of studies demonstrating a shelf-life extension of seafood products under MAP. Extracted from Masniyom (2011) and Odeyemi et al. (2018).

Seafood products	Storage Temp.	Atmosphere ($CO_2/O_2/N_2$)	Shelf-life (days)	References
Fish				
Cod	-0.9 °C	50/5/45	21	Wang et al., 2008
Sea bass	4 °C	60/10/30	13	Kostaki et al., 2009
	4 °C	60/0/40	18	Provincial et al., 2010
Atlantic salmon	2 °C	90/0/10	22	Fernández et al., 2009
	1.2 °C	60/0/40	15	Hansen et al., 2009
Mediterranean swordfish	4 °C	40/30/30	12	Pantazi et al., 2008
	4 °C	50/5/45	13	Kykkidou et al., 2009
Red drum	4 °C	50/0/50	29	Silbande et al., 2018a
Pearlspot	2 °C	60/40/0	10	Ravi Sankar et al., 2008
Lingcod	2 °C	60/0/40 and chitosan	21	Duan et al., 2010
Sutchi catfish	4 °C	50/0/50	14	Noseda et al., 2012
Seer fish	0-2 °C	70/30/0	32	Yesudhason et al., 2014
Crustacean				
Pacific white shrimp	4 °C	80/10/10	12	Qian et al., 2015
Norway lobster	1 °C	80/10/10	13	Gornik et al., 2013
Shellfish				
Blue mussel	4 °C	80/0/20	15	Goulas et al., 2005
	2 °C	80/0/20	12	Caglak et al., 2008
Stuffed mussel	4° C	50/0/50	15	Ulusoy and Özden, 2011
Green mussel	4 °C	80/10/10	15	Masniyom, 2011

6.8. Superchilling

Superchilling is a cooling method using lower temperatures than refrigeration applied on food commodities to maintain their freshness. It consists in chilling the product to a temperature close to or just below the initial freezing point, which is for most seafood comprised between -0.5 and -2.8 °C (Kaale et al., 2011; Sampels, 2015). Although initially described in 1920 by Edouard Le Danois, superchilling has only gained popularity over the last 20 years. This process can also be found under other denomination such as “deep-chilling” or “partial ice formation” (Kaale et al., 2011). Several reasons can explain the renewed interest for superchilling: maintaining products between -0.5 to -4 °C costs less energy than storage at -20 °C. Shipment costs are also reduced by space saving due to the absence of ice during storage and transportation. Finally, superchilled products benefit from a greater attractiveness than frozen equivalents and due to the lack of strict regulation, they are sometimes sold as fresh products (Claussen, 2011).

As for refrigeration and freezing process, superchilling retards the bacterial growth and enzymatic deterioration such as lipid oxidation and product discoloration (Figure 1.9) (Sampels, 2015). Compared to conventional chilling storage, products shelf-life can be extended by 1.5 up to 4 times, especially if combined with MAP or VP (Kaale et al., 2011). Seafood shelf-life extension by superchilling was demonstrated for prawn (Ando et al., 2004), swordtip squid (Ando et al., 2005), cod (Duun and Rustad, 2007) and atlantic salmon (Sivertsvik et al., 2003; Duun and Rustad, 2008). However, superchilling can cause organoleptic defects similar to those encountered for freezing (lipid oxidation, drip and texture loss) due to intra- and extracellular ice crystal formation (Kaale et al., 2011; Sampels, 2015). In some cases, an important myofibres detachment and breakage can also be observed during superchilled storage, caused by a significant proteolytic enzymes (cathepsin B and L) release in fish muscle (Kaale et al., 2011).

6.9. Chitosan

Chitosan is a modified, natural carbohydrate polymer derived by deacetylation of chitin (Figure 1.10), a major component of crustacean shells such as crab, shrimp and crawfish and the second most abundant natural polysaccharide after cellulose (No et al., 2007; Kong et al., 2010; Jianglian and Shaoying, 2013).

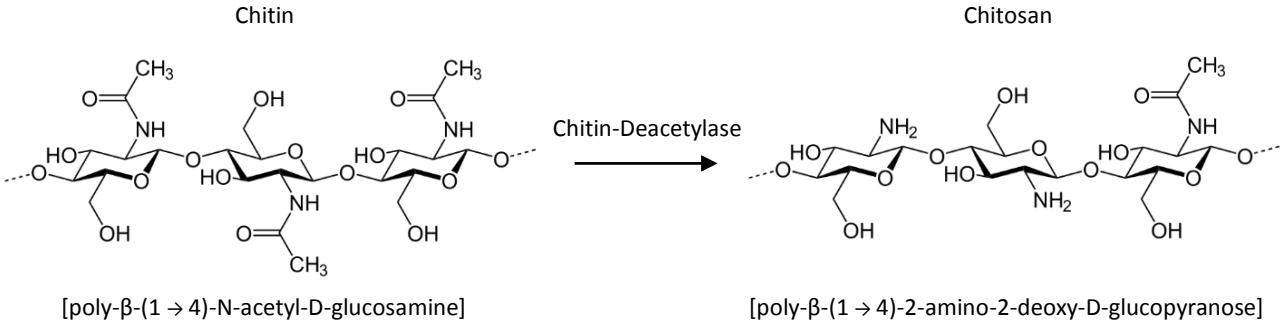

Figure 1.10: Chitosan synthesis from chitin (No et al., 2007; Kong et al., 2010).

Chitosan and its derivatives are water-insoluble, but soluble in weak organic acid solutions. Due to their multiple biological activities and chemical properties such as antimicrobial, antitumor, antioxidant, hypocholesterolemic and film-forming capacities, chitosans received these last decades a growing interest from pharmaceutical, medical and food industries (No et al., 2007; Kong et al., 2010).

Chitosan antimicrobial activity toward a broad range of Gram-positive and -negative bacteria, but also against fungi, is well described in the literature (No et al., 2007). However, this antagonistic activity can be affected by numerous factors such as the microorganisms species, environmental pH, molecular weight, deacetylation degree, pKa and the presence of metal cations (No et al., 2002; Wang et al., 2004; Kong et al., 2010). Although the chitosan inhibition mechanism is still unknown, it was suggested that it would be involved in cells membrane disruption and increase in permeability. Indeed, by being positively charged, chitosan molecules can interact with negatively charged microbial cell membranes, leading to the leakage of intracellular constituents (Figure 1.9) (No et al., 2007; Kong et al., 2010).

Numerous studies demonstrated the effectiveness of chitosan used as food preservative technique. Whether applied in solution by dipping or in the form of edible coating film, it allows the shelf-life extension of various food commodities such as fruit

and vegetable, meat and meat products, bread, milk, juice, seafood and fishery products (No et al., 2007; Kong et al., 2010; Jianglian and Shaoying, 2013). Some examples of chitosan use for seafood preservation are summarized in Table 1.8.

Table 1.8: Examples of studies demonstrating seafood shelf-life extension or better freshness qualities preservation by the use of chitosan.

Product	Storage temp.	Chitosan concen.	Reported effect	Reference
Tilapia	4 °C	2%	TVC reduction of 2-3 log CFU/g, *V. cholera* and *V. alginolyticus* growth delay	Chaparro-Hernández et al., 2015
Smoked tilapia	4 °C	1%	Decrease in TVBN and TBARS content, better texture, TVC reduction of 2 log CFU/g	da Silva Santos et al., 2017
Grass carp	4 °C	1.5% + 1% acetic acid	TVC reduction of 1 log CFU/g, decrease in TVBN, TBARS and K-value, better sensory score	Zhang et al., 2012
Atlantic salmon	4 °C	0.5%	Reduction in lipid oxidation	Kim and Thomas, 2007
Cod Herring	4 °C	1%	Decrease of moisture loss, important lipid oxidation reduction, Decrease in TVBN, TMA and hypoxanthine, TVC reduction of 2 log CFU/g	Jeon et al., 2002
Chum salmon	4 °C	1%	Shelf-life extension from 5 to 9 days, Decrease in TVBN and TVC (2 log CFU/g)	Tsai et al., 2002
Pink salmon	4 °C	1-2%	Reduction of TBARS, drip and moisture loss	Sathivel, 2005
Whiteleg shrimp	0 °C	1-1.5%	TVC reduction of 1 log CFU/g, decrease in TVBN, better sensory attributes	Huang et al., 2012
Shrimp	4 °C	3%	TVC reduction of 2 log CFU/g, decrease in TVBN and TMA, better color.	Aşik and Candoğan, 2014
	4 °C	0.4-1.6% + lactic acid	2.2 to 4.3 log CFU/g reduction of *V. parahaemolyticus*, no sensory adverse effect	Wang et al., 2013
Cooked shrimp	4°C	0.5-1%	Slight *L. monocytogenes* reduction (0.5-1 log CFU/g), better sensory scores	Li et al., 2013
Pacific oyster	5 °C	0.5%	Shelf-life extension from 8 to 15 days, TVC reduction of 2.5 log CFU/g.	Cao et al., 2009

TVC: total viable count.

6.10. Phage biocontrol

Initially studied for medical field purpose, the use of phages, as biocontrol approach to limit the growth of pathogenic and spoiling bacteria in food, is getting popular since early 2000s' (Bai et al., 2016; Letchumanan et al., 2016; Ronholm et al., 2016; Moye et al., 2018). Phages, also named bacteriophages, are viruses able to infect only bacterial cells, with high host specificity to the species or subspecies level. As all viruses, phages are unable to reproduce on their own and require infecting a host for their multiplication. The viral particle firstly binds specific receptors at the cell surface and injects its genetic material into the cytoplasm. If the phage is virulent (or strictly lytic), it will then hijack host biological machinery to produce new virions, as well as phage associated lytic enzymes (PAE or lysins) (Trudil, 2015; Letchumanan et al., 2016; Ronholm et al., 2016; Moye et al., 2018). PAEs rapidly cause bacterial cell lysis and death, leading to the leakage of brand new phage particles (Figure 1.9) (Trudil, 2015; Ronholm et al., 2016). This reproduction cycle is called the lytic cycle. Conversely, if the phage is temperate, the virus can reproduce using both lytic and lysogenic cycles. This second reproduction cycle consists in an integrating phase of a part of the phage genetic material into the bacteria's genome to form a prophage. The prophage can remain in latency for an indefinite period of time and is replicated when the bacterial cell multiplies. When the infected bacteria is submitted to an environmental stress, the prophage can be excised and therefore switch to the lytic cycle described above (Trudil, 2015; Letchumanan et al., 2016; Moye et al., 2018).

Nowadays, due to their high host specificity, phages application in food is essentially focused in foodborne pathogens way of control and detection (Bai et al., 2016; Letchumanan et al., 2016; Moye et al., 2018). Indeed, food spoilage can be a complex matter and may involve several bacterial species, representing a greater challenge. Several FDA approved phages are commercially available to ensure food safety against the major foodborne pathogens, as for example *E. coli* 0157:H7 (EcoShield™), *L. monocytogenes* (ListShield™ and PhageGard Listex™) or *Salmonella* spp. (SalmoPro®, PhageGuard S™, SalmoFresh™) (Moye et al., 2018). Some examples of phages biocontrol applications to seafood and fishery products are listed in Table 1.9.

Although the use of bacteriophages is a promising technique, benefiting from a good image as a green and natural method for food preservation (Moye et al., 2018), it has some drawbacks. As for antibiotics, bacteria can also develop and acquire resistance

to phages infections by simple single mutation changing bacterial surface receptors or notably via the clustered regularly interspaced short palindromic repeats system (CRISPR). Acquired resistance is a serious safety concern as it can be transmitted horizontally through the food chain (Nilsson, 2014; Letchumanan et al., 2016). In consequence, the use of PAE directly as food additives may be an interesting alternative, presenting only the benefits of using phage (Trudil, 2015).

Table 1.9: Examples of phage biocontrol applications in seafood and fishery products.

Target Host	Phage	Product	Results	Reference
Pathogenic bacteria				
V. parahaemolyticus	VPp1	Oyster	2.35-2.76 log CFU/g reduction in 36h	Rong et al., 2014
	pVp-1		Reduction of 3.3 log CFU/g	Jun et al., 2014
	OMN		Reduction of 2.5 log CFU/g	Zhang et al., 2018
L. monocytogenes	A511	Smoked salmon	Reduction of 2 log CFU/g	Guenther et al., 2009
	P100		Reduction of 0.5 log CFU/g	
	P100	Catfish	Reduction of 1.5 log CFU/g	Soni et al., 2010
	P100	Atlantic salmon	Growth inhibiton	Soni and Nannapaneni, 2010
S. typhimurium		Mixed seafood (shrimp, squid, shellfish)	Total inhibition after 1 day	Guenther et al., 2012
		Atlantic salmon Scallop	Total inhibiton	Xu et al., 2018
E. coli	phT4A	Cockle	Reduction of 2 log CFU/g	Pereira et al., 2017
	ECA2		Reduction of 0.6 log CFU/g	
Shigella sonnei	ShigaShield	Smoked salmon	Reduction of 1.1 log CFU/g	Soffer et al., 2017
Spoiling bacteria				
S. putrefaciens	Spp001	Olive flounder	Reduction of 2.0 log CFU/g, shelf-life extension.	Li et al., 2014
Serratia sp.	AZT6	Atlantic horse mackerel	Reduction of 1.0 log CFU/g	Hernández, 2017

6.11. Biopreservation

Biopreservation is defined as the use of microorganisms (mainly LAB) and/or their metabolites to extend shelf-life and enhance safety of food (Stiles, 1996). As consequence of consumer's demand for minimally processed food products without chemical additives, biopreservation, like phage biocontrol, represents an interesting preservative technique benefiting from natural image (Holzapfel et al., 1995; Calo-Mata et al., 2008; Ghanbari et al., 2013). Since the end of 90's, biopreservation represent a growing field of research, which is now widely studied. Currently a wide range of application examples can be found in literature for various types of foodstuff such as bakery products, fermented dairy products, meat and meat products, fruits, vegetables, seafood and fishery products, but also for grain and seed preservation (e. g. malting process, post-harvest storage) (Ghanbari et al., 2013; Zagorec and Christieans, 2013; Galvez et al., 2014; Axel et al., 2017; Leyva Salas et al., 2017; Rovira and Melero, 2018; Singh, 2018). The main bacteria used in biopreservation, their potential antagonistic mechanisms and their applications in seafood are detailed in the following part.

7. Biopreservation: protective cultures for food preservation

7.1. Lactic acid bacteria

The term lactic acid bacteria does not designate a taxonomic unit, but a set of 36 bacterial genera belonging to the *Firmicutes* phylum, whose main metabolite resulting from carbohydrates fermentation is the lactic acid (Holzapfel and Wood, 2013; Felis et al., 2015). Although they belong to *Actinobacteria* phylum, the genera *Bifidobacterium* and *Propionibacterium* are also sometimes associated with LAB (Pot, 2008; Holzapfel and Wood, 2013). LAB are Gram-positive, non-sporulating, non-motile, anaerobic but aero-tolerant, generally catalase negative (pseudo-catalases can sometimes be found), rod or cocci shaped bacteria. They are also characterized, for the genera belonging to the *Firmicutes*, by their low guanine and cytosine content (G+C%) in the DNA, inferior to 50% (Pot, 2008).

LAB can be separated into three different groups based on the metabolic pathways borrowed to ferment glucose and metabolize pentoses. The first group of LAB, defined as obligatively homofermentative, ferments glucose to exclusively lactic acid via the glycolytic pathway, but cannot ferment pentoses. *Lactococcus*, *Enteroccocus*,

Streptococcus, Pediococcus and some species from the *Lactobacillus* genus (Group I) are concerned. The second group or facultatively heterofermentative LAB, can ferment glucose to produce exclusively lactic acid through glycolysis and pentoses via the phosphoketolase pathway to produce lactic and acetic acid, but not CO_2. Genera such as *Lactovum*, *Paralactobacillus*, *Vagococcus, Carnobacterium* and few species belonging to *Lactobacillus* (Group II) are also included. LAB belonging to the third group, defined as obligately heterofermentative, are able to metabolize both glucose and pentoses via the phosphoketolase pathway into lactic acid, CO_2, acetic acid and ethanol. *Leuconostoc*, *Oenococcus*, *Weissella* and *Lactobacillus* species from the group III belong to this group (Kandler, 1983; Endo and Dicks, 2013). From a nutritional point of view, LAB are fastidious organisms which need rich and complex environment to grow, containing vitamins, amino acids, minerals, carbohydrates and nucleic acids (Desmazeaud, 1983; Endo and Dicks, 2013).

Due to their high nutritional requirements, LAB are commonly associated with food commodities, fermented or not, derived from plant and animal materials such as meat and dairy products, vegetables, fruits, grains and cereals. Many of them are also found as a part of humans and animals microbiome (e. g. digestive and genital tracts, buccal cavity). In the industry, LAB are widely used for their technological role, especially for food fermentation and for their involvement in flavors development via the production of diverse volatile compounds (aldehydes, alcohols, ketones, acids, etc.) (Rodgers, 2001; Giraffa, 2013). Beyond the technological aspect, LAB currently represent a growing interest as food protective cultures and probiotics. Indeed, LAB are widely described for their ability to produce a wide range of antimicrobial compounds (Caplice and Fitzgerald, 1999; Zagorec and Christieans, 2013; Leyva Salas et al., 2017) and many species are well adapted to grow at refrigerated temperatures (<10 °C) (Zagorec and Christieans, 2013). In addition, due to their ancestral history of use, most of them are generally recognized as safe for human consumption and benefit from a healthy and natural image from consumers (Holzapfel et al., 1995; Rodgers, 2001).

7.2. Lactic acid bacteria inhibitory properties

Since the last 30 years numerous molecules and mechanisms involved in LAB antimicrobial activity have been identified and exhaustively reviewed many times (Helander et al., 1997; Caplice and Fitzgerald, 1999; Schnürer and Magnusson, 2005; Reis et al., 2012; Crowley et al., 2013; Ghanbari et al., 2013; Leyva Salas et al., 2017; Singh, 2018). Among these antimicrobial compounds, we can find: organics acids, reuterine and reutericycline, hydrogen peroxide, diacetyl and bacteriocins (Figure 1.11).

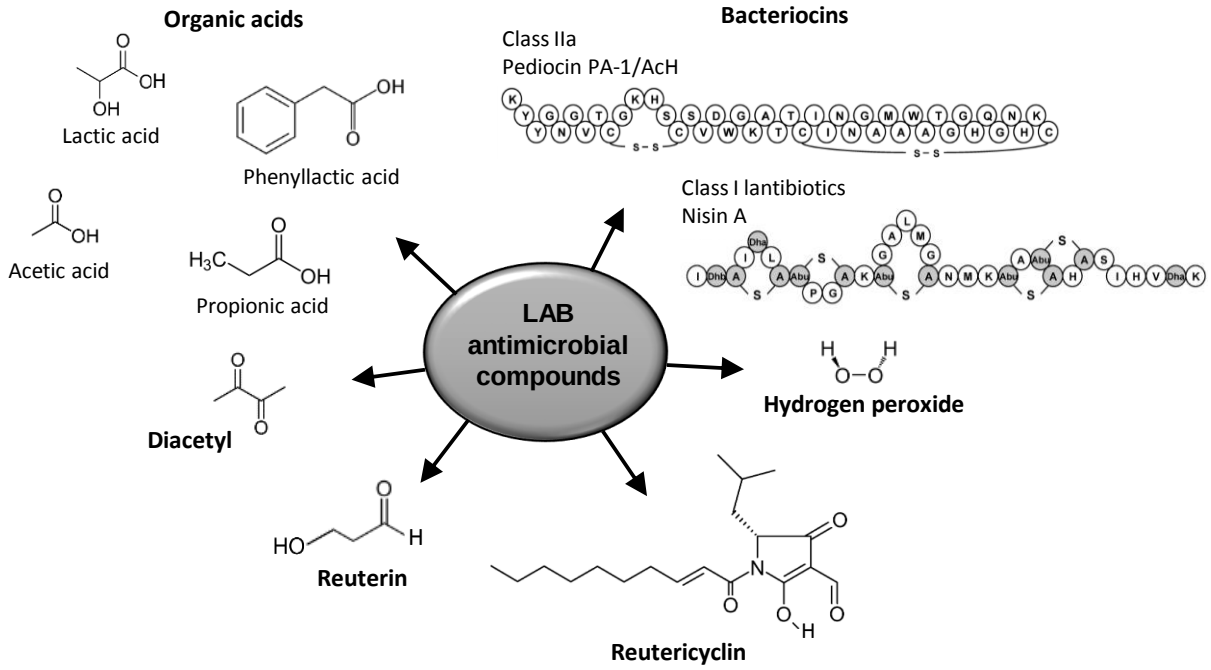

Figure 1.11: Antimicrobial compounds produced by LAB. Inspired from Crowley et al. (2013).

7.2.1. Nutritional competition and Jameson effect

The first inhibition mechanism involved in interaction between microorganisms of an ecosystem is nutritional competition. In the same environment, such as a food matrix, the different microbial biota will draw on available nutrient to ensure their respective growth. They might compete for a common substrate. Gram and Melchiorsen (1996) demonstrated that *S. putrefaciens* and *Pseudomonas* sp. were competing for iron capture in fish. In this example, *Pseudomonas* sp. was able to stop *S. putrefaciens* growth when it reached its maximum growth concentration (10^9 CFU/g). Nilsson et al. (2005) hypothesized that *L. monocytogenes* inhibition by a strain of *C. maltaromaticum*

(A9b), a non-bacteriocin producer, resulted in a major part from competition for glucose.

Nutritional competition, but also competition for space in an ecological niche, can be part of the Jameson effect. Firstly reported by Jameson (1962), this effect can be described “as a race between species to use the resources of the environment to maximize their growth and population numbers. When those resources are depleted, the race is over, and the growth of each species in the population stops” (Mellefont et al., 2008). In other terms, the Jameson effect corresponds to the simultaneous cessation of all microoganisms growth when the dominant population reaches its stationary phase (Ross et al., 2000; Gram et al., 2002; Mellefont et al., 2008; Irlinger and Mounier, 2009; Cornu et al., 2011; Christieans et al., 2013).

The Jameson effect might explain the inhibition obtained by Nilsson et al. (1999) against *L. monocytogenes* with *C. maltaromaticum* A9b in vacuum-packed cold-smoked salmon. *L. monocytogenes* was indeed inhibited when the protective strain reached its maximum concentration (8.5 log CFU/g) after 5 days of culture at 5 °C. This effect was also observed during challenge tests in cold-smoked salmon inoculated with a cocktail of LAB, enterococci, *Enterobacteriaceae* (all at 10^4 CFU/g) and *L. monocytogenes* (10^2 CFU/g). Enterococci and *L. monocytogenes* growth stopped when LAB reached their maximum growth concentration after 6 days of storage at 10 °C (Giménez and Dalgaard, 2004). The Jameson effect has also been highlighted in other products such as dairy, meat and plant based products (Christieans et al., 2013). It is now taken into account for modeling the growth of pathogens such as *L. monocytogenes* in naturally contaminated products (Giménez and Dalgaard, 2004; Powell et al., 2004; Mejlholm and Dalgaard, 2007; Cornu et al., 2011)

7.2.2. Organic acids

Organic acids are the main metabolites produced during fermentation by LAB. Although lactic acid is the main fermentation product (Stiles, 1996; Ross et al., 2002), other organic acids such as acetic, formic, propionic, hexanoic and benzoic acids are also produced in significant quantity by heterofermentative LAB (Schnürer and Magnusson, 2005; Crowley et al., 2013). The pH decrease of the surrounding food environment induced by the production of acids create the first barrier against non-acidophilic spoilage and pathogenic microorganisms (Stiles, 1996; Drouault and

Corthier, 2001; Singh, 2018). In addition, both dissociated and undissociated organic acid forms may also be involved in their antimicrobial activity, which is modulated by the environmental pH and their respective acid dissociation constant (pK_a) (Brul and Coote, 1999; Caplice and Fitzgerald, 1999). When the pH is below the pK_a, organic acids are mostly found in their undissociated form. In this form, acids are generally hydrophobic and can passively pass through bacterial cell membrane or interact with it. When inside the cell, as the intracellular pH is higher than the external environment, the acid dissociates by liberation of an H^+ proton. Protons accumulation in the cytoplasm then neutralizes the membrane electrochemical potential, resulting in active transport and metabolic functions inhibition (Piard and Desmazeaud, 1991; Brul and Coote, 1999; Caplice and Fitzgerald, 1999). Cells can, however, compensate for the addition of H^+ by exporting it outside the cell compartment, but at the cost of significant amount of energy (active transport), which is no longer mobilized to ensure growth and metabolic needs (Brul and Coote, 1999). Organic acids possess a broad spectrum activity, as they are able to inhibit both Gram-negative and -positive bacteria, yeasts and molds (Helander et al., 1997; Caplice and Fitzgerald, 1999; Ross et al., 2002; Schnürer and Magnusson, 2005; Crowley et al., 2013).

7.2.3. Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is produced by LAB in presence of oxygen (Ammor et al., 2006; Christieans et al., 2013). Several enzymes belonging to oxidase family are able to catalyze H_2O_2 synthesis, as for example: NADH (nicotinamide adenine dinucletotide) oxidase, pyruvate oxidase, lactate oxidase, glycerophosphate oxidase and superoxide dismutases (Piard and Desmazeaud, 1991). H_2O_2 antimicrobial activity is essentially limited to bacteria (Piard and Desmazeaud, 1991; Christieans et al., 2013) and its toxicity can be both direct and indirect. Hydrogen peroxide, through a peroxidation reaction on membrane lipids, can affect bacterial membranes integrity. It can then diffuse through the cytoplasmic membrane and damage all cellular components. In addition, H_2O_2 can also induce intracellular formation of reactive oxygen species such as superoxide ions (O_2^-) and hydroxyl radicals ($^{\bullet}OH$). These highly reactive species are also involved in DNA, proteins and membrane disruption, leading to cell death (Piard and Desmazeaud, 1991; Ross et al., 2002; Christieans et al., 2013).

7.2.4. Diacetyl

Diacetyl (or 2,3-butanedione), a ketone with a characteristic butter and nutty flavor, is produced mainly by lactic acid bacteria belonging the genera *Streptococcus*, *Leuconostoc*, *Lactobacillus* and *Pediococcus* (Jay, 1982; McKay and Baldwin, 1990) during citrate fermentation (McKay and Baldwin, 1990; Piard and Desmazeaud, 1991). In cells, citrate is converted into pyruvate under the successive action of the citrate lyase and oxaloacetate decarboxylase. Then two pyruvate molecules are condensed by the α-acetolactate synthase to form CO_2 and α-acetolactate. The α-acetolactate undergoes an oxidative chemical decarboxylation, releasing a CO_2 molecule to form diacetyl. As the α-acetolactate synthase has very little affinity for its substrate, diacetyl production reaction can only take place in excess of pyruvate (Monnet et al., 2008). Diacetyl antimicrobial spectrum includes Gram-negative bacteria (mostly), yeasts, but also filamentous fungi (Jay, 1982; Lanciotti et al., 2003; Aunsbjerg et al., 2015). Its mechanism of action seems to rely on its ability to interfere with arginine utilization (Caplice and Fitzgerald, 1999).

7.2.5. Reuterin and reutericyclin

Reuterin or 3-hydroxypropionaldehyde (3-HPA) by *Lactobacillus reuteri*, a digestive tract commensal bacterium (Vollenweider and Lacroix, 2004; Cleusix et al., 2007), was firstly described by Axelsson et al. and Chung et al. in 1989. In addition to *L. reuteri*, other LAB are also able to produce it, such as *L. brevis*, *L. buchneri*, *L. collinoides*, *and L. coryniformis* (Schnürer and Magnusson, 2005). Reuterin biosynthesis corresponds to the only pathway available for LAB to catabolize glycerol (Martín et al., 2005). 3-HPA production by glycerol dehydration involves a cobalamin-dependent enzyme: the glycerol dehydratase (Vollenweider and Lacroix, 2004; Martín et al., 2005; Cleusix et al., 2007). Reuterin is active toward a broad range of microorganisms, including Gram-negative and positive bacteria (*L. innocua, L. monocytogenes, Lactobacillus* sp., *C. difficile, B subtilis, E. coli, S. enterica, S. typhimurium, S. sonnei, Y. enterocolitica, V. cholearae, P. aeruginosa, C. jejuni, A. hydrophila*) (Vollenweider and Lacroix, 2004; Cleusix et al., 2007), yeasts (*Candida glabrata*, *Saccharomyces cerevisiae*, *Candida albicans*, *Kluyveromyces marxianus*, *Debaryomyces hansenii*) and fungi (*Aspergillus flavus*, *Fusarium samfucienum*), but also against parasites (*Trypanosoma cruzi*) (Axelsson et al., 1989; Chung et al., 1989). Reuterin action mechanism would be based on the inhibition of the ribonucleotide reductase, an enzyme involved in DNA

biosynthesis (Vollenweider and Lacroix, 2004), combined with the induction of an oxidative stress in cells (Schaefer et al., 2010).

L. reuteri is also able to produce another antimicrobial compound: the reutericyclin. Reutericyclin would act like a proton-ionophore on the target cell, leading to the membrane electrochemical potential dissipation. Its range of action includes mostly Gram-positive bacteria such as *E. faecalis*, *E. faecium S. aureus*, *B. cereus*, *B. subtilis*, *B. amyloliquefaciens* and *L. innocua* (Gänzle, 2004).

7.2.6. Bacteriocins

Mainly produced by Gram-positive bacteria, bacteriocins comprise a huge family of ribosomally synthesized peptides or proteins that have antibacterial activity towards closely related species and strains (De Vuyst and Leroy, 2007; Thonart and Dortu, 2009; Perez et al., 2014). This last decade, bacteriocins produced by LAB received a particular attention for their potential use in food industry as natural preservatives (De Vuyst and Leroy, 2007; Perez et al., 2014). With the constant discovery of new bacteriocins, classification for this family of very heterogeneous compounds, both in term of composition and action mechanism, is regularly discussed (Perez et al., 2014; Alvarez-Sieiro et al., 2016). Table 1.10 summarizes a usual classification based on bacteriocins properties.

Antimicrobial activity of bacteriocins towards Gram-positive pathogenic bacteria such as *S. aureus*, *L. innocua*, *L. monocytogenes*, *B cereus* and *C. difficile* is now widely described (De Vuyst and Leroy, 2007; Thonart and Dortu, 2009; Christieans et al., 2013; Singh, 2018). However, nisin (E234), produced by *L. lactis*, is currently the only bacteriocin authorized as food preservatives in EU (Thonart and Dortu, 2009; Christieans et al., 2013).

Table 1.10: Bacteriocins classification and examples from Perez et al. 2014, completed with information from Thonart and Dortu, 2009 and Alvarez-Siero et al, 2016.

Class	Features	Examples
I	Lantibiotics: heat-stable small (<5 kDa) peptides containing lanthionine and β-methyllanthionine (unusual amino acids with mono-sulfur bonds)	Nisine, Mersacidine,
II	Small (< 10 kDa), heat-stable, non-lanthionine-containing peptides	
	IIa: small heat-stable peptides, contains between 37 to 48 amino acids, active against *Listeria*, have a consensus sequence of YGNGVXC in the N-terminal part, hydrophobic or amphiphilic C-terminal part. Antagonistic activity by pores formation in cells membrane	Pediocin PA-1, Leucocin A, Enterocin A, Sakacin P
	IIb: two components system: two different peptides required to form an active poration complex, creating leakage of small molecules, cations and anions. Cationic, amphipathic or hydrophobic peptides	Lactococcin G, Enterocin NKR-5-3-AZ, Enterocin X
	IIc: N- and C- terminal parts are covalently linked, resulting in a circular structure	Lactocyclin Q, Leucocyclin Q
	IId: Other class II bacteriocins, including *sec*-dependent or leaderless bacteriocins. Increase cell membrane porosity	Lacticin Q and Z, Weissellicin Y, Leucocin Q
III	Large molecules heat sensitive peptides (> 30 kDa), suggested to be renamed bacteriolysins. Degrade peptidoglycan layer in cells wall	Enterolysin A, Millericin B

7.3. Biopreservation applied to seafood and fishery products

Although less documented than for dairy or meat products, seafood biopreservation is now widely studied (Ghanbari et al., 2013). An overview of scientific publications dealing with seafood biopreservation figures in Table 1.11.

Most of these studies are focused on *L. monocytogenes* inhibition, particularly in lightly processed and preserved products (high value-added products) such as cold-smoked salmon. For this purpose, LAB such as *Carnobacterium* species (*C. maltaromaticum* and *C. divergens*) and *L. sakei*, well known bacteriocins producers, have been particularly studied (Pilet and Leroi, 2011). In vacuum packed cold-smoked salmon, Nilsson et al. (1999), demonstrated that the strain *C. maltaromaticum* A9b, carnobacteriocin B2 producer, inhibited *L. monocytogenes* from 10^3 CFU/g to below 10 CFU/g after 32 days of incubation at 5 °C.

Table 1.11: Recent scientific works on seafood biopreservation. Updated from Ghanbari et al. (2013).

Product	PC/Bacteriocin	Reported effects	Reference
Fish fillets			
Catfish (air)	*L. lactis* spp. *cremoris* ATCC 19257 combined with sodium acetate	Improved odor and appearance	Kim and Hearnsberger, 1994
	Bifidibacterium adolescentis, *B. infantis, B. longum*	Shelf-life improvement	Kim et al., 1995
Indian oil sardine (air & sterilized)	*S. phocae* PI80	PV value and TVBN reduction, inhibition of *L. monocytogenes*, coliforms and *V. parahemolyticus*	Paari et al., 2011
Horse mackerel (VP)	*Pediococcus* spp.	Sensory improvement	Cosansu et al., 2011
Indian mackerel (air)	*P. acidilactici*, *P. pentosaceous*, *S. thermophilus*, *L. lactis, L. plantarum, L. acidophilus, L. helveticus*	TMA and TVBN reduction	Sudalayandi and Manja, 2011
Plaice (VP & MAP)	*Bifidobacterium bifidum*	Inhibition of *P. phosphoreum* and *S. putrefaciens*	Altieri et al., 2005
European bass (VP & minced)	*L. plantarum* 3, *L. pentosus* 7	Inhibition of coliform, decrease in TMA and TVBN content	El Bassi et al., 2009
Rainbow trout (VP)	Sakacin-A producing *L. sakei* lb706	Inhibition of *L. monocytogenes*	Aras et al., 2005
	L. sakei CECT 4808, *L. curvatus* CECT 904T	Shelf-life improvement, inhibition of *Enterobacteriaceae*, *Pseudomonas* spp. and H_2S- producing bacteria	Katikou et al., 2007
	Nisin	PV value, TBARS and TVBN reduction, inhibition of total flora and LAB	Behnam et al., 2015
Blue shark (air)	Pediocin ACCEL, nisin	Inhibition only in early stage of storage	Yin et al., 2007
Atlantic salmon (air)	*P. pentosaceus* T1	Inhibition of *L. monocytogenes*	Jang et al., 2015
	L. pentosus 39	Inhibition of *L. monocytogenes* and *A. hydrophila*	Anacarso et al., 2014
Atlantic salmon (VP)	*L. lactis* ssp. lactis	Sensory and Shelf-life improvement	Ibrahim and Vesterlund, 2014
Cooked tuna (VP)	*L. sakei* LHIS2885	Sensory improvement, inhibition of histamine production	Podeur, 2014
Tilapia (air)	*L. plantarum* 1.19	Sensory improvement	Cao et al., 2015
Pacu (VP)	Bacteriocin from *L. plantarum* LPBM10	Sensory improvement, bacteriostatic effect on coliforms	Suárez M et al., 2008
Smoked fish			
Cold-smoked salmon (VP)	*Carnobacterium* sp.	Shelf-life improvement inhibition of moulds and yeasts	Leroi et al., 1996
	C. maltaromaticum SF668, *C. divergens* V41	Inhibition of *L. monocytogenes*	Duffes et al., 1999
	C. maltaromaticum (A9b, A10a)	Inhibition of *L. monocytogenes*	Nilsson et al., 1999, 2004
	L. sakei Lb790, Sakacin P	Inhibition of *L. monocytogenes*	Katla et al., 2001
	C. maltaromaticum CS526	Inhibition of *L. monocytogenes*	Yamazaki et al., 2003
	C. divergens V41	Sensory improvement and shelf-life improvement, inhibition of *L. monocytogenes*	Brillet et al., 2005
	L. sakei 5754	Inhibition of *L. innocua*	Weiss and Hammes, 2006
	L. casei T3, *L. plantarum* Pe_2, *C. maltaromaticum* Sal_3	Inhibiton of *L. innocua*	Vescovo et al., 2006

Product	PC/Bacteriocin	Reported effects	Reference
	C. maltaromaticum M35 (divergicin M35 producer)	Inhibition of *L. monocytogenes*	Tahiri et al., 2009
	L. piscium (EU2229, CNCM I-4031), *L gelidum* (EU2247, EU2262)	Sensory improvement	Matamoros et al., 2009
	L. piscium CNCM I-4031	Sensory improvement	Leroi et al., 2015
	C. maltaromaticum A and B, nisin in alginate film	Inhibition of *L. monocytogenes*	Concha-Meyer et al., 2011
	Nisin	Inhibition of *L. monocytogenes*	Neetoo et al., 2008
Sterilized cold-smoked salmon (VP)	*C. maltaromaticum* V1, SF668, *C. divergens* V41	Inhibition of *L. monocytogenes*	Brillet et al., 2004
Cold-smoked rainbow trout (VP)	Nisin and sodium acetate	Inhibition of *L. monocytogenes*	Nykänen et al., 2000
Shrimp			
Brined shrimp (air)	Nisin Z, Carnocin UI49, Bavaricin A	Sensory improvement	Einarsson and Lauzon, 1995
Chilled shrimp	Nisin combined with lactic acid	Inhibition of *Pseudomonas* spp. and H_2S producing bacteria	Shirazinejad et al., 2010
Sterilized giant tiger prawn (air)	*S. phocae* PI80	PV value and TVBN reduction, inhibition of *L. monocytogenes*, coliforms and *V. parahemolyticus*	Paari et al., 2011
Cooked and peeled shrimp (MAP)	*L. piscium* CNCM I-4031	Sensory and shelf-life improvement, inhibition of *B. thermosphacta*	Fall et al., 2010a
	L. piscium CNCM I-4031, *C. divergens* V41 alone and in combination	Sensory and shelf-life improvement, inhibition of *Shewanella*, *Enterobacteriaceae* (*C. divergens*) and *L. monocytogenes* (both strains)	Saraoui et al., 2017
Cooked and peeled shrimp (VP)	*L. piscium* (EU2229, CNCM I-4031), *L. gelidum* (EU2247, EU2262)	Sensory and shelf-life improvement, inhibition of *S. aureus* and *L. monocytogenes*	Matamoros et al., 2009
Sterilized cooked and peeled shrimp (MAP)	*L. piscium* CNCM I-4031	Inhibition of *L. monocytogenes*	Fall et al., 2010b

Brillet et al. (2004) also showed that two strains of *Carnobacterium*, namely *C. divergens* V41 and *C. maltaromaticum* V1, producing respectively divercin V41 and piscicocin V1, were able to maintained *L. monocytogenes* to its initial inoculation level during the whole storage period. Tahiri et al. (2009) demonstrated that the strain *C. divergens M35*, producing divergicin *M35*, was able to inhibit *L. monocytogenes* growth by 3.0 log CFU/g after 21 days at 4 °C. Also in cold-smoked salmon, Katla et al. (2001) managed to maintained *L. monocytogenes* to its initial level of 10^3 CFU/g during 28 days at 10 °C, using the *L. sakei* strain Lb790 (sakacin P producer). Although *L. monocytogenes* inhibition is primarily obtained with bacteriocins producing LAB, some authors also demonstrated the ability of *L piscium* CNCM I-4031 (formerly EU2241), a non-bacteriocin producer, to inhibit this pathogen in cooked and peeled shrimp during chill storage (Matamoros et al., 2009a; Fall et al., 2010b; Saraoui et al., 2017). *L. piscium* CNCM I-4031 action mechanism against *L. monocytogenes* is still unknown,

but seems to be cell contact dependent and induced by quorum sensing (Saraoui et al., 2016a, 2018).

The biopreservation effect on seafood shelf-life is less documented. Indeed, spoilage is a complex matter that may involve several microorganisms and often requires, in order to be assessed, a polyphasic approach using biochemical analyses (pH, TVBN, TMA, PV measurement) and exhaustive sensory and microbiological analyses. For instance, Katikou et al. (2007) demonstrated in vacuum packed rainbow trout fillets that *L. sakei* CECT 4808 inhibited *Enterobacteriaceae*, *Pseudomonas* spp., H_2S-producing bacteria, yeast and mould and improved the sensory shelf-life by 5 days in comparison with the control. In refrigerated catfish stored in air, Kim et al. (1995) showed that a *Bifidobacterium infantis* strain extended the shelf-life by 3 days. In cold-smoked salmon, *L. piscium* EU2229 and CNCM I-4031, *L. gelidum* EU2247 and EU2262 greatly improved the sensory quality after 4 weeks of storage (1 week at 4 °C, 2 weeks at 6 °C and 1 week at 10 °C) (Matamoros et al., 2009a). In this study, all strains, excepted *L. gelidum* EU2262, totally prevent the *Enterobacteriaceae* growth. In challenge test in cooked and peeled shrimp, by inhibiting *B. thermosphacta* growth, *L. piscium* CNCM I-4031 totally prevent spoilage after 21 days of storage under MAP at 8 °C, (Fall et al., 2010a).

Purified bacteriocins can also be directly applied to improve seafood microbial quality and safety. By the use of bavaricin A and nisin Z, Einarsson and Lauzon (1995) improved the shelf-life of brined shrimp by 6 and 21 days respectively. However, that preservation effect was limited in comparison with the use of a benzoate-sorbate solution (common additives) which allowed a shelf-life extension of 49 days. Nykänen et al. (2000) and Neetoo et al. (2008) were able to obtain a slight inhibition of *L. monocytogenes* with nisin in cold-smoked trout and salmon respectively.

7.4. Commercial protective cultures

Despite numerous scientific studies about biopreservation use and its success to both extend the shelf-life and limit pathogenic risk in different food matrices, there are only relatively few commercialized protective cultures and real industrial applications (Le Fur et al., 2013; Rovira and Melero, 2018).

Several drawbacks can explain this situation. Many studies were done at the laboratory scale, on a limited product quantity or in model conditions, which can be far from a real industrial application. Industrial scaling-up can also be sometimes difficult or even infeasible (Rovira and Melero, 2018). Microbial culture introduction in manufacturing processes in a factory will necessarily involve rethinking the way it works, notably in term of staff training, equipment cleaning to avoid microorganisms' persistence, application method, new machine investment etc. Product initial microbial population and target bacteria intra-species variability (e. g. pathogenic bacteria such as *L. monocytogenes*) play an important role in biopreservation efficiency. All strains from the same species do not have the same sensitivity toward a protective culture (Rovira and Melero, 2018). For instance, Brillet et al. (2004) demonstrated that the sensitivity of 57 *L. monocytogenes* strains was variable toward 3 *Carnobacterium* spp. bacteriocin producer strains (*C. divergens* V41, *C. maltaromaticum* V1, *C. maltaromaticum* SF668) in agar spot test. Saraoui et al. (2018) also found similar results for 43 *L. monocytogenes* strains toward a strain of *L. piscium* non-bacteriocin producer in co-culture in shrimp model medium. Moreover, a bioprotective strain is often suitable to only one specific product or process, there is almost no transferability from one product type to another, as for instance from meat to seafood products. Indeed, if we follow that example, *C. maltaromaticum*, *L. gelidum* and *L. piscium*, strains commonly used in seafood biopreservation (Table 1.11), are often involved in meat and meat products spoilage (Zagorec and Champomier-Vergès, 2017a).

Strains used as bioprotective agent are often isolated from matrices that aimed to be biopreserved (Pilet and Leroi, 2011). Finally, biopreservation applied to unfermented food products such as seafood or meat, also remains an important challenge. Indeed, the protective cultures have to be growth competitive, to expressed antimicrobial activity without inducing through their metabolism any sensory adverse effects or medium acidification.

Despite these drawbacks, biopreservation as an efficient food preservation technique is now well established and recognized in food industry. This is particularly the case for fermented meat and dairy products, where several commercial cultures are currently available (Rovira and Melero, 2018). In seafood industry, only five commercial cultures or industrial applications currently exist so far. They are summarized in Table 1.12.

Table 1.12: Available commercial protective cultures or metabolites for seafood biopreservation

Company	Commercial name	Bacteria species or metabolite	Activity	Matrix
Biocéane	LLO®	*L. lactis* CNCM I-2716	Antimicrobial activity against coliforms, pseudomonads, *Staphylococcus* spp. and *L. monocytogenes*	All seafood. Mainly used for shrimp and scallop biopreservation.
Chr. Hansen	SafePro® Novalox	-	Antilisterial activity	Smoked salmon
Clerici-Sacco	Lyoflora FP-18 Lyoflora FP-50	*Carnobacterium* sp. bacteriocin producer	Antilisterial activity	Smoked salmon
Grizzly Fumoir	*BAC M35*	Bacteriocin from *C. divergens M35*	Antilisterial activity	Smoked salmon

For reference see Le Fur et al. (2013), Brousseau (2017), Rovira and Melero (2018) and companies' respective website.

7.5. Protective culture regulation in food

At the European scale, there is currently no regulation on the use of microbial cultures in human nutrition for biopreservation purposes (Vogel et al., 2011; Spinnler et al., 2013). Nevertheless some lines of thought are studied since early 2000s. This situation results mainly from the difficulty to categorize bioprotective microbial cultures among food components regarding the current regulation in place (Regulation (EC) No 1331/2008). Vogel et al. (2011) proposed a consensus definition for protective cultures (PC) as preparations consisting of living microorganisms that are added to foods with the aim of reducing risks by pathogenic or toxigenic microorganisms or extending shelf-life. According to this, PC do not clearly correspond to the definition of "ingredient", "processing aid" or "additive".

An ingredient, as defined by the Directive 2000/13/EC (2000), corresponds to any substance used in the manufacture or preparation of a foodstuff and still present in the finished product, even if in altered form. Starter cultures for fermented food production fall under that category. However, PC might also be concerned. Indeed, as emphasized by Vogel et al. (2011) and Spinnler et al. (2013), in addition to their antimicrobial properties, PC by their metabolism can also actively participate to fermented food production.

A processing aid is a substance intentionally used in the processing of raw material, foods or their ingredients, to fulfill a certain technological purpose, but are not present in the finished product or only as trace amount (Regulation (EC) No 1331/2008). PC do not really correspond to that definition as they are still present in finished product, during its commercialization and storage.

For the last category, additives, they are substances that are not normally consumed as food itself but are added to food intentionally for a technological purpose, such as the preservation of food (Regulation (EC) No 1333/2008). This category could therefore concern PC used in unfermented food products such as raw or cooked meat, vegetable and seafood (Spinnler et al., 2013).

This lack of regulation in human nutrition not only concerns PC, but also starter cultures with no long history of use (before May 1997, Regulation 258/97/CE, on Novel Food) and probiotics (Wessels et al., 2004; Vogel et al., 2011). However, in animal feed, paradoxically, the EU categorized microbial cultures as additives and they are subject to a full approval process (Directive 93/113/EC, 1993). Denmark is the only European country which has adopted a legal framework regarding microbial cultures in food. They are considered as additives and require notification and approval, including the documentation on safety and efficacy (Wessels et al., 2004; Vogel et al., 2011).

An appropriate and harmonized regulation is still required to answer the demand for PC industrial development and use in food. Emerging EU legislation might be based on existing regulation as for example for animal feed or might create a new category of food component which fits this intent of use (Wessels et al., 2004; Vogel et al., 2011; Spinnler et al., 2013).

7.6. Safety requirements

Despite a lack of regulations regarding the use of protective cultures in food, LAB must fulfill several safety requirements (Denis et al., 2013). PC and more broadly microbial food culture (MFC) are considered as foodstuff and therefore fall under the General Food Law, Regulation (EC) No 178/2002 (Spinnler et al., 2013; Laulund et al., 2017). The article 14 states that “food shall not be placed on the market if it is unsafe” meaning: “injurious to health or unfit for human consumption”. In addition, in the case of new application in food with no long history of use before 14 May 1997, MFC have to comply with the Novel Food regulation (Regulation 258/97/CE, 1997). That may imply to undergo a pre-market evaluation and authorization procedure including risk assessment before its commercialization (Spinnler et al., 2013; Laulund et al., 2017).

In Europe, to establish safety of microorganisms requiring market authorization such as probiotic strains used in feed or microorganisms used for plant biocontrol, the European Food Safety Authority has developed in 2005 the QPS approach (Qualified Presumption of Safety, EFSA, 2005). It takes into account 4 main elemets synthetized in Figure 1.12:

- the taxonomic unit definition (referring to the establishment of identity)
- the body of knowledge
- the intended end use
- the possible safety concerns (pathogenicity, antibiotic resistance, biogenic amines production)

In 2007, the EFSA scientific committee for biological hazards (BIOHAZ) introduced a QPS list of microorganisms. It includes “qualified” species after an exhaustive examination of literature data on virulence factors, toxic metabolites, possible antimicrobial resistance and transmissible antibiotic resistance. The QPS list is regularly updated and the last published version from March 2018 contains 88 species: 59 Gram-positive non-spore forming bacteria (mostly LAB) from genera such as *Bifidobacterium*, *Corynebacterium*, *Lactobacillus*, *Lactoccocus*, *Leuconostoc*, *Microbacterium*, *Oenococcus*, *Pasteuria*, *Pediococcus*, *Propionibacterium* and *Streptococcus*; 15 Gram-positive spore-forming bacteria from *Bacillus* and *Geobacillus* genera, 2 Gram-negative bacteria (*Gluconobacter oxydans*, *Xanthomonas campestris*) and 12 yeast species (EFSA, 2018).

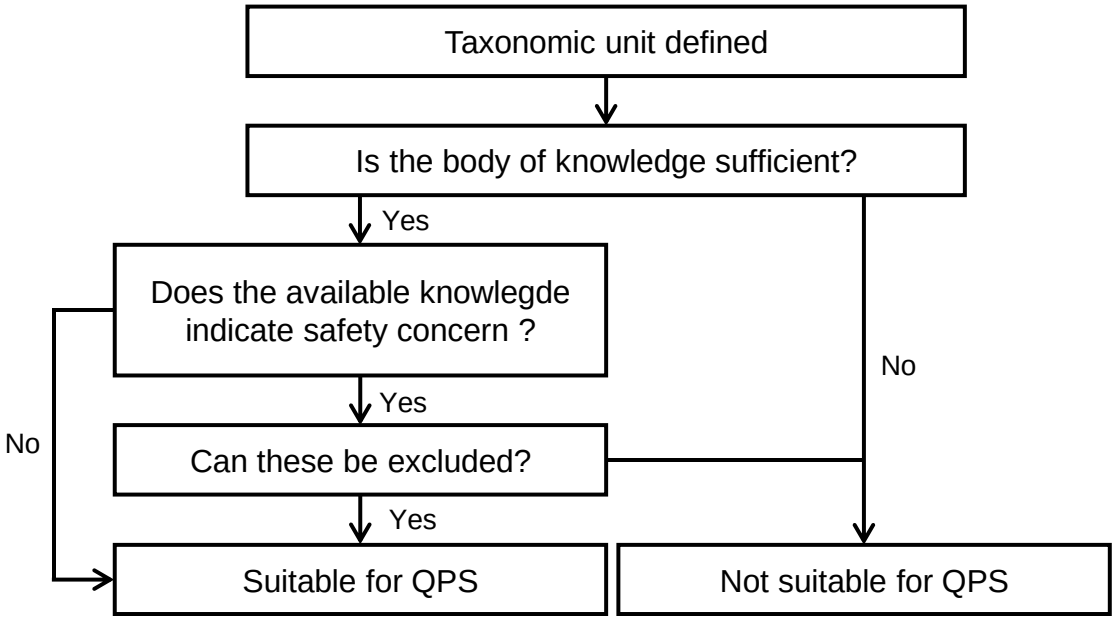

Figure 1.12: General scheme for assessing microorganisms' suitability for QPS status (EFSA, 2005).

Moreover, among this list, figure two LAB species well studied for seafood biopreservation, namely *L. sakei* and *C. divergens* (see 7.3). Currently, the QPS list is often wrongly perceived as a "positive list" of microorganisms intended to use in food commodities. However, microorganisms which are not on the QPS list are not necessarily considered as unsafe. Indeed, many species have not been yet evaluated by EFSA scientific committees (Laulund et al., 2017).

Complementary to the QPS list, and to fill the gap of information regarding microorganisms traditionally used in the EU before 1997 (Novel Food Regulation), the EFFCA (European Food and Feed Cultures Association) and the IDF (International Dairy Federation) jointly published an inventory of 113 microorganisms species with a documented history of use (Mogensen et al., 2002). This inventory was recently updated in 2012 and now includes 264 species (195 bacteria and 69 yeast and mold) from a wide range of food matrices such as dairy products, meat, fish, vegetables, cereals, beverages and vinegar (Bourdichon et al., 2012b, 2012a). As for QPS list, *L. sakei*, *C. divergens* but also *C. maltaromaticum* figure among the 2012 version of the IDF list.

In order to assess the safety of a strain, it is necessary to ensure the absence of pathogenicity, to assess antibiotics resistance and the ability to produce biogenic amines (Wessels et al., 2004; Vogel et al., 2011; Bourdichon et al., 2012b; Denis et al., 2013; Pariza et al., 2015; Laulund et al., 2017). For that purpose, whole genome sequencing offers new formidable insights as replacement of conventional molecular techniques like PCR amplification. With this tool it is possible to exhaustively screen genomes for the presence of virulence factors and antibiotics resistance genes (Pariza et al., 2015; Laulund et al., 2017). Several databases are currently available for genome-based safety assessment such as MvirDb (Zhou et al., 2007), Virulence Factor Database (VFDB) (Chen et al., 2016) and VirulenceFinder (Joensen et al., 2014) for microbial virulence factors screening, as well as CARD database (McArthur et al., 2013), ARDB (Liu and Pop, 2009) and ResFinder (Zankari et al., 2012) for antibiotic resistance screening.

7.6.1. Pathogenicity assessment

A strain selected as a ferment must not possess any virulence factors. Virulence factors refer to the properties (e. g. molecules) that enable a microorganism to establish itself on or within a host and enhance its potential to cause disease. Are included: bacterial endo- and exotoxins, hemolysins, cell surface proteins that mediate bacterial attachment (adhesins), cell surface carbohydrate and proteins for bacterial protection (capsule), invasion factors and enzymes such as invasins, hyaluronidase, collagenase, fibrinolytic enzymes, coagulase (van Reenen and Dicks, 2011; Pariza et al., 2015). *In vitro* tests against cells can also be performed to assess a microorganism cytotoxic potential (e. g. Vero cell cytotoxicity test) and adhesion ability (Laulund et al., 2017). Some physiological criteria such as the absence of growth or slow growth at 37 °C or the sensitivity to low pH and bile salts can reinforces the safety status of a strain (Ammor and Mayo, 2007; Denis et al., 2013).

However, by the lack of harmonization for safety assessment, coupled with a limited knowledge of how virulence factors work and incomplete databases, it is often difficult to interpret findings. For instance, surface molecules or enzymes promoting cells adhesion and colonization might be regarded as interesting factors for probiotic strains (Pariza et al., 2015), as well as for PC for growth competitiveness on food matrices.

7.6.2. Antibiotic resistance

The recent emergence of multidrug resisting pathogenic bacteria and the horizontal transfer possibility of resistance-conferring genes represent currently major health concerns. Intentionally added microbial cultures in food and feed should not act as an additional transferable antibiotic genes reservoir (Ammor and Mayo, 2007; Bourdichon et al., 2012b). The main hazard is related to acquired resistances carried by mobile genetic elements such as plasmids or transposons, which can be transferred to other bacteria (eventually pathogenic bacteria) through the food chain (van Reenen and Dicks, 2011).

Antibiotic susceptibility assessment is a prerequisite for a bacteria to obtain the QPS status (Denis et al., 2013). The EFSA published a guidance for antibiotic safety assessment based on a decision tree shown in Figure 1.13 (EFSA, 2012).

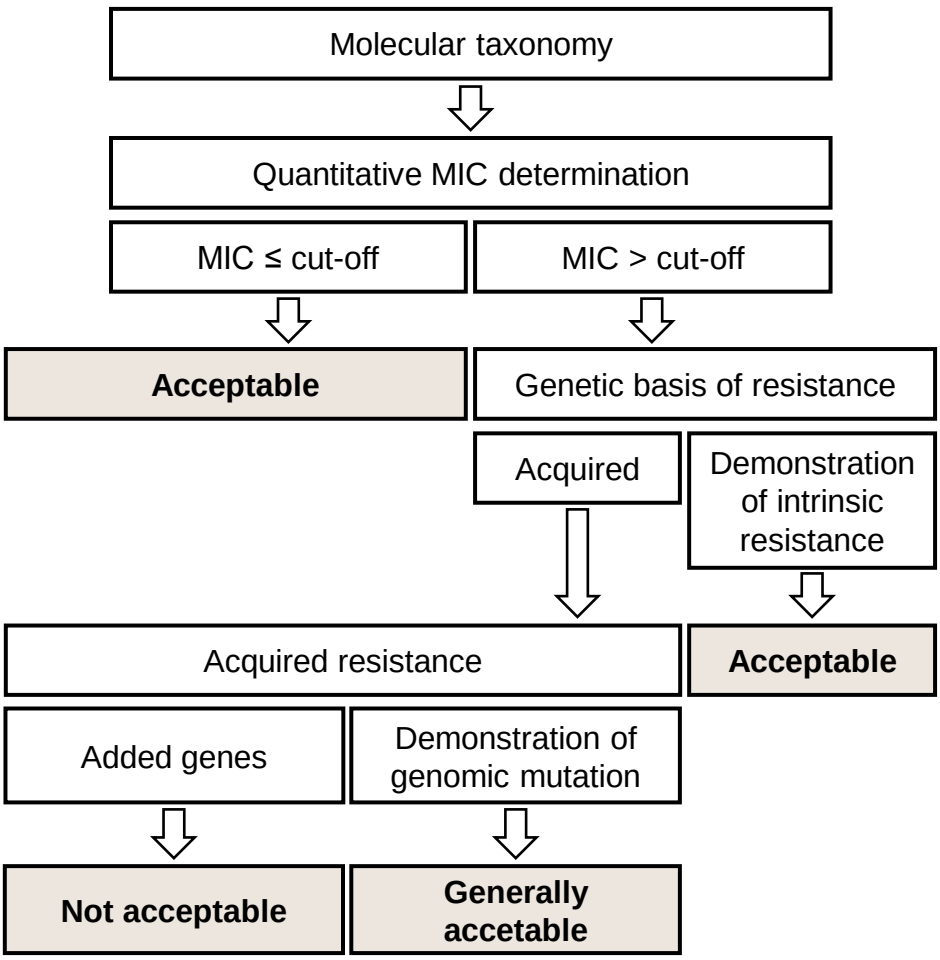

Figure 1.13: Scheme for antimicrobial resistance assessment of a bacterial strain (EFSA, 2012).

Bacterial strains are phenotypically tested against 9 clinically relevant antibiotics (ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline and chloramphenicol) to determine minimal inhibitory concentrations (MICs). Two internationally recognized standard methods for antibiotic resistance assessment are currently available. One proposed by the Clinical and Laboratory Standard Institute (CLSI, https://clsi.org) and the ISO standard 10932:2010. Obtained MICs are compared with cut-off values derived from published data, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and others national and European monitoring programs (EFSA, 2012; Laulund et al., 2017). If the MICs for one antibiotic is higher than the established cut-off value, the bacterial strain is considered as resistant. Two scenarios are then considered:

- When all strains from the same taxonomic group displayed a phenotypic resistance to an antimicrobial, it is considered as intrinsic resistance.
- In contrast, when a bacteria strain demonstrates higher resistance to a specific antimicrobial than the other strains from the same taxonomical unit, the presence of acquired resistance is indicated. Further additional information on genetic basis are needed to determine whether acquired genes are associated with genetic mobile elements or mutation.

Several examples of intrinsic resistance have been described in the literature. *Lactobacillus*, *Pediococcus* and *Leuconostoc* spp. have been reported to have high natural resistance to vancomycin. In addition to vancomycin, among the *Lactobacillus* genera, a high natural resistance to bacitracin, cefoxitin, ciprofloxacin, fusidic acid, kanamycin, gentamicin, metronidazole, nitrofurantoin, norfloxacin, streptomycin, sulphadiazine, teicoplanin and a mix of trimethoprim/sulphamethoxazole were also described (Mathur and Singh, 2005; van Reenen and Dicks, 2011).

Currently, cut-off values are only available for a few number of bacteria species or genera such as *Lactobacillus* (*L. acidophilus*, *L. reuteri*, *Lactobacillus plantarum/pentosus*, *L. rhamnosus*, *L. casei/paracasei*, homofermentative *Lactobacillus*, heterofermentative *Lactobacillus*, facultative heterofermentative *Lactobacillus Bifidobacterium*, *Pediococcus*, *Leuconostoc*, *Lactococcus lactis*, *Streptococcus thermophilus*, *Bacillus* spp. and *Propionibacterium* (EFSA, 2012). This lack of knowledge regarding antibiotic resistance within species can make it difficult to

interpret results and performed a relevant antibiotic safety assessment for a given microorganism (Laulund et al., 2017).

7.6.3. Biogenic amines production

As seen in 5.2.4, biogenic amines, especially histamine and tyramine can be a safety concern. Biogenic amines can be produced in a wide range of foodstuff including fish products and seafood, fermented products such as meat, dairy products, vegetables, beverages (wine, cider and beer) and can occasionally reach high concentrations (Spano et al., 2010; Denis et al., 2013). The ability to produce biogenic amines is also a key requirement for MFC safety assessment (Ammor and Mayo, 2007; Denis et al., 2013; Laulund et al., 2017).

MFC screening on their capability to produce biogenic amines can be assessed in specific medium enriched in amino acids precursors (e. g. histidine, tyrosine, lysine, agmatine, and ornithine). Biogenic amines are then quantified in supernatants by HPLC (Bover-Cid and Holzapfel, 1999). In addition, it is possible to confirm the ability of a strain to produce biogenic amines by genome screening or PCR by targeting genes encoding for enzymes involved in their formation (Spano et al., 2010; Laulund et al., 2017).

LAB ability to decarboxylate amino acids is highly variable and seemed to be strain specific (Masson et al., 1996; Bover-Cid and Holzapfel, 1999; Spano et al., 2010; Barbieri et al., 2019). Among PC strains studied in seafood biopreservation, *C. maltaromaticum* and *C. divergens* are well-known tyramine producers (Duffes et al., 1999a; Brillet et al., 2005; Barbieri et al., 2019). The *Lactobacillus* genus is also cited as having many species that can produce tyramine and putrescine. For instance, *L. sakei*, *L. curvatus*, *L. paracasei* and *L. brevis* are the main *Lactobacillus* tyramine producing species (Bover-Cid and Holzapfel, 1999; Barbieri et al., 2019). Unlike tyramine and putrescine, only very few LAB species were associated with histamine production (Masson et al., 1996; Bover-Cid and Holzapfel, 1999; Landete et al., 2007; Barbieri et al., 2019).

Chapter 2

Effect of the manufacturing process on the microbial ecosystem, organoleptic properties and volatilome of 3 salmon based products

Chapter 2. Effect of the manufacturing process on the microbial ecosystem, organoleptic properties and volatilome of 3 salmon based products

1. Preamble

In order to better adjust our approach for salmon gravlax biopreservation, we firstly had to characterize this poorly known product from a microbiological, physico-chemical and sensorial point of view. Indeed, in the literature, fish gravlax microbial flora was studied only by Leisner et al. (1994) and Lyhs et al. (2001). In addition, in both studies, the microbiological analyses was limited to cultural methods, without precise identification of bacteria genera or species present at the spoilage time. Moreover, before this thesis, the Ifremer intern sensory panel had never worked on this type of products. A part of this work constituted a preliminary study to identify relevant sensory descriptors (odors, flavors, visual aspect) related to salmon gravlax freshness or spoilage. Through this work, we also aimed to identify potential biochemical spoilage markers such as VOCs, TVBN and biogenic amines, in order to later assess the biopreservation effect on gravlax quality.

This chapter presents our results regarding salmon dill gravlax characterization through a polyphasic approach combining microbiological analyses (cultural and metabarcoding of 16S rRNA gene), biochemical analyses (ABVT, biogenic amines and VOCs) and sensorial description (profling test). This experiment was also extended to cold-smoked salmon stored under vacuum or modified atmosphere, to compare the manufacturing process effect on the microbiota and products conservation.

Effect of the manufacturing process on the microbial ecosystem, organoleptic properties and volatilome of 3 salmon based products: salmon gravlax and cold-smoked salmon packed under vacuum or modified atmosphere.

In Preparation

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2. Abstract

As traditionally consumed in Nordic countries, but also since few decades elsewhere in Europe, lightly processed and preserved seafood products such as cold-smoked fishes and fish gravlax are of considerable economic importance. This work aimed to extend the knowledge about 3 products, cold-smoked salmon (CSS) stored under vacuum (VP) or modified atmosphere packaging (MAP) and salmon dill gravlax, by comparing the effect of the manufacturing process on their microbial ecosystem, sensory properties and volatilome composition. From as same batch of fishes, VP CSS, MAP CSS and gravlax were produced at demand by a local company and stored in chilled condition for 49 and 35 days respectively (1 week at 4 °C, the rest at 8°C). For the 3 products, each week biochemical analyses (TMA, TVBN, biogenic amines, pH, and volatile organic compound), sensory analyses (profile test) and cultural microbiological analyses were performed. In addition, the microbial ecosystem composition was also monitored by metabarcoding approach (hyperviable region V4 of the 16S rRNA gene) the first day and after 14 and 28 days. At the end of the experiment no products reached the sensory rejection, especially MAP CSS with a

final spoilage score of 0.5 on a scale of 10 at 35 days. Despite a weak spoilage, several organoleptic changes were noticed for VP CSS and salmon gravlax such as an increase in amine and acid off-odors and flavors, in fatty appearance, a slight discoloration and a drop of firmness. An increase in concentration of fish spoilage associated volatile organic compounds (VOCs) such as decanal, nonanal, hexadecanal, benzealdehyde, benzenacetaldehyde, ethanol, 3-methyl-1-butanol, 2,3-butanediol, 1-octen-3-ol, 2-butanone and 1-octen-3-one was also observed. The initial microbiota of the 3 products was almost identical and mainly dominated by the *Photobacterium*, *Lactococcus* and *Lactobacillus* genera. The main microbial shift occurred between 14 and 28 days. VP CSS ecosystem was then mainly composed of *Photobacterium*, *Lactococcus* and *Lactobacillus* genera depending on the biological replicate, while *Lactobacillus* was the most abundant OTU (>95%) in MAP CSS. Salmon gravlax ecosystem was mainly dominated by *Enterobacteriaceae* (OTU multi-affiliated as *Serratia* or *Yersinia*), by *Photobacterium, Lactobacillus* and *Lactococcus*.

Keywords: Metabarcoding, 16S rRNA gene, seafood, volatile organic compounds, sensory analyses.

3. Introduction

Since the last decade, high value-added seafood products, including smoked fishes, fish gravlax and sea salads, are gaining popularity in Europe and often have a dedicated refrigerated shelves in our supermarkets. Among these lightly processed and preserved products, cold-smoked salmon is one of the best-selling delicatessen seafood products (FranceAgriMer, 2018). In European Union (EU), its market was estimated at 155 000 tons in 2013, Germany (28.0%) and

France (20.9%) being the first two countries in volume share (EUMOFA, 2016). EU domestic demand is currently mainly covered by France, United-Kingdom, Spain and Poland.

CSS and others lightly preserved fish products (LPFP) like fish gravlax are characterized by a low salt content (< 6% NaCl (w/w) in water phase) and a high pH (> 5), and are stored at refrigerated temperatures under vacuum or modified atmosphere packaging (Leroi, 2014; Løvdal, 2015). Their sensory shelf-life in chilled conditions, is usually comprised between 18 to 27 days for fish gravlax (Leisner et al., 1994; Lyhs et al., 2001) and between 2 to 6 weeks for smoked fishes, including CSS

(Leroi et al., 1998; Paludan-Müller et al., 1998; Truelstrup Hansen and Huss, 1998; Leroi et al., 2001; González-Rodríguez et al., 2002; Cardinal et al., 2004). Such variability in shelf-life for smoked products finds its origin in the raw material or processing environment microbial quality (Truelstrup Hansen and Huss, 1998; Jørgensen et al., 2000a; Leroi et al., 2001). CSS and gravlax are mainly consumed as ready-to-eat products without prior decontamination step such as heating by cooking, which makes them particularly sensitive to the development of pathogenic or spoilage microorganisms (Pilet and Leroi, 2011; Leroi, 2014).

CSS initial microbiota is often dominated by Gram-negative bacterial genera typically associated with fresh raw fish, such as *Brochothrix*, *Photobacterium*, *Shewanella*, *Vibrio* and *Yersinia* (Leroi et al., 1998; Paludan-Müller et al., 1998; González-Rodríguez et al., 2002; Olofsson et al., 2007; Leroi, 2014). During storage a microbial shift occurred and Gram-positive bacteria, especially lactic acid bacteria (LAB) such as *Carnobacterium* spp. (C. *maltaromaticum*, *C. divergens*) and *Lactobacillus* spp. (*L. sakei*, *L. curvatus, L. alimentarius*) became predominant with concentrations around $10^{7\text{-}9}$ CFU/g (Leroi et al., 1998, 2001; Paludan-Müller et al., 1998; Truelstrup Hansen and Huss, 1998; Jørgensen et al., 2000a; González-Rodríguez et al., 2002; Rachman et al., 2004; Olofsson et al., 2007; Leroi, 2014). However, *Enterobacteriaceae* (*Serratia liquefaciens*, *Hafnia alvei*), *P*. *phosphoreum* and *Brochothrix thermosphacta* can still be present in sufficient level (Truelstrup Hansen and Huss, 1998; Jørgensen et al., 2000a, 2000b; Cardinal et al., 2004; Rachman et al., 2004; Olofsson et al., 2007) to induced spoilage (Leroi et al., 1998; Stohr et al., 2001; Joffraud et al., 2006). Although rarely quantified, yeasts and molds can also be present all along storage time, but as subdominant microbiota (10^4 CFU/g) (Leroi et al., 1998). In salmon gravlax, Leisner et al. (1994) found similar trend with an ecosystem largely dominated after 7 days of storage by LAB belonging to the genera *Carnobacterium*. Conversely Lyhs et al., (2001) demonstrated in trout gravlax that H_2S-producing bacteria (including *Enterobacteriaceae*) was the main dominant microbial group, while LAB stayed in minority.

Almost all cited authors conducted CSS and gravlax microbiota study using conventional culture methods or by sequencing of all or a part of the 16S rRNA gene via isolated cultures. However, unfortunately these cultural tools often offer a biased or limited view the ecosystem due to several limitations: culture media unspecificity,

impossibility to recover low abundant or uncultivable microorganisms (Mayo et al., 2014; Rodrigues et al., 2018). In the two last decades, culture-independent methods, such as denaturing gradient gel electrophoresis (DGGE), temporal temperature gradient gel electrophoresis (TTGE) and in particular next gen sequencing technologies bring the study of microbial ecosystem to a new age (Rodrigues et al., 2018). By metagenetic approach (metabarcoding and metagenome sequencing), it is now possible to get a full and clear picture of microbial ecosystem composition and dynamics in food commodities (Cocolin et al., 2018; Rodrigues et al., 2018). To date, Chaillou et al., (2015) and Leroi et al. (2015) were the only authors to use metabarcoding approach to characterized CSS microbial ecosystem. The initial microbiota was mainly dominated by *P. phosphoreum, P. kishitanii, L. sakei, B. thermosphacta* and also in a lesser extend few LAB such as *Leuconostoc gasicomitatum*, *Lactococcus piscium*, *C. divergens* and *C. maltaromaticum*. At the spoiling date, composition was variable within samples. For some, the ecosystem was almost exclusively dominated by LAB such as *Staphylococcus equorum*, *L. curvatus*, *L. lactis*, *C. divergens* and *C. maltaromaticum*, or only by *P. phosphoreum* and *P. kishitanii,* or again by a mix of *B. thermosphacta* with *C. maltaromaticum* and *L. fuchuensis* (Chaillou et al., 2015). *Enterobacteriaceae* (*Serratia proteamaculans* and *H. alvei*) were also detected within samples. Leroi et al. (2015) described similar results with CSS microbial ecosystem mainly dominated by LAB (*Lactobacillus* spp., *Enterococcus* sp., *Vagococcus* spp., *Carnobacterium* spp., *Leuconostoc* spp.) and *Photobacterium* spp., (*P. phosphoreum,* and *P. kishitanii*).

Currently, VP CSS is a relatively well-described seafood products from a microbiological (Leroi et al., 1998, 2001, 2015; Paludan-Müller et al., 1998; Truelstrup Hansen and Huss, 1998; Jørgensen et al., 2000a; González-Rodríguez et al., 2002; Rachman et al., 2004; Olofsson et al., 2007; Chaillou et al., 2015), a sensorial (Jørgensen et al., 2000a; Leroi et al., 2001; Cardinal et al., 2004) and a biochemical point of view (Cardinal et al., 1997; Jørgensen et al., 2000a, 2001; Varlet et al., 2007; Jónsdóttir et al., 2008). This study aimed to extend that knowledge on two less known products, MAP CSS and dill salmon gravlax by measuring the effect of the manufacturing process on the microbial ecosystem, organoleptic properties and on the volatilome of these 3 LPFP products.

4. Material and methods

4.1. Salmon products, raw material process and storage

Dill salmon gravlax, MAP and VP CSS were produced simultaneously from the same batch of raw salmon (*Salmo salar*), purchased in Norway and processed on the same industrial site. Salmons were filleted in France, 4 days after slaughtering, stored at 4 °C and then processed the next day. For gravlax production, a part of the fillets was cured with a mix of salt, sugar, pepper and dill during 14 h at 6°C. Fillets were then rinsed, sliced and packed under vacuum as 120 g portions of 8 slices. The other part of the fillets was separately dry-salted during 10 h at 4 °C, followed by a rinsing and drying step of 2 h 30 at 23 °C. Fillets were then smoked during 6 h at 23 °C and let 2 days between 0-4 °C for maturation. Matured fillets were sliced and packed under vacuum as 150 g portions of 5 slices for the VP CSS, or cut into dices and packed under modified atmosphere (40% CO_2/60% N_2) as 500 g portion for MAP CSS. Immediately after the conditioning, products were transported to the laboratory under refrigerated conditions.

4.2. Sampling dates and type of analyses

MAP CSS and gravlax were stored 35 days and VP CSS for 49 days. All products were incubated at 4 °C for 1 week and 8 °C for the rest of storage period. From T0, which corresponds to first day of experiment, every 7 days samples were withdrawn for microbial enumeration, biochemical analyses (TMA TVBN, biogenic amines and pH), volatile organic compounds composition (VOCs) and sensory analyses. Ecosystem monitoring through metabarcoding approach was performed for the 3 products at T0 and after 14 and 28 days of storage. Except for the sensory assessment, all analyses were performed in triplicates.

4.3. Microbiological analysis

4.3.1. Microbial enumeration

At each sampling date, 20 g of product were aseptically withdrawn and stomached (Stomacher 400 circulator, Seward medical, London, UK) for 2 min with 80 ml of refrigerated sterile tryptone salt solution (Biokar Diagnostic, Beauvais, France) with 1% Tween 80 (Grosseron, Saint-Herblain, France). Prior to dilution, the stomached solution was left at room temperature for 30 min for bacterial revivification. Successive dilutions were realized in tryptone salt solution + Tween, and 1 ml of the appropriate

dilution was pour plated for *Enterobacteriaceae* enumeration and 100 µl were spread plated for the others microorganisms. Culture media and incubation conditions are listed in Table 2.1. To set anaerobic condition, Anaerocult A and Microbiology Anaerotest (Merck, Darmstadt, Germany) were added to hermetic jars. Detections threshold were 0.7 and 1.7 log (CFU/g) respectively for *Enterobacteriaceae* and other bacterial counts.

Table 2.1: Culture media and incubation conditions used for bacterial flora enumeration.

Target flora	Selective medium	Growth conditions
Total aerobic mesophilic bacteria (TAMB)	Long and Hammer	96 h at 15 °C, aerobic
Lactic acid bacteria (LAB)	Nitrite Actidione Polymyxin agar (NAP)	96 h at 20 °C , anaerobic
Brochothrix thermosphacta	Streptomycin-Thallous Acetate Actidione agar (STAA)	48 h at 20 °C, aerobic
Enterobacteriaceae	Violet Red Bile Glucose agar (VRBG)	48 h at 20 °C, double layer, aerobic

4.3.2. Total bacterial DNA extraction

Bacterial DNA was extracted from stomached solution following a modified and optimized protocol using MasterPure™ Gram-Positive DNA Purification kit (Epicentre, Illumina, Madison, USA). Four milliliters of stomached solution were spin down at 12,000 g for 10 min at 4 °C. After centrifugation, the supernatant was removed and cell pellet was re-suspended with 500 µl of TE buffer and treated with 1 µl of Ready-Lyse-Lysozyme for 1 h at 37 °C with homogenization every 20 min. After lysozyme treatment, the mixture was transferred into tubes containing 0.2 g of sterile 1.0 mm Zirconia/Silica beads (Biospec Products, Bartlesville, USA). Tubes were shaken twice during 2 min at 30 Hz with a beads beater (Retsch, Illkirch, France). Between each beads beating, samples were ice cooled for 2 min. Beads were briefly spun down and the supernatant was withdrawn into new tubes. Supernatant was then treated with 150 µl of Gram-Positive Lysis Solution containing 1 µl of Proteinase K (50 µg/µl) for 15 min at 65 °C. After incubation, samples were cooled at 37 °C for 5 min and then in ice for another 5 min. 175 µl of MPC Protein Precipitation reagent were added to the samples

followed by vigorous shaking for 10 seconds. Debris were pelleted by centrifugation at 4 °C for 10 min at 10,000 g. Supernatant was treated with 1 µl of RNase A (5 µg/µl) for 1 h at 37 °C. 500 µl of cold isopropanol (Carlo ERBA Reagents, Val de Reuil, France) was then added to the solution and homogenized by inverting tubes 40 times prior to an overnight incubation at -20 °C. DNA is recovered by centrifugation at 4 °C for 10 at 10,000 g. Supernatant was carefully removed and the pellet was then rinsed two times with 500 µl of ethanol at 70% (Carlo ERBA Reagents, Val de Reuil, France). DNA was re-suspended in 35 µl of TE buffer, and then quantified and checked for quality using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, USA). DNA samples were then stored at -20 °C.

4.3.3. Bacterial 16S rRNA Gene Sequencing

The hypervariable V4 region of the bacterial 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the EMP primers set (515f/806r) from Caporaso et al. (2011). PCR mixture was composed of 6.75 µl of nuclease-free water, 5 µl of 5X Q5 High GC Enhancer, 5 µl of 5X Q5 Reaction Buffer, 1.25 µl of each primer at 10 µM, 0.5 µl of dNTP at 10 mM, 0.25 µl of Q5 High-Fidelity DNA Polymerase (2 U/µl), and 5 µl of sample DNA diluted at 10 ng/µl, for a final volume of 25 µl. DNA template was amplified according to the following thermal conditions: 30s at 98 °C, 30 cycles composed by 10s at 98 °C, 30s at 50 °C, 30s at 72 °C, and a final extension step at 72 °C for 7 min. Each sample were amplified in triplicates and pooled into a single volume of 75 µl. PCR products were then cleaned-up, barcoded and normalized according to Illumina guideline and protocol “16S Metagenomic Sequencing Library Preparation”. Sequencing was performed with a MiSeq instrument with v3 chemistry and generated 300 bp paired-end reads which were demultiplexed by the Illumina run software.

4.3.4. Bioinformatics process of the data

Demultiplexed reads (around 300 pb) were firstly checked for quality using FastQC (Andrews, 2010), and trimmed with FASTX-trimmer from the FASTX-Toolkit (Hannon, 2010) as follow: reads R1 were trimmed after 280 pb and reads R2 after 230 pb. Reads were then processed using the FROGS pipeline (Escudié et al., 2018). Reads were merged using Flash (Magoč and Salzberg, 2011) with 10% mismatches authorized in overlapped region, and primers sequences removed with Cutadapt (Martin, 2011). Merged reads were clustered using Swarm (Mahé et al., 2014) according to Escudié

et al. (2018) recommendations, with a first execution with an aggregation parameter equal to 1, followed by a second execution on previous clusters seeds with an aggregation parameter equal to 3. Chimera detection and removal was performed using VSEARCH (Rognes et al., 2016). Clusters were then filtered on abundance and occurrence by representing a minimum 0.005% of all sequences and being present at least in 3 samples. Clusters affiliation was performed with blastn+ (Camacho et al., 2009) against 16S Silva database version 123 (Quast et al., 2013), and OTUs were filtered depending to an identity and coverage value of 100%.

Downstream analysis were performed on rarified counts with R version 3.4.4 (R Core Team, 2018) under RStudio environment version 1.1.442 (RStudio Team, 2016) using the R packages phyloseq (McMurdie and Holmes, 2013) and vegan version 2.4.6 (Oksanen et al., 2018). The R package DESeq2 (Love et al., 2014) was used to performed differential abundance analysis on samples raw counts normalized following a rlog transformation in base 2 with a pseudo-count of 1. All graphical visualizations were performed with the R package ggplot2 (Wickham, 2009).

4.4. Sensory analysis

At each sampling date, portions of salmon dill gravlax, VP and MAP CSS were collected and kept frozen at -80 °C until analysis. For each products a conventional sensory profiling test was conducted according to the ISO norm 13299 (2003). The sensory evaluation was performed by an internal trained panel of 17 judges experienced in seafood, especially in salmon products (Cardinal et al., 2004; Macé et al., 2013a). During sessions, panelists were asked to firstly assess the global spoilage based on aspect and off-odors perception. Then products were described according to a list of relevant sensory descriptors for the odor (fatty fish, acid, amine, smoke and dill), the appearance (fatty appearance, orange color), the texture (greasy film, firm, melting and pasty texture) and the flavor (acid, salty, amine, fish, smoked, sweet and dill). Both global spoilage and descriptors were scored depending on their intensity on continuous scale from 0 (low intensity) up to 10 (high intensity). A product was considered strongly spoiled and unfit for consumption when the global spoilage level exceeded a threshold value of 6.

The day before the sensory evaluation, samples were thawed overnight at 4 °C. Sessions were performed in individual partitioned booths, as described in the

procedure NF V-09-105 (ISO 8598, 2010) and equipped with a computerized system (Fizz, Biosystèmes, Couternon, France). Each panelist received one slice of salmon gravlax and 15 g of MAP CSS dices, both presented in covered plastic container. For VP CSS, a half slice (around 15 g) were repacked and presented in folded aluminum foil. Samples were assigned with three digit numbers and randomized for the order presentation within panelists. For each matrix odors and appearance were assessed for all sampling date, while flavors and textures were assessed only on 3 dates: on day 1, 21 and 28 for the salmon gravlax, on day 1, 21 and 35 for MAP CSS, and day 1, 28 and 35 for VP CSS.

Normalized principal component analyses (PCAs) were performed on sensory descriptors mean scores using the R package ggfortify (Horikoshi and Tang, 2016). In addition a two-way analysis of variance (ANOVA) was applied to the panelists' descriptors scores using products (in time) and panelists as independent factors. Significant differences between means were determined using Duncan's multiple range test ($p < 0.05$) (Fizz software, Biosystèmes).

4.5. Biochemical analysis

4.5.1. Physicochemical parameters

TVBN and TMA were quantified at each sampling date from 80 g of minced product, according to Conway micro-diffusion method (Conway and Byrne, 1933). The pH value was measured directly after microbiological analysis in the stomached solution with a pH-meter (Mettler Toledo AG, Schwerzenbach, Switzerland).

4.5.2. Biogenic amines measurement

Ten milliliters from stomached solution (1.2.1) were mixed with 5 mL of a Trichloroacetic acid solution at 12% (Panreac, Darmstadt, Germany). Samples were kept frozen at -20 °C until analysis. Eight biogenic amines (tryptamine, 2-phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine, spermine) were quantified by High Pressure Liquid Chromatography (HPLC) following the methodology described by Wiernasz et al. (2017).

4.6. Headspace-solid-phase microextraction (HS-SPME) and gas chromatography/mass spectrometry (GC/MS) analysis of the volatilome

For each sampling dates, for the 3 products, portions of 20 g were withdrawn and stocked under vacuum packaging at – 40 °C. Eight salmon flesh cylinders were sampled across the frozen product using a pre-cooled metal cork borer and immediately pooled to make up 1 g of analysis sample. Samples were kept frozen in 4 mL vials with screw cap and PTFE/silicone septum at -40 °C prior to extraction and analysis. For each sample (time point and treatment), three independent analysis samples (triplicate) were prepared.

Prior to volatile extraction, a 30% w/v NaCl solution (H_2O) was added to the sample, which was finally minced using a high-speed homogenizer. HS-SPME was applied for extraction of VOC using a manual SPME holder with a PDMS/DVB-coated 65 µm fiber (Supelco Inc., Bellefonte, PA). Prior to extraction, the SPME fiber was conditioned in the injection port of the GC according to the instructions provided by the supplier. The SPME fiber was exposed to the atmosphere in the closed sample vial for 30 min, while keeping the vial isothermally at 50 °C in a water bath. Samples were constantly agitated by a magnetic stirrer during extraction.

An Agilent 6890/5975 GC/MS (Agilent Technologies Inc., Palo Alto, CA) was used for all analyses. Analytes absorbed on the SPME fiber were desorbed in the injection port for 3 min under splitless conditions. GC separations were carried out using an apolar HP-5MS capillary column (30 m × 0.25 mm and film thickness 0.25 µm). Injection temperature was 220 °C, and the interface was set to 220 °C. The carrier gas was He at a constant flow rate of 1 ml/min. GC temperature was ramped from 40 °C to 211 °C at a rate of 4.5 °C/min, then raised at a rate of 50 °C/min and finally held at 220 °C (total run time: 40 min). The MS source was adjusted to 230 °C, and a mass range of *m/z* 35–350 was recorded. Mass spectra were acquired in electron impact ionization (EI) mode at 70 eV.

GC/MS chromatograms were visualized using the following GC/MS software packages: Agilent ChemStation software (Agilent Technologies, Waldbronn, Germany), AMDIS software (version 2.71; National Institute of Standards and Technology, Boulder, CO, USA), and the open source programme OpenChrom

Community Edition Alder (version 1.2.0) (Lablicate GmbH, Hamburg, Germany; http://www.openchrom.net).

Tentative identification of compounds was carried out using (a) MS libraries such as NIST05 spectral library (National Institute of Standards and Technology, Gaithersburgh, MD), the NIST Chemistry WebBook (https://webbook.nist.gov/chemistry) and a customized *in-house* MS library of VOCs, in combination with (b) linear retention indices (LRI), based on an homologous series of even numbered n-alkanes (C8 to C24), in combination with LRIs found in literature and NIST Chemistry WebBook. GC/MS data integration, normalization (total signal) and alignment was carried out using the Metalign software (PRI-Rikilt, Wageningen, The Netherlands). Detected analytes concentration were estimated quantitatively based on an internal standard (BHT) and expressed as µg/kg.

Multivariate analyses on VOCs composition were performed by hierarchical clustering analysis (HCA) coupled with a heatmap on log2(n)-median transformed data using the R package gplots (Warnes et al., 2016). The HCA was performed using the Ward clustering method on Euclidean distance.

5. Results

5.1. Natural microbiota

5.1.1. Cultural microbial analyses

For the 3 products, total aerobic mesophilic bacteria (TAMB), LAB, *Enterobacteriaceae*, *B. thermosphacta* growth kinetics are shown in Figure 2.1. Globally each enumerated microbial group started to increase quickly after 7 days, which corresponds to the temperature transition from 4 to 8 °C.

Salmon gravlax initial TAMB count was high with 4.0 ± 0.1 log CFU/g. In comparison, TAMB count was lower in MAP and VP CSS with respectively 3.1 ± 0.2 and less than 1.7 log CFU/g (detection threshold). TAMB counts reached their maximum value after 21 days for the gravlax (7.1 ± 0.4 log CFU/g) and 35 for the MAP and VP CSS (7.1 ± 0.4 log CFU/g and 6.9 ± 0.3 log CFU/g respectively). TAMB count in VP CSS appeared to be systematically 1.0 to 3.0 log CFU/g higher than the others enumerated groups, especially between 28 and 42 days.

On MAP CSS, LAB level started with a count below the detection threshold and quickly increased to fit after 14 day the exact TAMB growth trend, showing that lactic bacteria were the highly dominant microbial group from day 14 to 35. On VP CSS, LAB initial count was below the detection threshold until 14 days and reached its maximum after 35 days with 5.7 ± 0.6 log CFU/g. Salmon gravlax initial count in lactic flora was also below the detection threshold, but rapidly reached the TAMB level after 21 days with 6.9 ± 0.3 log CFU/g and remained constant until the end of storage.

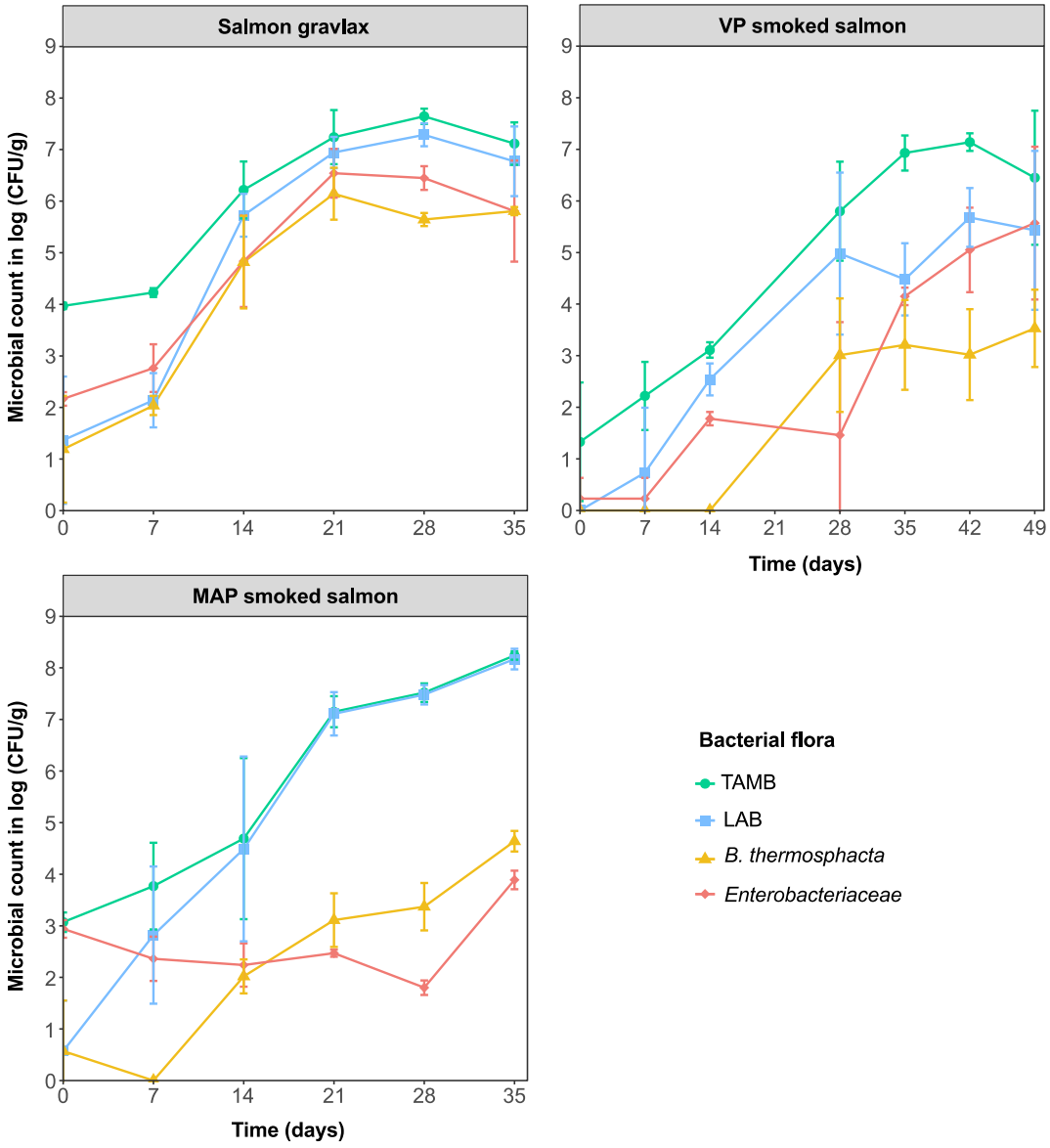

Figure 2.1: Salmon gravlax, MAP and VP CSS microbial kinetic during storage at 4°C for 1 week and then 8°C.

On salmon gravlax, *Enterobacteriaceae* increase was similar to the LAB, with a maximum reached after 21 days with 6.5 ± 0.5 log CFU/g. *Enterobacteriaceae* count was lower in VP CSS. It remained below the detection threshold until 28 days, but reached 5.4 ± 1.5 log CFU/g after 49 days. In contrast with gravlax and VP CSS, *Enterobacteriaceae* count in MAP CSS remained low during storage. The count was constant and comprised between 1.8 ± 0.4 to 2.9 ± 0.2 CFU/g during the first 4 weeks, and only increase after 35 days with a maximum concentration of 3.9 ± 0.2 log CFU/g.

B. thermosphacta count was the lowest microbial count among the enumerated microbial groups for the 3 matrices. In MAP CSS, *B. thermosphacta* concentration was below the detection level until 14 days. After that sampling date, the concentration increased slowly to reach a maximum of 4.6 ± 0.2 log CFU/g after 35 days of storage. Similarly, *B. thermosphacta* count was low in VP CSS. During the 2 first weeks, concentrations were below 1.7 log CFU/g. One week after *B. thermosphacta* growth reached a plateau, and its concentration remained constant until the end of storage with values around 3.0 ± 1.1 log CFU/g. In salmon gravlax, *B. thermosphacta* concentration was globally higher and reached its maximum after 21 days of storage with 6.1 ± 0.5 log CFU/g.

For all sampling dates, an important variability in counts was observable whatever the microbial group considered, in particular in the case of VP CSS. These differences in term of concentration between biological replicates were attributable to the very high heterogeneity among samples. Indeed, fillets were cut lengthwise and slices were gradually packed, resulting in plastic blister containing only slices from the head, belly or tail part of the fish, which are heterogeneous in thickness and fat content.

5.1.2. Ecosystem monitoring through metabarcoding

A total of 3 977 403 raw reads were obtained after Illumina sequencing. 1 746 144 reads passed through FROGS pipeline for an average of 63 270 reads per samples. The sequences number per samples ranged from 35 626 to 114 681. Thus, for each sample, reads number was normalized by rarefaction upon the lowest number of sequences per samples.

Salmon gravlax, MAP and VP CSS ecosystem composition over time are shown in Figure 2.2.

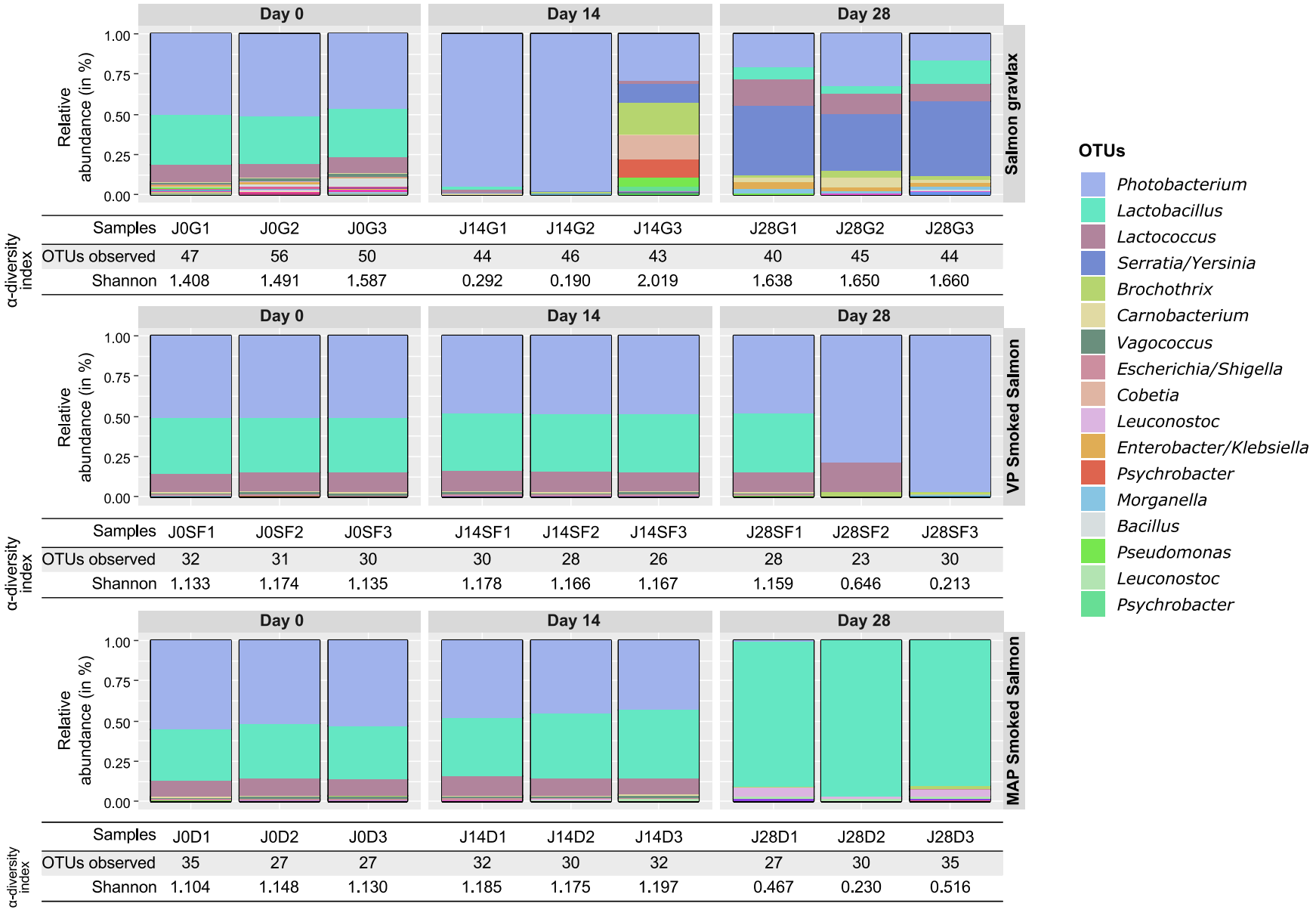

Figure 2.2: Salmon gravlax, MAP and VP CSS ecosystem relative composition and α-diversity. Only OTUs with a sequences number representing more than 0.1% of the total reads number appear on the legend and are ordered from the most abundant to the least abundant. For each sampling date, experiments were done in triplicates.

Whatever the product and for the 3 sampling dates, the microbial diversity was quite low. The richness, through the number of OTUs was between 27, for the lowest sample, to 56 for the highest (Figure 2.2). The evenness of OTUs abundance distribution, calculated with Shannon index, was for almost all samples represented by values comprised between 1.1 and 1.5. Values taken by these two indexes showed an ecosystem mainly composed by only a small number of highly dominant OTUs, which were affiliated to 9 main bacterial genera: *Photobacterium*, *Lactobacillus*, *Lactococcus*, *Serratia/Yersinia*, *Brochothrix*, *Carnobacterium*, *Vagococcus*, *Escherichia/Shigella*, and *Cobetia* with a representative sequences number superior to 0.5% of total reads.

At the beginning of storage, ecosystems were highly similar between products, as samples were closely clustered together on the Multidimentional scaling (MDS) ordination plot in Figure 2.3. *Photobacterium*, *Lactobacillus* and *Lactococcus* were the dominant genera, accounting for about 95% of the microbial composition, with respectively 50%, 29-34% and 8-11% (Figure 2.2). However, some differences were observable between products. Gravlax initial microbial diversity was slightly higher than in smoked products, and remained so until the end of storage. Indeed, twenty OTUs, specific to the gravlax, were found in addition, representing the fifteen following bacterial or archaea genera: *Halorubrum*, *Shewanella*, *Bacillus*, *Weissella*, *Hafnia/Obesumbacterium*, *Acinetobacter*, *Brachybacterium*, *Duganella*, *Terribacillus*, *Spelaeicoccus*, *Halohasta*, *Staphylococcus*, *Comamonas*, *Sphingobacterium* and *Brevibacterium*. *Staphylococcus* and *Acinetobacter* were the only shared genera between the 3 products (genera represented by several OTUs). Moreover, excepted *Halohasta*, *Staphylococcus* and *Comamomas*, all these genus specific to the salmon gravlax microbial ecosystem were detected until 28 days of storage. Conversely, a differential abundance analysis using DESeq2 R package, revealed no statistical difference between MAP and VP CSS microbial composition at day 0.

After 14 days of storage, gravlax microbial ecosystem was the only one which diverged from the first sampling date, with a diversity decreasing significantly over time (ANOVA on richness index, p value of 0.045). Except the third biological replicate, the ecosystem composition was largely dominated by *Photobacterium*, whose relative abundance increased from 50 to 97.5% (Figure 2.2). The third replicate microbial composition was more evenly distributed (highest Shannon index of 2.0) between 7 genera, namely: *Photobacterium* (29.0 %), *Brochothrix* (19.6%), *Cobetia* (14.4%),

Psychrobacter (13.8%), *Serratia/Yersinia* (11.9%), *Pseudomonas* (5.6%) and *Lactococcus* (1.7%). In the case of MAP and VP CSS, no statistical differences in term of OTUs abundance were noticed between day 0 and day 14 (Figure 2.2 and 2.3). Only a slight increase of 2 OTUs abundance belonging to *Leuconostoc* genera was observable for the MAP CSS. Moreover, in contrary to gravlax, the storage time showed no statistical effect on ecosystem richness of these 2 smoked products (ANOVA, p values of 0.854 and 0.196*).*

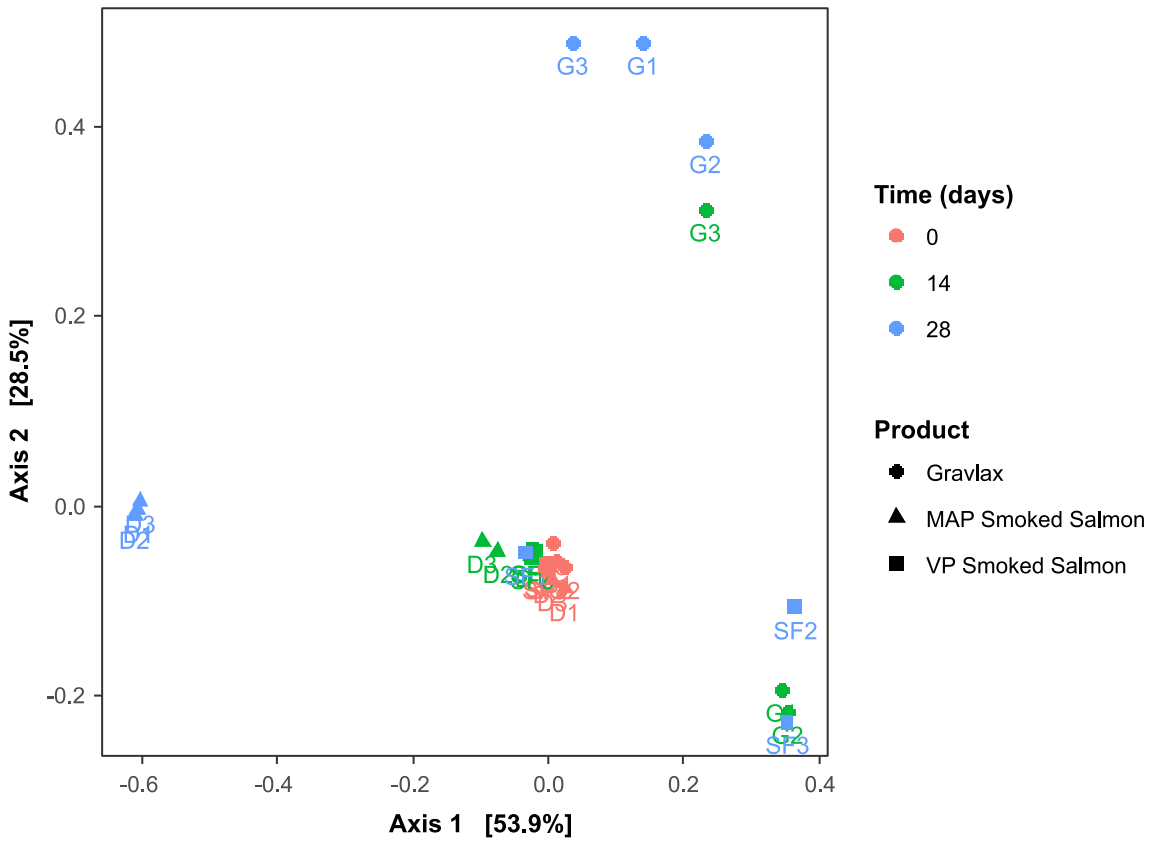

Figure 2.3: Multidimentional scaling representation of salmon gravlax, MAP and VP CSS samples microbial composition based on Bray-Curtis distance.

For the 3 products, the most important shift in ecosystem took place between 14 and 28 days of storage (Figure 2.3). After 28 days, salmon gravlax ecosystem composition was more diverse than the two smoked products (Figure 2.2). *Photobacterium* abundance dropped from 97.5 to 16.5-32.6% and *Enterobacteriaceae*, with *Serratia*/*Yersinia* and *Enterobacter*/*Klebsiella* genera, increased to reach 35.1-46.9% and 1.7-3.6% respectively. Some lactic acid bacteria genera, such as *Lactobacillus*, *Carnobacterium* and *Lactococcus,* weakly present at 14 days (<1.5%), also increased

after 28 days to reach, 4.1-14.3%, 1.9-6.6% and 10.8-16.4% respectively. For the MAP CSS, *Photobacterium* and *Lactococcus* disappeared (< 0.5% of abundance) in favor of *Lactobacillus* which became the only dominant genera with more than 90% of abundance and in a lesser extent *Leuconostoc* which represented 1 to 5% of abundance. Conversely, for VP CSS, despite variability between replicates, *Photobacterium* abundance increased to reach 78.4% and 96.8% in the second and third replicates respectively. *Lactococcus* and *Lactobacillus* disappeared from the third replicate (<0.05%). In the second replicate, *Lactococcus* remained at an abundance level similar to day 14, while the first replicate microbial composition was not different from samples from day 0 and 14.

5.2. Salmon based products sensory evolution

Until the end of the experiment, no product has exceeded the sensory rejection threshold (Global spoilage score < 6). The spoilage was low with maximum scores of 3.9 and 2.7 for the dill gravlax and VP CSS after 35 and 49 days respectively. MAP CSS demonstrated no sign of spoilage, even after 35 days of storage (global spoilage score of 0.5). The two PCA in Figure 2.4 illustrate the 3 products sensory characteristics. In Figure 2.4.A (odor and color), the 2 smoked products are discriminated from salmon gravlax by the second axis, while unspoiled samples are discriminated from lightly spoiled samples by the first axis of the PCA. In Figure 2.4.B (flavor and texture), it's the opposite, with the axis 2 discriminating unspoiled from lightly spoiled samples and axis 1 discriminating smoked products from salmon gravlax.

MAP CSS was characterized during the 35 days of storage by a high perception of smoke odor (3.8-5.4) and flavor (5.4-5.7), a stable fatty fish odor (2.2-2.5) and flavor (3.4-3.9), a firm texture with moderate development of fatty droplets (greasy film), and by the absence of spoilage notes such as amine odor (score < 0.5) (Figure 4). A strong butter odor, not figuring in the sensory analysis criteria, was also detected by panelists at the first sampling date and was never found later. Although not strongly spoiled after 35 days, MAP CSS, showed nevertheless, few minor sensory changes during storage. The panelist's perception of the smoke odor, orange color of the product, and melting texture significantly decreased after the first week of storage, while salty and acid flavor lightly increased after 35 days (Duncan test with p values < 0.05).

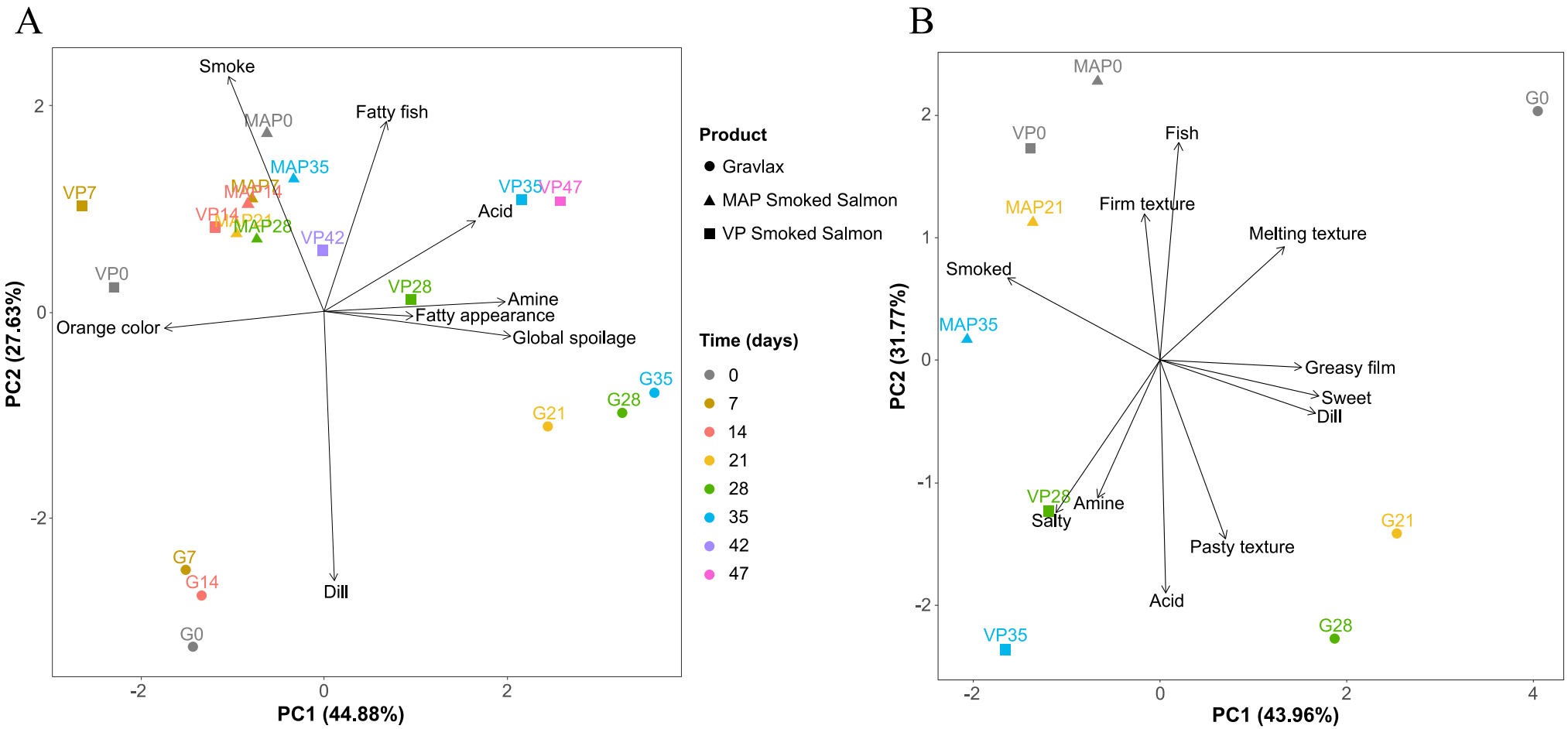

Figure 2.4: Normalized principal component analysis (PCA) representation, on dimension 1-2, of salmon dill gravlax, MAP and VP CSS sensory evolution, based on mean score of visual and odor descriptors (A) and flavor and texture (B).

The salmon gravlax was considered lightly spoiled after 21 days, with a global spoilage score increasing from of 0.2 at day 0, to 3.0. Until day 35, the spoilage score remained stable (no significant differences, Duncan test), reaching at its maximum 3.9. Gravlax "spoilage" was defined by a significant decrease in perception of the dill odor, of the orange color, the fish flavor, and melting texture of the product (Figure 2.4). Concomitantly, a significant increase was observable in the perception of the salty flavor, amine and acid flavor and odor. However, acid and amine odors, although associated to spoilage, never exceeded a score of 2.0 and 2.6 respectively after 35 days of storage.

VP CSS variability between samples was also observable during sensory analysis. Indeed, the product was considered slightly spoiled after 35 and 49 days of storage with a score of 2.5 and 2.6 respectively, but not at day 42 with a score of 1.0 (Duncan test, p value < 0.05) (Figure 2.4). The smoke flavor and odor intensity were highly perceived by panelist at the beginning of storage (6.1 and 5.3 respectively at day 0). As for the MAP CSS, panelists also described the presence of a strong butter odor, which disappeared after the first week. After one week of storage, the smoke odor intensity started to decrease from 5.6 and reached 2.3 after 49 of storage. Similarly, the orange color and the firmness of the product also decreased significantly after 28 days of storage. Although not statistically significant (ANOVA, p value of 0.051), a slight decrease in the perception of the smoke flavor was observable. Concomitantly, amine odor and flavor and acid flavor perception started to increase after 28 days to reach 2.8, 1.9 and 3.9 respectively. The fatty aspect of the product, characterized by fatty droplets at its surface, also increased significantly after 14 days, and reached a score of 5.2 after 47 days of storage. Nevertheless the important augmentation of this sensory criterion (2.9 at day 0 for comparison), was not noticed by panelists during tasting, as no significant difference during storage was found for the greasy film texture when masticating (p value of 0.31). An increase for the pastry texture of the VP CSS slices, although not significant (p value of 0.08), was also observable after 28 days of storage.

5.3. Chemical analyses

For the 3 products, the pH was almost identical and remained stable during the whole storage time with values between 5.47 and 5.93 (Table 2.2). Dill gravlax initial pH (5.87 ± 0.10) value was slightly higher than MAP and VP CSS initial pH (5.68 ± 0.02 and 5.63 ± 0.03 respectively. Initial TVBN contents were similar for the 3 products with values between 12.0 ± 1.8 and 14.5 ± 1.6 mg-N/100g (Table 2.2). Dill gravlax and VP CSS TVBN content increased during storage and reached their maximum at 28 (28.1 ± 1.0 mg-N/100g) and 35 (24.7 ± 2.5 mg-N/100g) days respectively. After 28 days, gravlax TVBN content lightly declined to reach 23.1 ± 1.3 mg-N/100g. No production of TVBN in MAP CSS was observed until the end of experiment. Whatever the product, no TMA was detected during the storage.

5.4. Biogenic amines content

On the 6 biogenic amines quantified, only histamine, cadaverine and tyramine were detected. For all samples, a very high variability was observed within the triplicates. Concentrations in gravlax increased after 14 days of storage (Table 2.2) and remained stable until 28 days within a range of 275.8 ± 30.6 to 404.1 ± 79.4 mg/kg for cadaverine, 42.2 ± 12.9 to 65.3 ± 60.9 mg/kg for tyramine and 55.7 ± 7.0 to 116.7 ± 104.3 mg/kg for histamine. As for the TVBN content, a decrease in biogenic amines content was observable after 35 days of storage. In VP CSS, biogenic amines production started after 28 days, and also remained more or less stable until 49 days (Table 2.2). Their respective range values were between 153 ± 160.5 to 218.9 ± 187.9 mg/kg for cadaverine, 26.7 ± 36.0 to 49.5 ± 78.5 mg/kg for tyramine and 10.8 ± 6.0 to 59.4 ± 45.2 mg/kg for histamine. In MAP CSS, no biogenic amine exceeded 10 mg/kg for the whole storage duration (Table 2.2). Although the mean concentration of histamine was inferior to 100 mg/kg, some replicates overpassed this limit (gravlax at day 14, VP CSS at days 35 and 49).

Table 2.2: Salmon gravlax, VP and MAP CSS pH evolution, TVBN (mg-N/100g) and biogenic amines (mg/kg) contents during storage at 4°C for 1 week and then 8 °C.

Sampling date (days)	0	7	14	21	28	35	42	49
Salmon gravlax								
pH	5.87 ± 0.10	5.93 ± 0.02	5.62 ± 0.04	5.81 ± 0.03	5.77 ± 0.02	5.84 ±0.02	-	-
ABVT	12.0 ± 1.8	15.1 ± 0.9	22.2 ± 5.3	24.9 ± 4.9	28.2 ± 1.0	23.1 ± 1.3	-	-
Cadaverine	2.0 ± 0.1	6.0 ± 3.6	294.2 ± 224.1	275.8 ± 30.6	404.1 ± 79.4	73.5 ± 58.2	-	-
Tyramine	2.0 ± 0.1	2.0 ± 0.1	65.3 ± 60.9	42.2 ± 12.9	63.5 ± 15.3	13.0 ± 12.0	-	-
Histamine	0.7 ± 0.6	1.3 ± 0.6	116.8 ± 104.3	57.7 ± 7	77.3 ± 24.0	17.1 ± 12.7	-	-
MAP CSS								
pH	5.68 ± 0.02	5.81 ± 0.01	5.53 ± 0.07	5.70 ± 0.02	5.65 ± 0.04	5.63 ± 0.02	-	-
ABVT	14.4 ± 0.7	14.6 ± 1.0	14.2 ±1.9	14.8 ± 1.0	13.3 ± 2.6	16.8 ±2.1	-	-
Cadaverine	2.3 ± 2.1	4.7 ± 0.6	9.0 ± 5.6	6.7 ± 2.0	6.2 ± 1.0	4.4 ± 0.4	-	-
Tyramine	0.0	0.0	3.3 ± 3.1	0.2 ± 0.4	0.3 ± 0.5	6.2 ± 2.7	-	-
Histamine	3.7 ± 3.2	6.0 ± 1.0	8.0 ± 2.0	5.3 ± 0.6	5.5 ± 1.5	5.2 ± 0.7	-	-
VP CSS								
pH	5.63 ± 0.03	5.81 ± 0.02	5.47 ± 0.04	-	5.64 ± 0.07	5.76 ± 0.01	5.60 ± 0.05	5.57 ± 0.013
ABVT	14.5 ± 1.6	17.1 ± 1.3	17.5 ± 1.3	-	21.0 ± 3.7	24.7 ± 2.5	24.6 ± 3.6	25.9 ± 5.0
Cadaverine	4.0 ± 1.0	4.0 ± 0.1	4.6 ± 0.9	-	153.4 ± 160.5	185.0 ± 186.3	185.3 ± 281.7	218.9 ± 187.9
Tyramine	0.0	0.0	0.0	-	26.7 ± 36.0	37.8 ± 43.9	49.5 ± 78.5	38.8 ± 33.7
Histamine	6.7 ± 1.2	5.7 ± 0.6	5.6 ± 0.2	-	33.4 ± 29.6	56.3 ± 61.9	10.8 ± 6.0	59.4 ± 45.2

-: not determined

5.5. Volatilome profile

For the 3 products, as for the biogenic amines and TVBN content, an important variability in VOCs composition was observed between replicates. Thus, the evolution of the volatilome composition for each product was visualized with a HCA heatmap based on log2(n) ratio of the mean concentrations amended by the median value (Figures 2.5, 2.6 and 2.7).

Salmon gravlax volatilome composition showed the lowest complexity with 59 compounds detected by SPME-GC/MS. Twenty-eight were identified as terpenes, probably related to the presence of spices (black pepper, dill) used in the curing step, and their concentration remained stable during the storage period. As the storage progressed, an increase in concentration of alcohols (ethanol, 3-methyl-1-butanol, 2,3-butanediol, 1-octen-3-ol, phenylethyl alcohol), aldehydes (nonanal, (E,E)-2,4-decadienal), aromatic aldehydes (benzaldehyde, benzenacetaldehyde), ketones (2-butanone, 1-octen-3-one) and 1-pentadecene was visible (Figure 2.5). Conversely, concentrations in alkane such as undecane, dodecane, tridecane, tetradecane decreased overtime.

MAP and VP CSS volatilome composition were much more complex than in gravlax, with 164 and 149 compounds detected respectively. For both products, more than half compounds were identified as furan and aromatic compounds related to the smoking process.

In the case of VP CSS, the heterogeneity was high between replicates at the different sampling dates was high. However, a trend emerged, characterized by an increase of the concentration of some aldehydes, such as decane, hexadecanal, in aromatic aldehydes, such as benzaldehyde, benzenacetaldehyde, in phenylethyl alcohol and 3,4-dimethyl-2-hydroxycyclopent-2-en-1-one (Figure 2.6). An increase in production of dimethylamine (at day 28 and 35) and ethanol (at day 35 and 42) was also visible. A decrease in concentration of some furan and aromatic compounds was also recorded, such as 2,4,6-trimethylphenol, furfural, acetylfuran, 3-methylfurfural, 5-methylfurfural and unidentified aromatic-5/19/20.

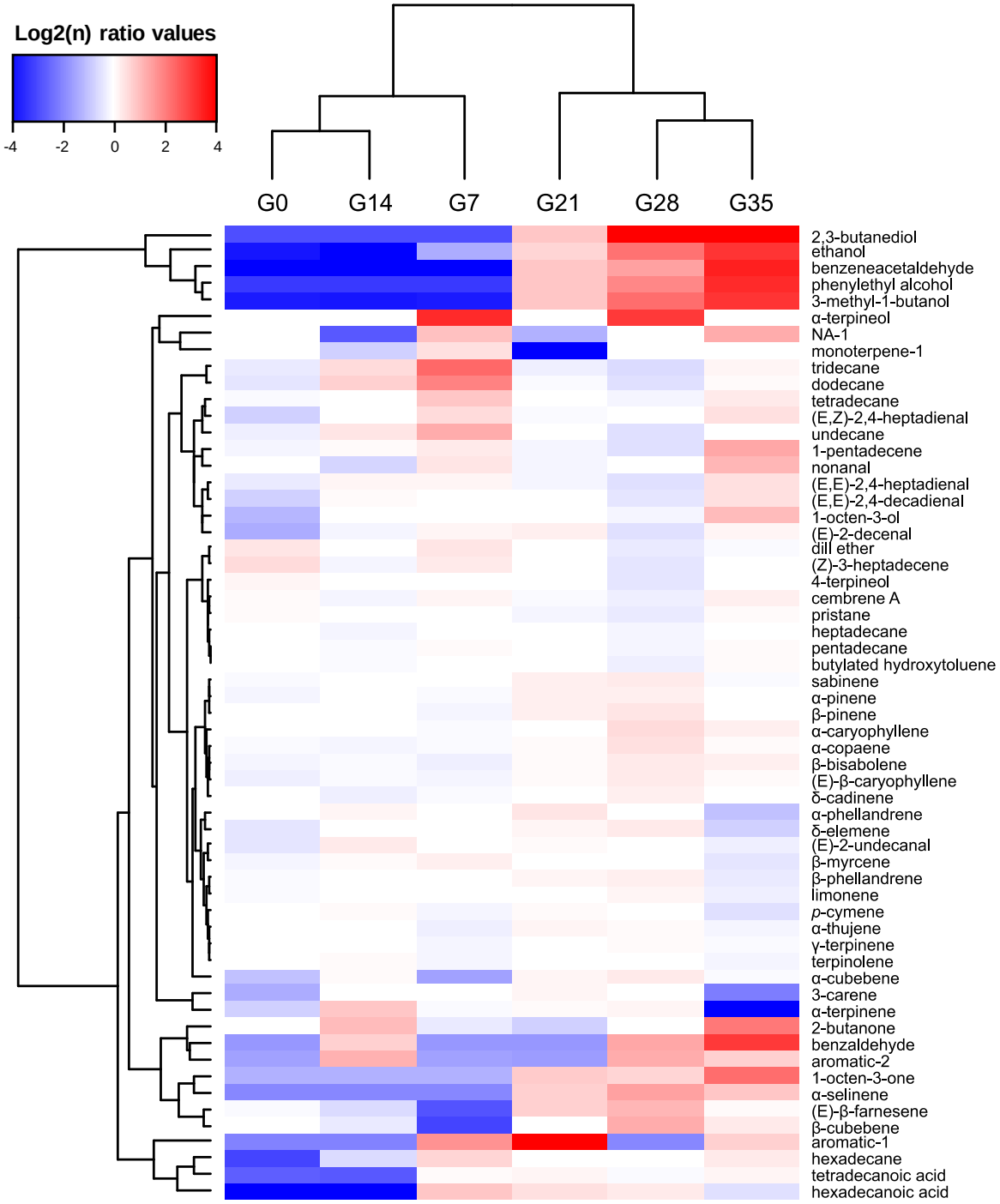

Figure 2.5: Salmon gravlax VOCs Hierarchical Cluster Analysis (HCA) heatmap based on Euclidean distance calculated from log2(n) transformation on mean concentration (n = 3) amended by the median. Sampling time points are represented in columns (0 to 35 days), while VOCs are depicted in rows. Blue colors indicate lower metabolite concentrations, while red colors show higher metabolite levels. See Supplementary Table 1, Table 2.4.

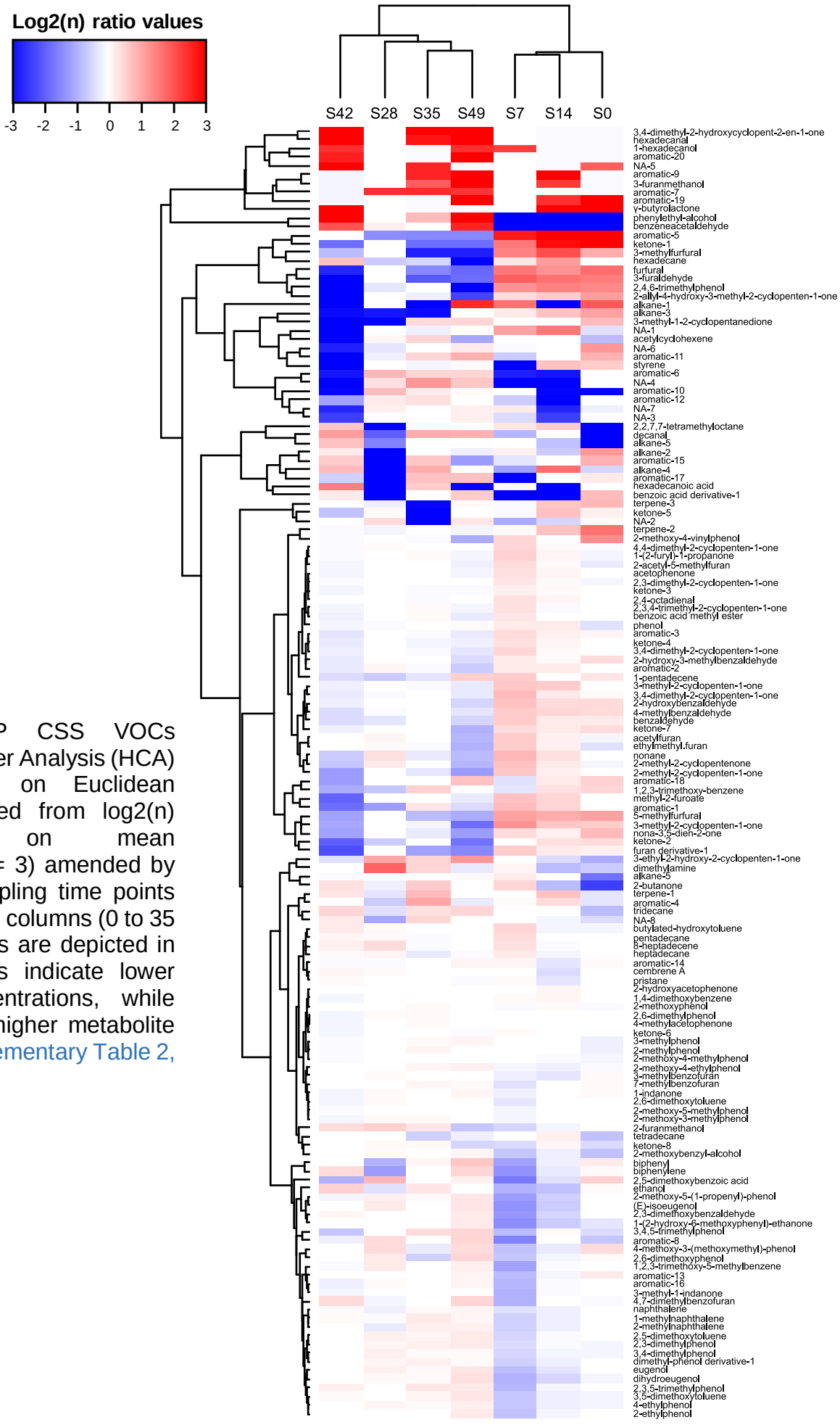

Figure 2.6: VP CSS VOCs Hierarchical Cluster Analysis (HCA) heatmap based on Euclidean distance calculated from log2(n) transformation on mean concentration (n = 3) amended by the median. Sampling time points are represented in columns (0 to 35 days), while VOCs are depicted in rows. Blue colors indicate lower metabolite concentrations, while red colors show higher metabolite levels. See Supplementary Table 2, Table 2.5.

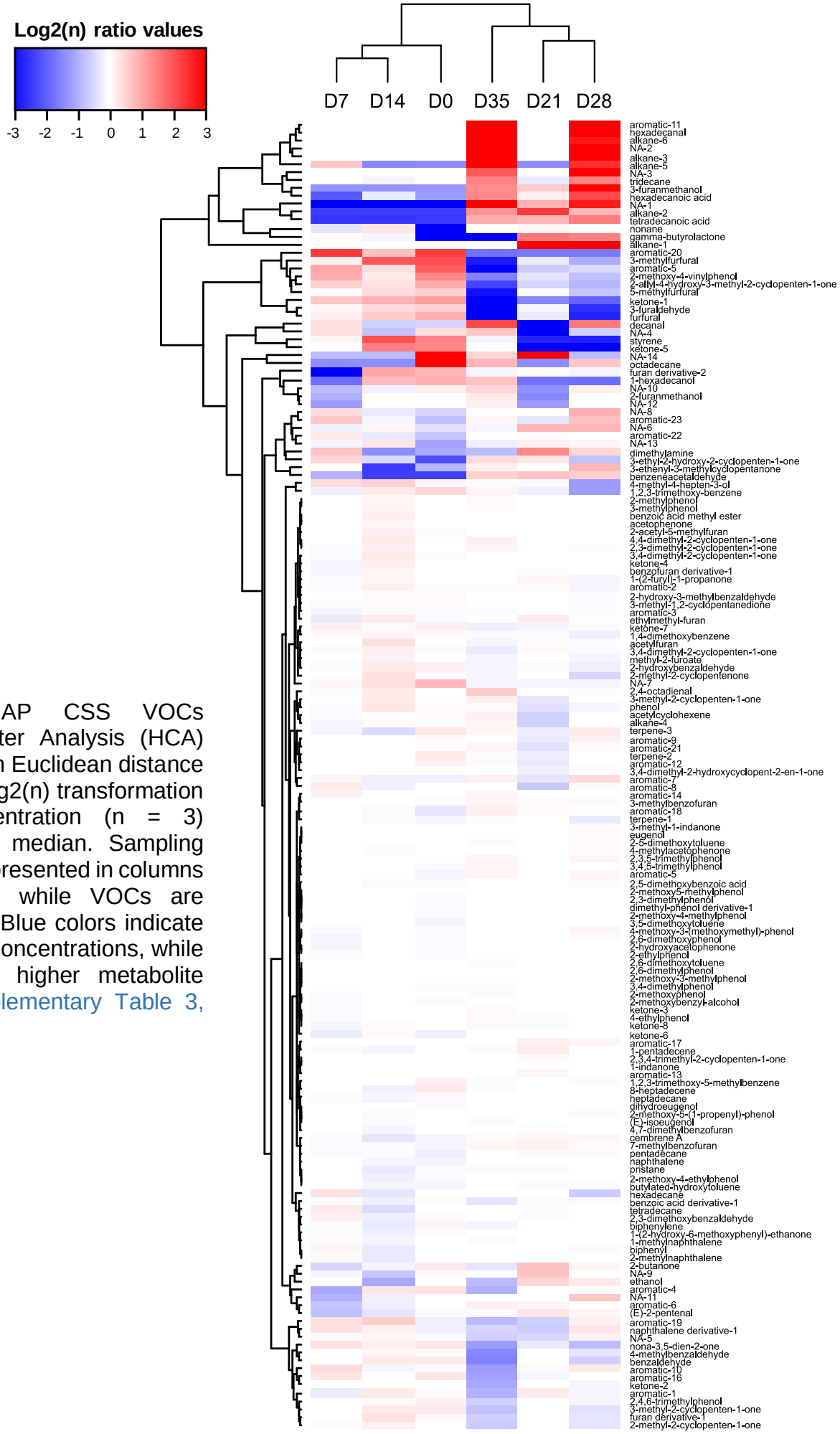

Figure 2.7: MAP CSS VOCs Hierarchical Cluster Analysis (HCA) heatmap based on Euclidean distance calculated from log2(n) transformation on mean concentration (n = 3) amended by the median. Sampling time points are represented in columns (0 to 35 days), while VOCs are depicted in rows. Blue colors indicate lower metabolite concentrations, while red colors show higher metabolite levels. See Supplementary Table 3, Table 2.6.

Despite the absence of spoilage, slight variations in MAP CSS VOCs concentration was observable during storage. An increase in tetradecanoic and hexadecanoic acid, ethanol (at day 21 and 28), decanal, hexadecal (after 28 days), tridecane, unidentified alkane-1/2/3/5/6, 3-furanmethanol and dimethylamine (at day 7, 21 and 28) were found (Figure 2.7). Conversely, a decrease in concentration overtime was visible for 3-furaldehyde, styrene, unidentified aromatic-5/11/20, 2-methoxy-4-vinylphenol, and as for the VP CSS, in furfural, 3-methylfurfural and 5-methylfurfural. Nonetheless, except for furan and aromatic compounds, concentrations were relatively low (Supplementary Table 3, Table 2.6), consequently the variations observed in the HCA heatmap representation were amplified by the median emendation and the ratio transformation in log2(n).

6. Discussion

The objective of this study was to compare the effect of the manufacturing process on the microbial ecosystem, organoleptic properties and on the volatilome of 3 salmon based products.

After the packaging step, the TAMB count of the 3 products ranged from values below 1.7 log CFU/g for the VP CSS, to 3.0-4.0 log CFU/g for the MAP CSS and salmon gravlax respectively. Such initial bacterial load are usually found in lightly preserved salmon products (Leisner et al., 1994; Leroi et al., 1998; Paludan-Müller et al., 1998; Truelstrup Hansen and Huss, 1998; Hoz et al., 2000; Leroi et al., 2001; González-Rodríguez et al., 2002), but may sometimes reach 5-6 log CFU/g depending on the raw material and smokehouse hygienic quality (Truelstrup Hansen and Huss, 1998; Leroi et al., 2001; Cardinal et al., 2004). MAP and VP CSS initial TAMB counts were lower than in gravlax probably due to the smoking process. Phenolic compounds are known to possess antimicrobial activity (Maqsood et al., 2013) and are responsible for bacterial growth delay or inhibition in CSS (Leroi et al., 2000; Giménez and Dalgaard, 2004; Porsby et al., 2008; Hwang, 2009). Initial bacterial concentration in MAP CSS were higher than VP CSS. As the *Enterobacteriaceae* count was similar to the TAMB, the supplementary cutting step to obtain dices was probably responsible for the recontamination before packaging. Despite a different initial contamination level, these 3 products microbial ecosystems were relatively closed in proportion, with *Photobacterium*, *Lactobacillus* and *Lactococcus* representing around 50%, 30% and 10% of its composition respectively. The initial microbial flora of lightly preserved

seafood under MAP or vacuum is mainly dominated by Gram-negative bacteria such as *Shewanella* spp., *Photobacterium* spp. and *Pseudomonas* spp. as a part of the natural fish microbiota (Leroi, 2014). However, LAB (*Carnobacterium* spp., *Lactococcus* spp., *Lactobacillus* spp.) and *Brochothrix* were also reported by some authors as the main bacteria in early stage of storage of raw and CSS stored under vacuum or MAP (Rudi et al., 2004; Olofsson et al., 2007; Powell and Tamplin, 2012).

Despite an overall composition similarity, salmon gravlax ecosystem diversity was initially higher than smoked products and remained so until 28 days, with 20 specific OTUs belonging to the bacterial or archaeal reign. Among these OTUs, *Shewanella* and *Hafnia*, especially *S. putrefaciens*, *S. baltica* and *H. alvei*, are commonly isolated from fish such as cod, salmon and mackerel (Dalgaard et al., 1993; Leroi et al., 1998; Truelstrup Hansen and Huss, 1998; Jørgensen et al., 2000b; Fletcher et al., 2005; Macé et al., 2012; Alfaro and Hernandez, 2013; Leroi, 2014). By metabarcoding approach, Chaillou et al. (2015) were able to detect 5 species of *Shewanella* (*S. putrefaciens*, *S. vesiculosa*, *S. morhuae*, *S. baltica*, *S. frigidimarina*) in cod fillets but not in raw or CSS. *Weissella* (*W. confuse*, *W. viridescens, W. kandleri*) was also isolated from salmon, halibut and mackerel gravlax (Leisner et al., 1994), CSS (González-Rodríguez et al., 2002), salted and dried fish (Thapa et al., 2006) and seafood salad (Andrighetto et al., 2009). This LAB, closed to the *Leuconostoc* genera, can be found in various types of food commodities such as meat products, plants material, raw vegetables and vegetable beverages (Björkroth and Holzapfel, 2006). *Staphylococcus* and *Bacillus* are ubiquitous bacterial genera, which can be isolated from a wide variety of ecological niches like soil, plant material, seafood, meat products, insects, water, feces, gut (Götz et al., 2006; Slepecky and Hemphill, 2006). All these bacterial genera are often encountered and isolated from various environments. Thus, in a seafood product containing spices (dill and black pepper), it is not unlikely to find them. On the other hand, *Duganella*, *Terribacillus*, *Spelaeicoccus*, *Comamonas*, *Sphingobacterium*, *Brevibacterium*, *Halorubrum* and *Halohasta* are for some of them relatively unknown and not frequently described. Table 2.3 summarizes some ecological origin of these uncommon bacterial or archaeal genera.

Table 2.3: Ecological origin of uncommon genera only detected by metabarcoding approach in salmon dill gravlax.

Genus	Ecological origin	References
Bacteria		
Brachybacterium	Salt-fermented seafood, lake sediment, soil, seawater, animal feces	Park et al., 2011; Liu et al., 2014; Kaur et al., 2016; Tak et al., 2018
Duganella	Soil, plant roots, water	Aranda et al., 2011; Madhaiyan et al., 2013; Haack et al., 2016
Terribacillus	Soil, salted lake sediment, plant material	An et al., 2007; Liu et al., 2010; Lu et al., 2015
Spelaicoccus	Soil	Lee, 2013
Brevibacterium	Soil, seawater, feces, compost, plant material, milk, cheese, poultry	Rattray and Fox, 1999; Onraedt et al., 2005; Srilekha et al., 2017; Choi et al., 2018; Valles et al., 2018
Comamonas	Freshwater, plant, compost, soil, fish gut	Wauters et al., 2003; Sun et al., 2013; Zhu et al., 2014; Dai et al., 2016; Kang et al., 2016
Sphingobacterium	Soil, permafrost, glacier, animal feces and gut, milk, compost, plant material, lichen, freshwater	Veress et al., 2017; Chatterjee et al., 2018; Chaudhary and Kim, 2018; Kaur et al., 2018; Niu et al., 2018; Sharma and Chatterjee, 2018; Van Le et al., 2018
Archaea		
Halorubrum	Salt-fermented seafood, rock salt, salted lake sediment, solar saltern	Yim et al., 2014; Corral et al., 2015; Kondo et al., 2015; Sánchez-Nieves et al., 2016
Halohasta	Salted lake water, aquaculture water	Mou et al., 2012

Although some genera have been isolated from a specific ecological niches (e.g. *Halohasta* and *Halorubrum* in salt) most of them seem ubiquitous and can therefore be brought with raw materials used for the salmon gravlax production (salt, spices). Next generation sequencing techniques are powerful tools to highlight such microbial population in minority within ecosystems, otherwise inaccessible by cultural method. For instance, Chaillou et al. (2015) detected *Sphingobacterium* sp., *Brachybacterium* sp. in diced bacon and *Comamonas* sp. in cod and salmon fillets with pyrosequencing technology.

For the 3 matrices, the most important shift in the microbial population occurred between 14 and 28 days, when the different bacterial groups reached their growth plateau.

In MAP CSS the microbial composition was then only dominated by LAB such as *Lactobacillus* (> 90%) and in a lesser extent by *Leuconostoc* (4-6%). These results

obtained by metabarcoding approach were in accordance with the results obtained by cultural method. LAB count was indeed identical to the TAMB. In VP CSS, depending on the biological replicate, *Lactobacillus* and *Lactococcus* were also detected in high proportion (13 to 37%). VP and MAP of food commodities are known to favor the LAB growth (Sivertsvik et al., 2002; Leroi, 2014; Bouletis et al., 2017). According to Silbande et al. (2018), the initial microbiota of red drum stored under VP and MAP (50%$C0_2$/50%N_2) was diverse, but quickly shifted over time and become progressively dominated by LAB (>75%) such as *Carnobacterium* (35-50%), *Vagococcus* (8-10%), *Leuconostoc* (1-13%), *Lactococcus* (8-10%). Many authors made the same observation in seafood, such as raw salmon steaks (Hoz et al., 2000; Rudi et al., 2004; Fletcher et al., 2005; Macé et al., 2012a), CSS (Leroi et al., 1998; Paludan-Müller et al., 1998; González-Rodríguez et al., 2002), cold-smoked trout (Lyhs et al., 1998), cooked and peeled shrimp (Mejlholm et al., 2005; Jaffrès et al., 2009) and in different fish gravlax (Leisner et al., 1994).

Lactobacillus and *Lactococcus* were the main LAB genera detected in the 3 products. Many species of *Lactobacillus* (*L. alimentarium*, *L. casei* subsp. *tolerans*, *L. coryneformis, L. curvatus, L. delbrueckii* subsp. *delbrueckii*, *L. farciminis, L. fuchuensis*, *L. homohiochii*, *L. malfermentans*, *L. plantarum*, *L. pentosus*, *L brevis*, *L. sakei* and *L. sanfranciscensis*) and *Lactococcus* (*L. piscium*, *L. lactis*, *L plantarum*) are frequently isolated from seafood products (Leroi, 2010; Ghanbari et al., 2013). However, among *Lactococcus* species, *L. piscium* seem to be the most detected in seafood (Matamoros et al., 2009b; Macé et al., 2012; Chaillou et al., 2015), and might therefore correspond to the OTU detected in our study. Although the spoilage potential of lactobacilli in seafood is strain dependent, some species such as *L. sakei*, *L. farciminis*, *L. fuchuensis* induced off-odor in shrimp, raw salmon and CSS (Stohr et al., 2001; Joffraud et al., 2006; Matamoros et al., 2009a; Leroi et al., 2015). As no sign of alteration were found in MAP CSS, even after 35 days, it's more likely that none of these 3 species correspond to the *Lactobacillus* OTUs.

In VP CSS, from day 14 to day 28, *Photobacterium* relative abundance increased and reached 78 to 97% of the microbial composition. In addition to be CO_2 resistant, *Photobacterium spp.* is also capable of anaerobic respiration by the use of trimethylamine-oxide, often present in fish, as final electron acceptor, making this bacteria able to grow under MAP and VP (Dalgaard et al., 1993; Leroi, 2014). Emborg

et al. (2002) and Macé et al. (2012) reported that *P*. *phosphoreum* dominated the spoilage microbiota of VP and MAP fresh salmon. *Photobacterium* was also found to be a part of the dominating microbiota of VP CSS (Olofsson et al., 2007; Leroi et al., 2015). However, in metabarcoding approach caution must be taken as its detection might be largely overestimated due to the use of the 16S rRNA gene as target. Indeed, according to the rrnDB database (Stoddard et al., 2015), *Photobacterium*, and especially *P. phosphoreum*, the most encountered species in seafood (Leroi et al., 1998; Emborg et al., 2002; González-Rodríguez et al., 2002; Rudi et al., 2004; Olofsson et al., 2007; Reynisson et al., 2009; Macé et al., 2012; Leroi, 2014) possess in its genome 15 copies of the 16S rRNA gene, where the average copy number for the bacterial reign is only 4.8.

After 28 days, in salmon gravlax, although *Photobacterium*, *Lactococcus* and *Lactobacillus* were detected in important proportion, *Enterobacteriaceae* mostly dominated the ecosystem, with an OTU corresponding to *Serratia*/*Yersinia* genera representing 35-47% of the total composition. In trout gravlax stored under VP at 3 and 8 °C, Lyhs et al. (2001) demonstrated that H_2S-producing bacteria (which includes some *Enterobacteriaceae* genera) represented a major part of the spoilage microbiota. Conversely, in VP salmon gravlax, Leisner et al. (1994) showed that H_2S-producing bacteria constituted at the spoiling date (18 days) only a small proportion of the microbioal ecosystem (10^6 CFU/g) compared to LAB (10^8 CFU/g).

The 3 products sensory quality remained acceptable for the whole storage duration, as the global spoilage score remained well below the sensory rejection threshold. Ordinarily, lightly preserved seafood products are declared spoiled and unfit for consumption after 3 to 4 weeks for fish gravlax (Leisner et al., 1994; Lyhs et al., 2001) and after 5 to 6 weeks for CSS (Leroi et al., 1998; Paludan-Müller et al., 1998; Truelstrup Hansen and Huss, 1998; Leroi et al., 2001; González-Rodríguez et al., 2002; Cardinal et al., 2004) and their final bacterial load can reached 8 to 9 log CFU/g (Leroi, 2014). In our study, the important shelf-life might be explained by the low TAMB count which never exceeded 7-8 log CFU/g. Moreover, the salt and the phenol contents, for the smoked products, were pretty high compared to the current production standards (Cardinal et al., 2004), with values of 2.6 g/100g and 3.2 mg/100g respectively (data not shown).

Despite weak spoilage in our products, in salmon gravlax and VP CSS, spoilage notes such as acid and amine off-odor and flavor increased a bit during storage, while the perception of the freshness related odors and flavors such as fresh fish, dill and smoke odor decreased a little over time. Similarly for the aspect of the product, the salmon characteristic orange color decreased, while the presence of fatty droplets increased at the products surface. Such spoilage sensory characteristics, in addition to sour/fermented, rancid, feet/cheese, sweet/nauseous, fruity and butter-like off-odors are commonly described for lightly preserved salmon products (Truelstrup Hansen and Huss, 1998; Paludan-Müller et al., 1998; Cardinal et al., 2004; Dondero et al., 2004). These spoilage notes could be induced by the presence of specific spoiling bacteria such as *B. thermosphacta*, *P. phosphoreum*, and *S. liquefaciens* (Joffraud et al., 2001; Stohr et al., 2001; Joffraud et al., 2006; Macé et al., 2013a), which were detected in our products.

Resulting from enzymatic reaction and microbial degradation, TVBN (Gram and Huss, 1996; Leroi et al., 2001; Cardinal et al., 2004) and biogenic amines (Jørgensen et al., 2000b; Biji et al., 2016; de la Torre and Conte-Junior, 2018) can be good seafood spoilage indicators. In salmon gravlax and VP CSS, TVBN and biogenic amines (tyramine, cadaverine and histamine) production started in both cases when the TAMB count reached 6 log CFU/g, after 14 and 28 days respectively. Although not known to be a fish with high histidine content, in both matrices, the histamine concentration exceeding, for some replicates, the regulatory limit of 100 mg/kg imposed by the European Regulation (EC) n°2073/2005 (2005) for some fish species (not including salmon). These values were higher than those described by some authors in similar products. (Hoz et al., 2000; Emborg et al., 2002; Brillet et al., 2005). After 4 weeks of storage, in one batch of CSS stored under VP, Brillet et al. (2005) found that cadaverine, histamine and tyramine level did not exceeded 25 mg/kg. Hoz et al. (2000) and Emborg et al. (2002) described similar results in spoiled raw salmon stored under MAP and air, with biogenic amines concentration not exceeding 120 mg/kg. On the other hand, some authors described equivalent or even higher concentrations for cadaverine, tyramine and histamine in spoiled CSS (Jørgensen et al., 2000b; Dondero et al., 2004). In VP CSS and salmon gravlax, despite a slight spoilage, high putrescine concentrations of up to 510 mg/kg (depending on replicates), support the hypothesis that biogenic amines are not reliable spoilage indicators in lightly processed and

preserved salmon based products. No production of biogenic amines was found in MAP CSS, despite a microbial load of 8.2 ± 0.1 log CFU/g. The microbial ecosystem was mainly dominated by LAB which are not strong biogenic amines producers in seafood (Masson et al., 1996; Bover-Cid and Holzapfel, 1999). In the same way, no TVBN production was found in MAP CSS, in comparison with salmon gravlax and VP CSS. In these two products TVBN increased slightly over time but did not exceed the European regulatory limit of 35 mg-N/100g for unprocessed salmon (Council Regulation (CE) N° 2074/2005, 2005).

VOCs composition of the 3 products, especially MAP CSS, did not change much over time. However an increase in concentration of alcohols, such as ethanol, 3-methyl-1-butanol, 2,3-butanediol, 1-octen-3-ol, phenylethyl alcohol, in aldehydes such as nonanal, (E,E)-2,4-decadienal, hexadecanal, benzaldehyde, benzenacetaldehyde, in ketones (2-butanone, 1-octen-3-one) and dimethylamine (VP CSS only) was observed. Aldehydes, amines, ketones, alcohols and organic acids production results mostly from microbial activity, and their concentration increases concomitantly with deterioration of seafood organoleptic properties (Jørgensen et al., 2001; Olafsdóttir et al., 2005; Varlet et al., 2006, 2007; Jónsdóttir et al., 2008). For instance, aldehydes, deriving from lipids oxidation by microorganisms, are especially good indicators of food degradation and actively participate in the characteristic rancid, cooked potatoes, fatty, floral, fruity, grassy odors of spoiled fish (Jørgensen et al., 2001; Varlet et al., 2007; Jónsdóttir et al., 2008). *P. phosphoreum*, *B. thermosphacta* and *S. liquefaciens*, common seafood spoilers, induced the production of many spoilage related volatiles compounds such as benzene ethanol, 2-methylpropanol, 3-methyl-2-butanol, 2/3-methyl-1-butanol, 1-propanol, 1-penten-3-ol, acetaldehyde, 2/3-methyl-1-butanal, pentanal, 2-methyl-1-propanal, benzaldehyde, benzacetaldehyde, 2,3-butanedione, 2-propanone, 3-pentanone, 3-methyl-2-butanone, 3-hydroxybutanone (acetoin), acetic acid, ethyl acetate, when added in cooked and peeled shrimp and raw and CSS (Joffraud et al., 2001; Jaffrès et al., 2011; Macé et al., 2013a). Otherwise, interestingly, for the 2 smoked products, an important decrease in furfural, 3-methylfurfural, 5-methylfurfural and 3-furaldehyde was observed during storage. Lots of furannic aldehydes, including these fours, are found in smoked fishes (Cardinal et al., 1997; Jørgensen et al., 2001; Guillen et al., 2006; Varlet et al., 2006; Jónsdóttir et al., 2008). Most of them are found in the smoke, but can also be generated through Maillard and Strecker reactions

between the wood smoke and the fish flesh during the smoking process (Varlet et al., 2007). Several bacteria, including species from *Pseudomonas*, *Acinetobacter* and *Serratia* genera are able to metabolize furfural and hydroxymethylfurfural compounds into furfuryl alcohols (2-furanmethanol and 3-furanmethanol) (Wierckx et al., 2011). Theses 2 alcohols were detected in MAP and VP CSS and found to slightly increase over time.

7. Conclusion

Within this work, we generated data on 3 salmon based products: VP CSS, MAP CSS and salmon dill gravlax. None of the 3 products has reached the sensory rejection during the whole experiment. The absence of spoilage was especially noticed in MAP CSS where the global spoilage score was of 0.5 on a scale of 10, even after 35 days. Despite a weak spoilage. Few sensory changes occurred during storage for VP CSS and salmon gravlax. In both products an increase in amine, acid flavors and odors, in discoloration and in fatty droplets (fatty appearance) was observed, while a decrease in dill odor perception was also noticed by the panelists for salmon gravlax and in smoked odor and product firmness for VP CSS. The volatilome analysis revealed than several VOCs commonly associated with seafood spoilage also increased in concentration during storage. Among these compounds, depending on the product, several aldehydes (decanal, nonanal, hexadecanal, benzealdehyde, benzenacetaldehyde), alcools (ethanol, 3-methyl-1-butanol, 2,3-butanediol, 1-octen-3-ol) and ketones (2-butanone, 1-octen-3-one) were concerned. The microbial ecosystem monitoring through metabarcoding approach targeting the hypervariable region V4 of the 16S rRNA gene showed that the initial composition of the 3 products was almost identical and mainly dominated by *Photobacterium, Lactococcus* and *Lactobacillus*. After 28 days of storage, VP CSS microbial ecosystem was largely dominated by *Photobacterium* but also by *Lactococcus* and *Lactobacillus* in a lesser extent. In contrast, MAP CSS microbial composition was exclusively dominated by LAB with an OTU representative of the *Lactobacillus* genera, while salmon gravlax microbial ecosystem was more diverse and mainly composed by a mix of *Enterobacteriaceae* (*Serratia* or *Yersinia*), *Photobacterium, Lactococcus* and *Lactobacillus*. Futher statistical analyses based on ComDim approach (Ghaziri et al., 2016) are currently studied to possibly find correlation between variables from different data blocks.

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Table 2.4: Supplementary Table 1 - Salmon gravlax VOCs composition.

		Average concentration (µg/kg)						Standard deviation (µg/kg)					
Compound	**Category**	**G0**	**G7**	**G14**	**G21**	**G28**	**G35**	**G0**	**G7**	**G14**	**G21**	**G28**	**G35**
tetradecanoic acid	acid	7	46	7	49	40	49	0	67	0	73	56	72
hexadecanoic acid	acid	7	281	7	221	199	105	0	473	0	192	331	169
ethanol	alcohol	62	312	7	1256	3649	7114	69	231	0	2163	5062	7433
3-methyl-1-butanol	alcohol	7	8	7	169	442	813	0	0	0	280	414	884
2,3-butanediol	alcohol	7	8	7	93	7632	2154	0	0	0	149	13206	3717
1-octen-3-ol	alcohol	62	132	137	142	115	279	94	159	34	30	93	175
(E,Z)-2,4-heptadienal	aldehyde	65	167	115	103	108	157	99	192	94	94	43	77
(E,E)-2,4-heptadienal	aldehyde	121	177	179	137	111	229	113	222	70	131	95	250
nonanal	aldehyde	129	172	76	107	122	274	108	173	120	172	99	123
(E)-2-decenal	aldehyde	75	214	167	226	137	219	117	249	143	227	115	186
(E,E)-2,4-decadienal	aldehyde	143	233	259	237	173	331	234	390	227	228	147	161
(E)-2-undecanal	aldehyde	76	105	132	110	97	82	119	169	113	177	80	129
undecane	alkane	455	1319	750	560	384	515	102	1285	134	376	37	110
dodecane	alkane	447	2453	1069	567	424	673	229	3304	406	498	154	69
tridecane	alkane	418	2808	813	461	352	635	249	4015	384	362	129	114
tetradecane	alkane	126	270	139	139	123	178	104	288	23	70	26	53
pentadecane	alkane	3857	4136	3596	3880	3328	4117	683	317	221	1495	1233	691
hexadecane	alkane	7	92	37	58	57	76	0	81	52	44	54	60
heptadecane	alkane	1836	1916	1606	1930	1613	1870	230	199	244	548	642	385
pristane	alkane	3164	2917	2799	2517	2251	3198	1056	475	311	574	660	630
1-pentadecene	alkene	43	64	55	44	36	135	62	98	83	64	48	21
(Z)-3-heptadecene	alkene	185	162	110	128	93	121	29	33	90	117	88	99
aromatic-1	aromatic	7	92	7	884	8	49	0	73	0	1439	0	71
benzaldehyde	aromatic	7	8	38	7	61	193	0	0	53	0	47	89
benzeneacetaldehyde	aromatic	7	8	7	267	388	1501	0	0	0	259	302	2300
aromatic-2	aromatic	7	8	49	7	55	35	0	0	72	0	41	47
phenylethyl alcohol	aromatic	7	8	7	119	231	631	0	0	0	193	289	1080
butylated hydroxytoluene	aromatic	504	508	437	461	404	523	166	111	42	107	175	66
2-butanone	ketone	122	100	279	76	135	550	102	83	381	119	221	771
1-octen-3-one	ketone	7	8	7	31	27	83	0	0	0	40	35	131
alpha-thujene	terpene	852	734	909	1015	940	772	29	46	261	219	353	283
alpha-pinene	terpene	281	301	335	411	413	325	46	75	91	102	172	37
sabinene	terpene	2860	3069	3269	3849	4153	2968	407	439	1187	723	1278	706
beta-pinene	terpene	1614	1513	1736	2128	2351	1667	192	24	616	373	806	304
beta-myrcene	terpene	1219	1689	1560	1371	1468	1025	68	355	226	191	433	145
alpha-phellandrene	terpene	1848	1856	2119	2501	1783	926	1385	473	940	1114	76	220
3-carene	terpene	98	243	248	278	254	61	157	18	70	35	37	93
alpha-terpinene	terpene	69	111	219	127	134	8	107	90	13	104	112	0
p-cymene	terpene	646	546	710	690	626	451	213	111	232	150	180	15

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		Average concentration (µg/kg)						Standard deviation (µg/kg)					
Compound	**Category**	**G0**	**G7**	**G14**	**G21**	**G28**	**G35**	**G0**	**G7**	**G14**	**G21**	**G28**	**G35**
limonene	terpene	5826	6364	6460	6794	7590	5280	432	1036	1876	778	2367	943
beta-phellandrene	terpene	896	929	989	1134	1143	761	44	146	295	109	319	135
gamma-terpinene	terpene	442	374	419	445	457	389	14	25	127	36	163	108
monoterpene-1	terpene	117	167	68	7	115	115	95	60	105	0	14	93
terpinolene	terpene	184	159	196	180	189	158	33	60	38	9	36	50
4-terpineol	terpene	393	357	362	338	256	330	4	39	25	61	65	193
dill ether	terpene	2530	2568	1929	1892	1493	1784	253	271	309	549	360	1047
alpha-terpineol	terpene	7	78	7	7	66	8	0	61	0	0	51	0
delta-elemene	terpene	129	176	173	205	228	106	105	8	4	40	66	170
alpha-cubebene	terpene	76	56	168	175	198	140	119	84	22	158	76	116
alpha-copaene	terpene	1182	1176	1143	1447	1840	1472	296	28	95	674	774	912
beta-cubebene	terpene	57	8	46	59	146	73	86	0	67	90	124	114
(E)-beta-caryophyllene	terpene	3286	3543	3716	4405	5210	4305	1171	89	240	1861	2279	2550
(E)-beta-farnesene	terpene	45	8	32	80	110	52	65	0	43	64	88	77
alpha-caryophyllene	terpene	356	333	354	360	532	445	91	39	36	318	277	255
alpha-selinene	terpene	7	8	7	47	78	51	0	0	0	68	61	76
beta-bisabolene	terpene	801	788	844	1035	1209	1128	361	40	25	420	526	682
delta-cadinene	terpene	427	414	374	472	544	469	197	30	28	233	207	249
cembrene A	terpene	441	478	362	378	339	498	88	199	60	57	102	90
NA-1	na	47	89	7	19	42	112	69	140	0	19	60	180

NA : Not identified

Table 2.5: Supplementary Table 2 - VP CSS VOCs composition.

		Average concentration (µg/kg)							Standard deviation (µg/kg)						
Compound	**Category**	**S0**	**S7**	**S14**	**S28**	**S35**	**S42**	**S49**	**S0**	**S7**	**S14**	**S28**	**S35**	**S42**	**S49**
hexadecanoic acid	acid	27	28	3	3	41	79	3	41	44	0	0	65	67	0
ethanol	alcohol	523	261	300	375	625	726	496	365	116	123	328	613	680	399
1-hexadecanol	alcohol	3	16	3	3	3	18	18	0	22	0	0	0	25	25
3-furaldehyde	aldehyde	173	223	182	59	15	3	18	147	53	5	58	21	0	26
2,4-octadienal	aldehyde	271	349	306	262	255	275	261	125	126	59	57	28	37	23
decanal	aldehyde	3	49	90	24	177	202	173	0	48	150	35	41	95	48
hexadecanal	aldehyde	3	3	3	3	23	77	77	0	1	0	0	35	13	44
alkane-1	alkane	665	540	3	171	3	3	949	1147	930	0	290	0	0	1038
nonane	alkane	404	734	535	525	340	261	232	354	307	51	29	292	225	204
alkane-2	alkane	97	47	27	3	41	50	36	26	75	41	0	66	80	56
alkane-3	alkane	49	28	38	3	3	3	23	79	42	60	0	0	0	34
2,2,7,7-tetramethyloctane	alkane	34	409	506	3	312	527	331	53	239	260	0	273	181	320
alkane-4	alkane	18	12	85	3	51	45	26	26	15	36	0	44	37	40
alkane-5	alkane	39	149	123	129	124	118	134	63	34	17	13	11	18	5
alkane-5	alkane	3	60	35	21	59	97	60	0	98	55	31	53	62	50
tridecane	alkane	59	100	102	77	131	147	147	21	70	70	27	30	45	36
tetradecane	alkane	63	108	125	108	73	109	93	5	68	97	18	10	26	15
pentadecane	alkane	1871	2619	1933	2054	1786	2152	1855	654	1423	417	359	36	105	356
hexadecane	alkane	25	31	56	16	18	42	3	19	24	26	21	25	33	0
heptadecane	alkane	898	1059	798	987	743	953	829	334	509	141	360	101	133	284
acetylcyclohexene	alkene	38	70	68	74	99	3	32	61	57	56	64	31	0	51
1-pentadecene	alkene	66	81	53	34	42	40	78	25	42	54	53	34	35	12
8-heptadecene	alkene	73	96	67	100	67	83	72	35	41	30	24	17	18	32
dimethylamine	amine	135	235	124	755	303	214	174	120	251	62	1063	408	365	104
styrene	aromatic	42	3	45	27	24	3	29	37	1	36	41	37	0	44
benzaldehyde	aromatic	440	561	440	287	365	264	255	107	132	33	60	34	58	58
phenol	aromatic	1928	2946	3003	2340	2489	2228	2616	701	368	266	811	858	298	262
aromatic-1	aromatic	106	187	154	47	133	32	79	90	76	4	76	19	51	70
benzeneacetaldehyde	aromatic	15	3	13	502	428	1759	2500	20	1	16	435	134	2631	2318
2-hydroxybenzaldehyde	aromatic	290	355	290	216	211	184	152	28	14	7	51	51	25	42
2-methylphenol	aromatic	1923	2199	2250	2277	2530	2118	2290	338	410	192	257	326	48	187
acetophenone	aromatic	1338	1748	1462	1319	1328	1194	1190	242	333	61	164	202	45	233
4-methylbenzaldehyde	aromatic	214	241	223	146	157	115	123	26	44	31	25	13	24	37
3-methylphenol	aromatic	2571	2982	3029	2982	3170	2809	3289	325	176	231	437	140	46	347
2-methoxyphenol	aromatic	17459	17708	19978	18578	19706	18517	19004	2975	5894	1200	2983	1314	929	989
benzoic acid methyl ester	aromatic	190	229	181	184	198	156	152	27	9	21	49	24	18	29
3-methylbenzofuran	aromatic	77	64	59	79	73	74	74	67	57	49	66	66	62	64
2,6-dimethylphenol	aromatic	620	629	654	673	663	558	602	158	82	56	70	38	19	67
7-methylbenzofuran	aromatic	184	134	174	171	195	176	186	59	123	33	64	45	45	85
phenylethyl alcohol	aromatic	3	3	3	114	198	1680	1434	0	1	0	192	30	2905	1367
aromatic-3	aromatic	184	228	194	167	156	130	140	24	28	25	26	14	7	15
aromatic-4	aromatic	30	40	52	25	79	38	32	46	63	43	37	14	60	49

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		Average concentration (µg/kg)							Standard deviation (µg/kg)						
Compound	**Category**	**S0**	**S7**	**S14**	**S28**	**S35**	**S42**	**S49**	**S0**	**S7**	**S14**	**S28**	**S35**	**S42**	**S49**
aromatic-5	aromatic	78	54	67	3	3	9	3	25	44	7	0	0	9	0
2-ethylphenol	aromatic	347	208	313	364	370	354	420	155	179	47	12	33	6	73
1,4-dimethoxybenzene	aromatic	241	256	268	240	242	219	244	27	9	25	10	30	26	24
2,3-dimethylphenol	aromatic	683	546	686	839	857	747	887	330	231	86	37	46	74	77
3,4-dimethylphenol	aromatic	609	494	626	779	725	689	746	233	212	92	31	91	45	82
2-hydroxy-3-methylbenzaldehyde	aromatic	219	200	175	164	161	134	121	75	75	32	51	8	11	34
2-hydroxyacetophenone	aromatic	318	306	355	315	324	303	302	104	77	41	62	35	22	61
4-ethylphenol	aromatic	590	405	563	719	683	652	758	314	227	50	40	77	96	62
2-methoxybenzyl alcohol	aromatic	220	233	313	393	382	369	412	22	105	32	59	31	59	99
dimethyl phenol derivative-1	aromatic	273	188	247	321	323	285	299	136	65	33	24	5	20	62
4-methylacetophenone	aromatic	360	357	361	371	376	325	350	83	51	29	29	21	46	102
2-methoxy-3-methylphenol	aromatic	2068	1902	2133	2279	2269	1882	2138	535	527	216	286	145	133	238
2-methoxy-5-methylphenol	aromatic	480	428	471	480	467	442	480	97	81	18	15	36	39	18
naphthalene	aromatic	1096	825	956	934	1189	1185	1205	394	348	133	279	305	243	375
2-methoxy-4-methylphenol	aromatic	11890	13155	12432	13708	13512	12066	13561	2027	763	501	707	456	442	278
2,4,6-trimethylphenol	aromatic	80	75	85	25	30	3	3	68	64	5	36	47	0	0
2,3,5-trimethylphenol	aromatic	170	99	162	177	219	205	210	90	85	6	57	39	7	47
aromatic-6	aromatic	21	3	3	39	31	3	30	31	1	0	32	47	0	47
4,7-dimethylbenzofuran	aromatic	119	69	135	116	130	178	188	113	59	18	101	111	37	54
aromatic-7	aromatic	3	3	3	19	19	3	18	0	1	0	27	28	0	26
aromatic-8	aromatic	55	32	90	119	88	75	127	90	48	1	29	75	63	25
aromatic-9	aromatic	3	3	35	3	20	3	39	0	1	29	0	29	0	62
3,4,5-trimethylphenol	aromatic	67	53	99	93	127	57	131	111	44	22	77	16	48	42
2,6-dimethoxytoluene	aromatic	691	552	703	760	716	640	726	205	181	44	48	94	77	97
aromatic-10	aromatic	3	24	3	41	29	3	26	0	35	0	32	44	0	39
aromatic-11	aromatic	65	21	33	29	48	3	64	17	30	26	45	39	0	3
aromatic-12	aromatic	68	42	3	79	86	30	66	60	66	0	12	5	47	55
aromatic-13	aromatic	210	103	158	182	178	172	194	103	98	14	39	32	38	62
2-methoxy-4-ethylphenol	aromatic	174	163	159	189	201	183	207	77	46	5	25	27	32	46
aromatic-14	aromatic	133	141	100	118	125	117	136	85	81	13	14	35	22	22
3,5-dimethoxytoluene	aromatic	490	340	467	544	601	593	673	267	155	43	52	89	113	131
aromatic-15	aromatic	87	36	46	3	73	70	20	91	56	38	0	13	21	30
2,5-dimethoxytoluene	aromatic	6022	4280	5714	7112	6861	6130	7390	3106	1658	610	484	987	774	900
1-indanone	aromatic	685	542	637	668	613	577	717	176	61	35	105	139	91	78
2-methylnaphthalene	aromatic	315	215	269	265	360	324	357	156	49	59	93	147	97	44
1,2,3-trimethoxy-benzene	aromatic	106	58	94	46	107	37	72	26	47	3	74	22	58	64
3-methyl-1-indanone	aromatic	255	133	236	260	257	226	254	101	112	39	27	26	25	16
2-methoxy-4-vinylphenol	aromatic	3620	2063	1422	1447	1340	1358	715	2388	875	489	583	161	358	565
1-methylnaphthalene	aromatic	193	130	168	177	234	204	212	88	16	32	87	36	65	38
aromatic-16	aromatic	183	101	171	190	181	153	202	69	85	15	32	23	30	37
aromatic-17	aromatic	40	3	33	3	55	22	54	64	1	27	0	52	33	45

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		Average concentration (µg/kg)							Standard deviation (µg/kg)						
Compound	**Category**	**S0**	**S7**	**S14**	**S28**	**S35**	**S42**	**S49**	**S0**	**S7**	**S14**	**S28**	**S35**	**S42**	**S49**
2,3-dimethoxybenzaldehyde	aromatic	145	62	104	145	149	164	177	79	52	38	25	63	18	23
2,6-dimethoxyphenol	aromatic	1423	836	1179	1619	901	1327	1866	1105	78	176	957	405	154	532
aromatic-18	aromatic	108	55	88	72	71	31	122	43	45	1	59	60	49	11
eugenol	aromatic	980	556	792	1135	1037	975	1250	720	273	98	206	215	182	223
1-(2-hydroxy-6-methoxyphenyl)-ethanone	aromatic	224	112	178	284	280	290	338	155	98	5	63	75	48	87
dihydroeugenol	aromatic	783	449	606	917	901	859	1122	649	211	38	180	238	92	202
aromatic-19	aromatic	31	3	19	3	3	3	46	47	1	27	0	0	0	38
aromatic-20	aromatic	3	3	3	3	3	20	58	0	1	0	0	0	30	47
biphenyl	aromatic	109	42	81	47	103	92	149	112	67	67	77	87	77	16
1,2,3-trimethoxy-5-methylbenzene	aromatic	125	57	115	149	122	113	142	47	47	6	30	32	16	42
2-methoxy-5-(1-propenyl)-phenol	aromatic	279	122	186	328	293	253	352	239	108	3	103	134	55	103
4-methoxy-3-(methoxymethyl)-phenol	aromatic	490	215	298	488	302	361	498	434	25	88	345	120	77	181
(E)-isoeugenol	aromatic	768	318	502	934	780	780	958	771	157	43	356	296	136	232
biphenylene	aromatic	94	36	74	39	89	120	128	96	56	61	62	74	27	13
butylated hydroxytoluene	aromatic	6956	11033	6878	8183	7778	9467	7804	1893	5176	1006	1765	989	231	1065
2,5-dimethoxybenzoic acid	aromatic	124	28	64	151	84	44	96	148	41	53	120	79	71	81
benzoic acid derivative-1	aromatic	55	3	3	3	31	37	47	52	1	0	0	48	58	38
furfural	furan	3174	2891	2324	970	362	168	282	1955	736	232	547	49	285	322
2-furanmethanol	furan	89	62	79	131	113	119	55	87	102	67	115	127	113	45
3-furanmethanol	furan	3	3	17	3	13	3	36	0	1	24	0	17	0	57
ethylmethyl furan	furan	116	242	179	163	145	154	82	101	119	7	11	45	23	74
acetylfuran	furan	1362	2376	1772	1705	1531	1544	845	768	996	208	122	455	408	741
furan derivative-1	furan	236	308	241	203	96	48	76	94	110	36	19	87	77	65
3-methylfurfural	furan	39	54	84	20	3	11	3	63	43	11	28	0	14	0
5-methylfurfural	furan	1898	1895	1748	893	513	420	457	953	323	199	503	88	366	457
methyl-2-furoate	furan	151	259	215	160	153	44	126	128	88	50	28	42	70	20
1-(2-furyl)-1-propanone	furan	317	521	394	350	368	324	309	126	179	54	12	53	41	55
2-acetyl-5-methylfuran	furan	468	744	603	556	556	498	480	213	267	54	81	83	102	56
2-butanone	ketone	26	178	70	106	194	161	124	39	97	62	91	111	68	60
2-methyl-2-cyclopentenone	ketone	880	1613	1186	1154	976	608	534	760	564	152	108	212	536	465
gamma-butyrolactone	ketone	55	3	86	3	3	69	3	89	1	143	0	0	114	0
3,4-dimethyl-2-cyclopenten-1-one	ketone	145	232	165	126	135	113	111	58	102	12	30	19	12	32
2-methyl-2-cyclopenten-1-one	ketone	135	215	150	132	104	60	60	42	100	9	5	38	49	53
ketone-1	ketone	109	33	78	11	3	3	3	92	50	3	13	0	0	0
3-methyl-2-cyclopenten-1-one	ketone	262	403	267	168	153	80	51	108	169	47	42	64	67	83
3-methyl-2-cyclopenten-1-one	ketone	253	415	398	260	241	216	210	225	174	59	223	213	192	180
4,4-dimethyl-2-cyclopenten-1-one	ketone	372	578	420	422	396	371	381	196	236	45	30	76	46	75
3,4-dimethyl-2-cyclopenten-1-one	ketone	495	713	527	498	462	426	398	198	299	52	24	60	80	60

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		Average concentration (µg/kg)							Standard deviation (µg/kg)						
Compound	**Category**	**S0**	**S7**	**S14**	**S28**	**S35**	**S42**	**S49**	**S0**	**S7**	**S14**	**S28**	**S35**	**S42**	**S49**
3-methyl-1,2-cyclopentanedione	ketone	174	99	101	3	136	3	143	167	143	169	0	116	0	229
2,3-dimethyl-2-cyclopenten-1-one	ketone	1405	1946	1655	1564	1547	1448	1486	655	604	235	191	273	137	152
3,4-dimethyl-2-hydroxycyclopent-2-en-1-one	ketone	3	3	3	3	68	96	51	0	1	0	0	56	82	83
2,3,4-trimethyl-2-cyclopenten-1-one	ketone	338	439	314	343	363	303	308	66	159	48	50	80	34	43
nona-3,5-dien-2-one	ketone	2212	1669	1433	1240	1040	566	544	1233	503	184	278	265	164	24
3-ethyl-2-hydroxy-2-cyclopenten-1-one	ketone	44	89	66	184	128	68	209	71	76	108	69	108	113	60
ketone-2	ketone	155	113	150	73	114	31	35	28	104	16	61	29	47	56
ketone-3	ketone	336	389	353	338	318	304	317	106	66	47	54	56	51	18
ketone-4	ketone	639	817	694	616	564	519	527	155	172	55	19	103	21	83
ketone-5	ketone	99	81	145	91	3	52	87	99	70	32	79	0	85	74
ketone-6	ketone	300	319	298	297	304	267	284	83	92	19	11	2	20	8
ketone-7	ketone	227	229	200	161	168	146	89	28	21	12	8	25	31	74
ketone-8	ketone	88	88	135	131	127	121	85	74	74	15	3	21	13	71
2-allyl-4-hydroxy-3-methyl-2-cyclopenten-1-one	ketone	237	149	165	106	91	3	23	62	31	14	39	24	0	35
terpene-1	terpene	43	55	91	45	98	70	55	69	50	11	71	20	27	45
terpene-2	terpene	95	32	50	27	30	27	27	30	50	41	40	46	42	41
terpene-3	terpene	44	22	38	21	3	24	26	36	32	33	30	0	36	39
pristane	terpene	1691	1829	1329	1671	1695	1837	1565	557	694	255	623	402	32	423
cembrene A	terpene	265	257	190	273	253	282	249	105	108	44	128	78	27	54
NA-1	na	24	67	92	31	27	3	31	35	60	27	48	41	0	49
NA-2	na	214	258	254	238	202	180	139	38	78	7	12	21	24	41
NA-3	na	63	30	42	81	3	59	73	61	46	35	68	0	49	64
NA-4	na	15	11	3	16	15	3	18	21	13	0	21	21	0	25
NA-5	na	30	3	3	39	70	3	48	46	1	0	35	17	0	41
NA-6	na	13	3	3	3	20	31	3	16	1	0	0	29	24	0
NA-7	na	45	18	19	16	20	3	23	45	25	28	21	29	0	35
NA-8	na	16	22	3	21	18	3	21	22	31	0	31	26	0	31
NA-9	na	35	50	44	23	69	59	49	56	41	35	34	57	53	48

NA : Not identified

Table 2.6: **Supplementary Table 3 - MAP CSS VOCs composition.**

		Average concentration (µg/kg)						Standard deviation (µg/kg)					
Compound	**category**	**D0**	**D7**	**D14**	**D21**	**D28**	**D35**	**D0**	**D7**	**D14**	**D21**	**D28**	**D35**
tetradecanoic acid	acid	2	2	2	21	35	23	0	0	0	33	56	36
hexadecanoic acid	acid	41	23	76	107	395	247	25	22	59	175	599	338
ethanol	alcohol	606	565	288	837	690	337	688	161	495	250	345	181
4-methyl-4-hepten-3-ol	alcohol	73	113	122	75	35	87	63	27	34	65	57	77
1-hexadecanol	alcohol	14	2	13	2	2	13	20	0	19	0	0	18
(E)-2-pentenal	aldehyde	267	148	201	322	275	237	140	127	95	38	43	57
3-furaldehyde	aldehyde	135	101	121	80	17	2	11	16	49	6	25	0
2,4-octadienal	aldehyde	155	144	184	148	144	230	136	125	157	133	123	24
decanal	aldehyde	49	89	46	2	204	307	57	49	39	0	224	26
hexadecanal	aldehyde	2	2	2	2	20	47	0	0	0	0	31	39
alkane-1	alkane	2	2	2	901	1291	2	0	0	0	1557	1162	0
nonane	alkane	2	66	108	93	84	86	0	111	183	158	141	144
alkane-2	alkane	2	2	2	55	20	28	0	0	0	15	31	44
alkane-3	alkane	2	2	2	2	45	32	0	0	0	0	37	37
alkane-4	alkane	102	91	111	69	102	115	7	16	18	58	10	34
alkane-5	alkane	2	10	2	2	33	80	0	13	0	0	40	20
tridecane	alkane	85	87	77	70	239	242	33	7	9	29	178	30
tetradecane	alkane	95	114	68	89	97	94	52	54	10	17	23	5
alkane-6	alkane	2	2	2	2	16	31	0	0	0	0	24	25
pentadecane	alkane	1667	1844	1815	2055	2039	2016	89	86	290	184	318	99
hexadecane	alkane	60	76	46	59	37	56	59	15	20	11	31	6
heptadecane	alkane	1007	896	850	958	959	933	365	131	119	142	123	140
octadecane	alkane	116	2	2	2	10	11	175	0	0	0	13	15
acetylcyclohexene	alkene	69	60	67	44	63	74	5	21	9	37	3	4
1-pentadecene	alkene	64	58	57	77	62	68	6	26	18	28	15	4
8-heptadecene	alkene	71	56	51	60	60	53	18	2	4	22	17	14
dimethylamine	amine	201	660	160	1066	569	228	190	187	272	262	419	146
styrene	aromatic	40	16	59	2	2	13	33	23	51	0	0	18
benzaldehyde	aromatic	345	279	353	301	193	107	22	78	91	5	59	25
3-methyl-2-cyclopenten-1-one	aromatic	244	257	306	201	245	294	95	66	97	33	36	84
phenol	aromatic	975	859	1159	707	808	985	346	247	347	163	101	196
aromatic-1	aromatic	125	94	138	139	105	60	15	13	17	14	15	55
benzeneacetaldehyde	aromatic	7	18	7	58	52	51	9	28	8	51	58	61
2-hydroxybenzaldehyde	aromatic	280	233	293	259	210	217	45	48	82	25	23	34
2-methylphenol	aromatic	1868	1892	2143	1900	1908	2068	197	199	186	110	41	241
acetophenone	aromatic	1137	1142	1270	1153	1109	1140	86	136	192	34	68	80
4-methylbenzaldehyde	aromatic	246	227	255	229	177	85	32	30	60	10	63	36
3-methylphenol	aromatic	2561	2586	2864	2576	2655	2800	488	322	130	125	72	247
2-methoxyphenol	aromatic	15328	14941	16376	15983	15934	17040	1501	1746	149	1705	1004	1453
benzoic acid methyl ester	aromatic	173	170	199	177	170	179	31	17	34	9	5	24
aromatic-2	aromatic	195	172	214	198	166	177	23	36	13	9	6	30
3-methylbenzofuran	aromatic	110	125	115	130	114	132	21	24	25	17	15	15

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		Average concentration (µg/kg)						Standard deviation (µg/kg)					
Compound	**category**	**D0**	**D7**	**D14**	**D21**	**D28**	**D35**	**D0**	**D7**	**D14**	**D21**	**D28**	**D35**
2,6-dimethylphenol	aromatic	579	589	629	610	600	629	49	46	27	26	29	101
7-methylbenzofuran	aromatic	217	228	226	276	257	267	65	45	40	32	34	10
aromatic-3	aromatic	172	145	176	170	156	151	30	37	24	7	21	7
aromatic-4	aromatic	68	25	67	53	52	27	6	39	12	45	44	43
aromatic-5	aromatic	74	42	26	13	15	2	19	38	41	18	21	0
benzofuran derivative-1	aromatic	98	92	112	106	101	101	6	23	17	7	16	4
2-ethylphenol	aromatic	326	324	364	345	359	358	23	41	36	19	8	23
1,4-dimethoxybenzene	aromatic	236	244	250	208	197	212	22	29	22	30	11	38
2,3-dimethylphenol	aromatic	714	750	751	768	761	780	14	69	34	52	100	76
3,4-dimethylphenol	aromatic	625	644	674	651	672	697	80	83	24	58	43	79
2-hydroxy-3-methylbenzaldehyde	aromatic	222	210	225	210	200	204	21	17	32	25	29	19
2-hydroxyacetophenone	aromatic	291	284	310	315	323	310	5	35	11	29	25	38
4-ethylphenol	aromatic	659	612	653	614	644	696	121	96	20	47	40	34
2-methoxybenzyl alcohol	aromatic	333	328	366	352	348	365	35	48	21	33	39	28
dimethyl phenol derivative-1	aromatic	280	296	287	292	297	301	38	42	22	23	37	16
4-methylacetophenone	aromatic	322	333	346	323	358	325	19	31	55	26	5	30
2-methoxy-3-methylphenol	aromatic	1911	1930	2073	1968	2029	2053	41	163	151	42	97	118
2-methoxy-5-methylphenol	aromatic	417	425	429	443	452	448	61	72	28	38	11	22
naphthalene	aromatic	1505	1693	1552	1753	1693	1771	519	187	249	241	108	23
2-methoxy-4-methylphenol	aromatic	10974	11212	11688	12123	11717	11962	793	889	802	806	250	344
2,4,6-trimethylphenol	aromatic	92	80	84	77	71	52	9	23	26	3	12	30
2,3,5-trimethylphenol	aromatic	209	201	210	209	232	231	48	7	22	27	12	33
aromatic-6	aromatic	95	59	82	103	93	110	34	52	9	4	16	25
aromatic-7	aromatic	47	61	47	44	76	62	40	3	39	36	24	13
4,7-dimethylbenzofuran	aromatic	276	282	246	262	285	290	121	37	44	14	35	27
aromatic-8	aromatic	39	45	32	23	40	37	39	39	28	35	33	30
aromatic-9	aromatic	114	105	105	91	127	112	21	20	14	32	8	9
aromatic-10	aromatic	42	60	40	47	54	19	34	10	34	42	45	30
3,4,5-trimethylphenol	aromatic	109	112	117	111	120	126	9	21	10	17	27	16
2,6-dimethoxytoluene	aromatic	613	619	646	615	626	654	25	26	35	73	50	35
aromatic-11	aromatic	2	2	2	2	20	59	0	0	0	0	31	9
aromatic-12	aromatic	68	57	58	49	59	61	8	19	4	9	14	18
aromatic-13	aromatic	80	81	79	88	76	79	12	1	7	12	7	7
aromatic-14	aromatic	226	263	237	227	227	256	28	40	24	20	4	16
2-methoxy-4-ethylphenol	aromatic	181	190	173	206	204	205	16	21	8	24	10	28
aromatic-15	aromatic	102	113	115	112	127	126	15	6	15	16	40	10
3,5-dimethoxytoluene	aromatic	577	629	609	646	636	635	111	57	78	28	71	79
aromatic-16	aromatic	124	126	104	92	98	47	23	12	22	20	14	39
2,5-dimethoxytoluene	aromatic	6205	6210	6174	6233	6585	6566	332	647	383	320	478	275
1-indanone	aromatic	580	570	580	599	594	572	47	26	67	65	20	29
2-methylnaphthalene	aromatic	541	586	466	571	567	533	243	62	88	61	106	21
1,2,3-trimethoxy-benzene	aromatic	102	62	86	62	32	80	22	52	6	52	51	7

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		Average concentration (µg/kg)						Standard deviation (µg/kg)					
Compound	**category**	**D0**	**D7**	**D14**	**D21**	**D28**	**D35**	**D0**	**D7**	**D14**	**D21**	**D28**	**D35**
3-methyl-1-indanone	aromatic	234	237	227	231	256	243	7	20	51	26	73	29
2-methoxy-4-vinylphenol	aromatic	3824	2941	1746	1179	868	529	407	324	154	163	119	51
1-methylnaphthalene	aromatic	317	341	283	315	328	300	161	18	67	24	42	30
aromatic-17	aromatic	160	162	156	190	181	163	6	37	17	4	30	28
aromatic-18	aromatic	49	63	59	66	60	72	40	9	1	2	13	6
aromatic-19	aromatic	22	33	39	17	29	14	34	27	9	25	26	21
aromatic-20	aromatic	35	35	12	2	2	2	30	29	17	0	0	0
2,3-dimethoxybenzaldehyde	aromatic	229	245	189	204	220	212	75	50	16	22	34	39
2,6-dimethoxyphenol	aromatic	1671	1609	1762	1754	1907	1772	170	187	455	279	364	600
aromatic-21	aromatic	93	93	93	71	99	105	5	16	20	59	16	14
eugenol	aromatic	1059	1057	1040	1037	1126	1070	138	127	40	66	147	74
1-(2-hydroxy-6-methoxyphenyl)-ethanone	aromatic	276	316	262	301	308	296	48	4	32	14	49	43
dihydroeugenol	aromatic	1002	937	914	929	989	966	147	181	97	27	96	61
aromatic-22	aromatic	39	74	53	66	61	65	33	16	4	20	11	5
aromatic-23	aromatic	24	67	37	32	69	46	38	4	31	52	23	39
biphenyl	aromatic	192	217	163	201	207	191	76	25	30	13	42	9
1,2,3-trimethoxy-5-methylbenzene	aromatic	126	103	106	107	113	102	10	22	15	21	12	12
2-methoxy-5-(1-propenyl)-phenol	aromatic	320	320	300	311	339	326	31	27	5	50	47	24
naphthalene derivative-1	aromatic	30	45	39	22	43	24	48	37	34	35	38	38
4-methoxy-3-(methoxymethyl)-phenol	aromatic	530	526	562	562	627	559	35	77	122	96	136	160
(E)-isoeugenol	aromatic	1010	961	905	955	1017	1033	165	180	50	110	118	85
biphenylene	aromatic	201	200	154	183	184	165	76	22	51	33	45	34
butylated hydroxytoluene	aromatic	7355	7867	7244	8628	8624	8575	594	679	1263	868	1599	627
2,5-dimethoxybenzoic acid	aromatic	147	161	149	158	170	158	19	48	37	34	8	23
benzoic acid derivative-1	aromatic	113	116	92	114	102	85	41	9	19	8	21	16
furfural	furan	2106	1305	1681	916	191	20	295	397	566	148	191	30
2-furanmethanol	furan	99	54	105	38	102	121	38	15	90	62	16	83
3-furanmethanol	furan	2	2	2	9	45	15	0	0	0	11	18	21
ethylmethyl furan	furan	91	80	116	117	92	99	19	27	16	14	9	27
acetylfuran	furan	944	870	1172	980	846	822	219	260	194	5	73	583
furan derivative-1	furan	173	161	206	161	129	110	32	51	35	4	11	39
gamma-butyrolactone	furan	2	59	50	162	149	2	0	99	83	144	133	0
3-methylfurfural	furan	61	17	56	12	7	2	12	26	24	17	9	0
5-methylfurfural	furan	1468	1054	1373	949	623	147	189	256	381	32	188	145
methyl 2-furoate	furan	137	134	159	139	121	121	12	28	39	17	6	12
1-(2-furyl)-1-propanone	furan	258	244	293	285	234	262	46	52	67	31	24	42
furan derivative-2	furan	35	2	40	21	18	17	29	0	33	32	26	26
2-acetyl-5-methylfuran	furan	378	386	464	406	391	416	58	41	69	37	26	82
2-butanone	ketone	130	76	99	182	120	85	100	66	88	67	102	27
2-methyl-2-cyclopentenone	ketone	712	653	898	703	475	606	95	154	116	37	340	426
3,4-dimethyl-2-cyclopenten-1-one	ketone	110	98	121	112	96	87	21	16	21	2	20	38
2-methyl-2-cyclopenten-1-one	ketone	101	107	118	100	80	64	14	31	20	11	14	54

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		Average concentration (µg/kg)						Standard deviation (µg/kg)					
Compound	**category**	**D0**	**D7**	**D14**	**D21**	**D28**	**D35**	**D0**	**D7**	**D14**	**D21**	**D28**	**D35**
ketone-1	ketone	93	68	80	16	12	2	8	15	24	23	16	0
3-methyl-2-cyclopenten-1-one	ketone	202	169	198	161	125	97	13	47	41	14	16	83
3-ethenyl-3-methylcyclopentanone	ketone	68	123	25	127	224	146	113	106	40	112	10	125
4,4-dimethyl-2-cyclopenten-1-one	ketone	303	294	359	303	294	344	42	23	37	25	54	96
3,4-dimethyl-2-cyclopenten-1-one	ketone	382	341	425	352	352	361	53	56	58	10	41	59
3-methyl-1,2-cyclopentanedione	ketone	427	368	411	408	364	377	110	70	94	129	14	117
2,3-dimethyl-2-cyclopenten-1-one	ketone	1187	1152	1402	1257	1168	1355	131	209	100	88	134	246
3,4-dimethyl-2-hydroxycyclopent-2-en-1-one	ketone	165	168	160	143	155	167	10	30	33	32	23	28
2,3,4-trimethyl-2-cyclopenten-1-one	ketone	292	282	301	307	288	286	52	16	25	20	58	20
nona-3,5-dien-2-one	ketone	1064	1084	982	703	481	378	188	147	147	91	130	59
3-ethyl-2-hydroxy-2-cyclopenten-1-one	ketone	23	141	70	123	57	134	36	20	75	107	95	53
ketone-2	ketone	138	135	138	135	123	66	6	25	31	7	34	21
ketone-3	ketone	298	275	308	294	299	317	44	53	29	17	9	37
ketone-4	ketone	563	498	642	571	534	541	105	86	103	10	36	78
ketone-5	ketone	116	45	126	2	2	43	99	74	107	0	0	71
ketone-6	ketone	221	215	277	257	258	262	40	27	19	18	22	16
ketone-7	ketone	200	197	188	155	160	150	10	31	8	7	19	33
ketone-8	ketone	125	112	132	117	126	135	15	5	19	3	18	5
2-allyl-4-hydroxy-3-methyl-2-cyclopenten-1-one	ketone	176	131	112	63	49	19	44	12	19	53	42	30
terpene-1	terpene	88	98	104	94	78	101	19	12	26	12	8	7
terpene-2	terpene	80	70	67	57	67	64	5	5	17	48	18	6
terpene-3	terpene	57	40	33	39	53	50	5	33	27	32	4	9
pristane	terpene	1253	1299	1136	1428	1414	1424	234	200	159	173	239	165
cembrene A	terpene	178	191	161	221	220	211	24	43	14	50	49	46
NA-1	na	2	2	2	43	144	183	0	0	0	71	44	47
NA-2	na	2	2	2	2	41	87	0	0	0	0	67	76
NA-3	na	2	2	2	2	34	9	0	0	0	0	28	12
NA-4	na	49	48	22	2	25	59	41	39	33	0	39	49
NA-5	na	83	95	100	62	102	60	10	13	11	52	21	53
NA-6	na	13	15	19	31	31	15	18	22	15	24	25	22
NA-7	na	76	49	58	39	39	40	18	40	11	33	32	34
NA-8	na	20	38	23	28	54	29	30	12	18	23	16	23
NA-9	na	18	15	10	27	17	16	27	22	14	22	26	24
NA-10	na	46	23	36	17	47	58	39	36	29	25	13	11
NA-11	na	31	16	27	31	49	31	25	24	21	25	12	25
NA-12	na	60	26	56	26	59	67	51	41	17	41	56	1
NA-13	na	26	49	70	64	62	48	41	81	23	53	54	40
NA-14	na	78	2	2	65	2	6	131	0	0	108	0	6

NA : Not identified

Chapter 3

Lactic acid bacteria selection for seafood biopreservation

Chapter 3. Lactic acid bacteria selection for seafood biopreservation

1. Preamble

Following the salmon gravlax characterization exposed in chapter 2, we generated enough information from a microbiological, sensorial and biochemical point of view to consider to biopreserve it. In particular, with the metabarcoding approach we detected in high proportion potential spoilage bacteria genera, namely *Photobacterium*, *Serratia/Yersinia* and *Brochothrix* (in a lesser extent).

Through their strong experience in seafood analysis, EM^{3}B (Ifremer) and Secalim (Oniris) laboratories constituted a mutualized large collection of bacteria isolated from these products. Among this collection, many LAB were studied for their antimicrobial properties and their use as protective cultures during these last 2 decades (Leroi et al., 1996, 2015; Duffes et al., 1999a, 1999b; Brillet et al., 2004, 2005; Matamoros et al., 2009b, 2009a; Fall et al., 2010b, 2010a, 2012; Saraoui et al., 2016a, 2017). Based on these studies we made a pre-selection of 35 LAB that showed the most interesting potential as biopreservative strains. From this pool of LAB we applied a screening strategy based on 7 criteria to further select promising protective cultures. Among these criteria, some such as the antimicrobial activity, spoilage potential, cross inhibition, biogenic amines production and antibiotics resistance, are commonly used for protective cultures assessment (Denis et al., 2013; Leroi et al., 2015). In addition, as the SAFEFISHDISH project had a work package dedicated to the study of the combined effect of chitosan coating, superchilling and biopreservation on whole fresh salmon and cold-smoked salmon fillets quality and safety, two additional technological criteria were added: LAB tolerance toward freezing and chitosan treatment.

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Lactic Acid Bacteria Selection for Biopreservation as a Part of Hurdle Technology Approach Applied on Seafood

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2. Abstract

As fragile food commodities, microbial and organoleptic qualities of fishery and seafood can quickly deteriorate. In this context, microbial quality and safety improvement during the whole food processing chain (from catch to plate), using hurdle technology, a combination of mild preserving technologies such as biopreservation, modified atmosphere packaging and superchilling, are of great interest. As natural flora and antimicrobial metabolites producers, lactic acid bacteria are commonly studied for food biopreservation. Thirty-five lactic acid bacteria (LAB) known to possess interesting antimicrobial activity were selected for their potential application as bioprotective agents as a part of hurdle technology applied to fishery products. The selection approach was based on 7 criteria including antimicrobial activity, alteration potential, tolerance to chitosan coating and superchilling process, cross inhibition, biogenic amines production (histamine, tyramine) and antibiotics resistance. Antimicrobial activity was assessed against 6 common spoiling bacteria in fishery products (*Shewanella baltica*, *Photobacterium phosphoreum, Brochothrix thermosphacta,*

Lactobacillus sakei, Hafnia alvei, *Serratia proteamaculans*) and 1 pathogenic bacterium (*Listeria monocytogenes*) in co-culture inhibitory assays miniaturized in 96-well microtiter plates. Antimicrobial activity and spoilage evaluation, both performed in cod and salmon juice, highlighted the existence of sensory signatures and inhibition profiles, which seem to be species related. Finally 6 LAB with no unusual antibiotics resistance profile nor histamine production ability were selected as bioprotective agents for further *in situ* inhibitory assays in cod and salmon based products, alone or in combination with other hurdles (chitosan, modified atmosphere packing and superchilling).

Keywords: antimicrobial activity, spoilage, screening, chitosan, fish juice, safety assessment

3. Introduction

Benefiting from a healthy image, as a source of valuable nutrients (proteins, vitamins, minerals, omega-3 fatty acids etc.), seafood and fishery products contribute to an important part of our alimentation with an average world consumption value of 20.1 kg/per capita in 2014 (FAO, 2016b). As the demand is increasing with the world population, total fisheries and aquaculture production for human consumption is expected to grow from 146.3 million tons (for a trade value of US$ 148 billion) in 2014 up to 181.1 million tons in 2022 (Lem et al., 2014; FAO, 2016b).

Fishery products are very fragile commodities with a short shelf-life not exceeding 1-2 weeks for fresh products to 3-4 weeks for lightly preserved ones. This is mainly due to a high *post-mortem* pH often superior to 6.0, combined with a high non-protein nitrogen fraction including trimethylamine oxide (TMAO) and amino acids such as methionine and cysteine which are related to strong off-odors and off-flavors molecules production (Gram and Huss, 1996). Thus, this intrinsic flesh composition makes an appropriate growth environment for specific spoilage microorganisms (SSO) (Dalgaard, 1995) involved in sensory degradation. Fresh and lightly preserved fish products are more likely spoiled by psychrotrophic Gram-negative bacteria such as *Shewanella* sp. (*S. putrefaciens*, *S. baltica*), *Aeromonas* sp., *Pseudomonas* sp. (*P. fragi*, *P. fluorescens*, *P. putida*, *P. lundensis,* etc.), *Photobacterium* sp. *(P. phosphoreum, P. illiopiscarium*), *Enterobacteriaceae* (*Serratia proteomaculans*, *Hafnia alvei,* etc.), or *Brochothrix thermosphacta* (Gram and Huss, 1996; Gram and Dalgaard, 2002; Cortesi et al., 2009;

Leroi, 2014; Løvdal, 2015). Lactic acid bacteria (LAB), mainly *Carnobacterium* sp. (*C. maltaromaticum*, *C. divergens*), *Lactobacillus* sp. (*L. curvatus*, *L. sakei*, *L. farciminis*, *L. plantarum*) can also be found in high proportion and may contribute to seafood spoilage (Cortesi et al., 2009; Leroi, 2010; Pilet and Leroi, 2011; Leroi, 2014).

Seafood products can also be important vectors for human illnesses, as 10 to 20% of food-borne diseases are attributed to fish consumption (Pilet and Leroi, 2011). The main microbial risk in seafood products is related to *Listeria monocytogenes, Vibrio* sp., *Salmonella* sp., *Staphylococcus aureus,* which are indigenous to the aquatic environment, or resulting from post-contamination during manufacturing processes (Huss et al., 1995, 2000; Pilet and Leroi, 2011; Leroi, 2014; Løvdal, 2015).

Thus, microbial food spoilage and pathogens growth control are currently representing a crucial challenge, as they have been evaluated to be responsible for the loss of 25% of all post-harvesting food production (Gram and Dalgaard, 2002). In addition to industrial traditional technologies and to face the consumers' demand for minimally processed food, new trends such as biopreservation, high hydrostatic pressure, pulsed electric fields, superchilling, chitosan coating and active packagings are promising complementary ways to extend food shelf-life and reduce microbial risks (Devlieghere et al., 2004; Cortesi et al., 2009).

Biopreservation corresponds to the use of bacteria (or their metabolites) with antimicrobial properties to prevent undesirable bacteria growth (Stiles, 1996). LAB are excellent candidates as they are naturally present in many food commodities (Rodgers, 2001; Ghanbari et al., 2013). They possess the ability to produce a wide range of broad spectrum antimicrobial compounds such as bacteriocins, organic acids, fatty acids, diacetyl, acetaldehyde, H_2O_2, reuterin (Caplice and Fitzgerald, 1999). They are generally recognize as harmless for human consumption and benefiting from a healthy and natural image from consumers (Holzapfel et al., 1995; Ghanbari et al., 2013). In food, biopreservation is mainly studied to control pathogens and many authors succeeded to inhibit *L. monocytogenes* or *S. aureus* growth in fresh or lightly processed products. On the contrary only few studies showed sensory quality improvement by targeting SSO (Devlieghere et al., 2004; Pilet and Leroi, 2011; Ghanbari et al., 2013).

This study aimed to select protective cultures (PC), from a collection of thirty-five LAB strains with interesting antimicrobial profiles, to be combined with others hurdles (MAP, chitosan, superchilling) for cod and salmon based products microbial quality and safety improvement. LAB strains were screened according to seven criteria (Figure 3.1).

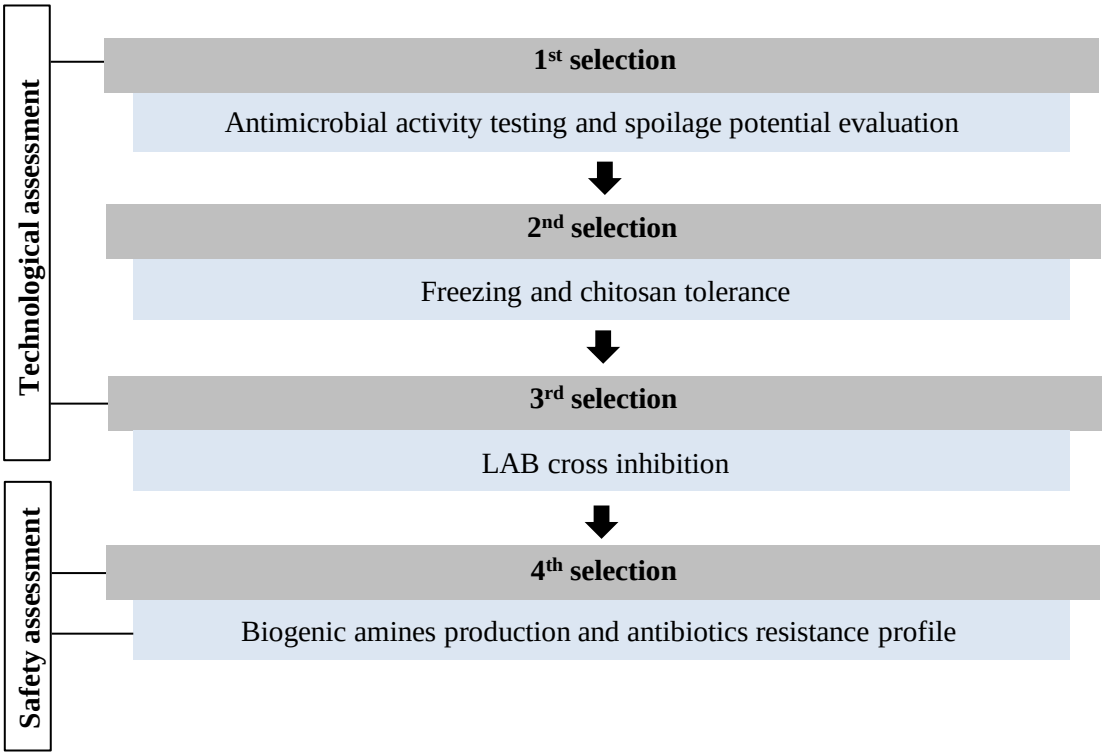

Figure 3.1: Protective cultures selection strategy for seafood products microbial safety and quality improvement.

5 criteria are commonly used for antimicrobial or functional cultures assessment and safety issue for human consumption (Holzapfel et al., 1995; Ammor and Mayo, 2007; Jones et al., 2011; Ghanbari et al., 2013; Leroi et al., 2015) and two specific criteria, chitosan and freezing tolerance, were especially designed for this study. Unlike conventional semi-quantitative antimicrobial methodologies, mainly based on agar diffusion tests (Jones et al., 2011), a miniaturized method in fish juice model was performed to quantify LAB inhibitory activity. To keep a bacterial diversity through the whole screening process and future experiments, at least one strain was selected per LAB species.

4. Materials and methods

4.1. Bacterial strains and subculture conditions

Bacterial strains were isolated from seafood matrices through previous collaborative projects between Ifremer and Oniris/UMR1014 SECALIM, INRA. Bacterial strains subculture conditions are listed in Table 3.1. All strains were then stored at -80 °C in their growth respective medium supplemented with 10% of sterile glycerol (Sigma-Aldrich, Steinheim, Germany).

Table 3.1: Lactic acid bacteria and target strains subculture conditions.

Species	Strains code	Origin	Subculture conditions
Lactic acid bacteria			
Carnobacterium maltaromaticum	SF1944, SF2009, SF2022, SF2094	Cold smoked salmon	48 h at 20 °C in Elliker broth
Lactococcus piscium	EU2229, EU2230, EU2231 EU2232, EU2233, EU2234, EU2235, EU2236, EU2238, EU2239, EU2244, EU2245, MIP2484, MIP2572, MIP2614	Fresh salmon	48 h at 20 °C in Elliker broth
Leuconostoc gelidum	EU2213, EU2214, EU2215, EU2247, EU2248, EU2249, EU2250, MIP2608	Fresh salmon	48 h at 20 °C in Elliker broth
	EU2251	Fresh seabream	
	EU2261, EU2262	Cold smoked salmon	
	LHIS2959	Fresh mackerel	
Vagococcus penaei	CD276		48 h at 20 °C in Elliker broth
Vagococcus fluvialis	CD264		48 h at 20 °C in Elliker broth
Aerococcus viridans	SF1044	Fresh salmon	48 h at 20 °C in Elliker broth
Carnobacterium inhibens	MIP2551	Cold smoked salmon	48 h at 20 °C in Elliker broth
Spoilage bacteria			
Photobacterium phosphoreum	EBP3067	Cold smoked salmon	48 h at 20 °C in BHI broth 20 g/l NaCl
Brochothrix thermosphacta	EBP3069		
Shewanella baltica	EU2187		
Serratia proteamaculans	EU2425	Fresh salmon	
Hafnia alvei	EU2438		
Lactobacillus sakei	SF841		48 h at 20 °C in MRS broth
Pathogenic bacteria			
Listeria monocytogenes	RF191	Shrimps	48 h at 20 °C in BHI broth 20 g/l NaCl

Brain Heart Infusion (BHI), de Man, Rogosa and Sharpe (MRS) and Elliker broth were purchased from Biokar Diagnostic (Beauvais, France).

4.2. Fish juice preparation

Salmon and cod juices were prepared according to an adapted method from Dalgaard (1995) and Leroi et al. (1998). 500 g of fresh cod or salmon filet obtained from local retailer (Carrefour, Nantes, France) were added with 1 l of distilled water prior to a blending step using a blendor (Waring, Torrington, Connecticut, USA). The mixture was then boiled for 2 min before being filtered through a pleated filter (Whatman, Maldstone, England). Fish juice extracts was sterilized at 100 °C for 30 min and stored at -20°C.

4.3. Co-culture inhibitory assay in 96-well plates

Bacteria were precultured in their respective broth medium for 48 h at 15 °C and then diluted in tryptone salt (Biokar Diagnostic, Beauvais, France) to reach respectively 10^8 CFU/ml for PC and 10^4 for the target bacteria. Prior to use, fish juices were thawed at room temperature, 90 ml were supplemented with 10 ml of 1 M K_2HPO_4/KH_2PO_4 (Merck, Darmstadt, Germany) buffer solution at pH 6.7, 1 g of D-glucose (Merck) and 1.5 g of NaCl (Merck). Enriched fish juices were then sterilized on 0.45 µm PTFE filter (Merck). Co-cultures were performed in a 96-well plate containing 196 µl of fish juice and 2 µl of both PC and target diluted suspension. Thus, initial bacterial concentration in co-cultures was 10^6 CFU/ml for PC and 10^2 CFU/ml for targets. After 96 h at 15 °C, co-culture products were diluted following a 10-fold serial dilution in tryptone salt in a 96-well plate. 5 µl of each dilution were then platted on target selective agar medium in 12 cm² Petri dishes. Selective media and growth conditions are summarized in Table 3.2. After incubation period, colonies were enumerated for each spot with a maximum of fifty colonies to avoid overlap. Inhibition was then quantified by comparing target concentration in co-culture with pure culture. According to the deposit volume (5 µl) for enumeration, the method threshold was 2.30 log CFU/ml. A principal component analysis (PCA) was firstly performed on PC inhibition scores against the 7 targets. Then a Ward's hierarchical clustering method with squared Euclidian distance was performed on PCA components to separate the thirty-five PC into clusters according to their antimicrobial profile (R, Vienna, Austria).

Table 3.2: Targets selective culture media and growth condition.

Target species	Selective medium	Growth conditions
Serratia proteamaculans	VRBG	24-36 h at 20 °C
Hafnia alvei	VRBG	24-36 h at 20 °C
Photobacterium phosphoreum	BHI desoxycholate	48 h at 15 °C
Shewanella baltica	BHI desoxycholate	48-72 h at 20 °C
Brochothrix thermosphacta	STAA	48 h at 20 °C
Lactobacillus sakei	MRS	24 h at 37 °C in anaerobic condition
Listeria monocytogenes	Palcam	24 h at 37 °C

VRBG: Violet Red Bile Glucose agar medium (Biokar Diagnostic, Beauvais, France), STAA: Streptomycin-Thallous Acetate-Actidione agar medium (Oxoid, Basingstoke, England). BHI desoxycholate: Brain Heart Infusion Agar medium added with 15 g/l NaCl and 0.5 g/l sodium desoxycholate (Sigma-Aldrich, Steinheim, Germany). Palcam medium was purchased from Oxoid

4.4. Spoilage potential evaluation

PC strains spoilage potential was evaluated both in cod and salmon juice prepared as described in 2.3 and supplemented with 0.1 g/l of L-cysteine, 0.1 g/l of L-methionine and 0.1 g/l of TMAO (Sigma-Aldrich). 450 µl of PC preculture (Elliker 48 h at 15 °C) were inoculated into 45 ml of fish juice and incubated at 15 °C for 96 h. For each PC strain, twelve trained panellists, experienced in seafood sensory evaluation and spoilage assessment (Macé et al., 2012, 2013, 2014), carried out a conventional profiling test (ISO, 2003) on 7 relevant descriptors (spoilage intensity, fish, pungent acid, sour, acid/lemon, feet/banana, and sulfur). After a sniffing step, descriptors were scored on a continuous scale anchored by low intensity (score 0) up to high intensity (score 10). Sessions were performed in individual partitioned booths, as described in the procedure NF V-09-105 (ISO, 2010) and equipped with a computerized system (Fizz, Biosystèmes, Couternon, France). All the inoculated juices were kept frozen at -80 °C until sensory sessions and thawed one hour in cold water prior to use. During sensory evaluation, panellists received 5 ml from samples. For each session, a sample of non-inoculated cod or salmon juice was set as a reference of non-spoiled sample. This sample was not scored but served as a baseline for sensory evaluation. The thirty-five samples per fish juice (cod and salmon) were divided in 4 profiling sessions, with 9 samples per session. Strains choice was balanced all over the sensory evaluation

by presenting the same bacterial groups diversity at each session. Samples were assigned with three digit numbers and randomized for the order presentation within panellists. PCA with standardization was performed on scores mean for each sensory descriptor.

4.5. Freezing impact on cells viability

Freezing tolerance test was performed in salmon fish juice prepared as 2.3. PC were precultured in Elliker broth 48 h at 15 °C and then 48 h at 15°C in salmon juice. For each bacterial strain, 1 ml of preculture was frozen in eppendorf tube at -80 °C for 3 h and thawed at room temperature. Bacterial enumeration was performed prior and after the freezing period using the micro-enumeration method described above in 2.3. All strains were enumerated after 48-72 h at 20 °C on Elliker agar medium, except for *Leuconostoc gelidum* strains, which were instead counted on BHI 20 g/l NaCl agar medium to avoid exopolysaccharides production.

4.6. Chitosan treatment tolerance

PC chitosan tolerance was evaluated at two different pH values (6.0 and 6.6) in 10-fold diluted salmon juice prepared as described in 2.2 and supplemented with less sugar and salt, 0.15% (w/v) and 0.30% (w/v) respectively, to avoid chitosan precipitation. pH was adjusted with NaOH or HCl solution addition (Grosseron, Nantes, France) and was then filter-sterilized. Prior to use, juices were supplemented with 0.02% (v/v) of chitosan D (Primex, Siglufjordu, Iceland). PC strains were pre-cultivated twice successively in Elliker broth 48 h at 15 °C followed by 48 h at 15 °C in salmon juice (prepared as 2.3). 198 µl of diluted salmon juice containing chitosan were inoculated in 96-well plates with 2 µl of PC preculture. Microtiter plates were incubated 3 h at 15 °C. PC concentration was determined just after inoculation and every hour with micro-enumeration method described in 2.3.

4.7. Lactic acid bacteria cross inhibition assay

The cross inhibition was evaluated by the double layer method used by Matamoros et al. (2009a). PC were precultured 48 h at 15 °C in Elliker broth. 10 µl were spotted onto Elliker agar Petri dish. Plates were incubated 48 h at 15 °C under anaerobic conditions. A second preculture was performed at 15 °C for 48 h. Bacterial suspensions were 100-fold diluted in physiological water. 1 ml of bacterial dilution was transferred into 15 ml of molten Elliker medium containing 1% agar, which were quickly spread to form a

double layer onto previously inoculated Elliker plates containing PC spots. Plates were incubated 96 h at 20 °C and cross inhibition was quantified by measuring inhibition diameters from the colonies center.

4.8. Biogenic amines production

After preculture (Elliker 48 h at 15 °C), each PC was cultivated 96 h at 15 °C in salmon juice prepared as 2.3 and supplemented with histidine or tyrosine (Sigma-Aldrich) at a final concentration of 350 µg/ml. Histamine and tyramine were quantified following a protocol adapted from Duflos et al. (1999). 10 ml from sample were added with 5 ml of TCA (Panreac, Darmstadt, Germany) at 12% and kept frozen at -20 °C until analysis. After thawing, samples were spiked with 100 µl of intern standard (1,7 diaminoheptane dihydrochloride at 2 mg/ml) and centrifuged 15 min at 7000 g. 100 µl of supernatant were mixed with 300 µl of satured Na_2CO_3 (Sigma-Aldrich) solution, 400 µl of dansyl chloride solution at 7.5 mg/ml (Sigma-Aldrich) and incubated in obscurity in water bath at 40 °C for 45 min. Tubes were left at room temperature for 10 min before the addition of 100 µl of ammoniac solution (Carlo Erba Reagents, Val de Reuil, France) at 25% and kept in obscurity for 30 min. Biogenic amines were then recovered as following Duflos et al. (1999) methodology. For each sample, 20 µl were injected in a HPLC chain (Shimadzu, France) equipped with a C_{18} Kinetex column (5 µm, 100A, 250 x 4.6 mm, Phenomenex, Le Pecq, France). Elution gradient started with 60% acetonitrile (Fisher Chemical, Loughborough, UK) and 40% water and ended with 95% acetronitrile and 5% water after 18 min. Biogenic amines were detected with UV detector. Chromatograms were analyzed with LabSolutions software (Shimadzu).

4.9. Antibiotics resistance profile

PC antimicrobial profile was evaluated according to the standard method procedure ISO 10932:2010. A minimum inhibitory concentration (MIC) was determined for the 9 following antimicrobial agents as recommended by EFSA, 2012. For each agent, the concentration range expressed in µg/ml is given in brackets: gentamicin (0.5 – 256), kanamycin (2 – 1024), streptomycin (0.5 – 256), tetracycline (0.125 – 64), erythromycin (0.016 – 8), clindamycin (0.032 – 16), chloramphenicol (0.125 – 64), ampicillin (0.032 – 16), vancomycin (0.25 – 128). MICs were determined for all strains after 48 h in anaerobic conditions at 28 °C, except for *L. gelidum* strains, incubated at 26 °C.

Lactobacillus plantarum ATCC® 14917 was used as reference strain for quality control (ISO 10932, 2010).

5. Results

5.1. First selection

5.1.1. Antimicrobial activity

The thirty-five LAB strains antimicrobial activity was evaluated in co-culture in fish juice (cod and salmon) miniaturized in 96-well plates against 7 targets frequently isolated from seafood (*Shewanella baltica*, *Photobacterium phosphoreum, Brochothrix thermosphacta, Lactobacillus sakei, Hafnia alvei*, *Serratia proteamaculans* and *Listeria monocytogenes*).

All LAB strains demonstrated an antagonist effect against at least one target in fish juice model. Inhibitory results are summarized in Table 3.3.

L. piscium isolates showed the widest antimicrobial spectrum by demonstrating inhibition activity for all target bacteria in both fish juices. *P. phosphoreum*, *B. thermosphacta*, *S. baltica* and *L. monocytogenes*, were strongly inhibited (count <2.3 log CFU/ml). *S. proteamaculans*, *H. alvei* and *L. sakei* were also broadly inhibited with 2.5 to 7.0 log CFU/ml reduction depending on the *L. piscium* strain.

Carnobacterium inhibens MIP2551, *Vagococcus fluvialis* CD264 and *Aerococcus viridans* SF1044 were the most active, by almost totally inhibiting all target bacteria (count <2.3 log CFU/ml). There were exceptions such as *B. thermosphacta* which was never inhibited, *L. sakei* which was not inhibited by *C. inhibens* MIP2551 and *A. viridans* SF1044 (only in cod juice) or strongly inhibited (4.7 log CFU/ml reduction) by *V. fluvialis* CD264 only in salmon juice. Among these three strains, *V. fluvialis* CD264 did not totally inhibit *S. proteamaculans* in salmon juice, but still displayed a strong activity with 6.4 log CFU/ml reduction.

Vagoccoccus penaei strain CD272 exhibited the weakest inhibition properties. This isolate inhibited only *S. baltica* (count <2.3 log CFU/ml), and was slightly active against *P. phosphoreum* and *L. monocytogenes* with 2.0 log CFU/ml reduction.

Table 3.3: Growth of the 7 targets strains in pure culture (control) and in co-culture with the thirty-five LAB in cod and salmon juice after 96 h at 15 °C. The method detection threshold was 2.3 log CFU/ml.

		Targets (log CFU/ml)													
		P. phosphoreum		***S. proteamaculans***		***H. alvei***		***B. thermosphacta***		***S. baltica***		***L. sakei***		***L. monocytogenes***	
	Strains	**Salmon**	**Cod**	**Salmon**	**Cod**	**Salmon**	**Cod**	**Salmon**	**Cod**	**Salmon**	**Cod**	**Salmon**	**Cod**	**Salmon**	**Cod**
Control		5.7±0.5	6.9±0.6	9.7±0.1	9.5±0.2	9.4±0.3	9.4±0.3	8.4±0.5	8.7±0.5	8.6±0.3	8.3±0.9	7.2±0.3	7.1±0.3	8.1±0.3	8.1±0.1
C. maltaromaticum	SF1944	3.8	5.8	8.6	8.9	6.7	8.2	5.1	6.3	<2.3	5.8	<2.3	2.6	<2.3	<2.3
	SF2009	4.6	5.9	8.9	8.4	7.6	7.7	7.7	7.2	4.0	5.8	<2.3	2.6	<2.3	<2.3
	SF2022	5.0	4.4	10.1	8.9	7.4	8.3	7.3	7.3	4.1	6.3	<2.3	<2.3	<2.3	<2.3
	SF2094	4.9	4.7	9.3	8.2	8.8	7.6	6.2	6.3	3.1	5.6	<2.3	3.1	<2.3	<2.3
L. piscium	EU2229	<2.3	<2.3	3.1	5.1	4.5	4.6	2.3	<2.3	<2.3	<2.3	3.7	3.3	<2.3	3.8
	EU2230	<2.3	<2.3	3.4	5.4	3.8	4.6	2.3	<2.3	<2.3	<2.3	3.6	3.9	<2.3	3.5
	EU2231	<2.3	<2.3	4.4	5.1	4.0	4.2	<2.3	<2.3	<2.3	<2.3	4.3	5.0	<2.3	3.6
	EU2232	<2.3	<2.3	5.3	5.9	3.9	4.5	<2.3	4.4	<2.3	<2.3	3.7	3.4	<2.3	3.8
	EU2233	<2.3	<2.3	6.5	7.1	4.6	5.4	<2.3	5.7	<2.3	<2.3	5.5	4.9	<2.3	4.3
	EU2234	<2.3	<2.3	4.5	5.5	3.8	4.2	<2.3	2.3	<2.3	<2.3	3.5	2.9	<2.3	3.4
	EU2235	<2.3	<2.3	4.2	5.4	5.0	4.3	<2.3	3.5	<2.3	<2.3	4.7	2.3	2.8	3.3
	EU2236	<2.3	<2.3	4.5	5.3	4.3	4.5	<2.3	4.3	<2.3	<2.3	3.8	3.2	<2.3	3.5
	EU2238	<2.3	<2.3	2.8	6.7	4.1	5.8	<2.3	4.3	<2.3	<2.3	3.8	3.3	<2.3	3.5
	EU2239	<2.3	<2.3	4.7	5.7	4.3	4.9	<2.3	4.3	<2.3	<2.3	4.4	2.8	<2.3	3.8
	EU2244	<2.3	<2.3	4.4	5.5	3.9	4.0	<2.3	4.0	<2.3	<2.3	5.0	2.8	<2.3	3.0
	EU2245	<2.3	<2.3	4.3	5.3	4.1	4.5	<2.3	3.9	<2.3	<2.3	4.9	2.9	<2.3	2.9
	MIP2484	<2.3	<2.3	6.8	7.0	5.0	5.6	3.3	4.5	<2.3	<2.3	4.6	2.6	3.3	3.6
	MIP2572	<2.3	<2.3	3.6	5.5	4.5	5.1	2.3	4.3	<2.3	<2.3	4.9	<2.3	<2.3	3.5
	MIP2614	<2.3	<2.3	4.3	5.3	4.5	4.4	<2.3	2.6	<2.3	<2.3	4.5	<2.3	<2.3	3.6

Table 3.3 (continued) -

		Targets (log CFU/ml)													
		P. phosphoreum		*S. proteamaculans*		*H. alvei*		*B. thermosphacta*		*S. baltica*		*L. sakei*		*L. monocytogenes*	
	Strains	Salmon	Cod	Salmon	Cod	Salmon	Cod	Salmon	Cod	Salmon	Cod	Salmon	Cod	Salmon	Cod
L. gelidum	EU2213	4.8	6.0	8.9	9.6	6.9	8.5	6.2	5.7	<2.3	7.6	4.7	3.4	3.8	3.4
	EU2214	4.7	6.7	7.8	9.3	7.1	8.6	5.3	5.2	<2.3	3.4	4.6	3.7	3.7	3.2
	EU2215	4.8	6.5	8.8	9.6	6.7	8.9	5.4	5.2	<2.3	6.4	3.4	3.8	3.5	3.0
	EU2247	6.0	6.0	6.6	9.3	5.4	8.9	5.2	4.4	<2.3	6.6	3.5	3.6	<2.3	<2.3
	EU2248	5.4	6.5	8.7	9.3	5.6	8.0	5.7	5.4	<2.3	5.8	4.4	3.4	3.6	2.9
	EU2249	6.0	5.6	6.8	9.2	5.9	9.0	4.9	4.6	<2.3	6.5	3.5	3.1	<2.3	<2.3
	EU2250	5.8	5.8	8.0	9.4	5.0	9.0	5.1	3.6	<2.3	6.6	3.8	3.8	<2.3	<2.3
	EU2251	4.5	7.6	9.2	10.2	6.5	4.9	8.2	6.0	<2.3	7.9	4.7	3.5	5.6	3.5
	EU2261	5.4	6.5	8.9	9.8	7.3	8.9	7.0	5.4	<2.3	7.2	5.2	3.8	4.1	3.5
	EU2262	5.7	6.7	10.3	10.2	8.0	8.8	6.8	5.6	<2.3	8.0	5.1	3.6	4.3	2.6
	LHIS2959	4.8	6.7	9.0	9.3	8.3	9.3	5.3	5.4	<2.3	<2.3	4.1	3.6	3.6	3.5
	MIP2608	3.8	6.8	8.3	9.5	8.1	8.9	4.9	4.7	<2.3	<2.3	4.0	3.9	3.4	3.3
V. fluvialis	CD264	<2.3	<2.3	3.3	<2.3	<2.3	<2.3	8.6	8.4	<2.3	<2.3	2.5	<2.3	<2.3	<2.3
V. penaei	CD276	4.6	4.6	8.6	8.8	8.5	9.2	8.2	8.5	<2.3	<2.3	8.3	8.1	6.2	5.4
C. inhibens	MIP2551	<2.3	<2.3	<2.3	<2.3	<2.3	<2.3	7.3	8.0	<2.3	<2.3	5.7	5.5	<2.3	<2.3
A. viridans	SF1044	<2.3	<2.3	<2.3	<2.3	<2.3	<2.3	8.4	9.0	<2.3	<2.3	<2.3	5.3	<2.3	<2.3

In presence of *C. maltaromaticum* strains, both in cod and salmon juice, *L. sakei* and *L. monocytogenes* were strongly inhibited with respectively 4.0-4.5 and 5.5 log CFU/ml reduction. *C. maltaromaticum* strains also demonstrated a strong antagonist activity against *S. baltica* in salmon juice (4.5-6.3 log CFU/ml reduction), but not in cod juice, which was drastically lower (2.0 log CFU/ml). *P. phosphoreum* and *H. alvei* were only slightly inhibited (1.0-2.0 log CFU/ml reduction), while *S. proteamaculans* growth was not significantly reduced in comparison with the control culture (<1 log CFU/ml reduction).

L. gelidum strains showed contrasting inhibitory activities against *S. baltica*, *S. proteamaculans* and *H. alvei* according to the fish juice and the strain. For instance, all strains strongly inhibited *S. baltica* (count <2.3 log CFU/ml) in salmon juice, but lost their activity in cod juice, except EU2214, LHIS2959 and MIP2608. *L. monocytogenes* was highly inhibited with more than 4.0 log CFU/ml reduction. Especially in the presence of the strains EU2247, EU2249 and EU2250, *L. monocytogenes* bacterial count was under the detection threshold. *B. thermosphacta* and *L. sakei* were only slightly inhibited. According to the fish juice and for some *L. gelidum* strains, *P. phosphoreum* was also slightly inhibited with less than 1.9 log CFU/ml reduction.

Figure 3.2, representing Ward's hierarchical classification based on PCA components of the thirty-five PC inhibition scores against the 7 targets, showed that PC strains were strongly clustered together according to the species. Fish juice type played minor effect with antimicrobial activity generally slightly lower in cod juice compared to salmon juice. *V. fluvialis* CD264, *C. inhibens* MIP2551 and *A. viridans* SF1044 antimicrobial spectra were found to be closed to *L. piscium* strains activity, while *L. gelidum* EU2247, EU2249, EU2250 antimicrobial activity were closed to *C. maltaromaticum* strains antimicrobial activity.

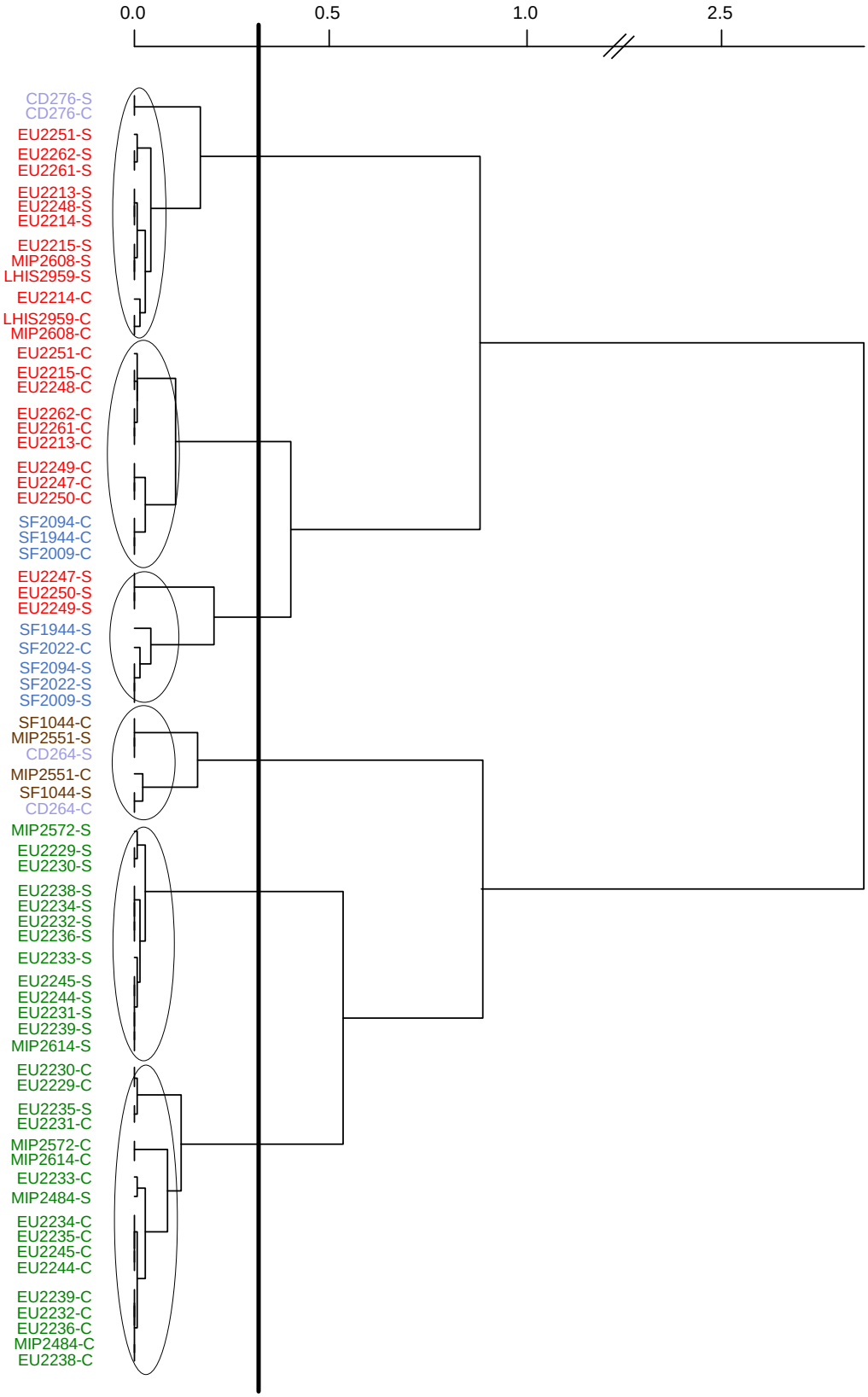

Figure 3.2: Ward's hierarchical classification realized with squared Euclidian distance on PCA components of the thirty-five protective cultures inhibition scores against the 7 targets in both salmon and cod juices. Strain code followed by a letter S: Salmon juice, a letter C: cod juice. Strains in red: *L. gelidum*, in blue: *C. maltaromaticum*, in green: *L. piscium*, in brown: *C. inhibens* MIP2551 and *A. viridans* SF1044, in violet: *V. fluvialis* CD264 and *V. penaei* CD276.

5.1.2. Spoilage potential evaluation

The thirty-five PC spoilage potential was investigated in cod and salmon juice supplemented with 0.1 g/l of L-cysteine, 0.1 g/l of L-methionine and 0.1 g/l of TMAO, precursors of bad smell molecules related to seafood spoilage. For each strain, sensory descriptors were scored from 0 to 10 according to their intensity during a profiling test.

For both juices, sensory results are synthetized in Figure 3.3, which shows normalized PCA, performed on sensory descriptors mean scores. Reference samples were mainly characterized by fresh fish, dried fish, fish flour or crustacean odors. According to their sensory profile, all PC strains were found to be distinctly clustered according to the species.

C. maltaromaticum strains were considered as the most spoiling bacteria group in both fish juices. They were able to reach a spoilage level of 3.0 to 4.5 in salmon juice, and even exceeding 5.0 for strains SF1944 and SF2009 in cod juice. *C. maltaromaticum* spoilage was mainly characterized by pungent acid and feet/banana odor production, also described as malt or rhubarb by some panelists.

L. piscium strains spoilage level were found to be slightly lower in salmon juice (0.5 to 2.0) in comparison with cod juice (2.0 to 3.5). In salmon juice, these strains produced weak sour and fish odors, also associated with a slight sulfur smell for isolates EU2231, EU2234, EU2239, EU2244, EU2245 and MIP2484. *L. piscium* EU2229 was the only exception with acid/lemon odor production. In cod juice, no *L. piscium* strains produced sulfur, but were associated with a higher sour and pungent acid smell.

In both juices, *L. gelidum* strains constitute the LAB group identified to possess the weakest alteration potential, by never exceeding 1.5 as spoilage score. In salmon juice, they were mostly related to fish odor close to the reference, and for some isolates such as EU2215, EU2247, EU2249, EU2251, and LHIS2959, with a small acid/lemon smell. In cod juice, *L. gelidum* strains were the most neutral, with same sensory profile than the non-inoculated control.

As for *L. gelidum*, *C. inhibens* MIP2551, *V. fluvialis* CD264 and *A. viridans* SF1044 were not different from the reference in cod juice. On the contrary, *V. penaei* CD276 was considered as the most spoiling bacteria with a high sulfur smell and a spoilage level reaching 6.6. In salmon juice, *C. inhibens* MIP2551 was associated with slight

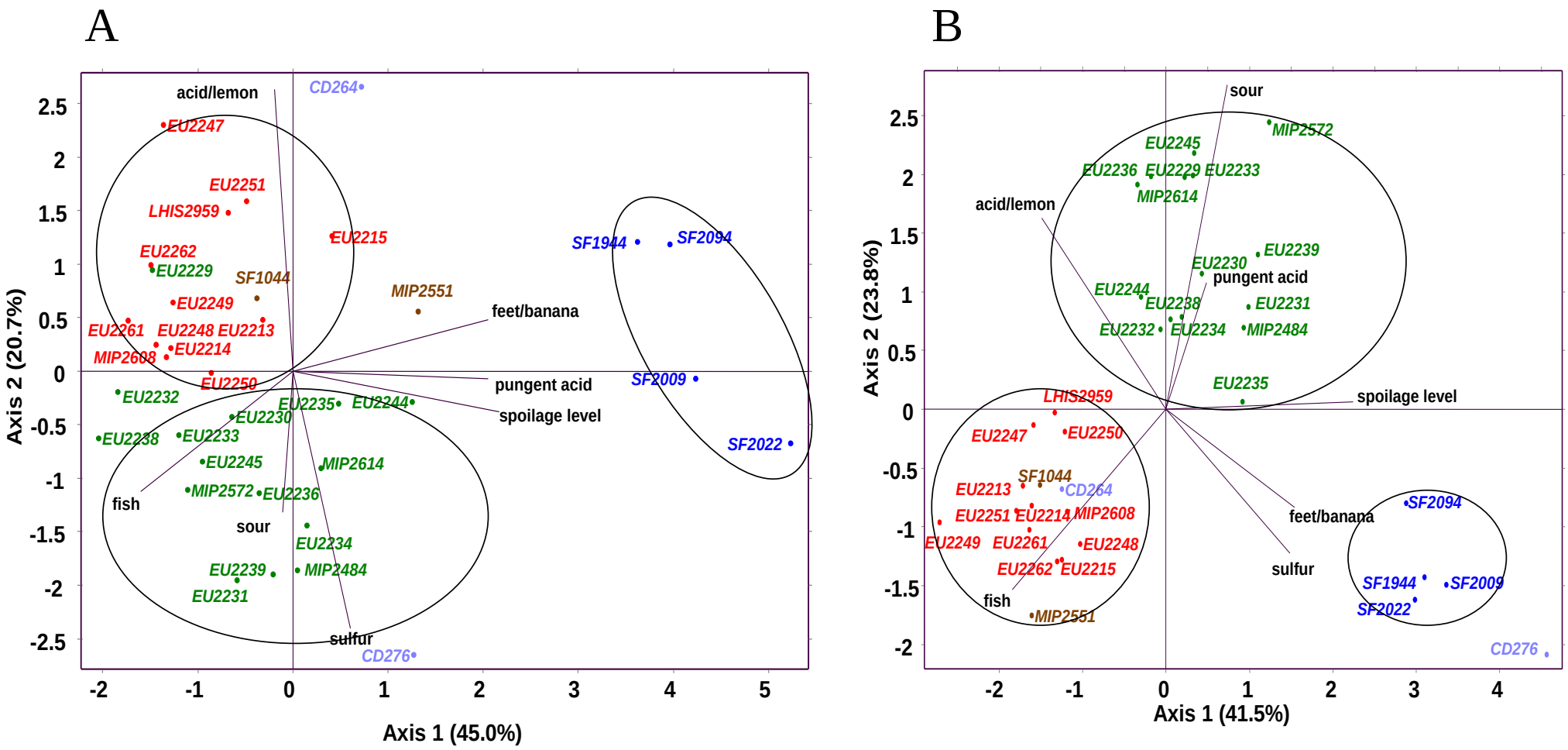

Figure 3.3: Normalized principal component analysis representation of inoculated salmon juice (A) and cod juice (B) based on odor descriptors, on dimension 1-2. Strains in red: *L. gelidum*, in blue: *C. maltaromaticum*, in green: *L. piscium*, in brown: *C. inhibens* MIP2551 and *A. viridans* SF1044, in violet: *V. fluvialis* CD264 and *V. penaei* CD276.

feet/banana odor, *V. fluvialis* CD264 and *A. viridans* SF1044 to a small acid/lemon smell, while *V. penaei* CD276 was still related to a sulfur smell, but in much less proportion than in cod juice.

Although almost all PC produced odors non-specific to seafood or fishery products, it was always in very small proportions and not always perceived by panelists as unpleasant smells related to spoilage, as for instance for acid/lemon odor production. According to these two first screening steps and by maintaining species diversity, fourteen LAB strains were selected as the most interesting LAB in terms of antimicrobial activity with the less spoilage potential (per species): 2 strains of *C. maltatomaticum* (SF1944, SF2094), 5 strains of *L. piscium* (EU2229, EU2231, EU2234, EU2238, MIP2614), 4 strains of *L. gelidum* (EU2247, EU2249, EU2250, MIP2608), *C. inhibens* MIP2551, *A. viridans* SF1044 and *V. fluvialis* CD264.

5.2. Second selection

5.2.1. Freezing tolerance assay

The fourteen previously selected PC were enumerated before and after 3 h at -80 °C. The bacterial count revealed no impact of the freezing step on strains viability, as the bacterial concentration was identical in salmon juice before and after the freezing period (data not shown).

5.2.2. Chitosan tolerance assay

The fourteen PC tolerance to chitosan D treatment was evaluated at a concentration of 0.02% at pH 6.0 and 6.6 in salmon juice during 3 hours. Bacterial enumeration was realized after each hour of treatment.

Chitosan D at 0.02% showed a variable antimicrobial activity against the fourteen LAB at pH 6.0 (Figure 3.4).

C. inhibens MIP2551 and *A. viridans* SF1044, were the two most sensitive strains to chitosan treatment. Their count was below the detection threshold after only 1 h. In the same way *L. gelidum* isolates, were also highly sensitive but still persistent after 2 h of treatment, with an exception for the strain MIP2608 inhibited after only 1 h. All *L. gelidum* counts were below the detection threshold after 3 h of chitosan treatment.

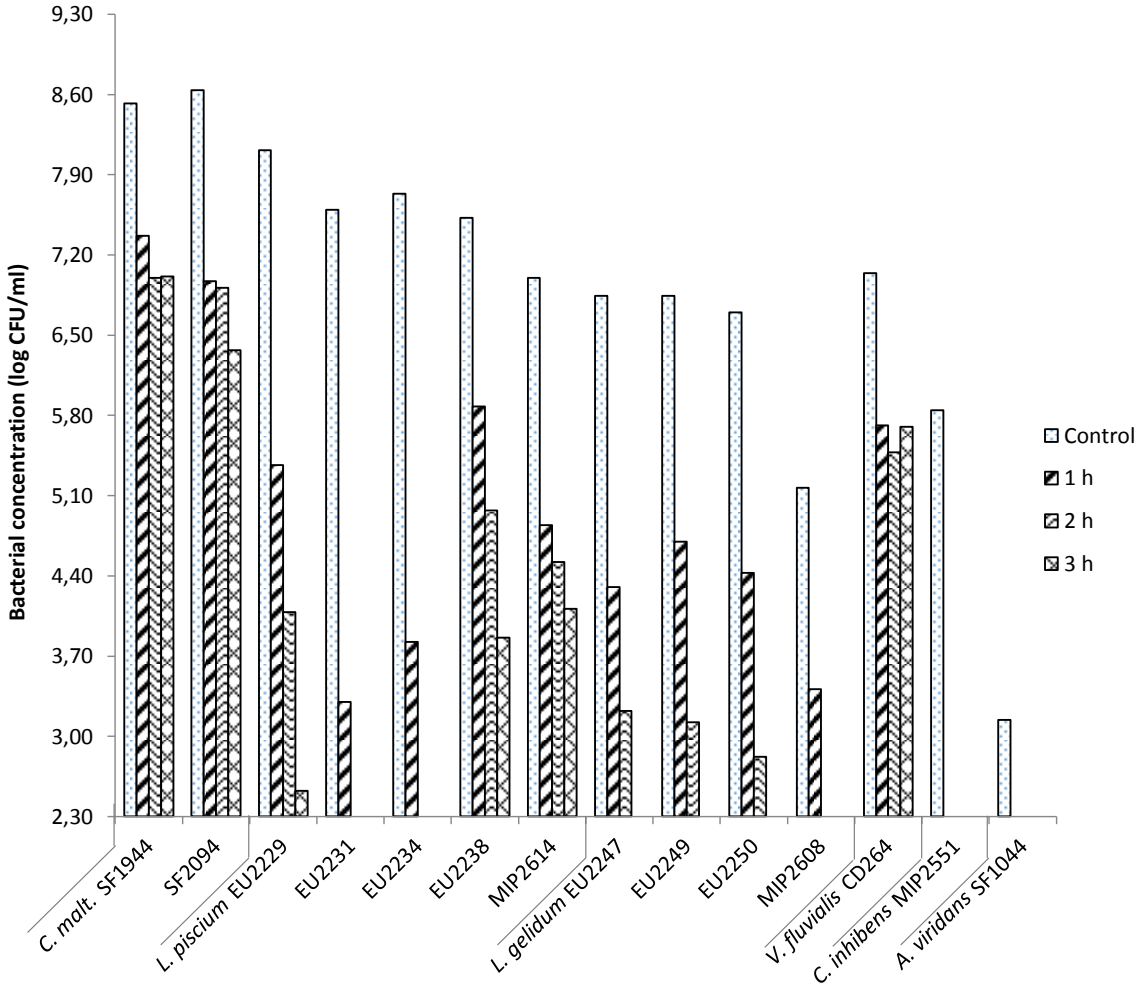

Figure 3.4: Chitosan D antimicrobial activity at 0.02% in salmon juice at pH 6.0 towards protective cultures strains after 1, 2 and 3 h of treatment.

C. maltaromaticum strains and *V. fluvialis* CD264 were the most tolerant PC. They were slightly inhibited after 1 h of treatment, with 1.2 to 1.7 log CFU/ml reduction, then no differences in microbial count was observable after 2 and 3 h.

L. piscium tolerance to chitosan was heterogeneous. Isolates EU2231 and EU2234 were drastically inhibited after 1 h of treatment, with respectively 4.3 and 3.9 log CFU/ml reduction in comparison with the initial bacterial load. After 2 h, the bacterial concentrations were below 2.3 log CFU/ml. In contrast, *L. piscium* EU2229, EU2238 and MIP2614 bacterial concentration were respectively 2.5, 3.9 and 4.1 log CFU/ml after 3 h of treatment.

At pH 6.6 chitosan sensitivity was identical to pH 6.0 for all PC, with an exception for *L. gelidum* EU2249 and EU2250 which were less sensitive at pH 6.6 (data not shown).The chitosan tolerance assay allowed to refine the LAB screening by

eliminating *L. piscium* strains EU2231, EU2234, *L. gelidum* EU2247 and MIP2608, as the most sensitive LAB. Although very sensitive to chitosan treatment, *C. inhibens* MIP2551 and *A. viridans* SF1044 were retained for further screening steps as they showed the broadest and the most interesting antimicrobial profiles.

5.2.3. PC cross inhibition activity

PC antagonist activity between them was evaluated for potential association for food biopreservation. Inhibitory results are exposed in Table 3.4.

All PC demonstrated inhibitory activity against the others, including strains from the same species. This was the case for *L. piscium*, *C. maltaromaticum* and *L. gelidum* strains, which displayed the strongest activity characterized by large inhibition halos with diameters greater than 15 to 30 mm.

On the contrary, *V. fluvialis* CD264, *C. inhibens* MIP2551 and *A. viridans* SF1044 showed the weakest antagonist activity. *V. fluvialis* CD264 displayed only small inhibition diameters (< 15 mm) against *C. maltaromaticum* strains, *L. piscium* EU2229 and a strong inhibition against *C. inhibens* MIP2551. *C. inhibens* MIP2551, was only lightly active against *L. piscium* strains and *V. fluvialis* CD264, while *A. viridans* SF1044 only displayed strong inhibition against *C. inhibens* MIP2551 and *V. fluvialis* CD264.

L. gelidum strains were the less inhibited PC, being only slightly sensitive between them and to *C. maltaromaticum* strains, while *C. inhibens* MIP2551 was the most sensitive one, with inhibition halos almost all superior to 30 mm.

According to this criterion, for each species the strain displaying the lowest inhibition activity towards LAB was selected. Thus *C. maltaromaticum* SF1944, *L. piscium* EU2229, *L. gelidum* EU2249, *V. fluvialis* CD264, *C. inhibens* MIP2551 and *A. viridans* SF1044 were selected to be screened further on risk assessment criteria.

Table 3.4: Protective cultures cross inhibition activity.

		Lactic acid bacteria targets										
	Strains	**SF1944**	**SF2094**	**EU2229**	**EU2238**	**MIP2614**	**EU2249**	**EU2250**	**MIP2608**	**CD264**	**MIP2251**	**SF1044**
C. maltaromaticum	SF1944	nd	++	+	++	++	++	+	+	++	+++	+
	SF2094	++	nd	++	++	++	++	++	-	+++	+++	+
L. piscium	EU2229	++	++	nd	++	++	-	-	-	++	+++	++
	EU2238	++	++	+	nd	+	-	-	-	++	++	++
	MIP2614	++	++	++	++	nd	-	-	-	++	+++	++
L. gelidum	EU2249	++	++	++	++	++	nd	-	++	++	+++	++
	EU2250	++	++	++	++	++	+	nd	++	++	+++	++
	MIP2608	++	++	++	++	++	+	-	nd	++	+++	++
V. fluvialis	CD264	+	+	+	-	-	-	-	-	nd	++	-
C. inhibens	MIP2551	-	-	+	+	+	-	-	-	++	nd	-
A. viridans	SF1044	-	-	-	-	-	-	-	-	++	++	nd

Nd: not determined; -: no inhibition, +: inhibition diameter ≤15 mm, ++: inhibition diameter between 15 and 30 mm, +++: inhibition diameter ≥30 mm

5.3. LAB strains safety assessment

5.3.1. Biogenic amines production evaluation

The 6 PC were assessed on their ability to produce histamine and tyramine in fish juice model containing precursors. No PC produced histamine and tyramine in these experimental conditions, with an exception for *C. maltaromaticum* SF1944 and *V. fluvialis* CD264 which produced respectively 52 ± 2 and 164 ± 1 mg/l of tyramine.

5.3.2. Antibiotic resistance profile

PC resistance profile was assessed following the ISO procedure 10932:2010. MICs for 9 antimicrobials against PC strains are summarized in Table 3.5. The MICs for the quality control *L. plantarum* ATCC® 14917 were similar to the results expressed in the procedure ISO 10932:(2010). There was an exception for streptomycin, where the MIC was slightly lower than in the standard procedure (4 µg/ml versus 16 to 256 µg/ml), but also for tetracycline whose MIC was somewhat higher (64 µg/ml versus 8 to 32 µg/ml). Overall, MICs ranges for all antibiotics were variable depending on the strain. Some strains, such as *L. piscium* EU2229, *C. inhibens* MIP2551 and *A. viridans* SF1044 were more sensitive to all antimicrobials. On the contrary *C. maltaromaticum* SF1944 was less sensitive to antibiotics, especially to clindamycin and ampicillin (not inhibited in the concentration range tested).

Table 3.5: Protective cultures susceptibility towards 9 antibiotics.

Antibiotics	**MIC range (µg/ml)**						
	L. plantarum ATCC 14917	*C. malta.* SF1944	*L. piscium* EU2229	*L. gelidum* EU2249	*V. fluvialis* CD264	*C. inhibens* MIP2551	*A. viridans* SF1044
Gentamicin	4	4	<0.5	<0.5	4	1	<0.5
Kanamycin	64	16	<2	8	16	4	4
Streptomycin	4	4	<0.5	1	2	1	1
Tetracycline	64	0.5	<0.125	64	16	0.5	0.5
Erythromycin	2	2	0.5	1	2	0.5	1
Clindamycin	8	>16	0.125	0.25	>16	0.5	1
Chloramphenicol	16	2	1	4	2	2	4
Ampicillin	0.25	>16	<0.032	0.5	0.5	<0.032	<0.032
Vancomycin	>128	1	<0.25	>128	2	<0.25	<0.25

6. Discussion

This study presents a new strategy, based on 7 criteria, implemented to finally select PC of interest for cod and salmon based products biopreservation. A collection of thirty-five isolates with antimicrobial properties and represented by 7 species was screened. To our knowledge, *C. inhibens*, *V. fluvialis*, *A. viridans* and *V. penaei* have never been tested for food preservation. Although, *C. maltaromaticum*, *L. piscium*, *L. gelidum* antimicrobial activity has been studied (Pilet and Leroi, 2011; Ghanbari et al., 2013), the screening of several isolates at the same time (respectively 4, 15 and 12) allowed to determine their intra-species biodiversity. The possibility for combining PC with other hurdles was also investigated.

6.1. Technological assessment

The first selection was made on two crucial aspects for food biopreservation: presence of antimicrobial activity without sensory adverse effects when added to food commodities (Jones et al., 2011; Leroi et al., 2015). Instead of usual semi-quantitative method to study LAB antimicrobial activity, based on commercial culture media, a quantitative miniaturized method was developed in two fish juices, allowing a rapid and precise comparison of several isolates.

According to these two first criteria, all PC strains could be clustered distinctly per species, based on their specific sensory signature and antimicrobial profile. Intra-species variability was quite weak.

C. maltaromaticum were strongly active against *L. sakei*, *L. monocytogenes* and *S. baltica* (in salmon juice) with more than 4.5 to 5.0 log CFU/ml reduction, but also to a lesser extent against *P. phosphoreum* and *H. alvei*. Antilisterial activity is largely described among *Carnobacterium* species, as they are known to produce a wide range of bacteriocins targeting more specifically Gram-positive bacteria (Leisner et al., 2007). Thus, many studies reported *C. maltaromaticum* (formerly *C. piscicola*), *C. divergens* and *C. alterfunditum* strong inhibitory activity against *L. monocytogenes*, *in vitro* and in seafood (Duffes et al., 1999a, 1999b; Brillet et al., 2004; Nilsson et al., 2004; Tahiri et al., 2004; Brillet et al., 2005; Vescovo et al., 2006; Tahiri et al., 2009). *C. maltaromaticum* and *C. divergens* were also found to inhibit, in supernatant spot assays, several other Gram-positive bacteria, such as *L. innocua*, and LAB (*L. sakei, E. faecalis, C. maltaromaticum* and *C. divergens*) (Duffes et al., 1999b). On the

contrary, *Carnobacterium* sp. are not or weakly active toward Gram-negative bacteria. When inoculated at 8.0 log CFU/g in cold smoked salmon (CSS), *Carnobacterium* spp. 10 and 39 did not show any H_2S-producing bacteria inhibition (Leroi et al., 1996). *C. maltaromaticum* V1 and *C. divergens* V41 did not demonstrate antimicrobial activity against *Escherichia coli* or *S. putrefaciens* (Duffes et al., 1999b). *C. alterfunditum* EU2257 is the only exception, which exhibited a broad-spectrum antimicrobial profile by inhibiting in double layer assay *C. sporogenes*, *L. monocytogenes*, *B. thermosphacta*, *L. farciminis*, but also Gram-negative bacteria such as *Pseudomonas* sp., *S. liquefaciens*, *Psychrobacter* sp., *S. putrefaciens* and *P. phosphoreum* (Matamoros et al., 2009a). When inoculated in cooked and peeled shrimps stored under vacuum packaging, this strain decreased the total Enterobacteriaceae count by 2 log CFU/g in comparison with control (Matamoros et al., 2009b). In our screening approach *C. maltaromaticum* was considered as the most spoiling bacterium in both fish juices by producing feet/banana, pungent acid, malt or rhubarb off-odor. However, the spoilage score remained acceptable in all cases and the 4 *C. maltaromaticum* strains might be suitable in salmon and cod based products. As for all LAB, *Carnobacterium* sp. spoilage activity is food matrices and strains dependent (Leisner et al., 2007; Leroi, 2010). Some authors did not find spoilage evidence in seafood products (Leroi et al., 1996; Duffes et al., 1999a; Brillet et al., 2005; Joffraud et al., 2006; Laursen et al., 2006; Vescovo et al., 2006; Jaffrès et al., 2011; Matamoros et al., 2009b). On the other hand, Macé et al. (2013, 2014) showed that *C. maltaromaticum* strains, isolated from spoiled seafood products, were related to fresh salmon and cooked peeled shrimps alteration, with strong feet/cheese, bitter, sour and acid smell. Malt, burnt, herring, oxidized, sweet/nauseous, nutty, chlorine, grass/hay, butter and plastic were also described for *C. maltaromaticum* and *C. divergens* (Duffes et al., 1999a; Leroi et al., 1998; Paludan-Müller et al., 1998; Truelstrup Hansen and Huss, 1998; Joffraud et al., 2001; Stohr et al., 2001; Brillet et al., 2005; Laursen et al., 2006; Saraoui et al., 2017), but never at a level that could jeopardize their use in seafood.

L. piscium showed the broadest antimicrobial activity towards targets in both fish juices. *L. monocytogenes*, *B. thermosphacta*, *S. baltica* and *P. phosphoreum* were totally inhibited, while *S. proteamaculans*, *H. alvei*, *L. sakei* bacterial count were reduced from 2.5 to 7.0 log CFU/ml, depending on the strain. In double layer assays on Elliker medium, *L. piscium* EU2229 (also screened in this study) and CNCM I-4031 (formerly

EU2241) displayed such wide antimicrobial spectrum by inhibiting *C. sporogenes*, *L. monocytogenes*, *Pseudomonas* sp., *S. liquefaciens*, *B. thermosphacta*, *S. aureus*, *Psychrobacter* sp., *S. putrefaciens*, *P. phosphoreum*, *E. coli* and *Salmonella* sp., (Matamoros et al., 2009a; Fall et al., 2010a). When inoculated in MAP cooked peeled shrimps and CSS under vacuum packaging, *L. piscium* CNCM I-4031 also reduced *L. monocytogenes* by 2-4 log CFU/g and totally inhibited *B. thermosphacta* and *Enterobacteriaceae* (Matamoros et al., 2009b; Fall et al., 2010a, 2010b; Leroi et al., 2015). *L. piscium* strains did not exhibit a strong spoilage potential in both juices. Only very slight sour, acid/lemon and for some strains very light sulfur odors were produced. *L. piscium* is not considered as spoiler in seafood product (Matamoros et al., 2009b; Fall et al., 2010a; Macé et al., 2012; Leroi et al., 2015; Saraoui et al., 2016), although some strains have sometimes been related to light butter/fatty fish, floor cloth, sour and seaweed/iodine-like odors (Matamoros et al., 2009b; Macé et al., 2013).

L. gelidum strains did not display a strong inhibitory activity, except against *S. baltica* (only in salmon juice) and *L. monocytogenes*. *B. thermosphacta*, *L. sakei*, *H. alvei*, and *S. proteamaculans* were also slightly inhibited by some strains. In *in vitro* conditions, Matamoros et al. (2009a) demonstrated similar inhibition spectra for *L. gelidum* EU2213, EU2247 and EU2262 (also screened in this study). Harding and Shaw (1990) demonstrated for the strain *L. gelidum* IN139, in double layer and supernatant assays, an antagonist activity only against *L. monocytogenes* on a pool of 21 targets including pathogenic or spoilage bacteria such as *B. thermosphacta*, *H. alvei* and *S. liquefaciens*. In challenge test in CSS and cooked peeled shrimps, *L. gelidum* was also able to slightly inhibit *P. phosphoreum* (1.5 log CFU/g), *B. thermosphacta* (4.0 log CFU/g), *S. proteamaculans* (2.5 log CFU/g), without inducing any sensory adverse effect (Matamoros et al., 2009b; Leroi et al., 2015) and in some cases, leading to sensory shelf-life extension. In the same way, in our study, *L. gelidum* strains were the most neutral LAB, with for some strains an appreciated acid/lemon smell.

C. inhibens MIP2551, *V. fluvialis* and *A. viridans* SF1044 did not induce spoilage off-odor and showed the strongest antimicrobial activity among LAB strains. Regardless the fish juice type, except *B. thermosphacta*, all target bacteria were inhibited. No data are available concerning their antimicrobial activity or spoilage potential, thus, this study constitutes the first report for these three species. In contrast to *V. fluvialis* CD264, *V. penaei* CD276 displayed the lowest antimicrobial activity by inhibiting only

S. baltica and induced a strong spoilage of cod juice by producing an important sulfur odor. *V. penaei*, is a recently described species (Jaffrès et al., 2010) which was not associated with spoilage in sterile cooked and peeled shrimps (Jaffrès et al., 2011).

PC cross inhibition activity was also investigated for mixed culture applications in food biopreservation. LAB antimicrobial activity is not commonly determined against other LAB, except for some species which might be related to spoilage such as *L. farciminis* and *L. sakei* (Stohr et al., 2001; Joffraud et al., 2006; Leroi et al., 2015). In this study, the 10 selected PC displayed antagonist activity between them, including strains from the same species. This might act as a brake for their use in combination. These results are not surprising for LAB that can be bacteriocins or antimicrobial peptides producers such as *Carnobacterium* sp. (Leisner et al., 2007). Bacteriocins are known to be active against closely-related species (Thonart and Dortu, 2009). Moreover, many authors observed LAB strains possessing antimicrobial activity towards a wide range of other LAB (Harding and Shaw, 1990; Jöborn et al., 1999; Duffes et al., 1999b; Fall et al., 2010a). According to our results, *C. maltaromaticum* strains could only be used in combination with *A. viridans* SF1044. Among *L. gelidum* strains, EU2249 and EU2250 can only be combined together, while *L. piscium* strains, *V. fluvialis* CD264 and *C. inhibens* MIP2551 cannot be combined with any other PC strains. However, as Saraoui et al. (2017) improved cooked and peeled shrimp quality and safety using in combination *C. divergens* V41 with *L. piscium* CNCM I-4031 without any adverse effect on their respective growth, antagonist activity in model conditions might be moderated.

In order to be combined with chitosan coating and superchilling, PC were also screened for their chitosan and freezing tolerance, as part of an entire hurdle technology strategy. A rapid freezing step of 1 ml of subculture in salmon juice at -80 °C, used to mimic superchilling conditions (-37/-40 °C for 2 min to rapidly decrease internal fish flesh temperature at -1.5 to -2 °C) did not affect PC viability. Thus, although superchilling tolerance in seafood still needs to be confirmed, the freezing tolerance was not a discriminant criterion in our screening approach. Several hypotheses could explain these results: fish juice may contain cryoprotectants (in addition to 1% glucose added in our study) and 3 h at -80 °C may not be sufficient to induce bacterial viability decrease. 15 °C as preculture temperature may induce cold-shock proteins (CSP) production. Indeed, LAB are generally naturally tolerant to freezing. Several studies demonstrated a correlation between CSP (CspL and CspP) produced after a cold-

shock at suboptimal temperatures and cryotolerance (Kim and Dunn, 1997; Derzelle et al., 2003; Song et al., 2014).

Chitosan tolerance among PC was both linked to the species and to the strain. Chitosan and derivatives (metal complexes or oligomers) are well known for their antimicrobial activity against a broad range of bacteria and fungi (Kong et al., 2010). The activity may depend on numerous factors like microorganism species, environmental pH, molecular weight, deacetylation degree, pKa and presence of metal cations (No et al., 2002; Wang et al., 2004; Kong et al., 2010). No et al. (2002) observed that chitosans and chitosans oligomers antimicrobial activity, within a wide range of molecular weights, was highly variable depending on the target bacteria. For instance, a 746 kDa chitosan molecule (0.1% in MHB or MRS broth, pH 5.9, 24 h at 37 °C) was able to strongly inhibit *L. plantarum* (8.0 log CFU/ml), while other LAB such as *L. brevis* and *L. bulgaricus* were much less sensitive. Likewise, *S. aureus*, *B. cereus* and *L. monocytogenes* were totally inhibited, while *E. coli*, *P. fluorescens*, *S. typhimurium*, *V. parahaemolyticus*, *Bacillus megaterium* growth was variably inhibited from 3.4 to 7.2 log CFU/ml.

6.2. Safety assessment

Despite the lack of strict regulations regarding the use of microbial cultures as protective agents in food for human consumption (Zagorec and Christieans, 2013), LAB must fulfill several safety requirements (Wessels et al., 2004). Based on existing safety regulation for the use of microbial cultures in animal feed or food fermentation and on the Quality Presumption of Safety status of species (EFSA, 2007), LAB should not produce any toxins such as biogenic amines (tyramine, histamine) and should not possess enterotoxic activity nor transmissible antibiotic resistance (Holzapfel et al., 1995; Holzapfel, 2002; Ammor and Mayo, 2007; Jones et al., 2011; Vogel et al., 2011; Bourdichon et al., 2012b). Among the 6 LAB strains selected so far, no histamine production was detected, and only *C. maltaromaticum* SF1944 and *V. fluvialis* CD264 were able to produce tyramine. *Carnobacterium* spp. are known to be important tyramine producers (Masson et al., 1996; Bover-Cid and Holzapfel, 1999; Duffes et al., 1999a; Brillet et al., 2005; Leisner et al., 2007). When inoculated in sterile CSS, *C. maltaromaticum* V1, SF668 and *C. divergens* V41 produced 37, 63 and 122 mg/kg after 4 weeks of storage at 4-8 °C (Brillet et al., 2005). Higher concentrations were even observed (up to 260 mg/kg) by Duffes et al. (1999a). Tyramine-producing

microbial cultures addition to food might be a safety concern, as it may cause headaches and hypertensive effects at concentration superior to 100-800 mg/kg (ten Brink et al., 1990; Halász et al., 1994). Nevertheless, there is no legal regulation for tyramine in food. Unlike tyramine, only few LAB species were associated with histamine production (Masson et al., 1996; Bover-Cid and Holzapfel, 1999).

The use of microbial cultures as active agents in food and feed might represent a risk for antimicrobials resistance genes transfer to gut bacterial population through the food chain. Thus, it is important to assess the absence of acquired antibiotics resistance genes for each LAB strain intended for use in food (EFSA, 2012). A standardized procedure already exists for antimicrobials susceptibility testing (ISO 10932, 2010) and data are available for results interpretation based on MICs cut-off values (Klare et al., 2007; EFSA, 2012). Nevertheless, these guidelines mainly focus on LAB genera or species found and/or intended for use in dairy or fermented dairy products. The species used in the present study have never been extensively studied for antibiotic resistance, in consequence, no data are available. Only *L. gelidum* EU2249 could be compared with *Leuconostoc* sp. MICs cut-off values from EFSA (2012). For the nine antimicrobials tested, *L. gelidum* EU2249 MICs values were below the recommended values, except for tetracycline where the MIC was well above (64 µg/ml versus 8 µg/ml). A deeper look at the genetic level may be necessary to determine whether this resistance might be acquired or intrinsic to the species. Concerning *V. fluvialis*, Teixeira et al. (1997) investigated the susceptibility of seven isolates to 18 antibiotics. MICs values were similar to those found in our study with *V. fluvialis* CD264. Martín et al. (2007) tested 58 *A. viridans* isolates for their susceptibility to 12 antimicrobials using the disk diffusion method. They found that *A. viridans* isolates were slightly resistant to streptomycin, erythromycin, clindamycin and tetracycline. On the contrary, in our study, *A. viridans* SF1044 growth was inhibited with very low concentrations. The other PC (*L. piscium* EU2229, *C. inhibens* MIP2551 and *C. maltaromaticum* SF1944) were sensitive to all antibiotics with very low MICs values, except *C. maltaromaticum* which was not inhibited by the highest concentrations in clindamycin and ampicillin.

As no adverse health effects have been reported so far for the consumption of LAB added into food commodities (Wessels et al., 2004) and as *L. piscium*, *L. gelidum*, and *C. maltaromaticum* are commonly isolated from meat and seafood products (Leisner et al., 2007; Leroi, 2010; Saraoui et al., 2016b), our strains seems to be suitable for

biopreservation strategy. Moreover, one strain of *C. maltaromaticum* is already commercialized by Sacco S.r.l (Italy). However, caution must be taken in the case of *V. fluvialis* CD264 and *A. viridans* SF1044. Indeed, in our study, *V. fluvialis* CD264 was found to be able to grow at 37 °C and showed a β hemolytic activity on sheep blood Columbia agar medium (data not shown). Pot et al. (1994) and Teixeira et al. (1997) highlighted similar phenotype results for *V. fluvialis* isolates from various environment including clinical sources. This character may not lead to the species rejection but should be investigated deeper. *A. viridans* SF1044, although not resistant to antibiotics, was able to grow at 37 °C and showed α hemolytic activity (data not shown). Kerbaugh and Evans (1968) also underlined such phenotype for *A. viridans* isolated from hospital environment. Moreover, *A. viridans* is a crustacean pathogen, causing the lobster's gaffkemia disease, and has been associated with different human infections such as endocarditis, urinary tract infections, arthritis, or meningitis (Kerbaugh and Evans, 1968; Martín et al., 2007).

7. Conclusion

According to this screening approach, 6 out of thirty-five LAB strains were selected for being suitable as bioprotective agents in a hurdle technology strategy applied to cod and salmon based products. Nevertheless, for some strains, more investigations are needed to entirely assess their safety. Strains whole genome sequencing will provide more information on this aspect. This screening approach also permitted to highlight the existence of species related sensory signatures and antimicrobial profiles. Consequently, we finally selected one strain per species in order to ensure a bacterial diversity for future experiments. *C. maltaromaticum* SF1944, *L. piscium* EU2229, *L. gelidum* EU2249 and *V. fluvialis* CD264 seem to be suitable for combining with chitosan coating treatment, freezing or superchilling, while *C. inhibens*, MIP2551 and *A. viridans* SF1044 should not be combined with chitosan.

8. Authors contributions

NW: Ph.D. student, in charge of experimentations, data processing and writing of the article. JC and MC in charge of sensory analyses and data processing. MFP, DP and FL: Co-Directors of the Ph.D. student.

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Chapter 4

Salmon gravlax biopreservation by six lactic acid bacteria

Chapter 4. Salmon gravlax biopreservation by six lactic acid bacteria

Salmon Gravlax Biopreservation with Six Lactic Acid Bacteria: a Polyphasic Approach to Assess the Impact on Organoleptic Properties, Microbial Ecosystem and Volatilome Composition

In Preparation

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1. Abstract

Seafood and fishery products are very fragile commodities with a short shelf-life as the consequence of organoleptic and microbiological qualities quick deterioration. Microbial growth and activity, is responsible for up to 25% of food losses in the fishery industry. In this context and to meet the consumer's demand for minimally processed food, developing mild preserving technologies such as biopreservation represents a crucial challenge. In this work, we studied the use of six lactic acid bacteria (LAB), previously selected for their properties as bioprotective agent, for salmon dill gravlax biopreservation. Naturally contaminated salmon dill gravlax slices, with a commercial shelf-life of 21 days, were purchased from a French industrial company and inoculated by spraying the protective cultures (PC) to reach an initial concentration of 10^6 log CFU/g. PC impact on gravlax microbial ecosystem (cultural and acultural methods), sensory properties (sensory profiling test), biochemical parameters (pH, TMA, TVBN,

biogenic amines) and volatilome was followed during 25 days of storage at 8° C under vacuum packaging. PC antimicrobial activity was also assessed *in situ* against *Listeria monocytogenes*. This polyphasic approach underlined two scenarios depending on the protective strain. *Carnobacterium maltaromaticum* SF1944, *Lactococcus piscium* EU2229 and *Leuconostoc gelidum* EU2249, were very competitive in the product, dominated the microbial ecosystem, displayed antimicrobial activity against the spoilage microbiota and *L. monocytogenes.* They also expressed their own sensory signatures and had a complex volatilome. However, among these 3 strains, *C. maltaromaticum* SF1944 did not induce strong spoilage and seemed to be suitable for *L. monocytogenes* growth control in gravlax. Conversely, *Vagococcus fluvialis* CD264, *Carnobacterium inhibens* MIP2551 and *Aerococcus viridans* SF1044 were not competitive, did not express strong antimicrobial activity and produced only few organic volatile compounds (VOCs). However, *V. fluvialis* CD264 was the only strain to extend the sensory quality even after 25 days. This study shows that *C. maltaromaticum* SF1944 and *V. fluvialis* CD264 have both a promising potential as bioprotective cultures to respectively ensure salmon gravlax microbial safety and sensorial quality.

Keywords: Metabarcoding, 16S rRNA gene, seafood, antilisterial activity, volatile organic compounds, sensory analyses.

2. Introduction

Originally from Scandinavia, and widely consumed in Nordic countries, fish gravlax are gaining popularity elsewhere in Europe (FranceAgriMer, 2018). These products, mainly based on fishes like salmon (*Salmo salar*), whitefish (*Coregonus lavaretus*), rainbow trout (*Oncorhyncus mykiss*), herring (*Clupea harengus*) mackerel (*Scomber scombrus*) (Leisner et al., 1994; Lyhs et al., 2001), are now commonly found on supermarkets refrigerated shelves as Ready-to-Eat products. Although there are as many recipes as chefs, fish gravlax are usually prepared by curing fish fillets in a mixture of dry salt-sugar base often added with spices such as dill and black pepper, without pre-heating treatment or smoking process. They are mainly characterized by a salt content comprise between 3 to 6% and by a pH comprise between 5 and 6 (Morzel et al., 1999; Lyhs et al., 2001). Commercial products are stored in chilled conditions, often sliced and vacuum-packed.

Considering the limited technologies involved in their preservation (salting, vacuum-packaging and cooling storage), fish gravlax are considered as lightly preserved fish products (LPFP), with a shelf-life often not exceeding 18 to 27 days (Leisner et al., 1994; Lyhs et al., 2001). They offer an ideal growth environment for psychrotrophic pathogenic bacteria or specific spoilage microorganisms involved in sensory degradation (Gram and Huss, 1996). The microbial ecosystem of LPFP stored under vacuum-packaging is usually dominated by spoiling psychrotrophic Gram-negative bacteria such as *Photobacterium* spp. (*P. phosphoreum*, *P. illiopiscarium*), *Vibrio* spp., *Shewanella* spp. (*S. baltica*, *S. putrefaciens*), but also *Enterobacteriaceae* such as *Serratia proteomaculans* and *Hafnia alvei,* which can easily reached $10^{7\text{-}8}$ CFU/g during storage (Paludan-Müller et al., 1998; Lyhs et al., 2001; Cardinal et al., 2004; González-Rodríguez et al., 2002; Leroi, 2014). *Brochothrix thermosphacta*, as many lactic acid bacteria (LAB) such as *Carnobacterium* spp. (*C. maltaromaticum*, *C. divergens*), *Lactobacillus* spp. (*L. farciminis*, *L. alimentarius*, *L. plantarum*, *L. delbrueckii*, *L. sakei*), *Leuconostoc* spp. (*L. mesenteroides*, *L. gelidum*), *Vagococcus* sp. (*V. fluvialis*, *V. penaei*) and *Weissella* spp. can also be found as dominant microbial group and may contribute to spoilage (Leisner et al., 1994; Morzel et al., 1999; Lyhs et al., 2001, 2002; Leroi, 2010, 2014; Pilet and Leroi, 2011). Moreover, as many LPFP such as smoked fishes, marinated fish, seafood salads and insufficiently cooked products, fish gravlax also represent a high potential risk for listeriosis transmission (Cruz et al., 2008; Pilet and Leroi, 2011; Jami et al., 2014).

Nowadays, to face the consumer's demand for minimally processed high quality food, without chemical additives, new trends such as biopreservation and phage biocontrol appeared as promising green solutions (Ghanbari et al., 2013; Le Fur et al., 2013). Biopreservation, defined in 1996 by Stiles, refers to the use of microorganisms or their metabolites to extended shelf-life and enhanced safety of food commodities. Possessing antimicrobial properties and as natural dominant microbiota of many food products, LAB are now widely studied for the biopreservation of fruits, fermented and raw vegetables, dairy and bakery products, meat and seafood (Pilet and Leroi, 2011; Ghanbari et al., 2013; Zagorec and Christieans, 2013; Axel et al., 2017; Leyva Salas et al., 2017; Singh, 2018).

In seafood, most of the scientific works dealing with biopreservation are mainly focused on microbial safety control, especially for *L. monocytogenes* growth inhibition. For an

exhaustive review see Ghanbari et al., 2013 (and 7.3 in chapter 1). For that purpose, bacteriocins producing LAB, such as *Carnobacterium* species (*C. maltaromaticum* and *C. divergens*) and *L. sakei* have been particularly studied (Pilet and Leroi, 2011). Tahiri et al. (2009) demonstrated that the strain *C. divergens* M35, producing the divergicin M35, was able to inhibit *L. monocytogenes* growth by 3.0 log CFU/g in cold-smoked salmon after 21 days at 4 °C. In the same food matrix, Katla et al. (2001) managed to maintained *L. monocytogenes* to its initial level of 103 CFU/g during 28 days at 10 °C, using the *L. sakei* strain Lb790 (sakacin P producer).

Compared to microbial safety control, the biopreservation use for seafood shelf-life extension is far less documented. Indeed, spoilage is a complex matter that may involve several microorganisms and often required, in order to be assessed, a polyphasic approach using biochemical analyses (pH, TVBN, TMA, PV measurement), an exhaustive sensory evaluation and microbiological analyses. For instance, by the use of the *L. piscium* strain CNCM I-4031, Matamoros et al. (2009) and Fall et al. (2010), greatly improved the sensory shelf-life of cold-smoked salmon and cooked and peeled shrimp respectively.

In a previous work, 6 protectives cultures: *Carnobacterium maltaromaticum* SF1944, *Lactococcus piscium* EU2229, *Leuconostoc gelidum* EU2249, *Vagococcus fluvialis* CD264, *Carnobacterium inhibens* MIP2551 and *Aerococcus viridans* SF1044, were screened and selected as promising PC for seafood (Wiernasz et al., 2017). The present work aimed to study the use of these 6 PC for salmon dill gravlax safety and quality improvement. Their biopreservation potential was assessed with an approach combining cultural microbial analyses, sensory evaluation, biochemical analyses (pH, TMA, TVBN, biogenic amines). Moreover, a focus on their metabolic activity was made with the characterization of the volatilome. Their implantation in the products and their impact on the gravlax endogenous microbial ecosystem was monitored through metabarcoding. To our knowledge, this is the first study using such polyphasic approach to monitor the effect of bioprotective LAB addition in food. This also constitutes the first report on fish gravlax biopreservation, which differs from other seafood due to its content in sucrose and on the use of LAB species such as *V. fluvialis*, *C. inhibens* and *A. viridans*.

3. Material and methods

3.1. Gravlax production and storage

Gutted salmons (*Salmo salar*) from a same batch were purchased in Norway and processed on the same industrial site in France. Salmons were filleted and cured with a mix of dry salt, sugar, pepper and dill for 14 h at 6 °C. Fillets were then rinsed, sliced and packed under vacuum as 120 g portion of 8-10 slices. After conditioning, salmon gravlax portions were transported to the laboratory under refrigerated conditions and then stored at 0 °C until the experiment start (the same day).

3.2. Bacterial strains and media

Information on the six PC and *L. monocytogenes* strains are listed in Table 4.1. PC and *L. monocytogenes* strains were respectively subcultured in Elliker broth and Brain Heart Infusion broth (Biokar Diagnostic, Beauvais, France) for 48 h at 15°C before being stored at -80 °C in their growth medium supplemented with 10% of sterile glycerol (Sigma-Aldrich, Steinheim, Germany).

Table 4.1: Protective cultures and *L. monocytogenes* strains information.

Species	Strains code	Origin
Protective cultures		
Carnobacterium maltaromaticum	SF1944	Cold smoked salmon
Lactococcus piscium	EU2229	Fresh salmon
Leuconostoc gelidum	EU2249	Fresh salmon
Vagococcus fluvialis	CD264	Cooked and peeled shrimp
Carnobacterium inhibens	MIP2551	Fresh salmon
Aerococcus viridans	SF1044	Cold smoked salmon
Pathogenic bacteria		
Listeria monocytogenes	RF107, RF114, RF131, RF151, RF191	Cooked and peeled shrimp

3.3. Gravlax inoculation

PC were precultured in Elliker broth medium for 48 h at 15 °C before the experiment and were then diluted in tryptone salt (Biokar Diagnostic) to reach 10^8 CFU/ml. In order to get totally rid of the growth medium, diluted suspension were centrifuged 10 min at 8000 g and pelleted bacterial cells were homogenized in the same volume of a new tryptone salt solution. Gravlax slices were inoculated at 2% (v/w) (1% on each face) with PC by spray, using an airbrush (Paasche airbrush H202S model, Paasche Airbrush Company, Chicago, USA) in order to obtain an initial concentration in the product around 10^6 CFU/g. Slices were then packed under vacuum and stored at 8 °C for 25 days. Non inoculated gravlax packed portions were used as negative control. Except sensory analysis, all measures were done in triplicate (biological replicates). T0 was set as the first experimentation day when gravlax was inoculated.

3.4. Microbiological analysis

3.4.1. Bacterial enumeration

Total viable count (TVC), LAB, *Enterobacteriaceae* and *Brochothrix thermosphacta* were enumerated at T0 and after 7, 14, 18, 21 and 25 days by cultural method. At each sampling date, 20 g of product were aseptically withdrawn and stomached (Stomacher 400 circulator, Seward medical, London, UK) for 2 min with 80 ml of refrigerated sterile tryptone salt solution (Biokar Diagnostic) with 1% Tween 80 (Grosseron, Saint-Herblain, France). Prior to dilution, the stomached solution was left at room temperature for 30 min for bacterial revivification. Successive dilutions were realized in tryptone salt solution + Tween, and 1 ml of the appropriate dilution was pour plated for *Enterobacteriaceae* enumeration and 100 µl were spread plated for the others microorganisms. For the different bacterial groups enumerated, culture media and growth conditions are listed in Table 4.2. To set anaerobic condition, Anaerocult A and Microbiology Anaerotest (Merck, Darmstadt, Germany) were added to hermetic jars. Detections threshold were 0.7 and 1.7 Log CFU/g respectively for *Enterobacteriaceae* and other counts.

Table 4.2: Culture media and growth conditions used for bacterial enumeration.

Target flora	Selective medium	Growth conditions
Total viable count	Long and Hammer (LH)	96 h at 15 °C, aerobic
Lactic flora	Nitrite Actidione Polymyxin agar (NAP)	96 h at 20 °C , anaerobic
Brochothrix thermosphacta	Streptomycin-Thallous Acetate Actidione agar (STAA)	48 h at 20 °C, aerobic
Enterobacteriaceae	Violet Red Bile Glucose agar (VRBG)	48 h at 20 °C, double layer, aerobic

VRBG was purchased from Biokar Diagnostic (Beauvais, France) and STAA from Oxoid (Basingstoke, England)

3.4.2. Total bacterial DNA extraction

Bacterial DNA was extracted from stomached solution following a modified and optimized protocol using MasterPure™ Gram-Positive DNA Purification kit (Epicentre, Illumina, Madison, USA). Four milliliters of stomached solution were spin down at 12,000 g for 10 min at 4 °C. After centrifugation, the supernatant was removed and cell pellet was re-suspended with 500 µl of TE buffer and treated with 1 µl of Ready-Lyse-Lysozyme for 1 h at 37 °C with homogenization every 20 min. After lysozyme treatment, the mixture was transferred into tubes containing 0.2 g of sterile 1.0 mm Zirconia/Silica beads (Biospec Products, Bartlesville, USA). Tubes were shaken twice during 2 min at 30 Hz with a beads beater (Retsch, Illkirch, France). Between each beads beating, samples were ice cooled for 2 min. Beads were briefly spun down and the supernatant was withdrawn into new tubes. Supernatant was then treated with 150 µl of Gram-Positive Lysis Solution containing 1 µl of Proteinase K (50 µg/µl) for 15 min at 65 °C. After incubation, samples were cooled at 37 °C for 5 min and then in ice for another 5 min. 175 µl of MPC Protein Precipitation reagent were added to the samples followed by vigorous shaking for 10 seconds. Debris were pelleted by centrifugation at 4 °C for 10 min at 10,000 g. Supernatant was treated with 1 µl of RNase A (5 µg/µl) for 1 h at 37 °C. 500 µl of cold isopropanol (Carlo ERBA Reagents, Val de Reuil, France) was then added to the solution and homogenized by inverting tubes 40 times prior to an overnight incubation at -20 °C. DNA is recovered by centrifugation at 4 °C for 10 at 10,000 g. Supernatant was carefully removed and the pellet was then rinsed two times with 500 µl of ethanol at 70% (Carlo ERBA Reagents, Val de Reuil, France). DNA was

re-suspended in 35 µl of TE buffer, and then quantified and checked for quality using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, USA). DNA samples were then stored at -20 °C.

3.4.3. Bacterial 16S rRNA gene sequencing

The hypervariable V4 region of the bacterial 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the EMP primers set (515f/806r) from Caporaso et al., 2011. PCR mixture was composed of 6.75 µl of nuclease-free water, 5 µl of 5X Q5 High GC Enhancer, 5 µl of 5X Q5 Reaction Buffer, 1.25 µl of each primer at 10 µM, 0.5 µl of dNTP at 10 mM, 0.25 µl of Q5 High-Fidelity DNA Polymerase (2 U/µl), and 5 µl of sample DNA diluted at 10 ng/µl, for a final volume of 25 µl. DNA template was amplified according to the following thermal conditions: 30s at 98 °C, 30 cycles composed by 10s at 98 °C, 30s at 50 °C, 30s at 72 °C, and a final extension step at 72 °C for 7 min. Each sample were amplified in triplicates and pooled into a single volume of 75 µl. PCR products were then cleaned-up, barcoded and normalized according to Illumina guideline and protocol “16S Metagenomic Sequencing Library Preparation”. Sequencing was performed with a MiSeq instrument with v3 chemistry and generated 300 bp paired-end reads which were demultiplexed by the Illumina run software.

3.4.4. Bioinformatics processing of the data

Demultiplexed reads (around 300 pb) were firstly checked for quality using FastQC (Andrews, 2010), and trimmed with FASTX-trimmer from the FASTX-Toolkit (Hannon, 2010) as follow: reads R1 were trimmed after 280 pb and reads R2 after 230 pb. Reads were then processed using the FROGS pipeline (Escudié et al., 2018). Reads were merged using Flash (Magoč and Salzberg, 2011) with 10% mismatches authorized in overlapped region, and primers sequences removed with Cutadapt (Martin, 2011). Merged reads were clustered using Swarm (Mahé et al., 2014) according to Escudié et al. (2018) recommendations, with a first execution with an aggregation parameter equal to 1, followed by a second execution on previous clusters seeds with an aggregation parameter equal to 3. Chimera detection and removal was performed using VSEARCH (Rognes et al., 2016). Clusters were then filtered on abundance and occurrence by representing a minimum 0.005% of all sequences and being present at least in 3 samples (referring to the number of biological replicates). Clusters affiliation was performed with blastn+ (Camacho et al., 2009) against 16S Silva database version

123 (Quast et al., 2013), and OTUs were filtered depending to an identity and coverage value of 100%.

Downstream analysis were performed on rarified counts with R version 3.4.4 (R Core Team, 2018) under RStudio environment version 1.1.442 (RStudio Team, 2016) using the R packages phyloseq (McMurdie and Holmes, 2013) and vegan version 2.4.6 (Oksanen et al., 2018). The R package DESeq2 (Love et al., 2014) was used to performed differential abundance analysis on samples raw counts normalized following a rlog transformation in base 2 with a pseudo-count of 1. In these samples, prior to the normalization, only OTUs presenting a sequences number superior to 0.05% of the sum of all sequences were kept. All graphical visualizations were performed with the R package ggplot2 (Wickham, 2009).

3.5. Challenge test against L. monocytogenes

In a second trial, PC antilisterial activity was determined *in vivo* against a cocktail of 5 *Listeria monocytogenes* strains on another salmon gravlax batch from the same company and produced in the same conditions. *L. monocytogenes* strains were cultivated individually in Brain Heart Infusion broth for 48 h at 15 °C. Cultures were then pooled and diluted to reach a concentration of 10^6 CFU/ml in tryptone salt. PC were cultivated and diluted as described in 1.3. Prior to inoculation, growth culture medium was removed from diluted bacterial suspension by centrifugation as in 1.3. Dill gravlax slices were firstly inoculated at 1% (v/w) on only one face with *L. monocytogenes* strains suspension to reach a concentration of 10^4 CFU/g. To allow bacterial adhesion, inoculated slices were stored 1 h at 4 °C. Gravlax slices were then inoculated a second time with PC diluted suspension at 1% (v/w) on the same face to reach an initial count of 10^6 CFU/g. A control was realized by inoculating gravlax slices only with *L. monocytogenes*. Slices were store under vacuum packaging at 8 °C for 21 days. *L. monocytogenes* and LAB were enumerated at T0 and after 14 and 21 days, by spread plating on Palcam (Biokar Diagnostic) and NAP medium respectively, after following the procedure described in 1.4. *L. monocytogenes* was enumerated after 48 h at 37 °C.

3.6. Salmon gravlax sensory evaluation

Sensory evaluation of salmon gravlax was performed in parallel to the microbiological analyses. For each conditions (control and inoculated samples), and at each sampling

dates (T0 to 25 days), a conventional sensory profiling test was conducted on gravlax slices odors, according to the ISO norm 13299 (2003). The sensory evaluation was performed by an internal trained panel of 17 judges experienced in seafood, especially in salmon products (Joffraud et al., 2006; Macé et al., 2013a). During session, panelists were asked to firstly assess the global spoilage based on off-odors perception. Then they had to characterized it using a list of relevant sensory descriptors specific to seafood spoilage (salmon products mainly) and PC sensory signature (Wiernasz et al., 2017). Among the list of descriptors figured: butter, acid, sour, amine, foot/banana, sulfur, dill and fish odors. Both global spoilage and descriptors were scored depending on their intensity on continuous scale from 0 (low intensity) up to 10 (high intensity). The product was considered strongly spoiled and unfit for consumption when the global spoilage level exceeded a threshold value of 6.

Sessions were performed in individual partitioned booths, as described in the procedure NF V-09-105 (ISO 8598, 2010) and equipped with a computerized system (Fizz, Biosystèmes, Couternon, France). For the practical part, each panelist received one slice of salmon gravlax presented in a covered plastic container. Samples were assigned with three digit numbers and randomized for the order presentation within panelists. A normalized principal component analysis (PCA) was performed on sensory descriptors mean scores using the R package ggfortify (Horikoshi and Tang, 2016).

3.7. Biochemical analysis

3.7.1. Physicochemical parameters

Total Volatile Basic Nitrogen (TVBN) and Trimethylamine (TMA) were quantified, at T0 (control only) and after 14 days, from 80 g of minced product, according to Conway micro-diffusion method (Conway and Byrne, 1933). The pH value was measured directly after microbiological analyses in the stomached solution with a pH-meter (Mettler Toledo AG, Schwerzenbach, Switzerland).

3.7.2. Biogenic amines quantification

Biogenic amines concentrations were determined at T0 after 14 and 21 days. Ten milliliters from stomached solution (1.2.1) were added with 5 mL of a trichloroacetic acid solution at 12% (Panreac, Darmstadt, Germany). Samples were kept frozen at -20 °C until analysis. Tree biogenic amines (putrescine, cadaverine, and tyramine) were

quantified by High Pressure Liquid Chromatography (HPLC) following Wiernasz et al. (2017) methodology.

3.8. Volatilome investigation by Headspace SPME-GS/MC

For each sampling dates, 20 g of gravlax were withdrawn and stocked under vacuum packaging at – 40 °C before analysis. Eight salmon flesh cylinders were sampled across the frozen product using a pre-cooled metal cork borer and immediately pooled to make up 1 g of analysis sample. Samples were kept frozen in 4 mL vials with screw cap and PTFE/silicone septum at -40 °C prior to extraction and analysis. For each sample (time point and treatment), three independent analysis samples (triplicate) were prepared.

Prior to volatile extraction, a 30% w/v NaCl solution (H_2O) was added to the sample, which was finally minced using a high-speed homogenizer. HS-SPME was applied for extraction of volatile organic compounds using a manual SPME holder with a PDMS/DVB-coated 65 µm fiber (Supelco Inc., Bellefonte, PA). Prior to extraction, the SPME fiber was conditioned in the injection port of the GC according to the instructions provided by the supplier. The SPME fiber was exposed to the atmosphere in the closed sample vial for 30 min, while keeping the vial isothermally at 50 °C in a water bath. Samples were constantly agitated by a magnetic stirrer during extraction.

An Agilent 6890/5975 GC/MS (Agilent Technologies Inc., Palo Alto, CA) was used for all analyses. Analytes absorbed on the SPME fiber were desorbed in the injection port for 3 min under splitless conditions. GC separations were carried out using an apolar HP-5MS capillary column (30 m × 0.25 mm and film thickness 0.25 µm). Injection temperature was 220 °C, and the interface was set to 220 °C. The carrier gas was He at a constant flow rate of 1 ml/min. GC temperature was ramped from 40 °C to 211 °C at a rate of 4.5 °C/min, then raised at a rate of 50 °C/min and finally held at 220 °C (total run time: 40 min). The MS source was adjusted to 230 °C, and a mass range of *m/z* 35–350 was recorded. Mass spectra were acquired in electron impact ionization (EI) mode at 70 eV.

GC/MS chromatograms were visualized using the following GC/MS software packages: Agilent ChemStation software (Agilent Technologies, Waldbronn, Germany), AMDIS software (version 2.71; National Institute of Standards and Technology, Boulder, CO, USA), and the open source programme OpenChrom

Community Edition Alder (version 1.2.0) (Lablicate GmbH, Hamburg, Germany; http://www.openchrom.net).

Tentative identification of compounds was carried out using (a) MS libraries such as NIST05 spectral library (National Institute of Standards and Technology, Gaithersburgh, MD), the NIST Chemistry WebBook (https://webbook.nist.gov/chemistry) and a customized *in-house* MS library of VOCs, in combination with (b) linear retention indices (LRI), based on an homologous series of even numbered n-alkanes (C8 to C24), in combination with LRIs found in literature and NIST Chemistry WebBook. GC/MS data integration, normalization (total signal) and alignment was carried out using the Metalign software (PRI-Rikilt, Wageningen, The Netherlands). Detected analytes concentration were estimated quantitatively based on an internal standard (BHT) and expressed as µg/kg.

Multivariate analyses on VOCs composition were performed by normalized principal component analysis (PCA) and by hierarchical clustering analysis (HCA) coupled with a heatmap on concentrations corrected by the median and transformed into log2(n). The HCA was carried out with the R package gplots (Warnes et al., 2016) using the Ward clustering method on Pearson correlation distance.

4. Results

4.1. Impact on microbial ecosystems

4.1.1. Cultural microbial analyses

Growth kinetics of the four microbial groups during storage at 8 °C are presented in Figure 4.1. For the control, at the beginning of the experiment, the TVC and LAB count were 4.3 ± 0.2 and 2.2 ± 0.3 log CFU/g respectively. Subsequently, both microbial groups quickly grew and reached its maximum after 14 days of storage with 8.3± 0.3 and LAB after 25 days with 7.1 ± 0.7 log CFU/g. In the inoculated samples, the initial PC count enumerated on NAP medium was closed to the expected concentration, with values between 6.1 and 6.8 log CFU/g. Identical values were also found on LH medium, for TVC. By comparing TVC and LAB count, *C. maltaromaticum* SF1944, *L. piscium* EU2229 and *L. gelidum* EU2249 strains seemed to be more competitive in the product than *V. fluvialis* CD264, *A. viridans* SF1044 and *C. inhibens* MIP2551.

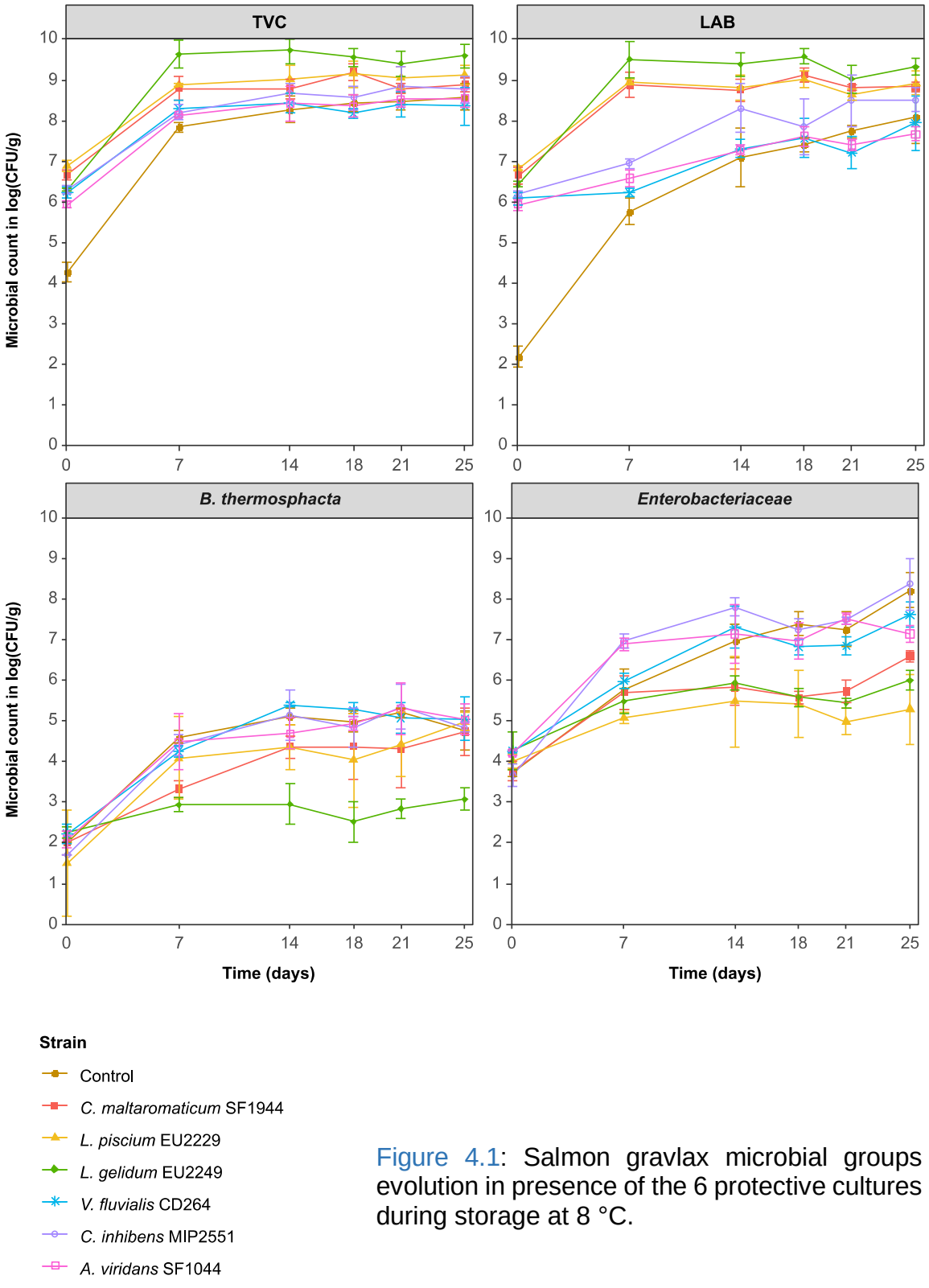

Figure 4.1: Salmon gravlax microbial groups evolution in presence of the 6 protective cultures during storage at 8 °C.

Their growth was indeed faster and they reached their maximum concentration in 7 days with values between 8.9 and 9.5 log CFU/g. Moreover, TVC and LAB count were identical over time and always 1 to 2 log CFU/g higher than in control, suggesting that

PC remained dominant during storage. Conversely, in presence of *V. fluvialis* CD264, *A. viridans* SF1044, and *C. inhibens* MIP2551, LAB counts reached their maximum concentration after 14 days and remained in a range of concentrations closed to the control, never exceeding 7.9 log CFU/g. On LH medium, in their presence, the TVC reached its maximum after 7 day with concentrations 1.0 log CFU/g higher than LAB count.

Salmon gravlax *Enterobacteriaceae* initial population was around 4.0 log CFU/g and reached its maximum concentration of 8.2 ± 0.4 after 25 days of storage. *C. maltaromaticum* SF1944, *L. piscium* EU2229 and *L. gelidum* EU2249 inhibited their growth after 14 days and until the end of storage with a reduction after 25 days of 1.6, 2.9 and 2.2 log CFU respectively. In presence of *V. fluvialis* CD264, *C. inhibens* MIP2551 and *A. viridans* SF1044, *Enterobacteriaceae* count was not different from the control. An exception for *A. viridans* SF1044 was observable, which displayed a slight inhibition (1.1 log CFU/g) after 25 days.

B. thermosphacta initial concentration in gravlax was around 2.0 log CFU/g and stay low and never overpassed 5.1 ± 0.2 log CFU/g. A significant inhibitory effect was observed in presence of *L. gelidum* EU2249, as the *B. thermosphacta* count was maintained below 3.0 log CFU/g during the whole experiment.

4.1.2. Microbial composition through metabarcoding

A total of 8 430 429 raw reads were obtained after Illumina sequencing. 5 131 274 reads passed through FROGS pipeline for an average of 61 087 reads per samples. The sequences number per samples ranged from 6 895 to 176 787. All samples, except two, were normalized by rarefaction on a read number of 14 375. These two specific samples corresponded to 2 replicates from the control condition at T0 (sample T2D0 and T3D0) which were significantly below the others with respectively 6 895 and 9 674 reads. Thus, these two replicates were kept unormalized only for ecosystem composition exploration and visualization, but were systematically removed from downstream analysis (α-, β-diversity and statistical analyses). An exception was made for DESeq2 differential abundance analysis, where reads count per sample was rlog normalized.

In control, at the beginning of the experiment, diversity was high with 53 OTUs observed (Figure 4.2). The ecosystem was mainly dominated by *Photobacterium* (49%) and *Pseudomonas* (13%), but also by many subdominant OTUs representing each 0.5 to 2.5% of the total composition (Figure 4.3). After one week of storage, that number decreased drastically and significantly (HSD-Tukey multiple comparison test after ANOVA, p value < 0.05) to 28 OTUs in average (Figure 4.2). Indeed, more than 20 OTUs belonging to the following bacterial or archaea genera disappeared from the ecosystem after the first week: *Pseudoalteromonas*, *Duganella*, *Pantoea*, *Enterococcus*, *Hafnia/Obesumbacterium*, *Spelaeicoccus*, *Escherichia/Shigella*, *Halorubrum*, *Microbacterium*, *Staphylococcus*, *Brevibacterium*, *Pelomonas*, *Stenotrophomonas*, *Terribacillus*, and *Haloplanus*.

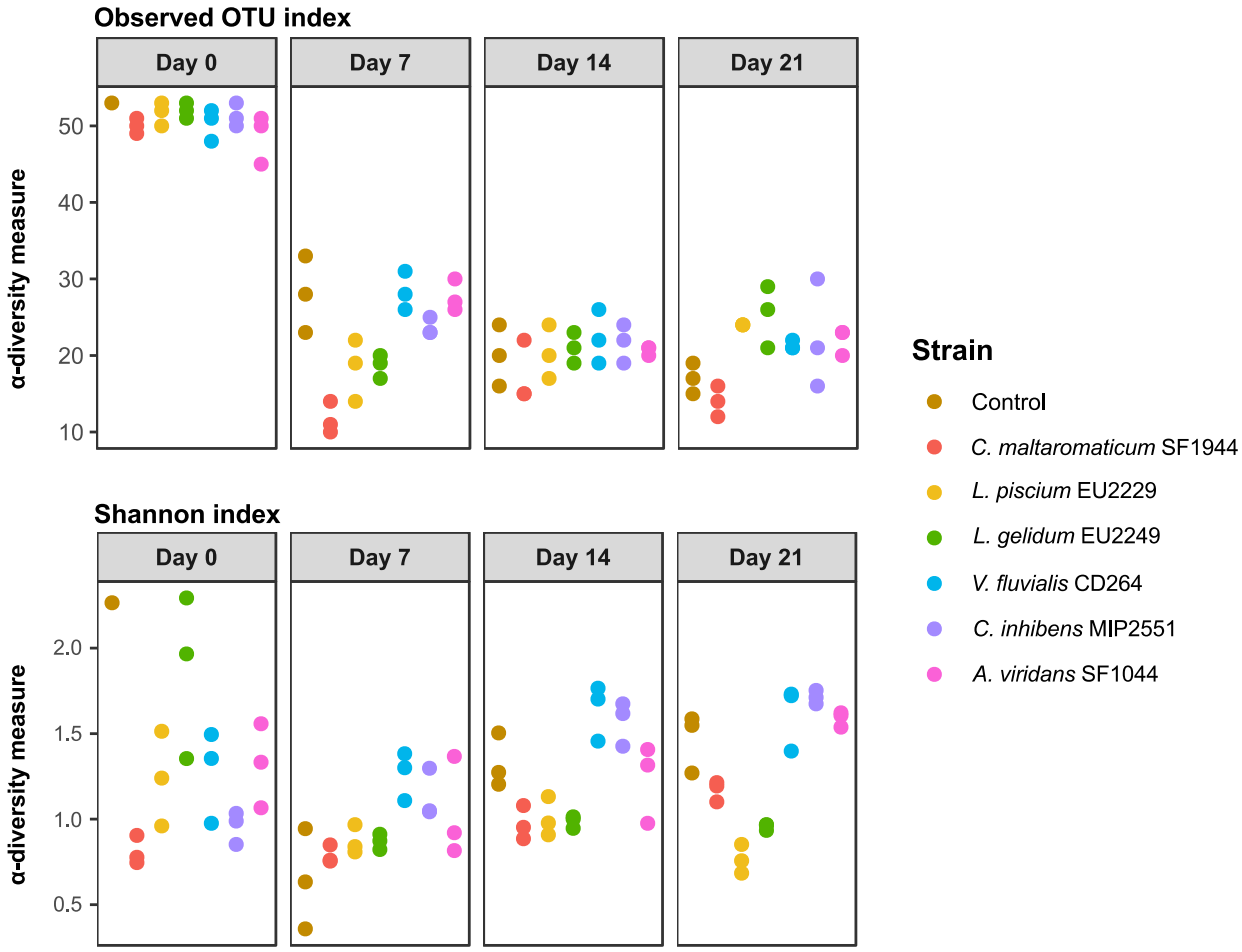

Figure 4.2: Microbial ecosystem α-diversity measures at each sampling dates in presence of the 6 protective cultures.

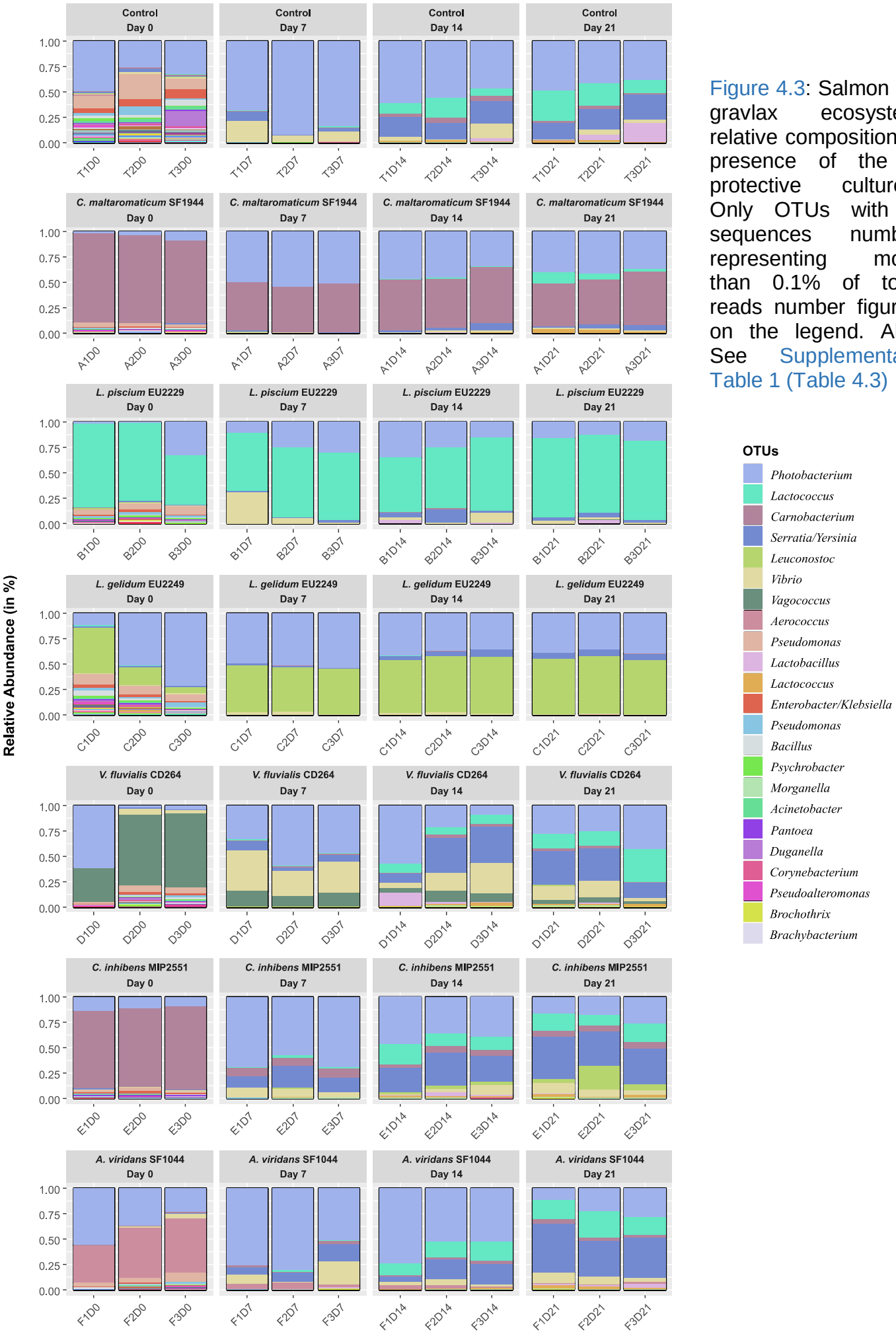

Figure 4.3: Salmon dill gravlax ecosystem relative composition in presence of the 6 protective cultures. Only OTUs with a sequences number representing more than 0.1% of total reads number figured on the legend. Also See Supplementary Table 1 (Table 4.3)

The ecosystem was then largely dominated by *Photobacterium* (68-92%), *Vibro* (56-21%) and *Serratia/Yersinia* (1-8%) in a lesser extent, while *Pseudomonas* abundance decreased to less than 0.5% of the total composition. After 14 days of storage, the microbial diversity continued to significantly decrease and reached a minimum of 17 OTUs observed after 21 days. At day 14 the ecosystem composition was still dominated by *Photobacterium* (38-60%), *Serratia/Yersinia* (16-23.5%), *Vibrio* (3-14%). In addition, *Lactococcus* and *Carnobacterium*, previously in minority, became dominant. (Figure 4.3). Their abundance were significantly higher (DESeq2 pairwise differential abundance analysis, p value < 0.05) after 14 days of storage, representing respectively 7.5-29.5% and 2-5% of the total composition depending on the replicate. Between 14 and 21 days, ecosystems composition was almost identical. Only two OTUs, corresponding to *Lactobacillus* and *Aerococcus,* appeared to be more abundant at day 21 (p value < 0.05), representing respectively 0.5-18.5% and 0.5% of the total composition depending on the replicate.

For each PC, the corresponding OTU was determined by comparison with the control ecosystem composition at T0. As expected with an initial inoculum of 10^6 CFU/g, OTUs which corresponded to the PC genus were largely dominant within the microbiota and significantly much more abundant than in control (Figure 4.3). In consequence, PC strains have been presumptively identified as corresponding to these OTUs. Furthemore the hyperviable region V4 of the 16S rRNA gene was not enough discriminant to distinguish the two *Carnobacterium* species inoculated on the gravlax. Indeed, *C. maltaromaticum* SF1944 and *C. inhibens* MIP2551 appeared as one unique OTUs belonging to the *Carnobacterium* genus.

Despite a concentration 2.0 log CFU/g higher than the TVC in non-inoculated product (Figure 4.1), no statistical differences were observed for the diversity with the control (ANOVA followed by HSD-Tukey multiple comparison test, p values > 0.05). After 7 days, as for the control, the diversity drastically dropped with 11 to 28 OTUs observed (Figure 4.2). Until 21 days of storage no significant evolution in terms of diversity was observed within samples (p values > 0.05). From the 7^{th} day, two scenarios were observed (Figure 4.3).

The first one corresponded to the samples inoculated with *V. fluvialis* CD264, *C. inhibens* MIP2551 and *A. viridans* SF1044. The abundance of their corresponding OTUs decreased drastically after 7 days. However, they remained present over time

among the minority OTUs, with constant proportions varying between 0.7% up to 13% (Figure 4.3). However caution must be taken in the case of *C. inhibens* MIP2551 for results interpretation,, as an OTU corresponding to *Carnobacterium* was also detected in the control. Until 21 days, in their presence, the ecosystem composition was very similar to the control. It was confirmed by a differential abundance analysis made in pairwise for each sampling time against the control condition (Supplementary Table 2, Table 4.4). Except for *C. inhibens* MIP2551, the only difference with control was the presence of the bioprotective strain itself and a higher proportion of *Leuconostoc* in all samples.

In the second scenario, *C. maltaromaticum* SF1944, *L. piscium* EU2229 and *L. gelidum* EU2249 grew well in the product and remained largely dominant until the end of experiment, representing more than 50% of the total ecosystem. Nevertheless, as for the control, *Photobacterium* and *Serratia/Yersinia* were also major genera all along the storage representing respectively 15-50% and 1.7-14% of the ecosystem. By comparing the microbial ecosystem in presence of these 3 PC strains with control by a pairwise differential abundance analysis at each sampling date, only few differences in composition were recorded (including the strain itself) (Supplementary Table 2, Table 4.4). In the case of *C. maltaromaticum* SF1944, after 7 days a significant decrease in abundance was observed for *Psychrobacter* and *Brochothrix*, while an increase was recorded for *Leuconostoc* and *Vagococcus*. After 14 and 21 days the only differences with the control was a decrease in *Lactococcus* and *Lactobacillus* abundance respectively. After 7 days, for *L. piscium* EU2229, the differences in terms of abundance compared to the control was a decrease in *Pseudomonas* and an increase in *Leuconostoc* and *Vagococcus*. At day 14 and 21 only a significant increase in *Leuconostoc* and a decrease in *Carnobacterium* abundance were recorded respectively. In presence of *L. gelidum* EU2249, the differences in comparison with the control was at 7 days an increase in *Vagococcus* abundance and a decrease in *Brochothrix*. After 14 an increase in *Corynebacterium* and *Psychrobacter* and a decrease in *Serratia*/*Yersinia*, *Lactococcus*, *Carnobacterium* and *Brochothrix* was recorded. After 21 days the abundance of OTUs corresponding to *Photobacterium*, *Duganella*, *and Corynebacterium* increased, while a decrease in the abundance of *Lactococcus*, *Lactobacillus*, *Carnobacterium* and *Brochothrix* was recorded. These results suggest that, although largely dominant, *C. maltaromaticum* SF1944, *L. piscium*

EU2229 and *L. gelidum* EU2249 had only a slight effect on the gravlax natural microbial ecosystem, at least on the most abundant OTUs.

These two scenarios in presence of the PC strains are well summarized on the Figure 4.4 showing the distance between samples on a MDS ordination plot using the Weighted Unifrac distance. Samples inoculated with *C. maltaromaticum* SF1944, *L. piscium* EU2229 and *L. gelidum* EU2249 are respectively tightly clustered together, while samples inoculated with *V. fluvialis* CD264, *C. inhibens* MIP2551 and *L. gelidum* EU2249 followed the exact same evolution as the control.

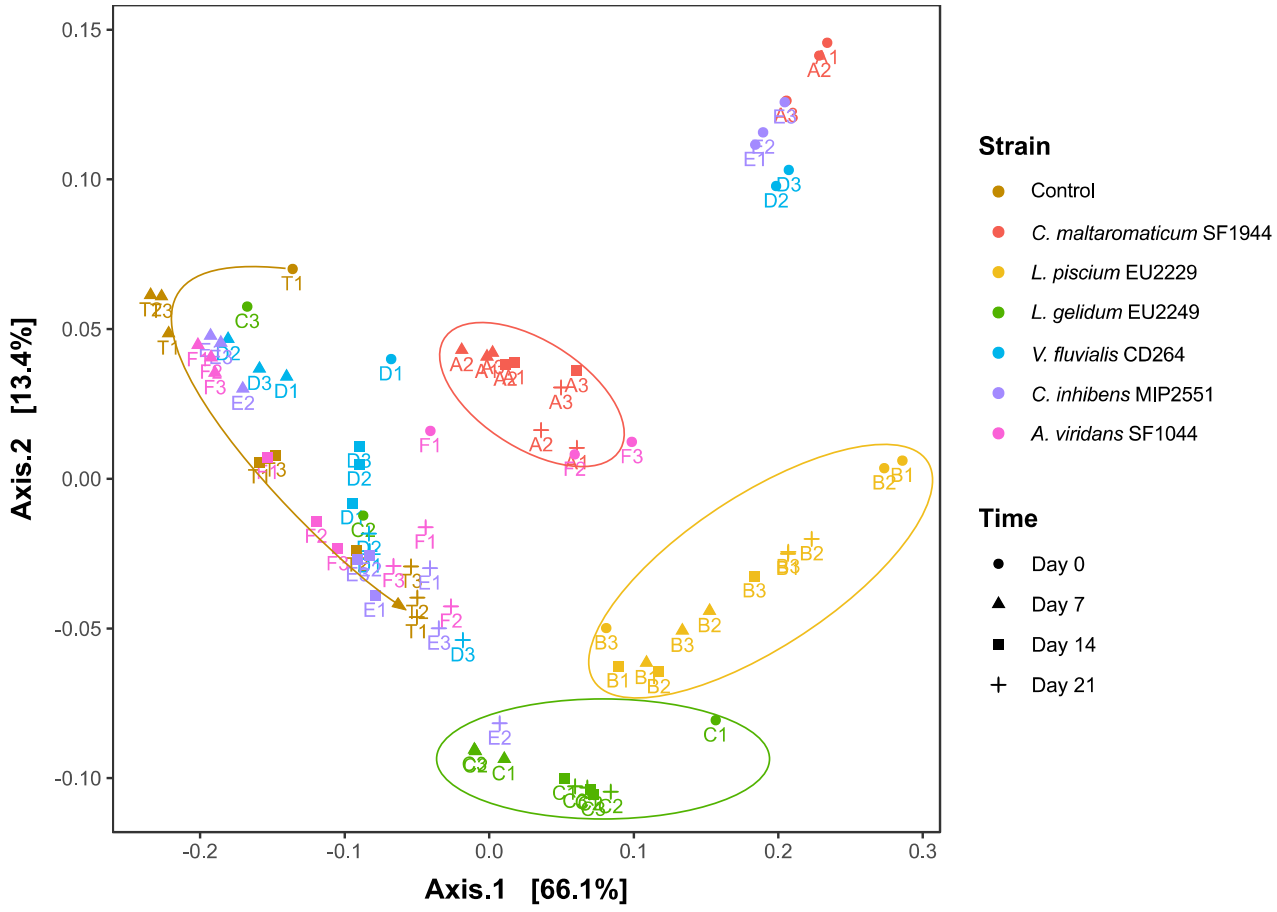

Figure 4.4: Multidimentional scaling representation of the samples microbial composition on dimension 1-2, based on Weigthed Unifrac distance.

4.2. PC antilisterial activity

Figure 4.5 summarized the antilisterial activity of the 6 PC on salmon gravlax against a cocktail of 5 *L. monocytogenes* strains.

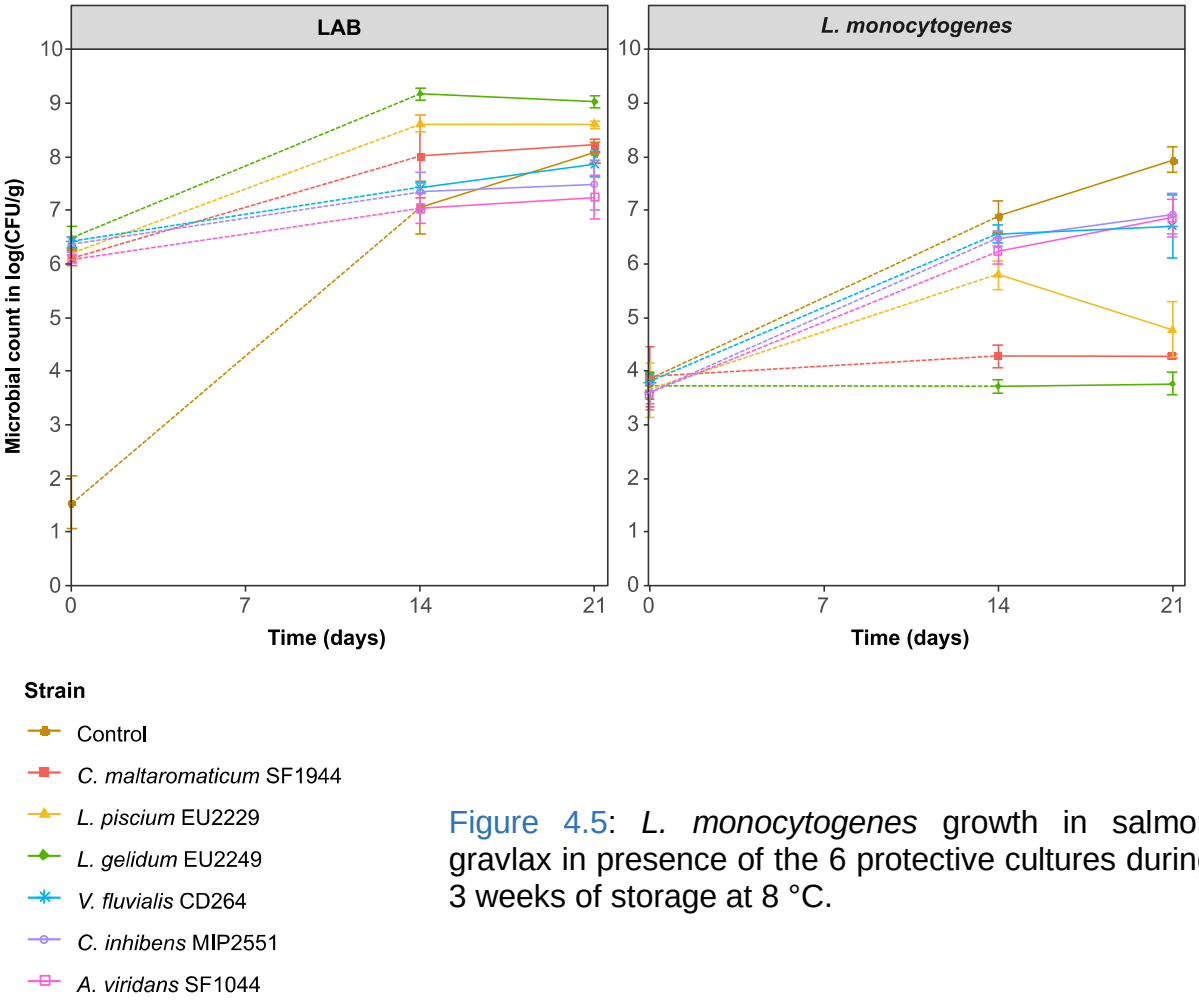

Figure 4.5: *L. monocytogenes* growth in salmon gravlax in presence of the 6 protective cultures during 3 weeks of storage at 8 °C.

Both for control and PC inoculated samples, the lactic flora behaved as previously shown in 2.1.1. In the control, the initial LAB count started at 1.6 ± 0.5 and reached a maximum concentration of 8.1 ± 0.2 log CFU/g after 21 days. The initial PC concentrations were closed to the expect count with value comprised between 6.1 to 6.5 log CFU/g. As noticed before, *C. maltaromaticum* SF1944, *L. piscium* EU2229 and *L. gelium* were more competitive in the product with concentrations ranging from 8.2 to 9.0 log CFU/g, while LAB only reached concentrations around 7.3 to 7.9 log CFU/g after 21 days in presence of *V. fluvialis* CD264, *C. inhibens* MIP2551, *A. viridans* SF1044. The initial level of *L. monocytogenes* inoculated in samples ranged from 3.6 to 3.9 log CFU/g. In control, *L. monocytogenes* reached a concentration of 6.9 ± 0.3 log CFU/g and 7.9 ± 0.2 log CFU/g respectively after 14 and 21 days of storage.

During this experiment, 3 inhibition patterns against *L. monocytogenes* were observed. (1): *C. maltaromaticum* SF1944 and *L. gelidum* EU2249 totally prevented the growth of *L. monocytogenes* during storage, maintaining it to its initial concentration (bacteriostatic effect). The inhibitions were 3.6 and 4.1 log CFU/g after 21 days for *C. maltaromaticum* SF1944 and *L. gelidum* EU2249 respectively. (2): *L. piscium* EU2229 strain also displayed an important inhibition, with 3.1 log CFU/g reduction at day 21, but with a different behavior. A bactericidal effect appeared after 14 days when the PC reached its maximum population. Thus, *L. monocytogenes* count decreased from 5.8 ± 0.3 at day 14, to 4.8 ± 0.5 log CFU/g at day 21. (3): In presence of *V. fluvialis* CD264, *C. inhibens* MIP2551 and *A. viridans* SF1044 a slight inhibition (1.0 log CFU/g) was visible only after 21 days of storage.

4.3. Impact on salmon dill gravlax organoleptic properties

During sensory sessions panelists had firstly to score the global spoilage level of the product, based off-odors perception. Results are shown in Figure 4.6.

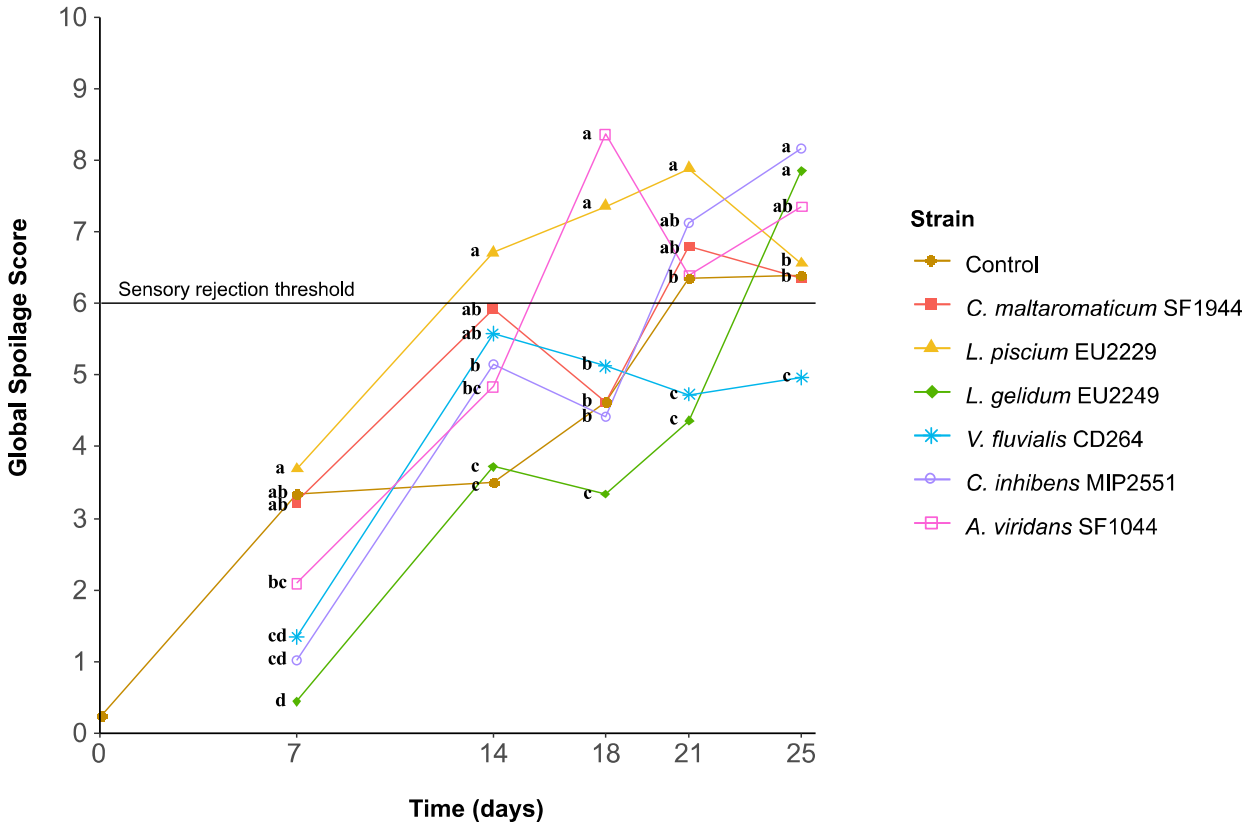

Figure 4.6: Sensory global spoilage score of salmon gravlax slices inoculated with the 6 protective cultures. Samples associated with identical letters are not significantly different, according to a HSD-Tukey test with a p value of 0.05 performed after an ANOVA.

Control gravlax quickly shown signs of spoilage with a global spoilage score of 3.3 after 1 week of storage. Samples were strongly spoiled and rejected by panelist at 21 days with a score of 6.3. Details on sensory profiles are illustrated on the PCA in Figure 4.7. As the storage progressed, control samples evolved from the left part of the PCA, characterized by unspoiled products with strong fish and dill odor, to the right part corresponding to spoiled products. Control sensory profile were mainly driven by amine, acid, sour odors. A strong butter-like odor was also recorded at 18 days but not later.

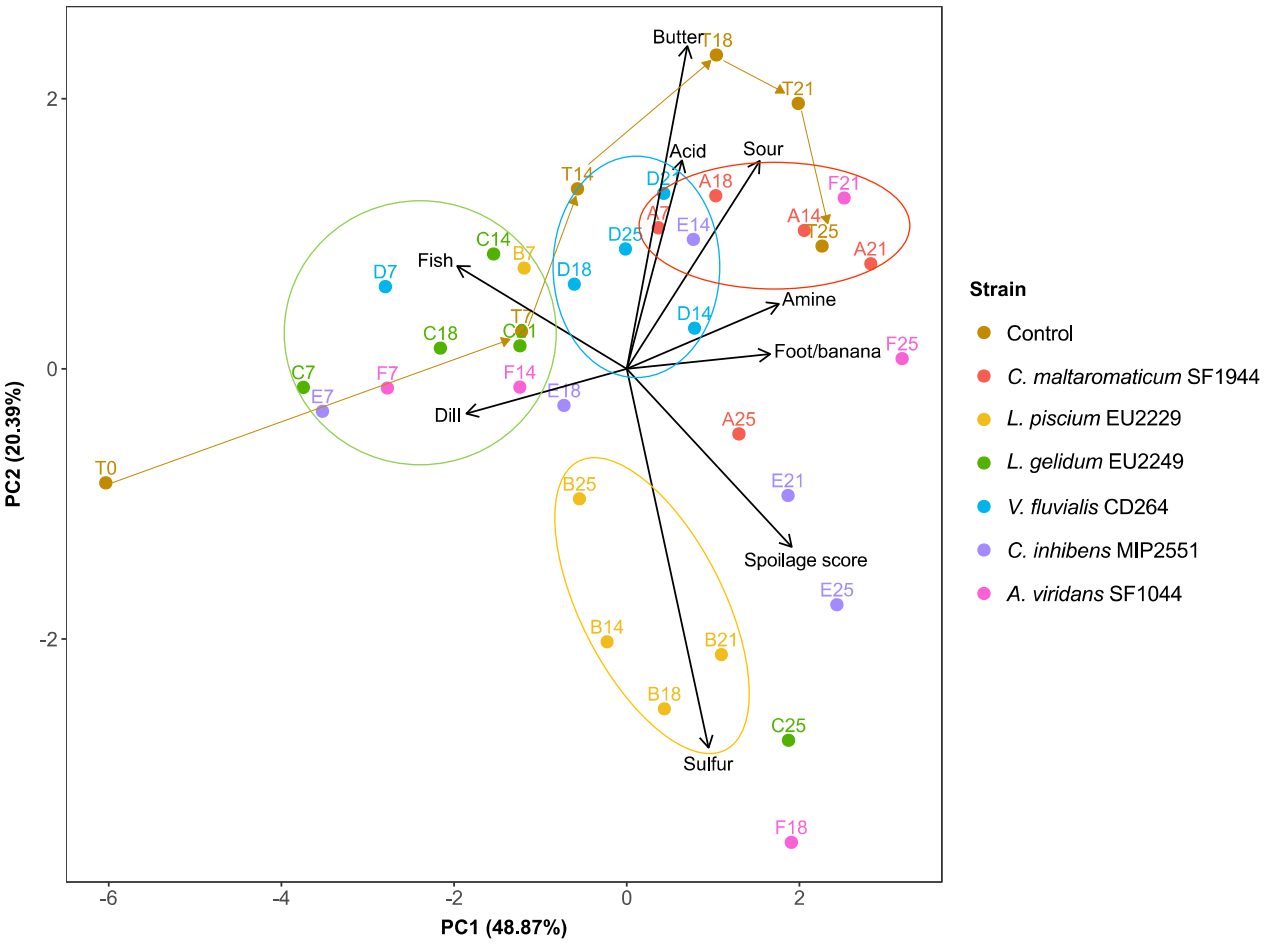

Figure 4.7: Normalized principal component analysis (PCA) representation in dimension 1-2 of the salmon dill gravlax sensory descriptors in presence of the 6 protective cultures. The PCA is based on mean scores of each descriptor. Numbers following letters are referring to the sampling date.

In presence of *C. maltaromaticum* SF1944, the spoilage score was not different from the control except at 14 days where it almost reached the sensory rejection (score of 5.9). On the PCA, samples inoculated with this strain were clustered together and characterized by odors such as sour, amine and foot/banana or malty/rhubarb depending on the panelist perception.

Gravlax slices inoculated with *L. piscium* EU2229 were rapidly spoiled and rejected by panelists after only 14 days (score of 6.7) due to important sulfur off-odors. After leaving the plastic containers opened for few seconds, the sulfur odor disappeared in favor of acid and amine smells. In addition, from day 14, salmon gravlax slices color moved from orange to pink with a cooked-like aspect.

In presence of *L. gelidum* EU2249, the global spoilage remained low from 7 days (0.4) to 18 (3.3) and still acceptable after 21 days (4.4), with fatty fish and dill odors still strongly perceived and a slight acid smell. After 25 days, slices were strongly spoiled (7.9), characterized by an important sulfur and vinegar off-odors. Moreover, from day 14, the product had a pink cooked-like appearance and a slight formation of gas and yellow slime were visible in the package.

From a low spoilage score of 1.3 at 7 days, samples inoculated with *V. fluvialis* CD264 almost reached the sensory rejection threshold after 14 days of storage (5.6). After this sampling date the score slightly decreased at 18 days and then remained more or less constant until 25 days of storage with a value of 5.0. Similarly to the control, gravlax slices inoculated with *V. fluvialis* CD264 were characterized by amine, sour and acid off-odors, but in lower intensity. *V. fluvialis* CD264 was the only strain whose global spoilage score remained below the sensory rejection even after 25 days.

Until 18 days, in presence of *C. inhibens* MIP2551 the spoilage score followed a pattern similar to that described for *V. fluvialis* CD264. After 21 days samples were judged strongly spoiled and rejected by panelists with a score of 7.1. Slices sensory profile was mainly driven by a strong amine odor after 14 days, combined with a sulfur smell after 21 days.

Spoilage score for samples inoculated with *A. viridans* SF1044 increased quickly after 7 days and exceeded the sensory threshold between 14 and 18 days. Gravlax slices were characterized by strong amine odor from day 14 combined with sulfur and sour smells after 18 days

5. Biochemical changes

At the beginning of the experiment, the TVBN content in control was very low (3.8 ± 0.1 mg-N/100 g) (Figure 4.8). After 14 days, it increased and reached 29.3 ± 0.8 mg-N/100g. A significant reduction was observed for the samples inoculated with *L. piscium* EU2229, *L. gelidum* EU2249, *C. inhibens* MIP2551, and *A. viridans* SF1044 (HSD-Tukey multiple comparison test after ANOVA, p values < 0.05), with concentration of 22.5 ± 0.4, 21.4 ± 0.8, 19.2 ± 0.8, 17.9 ± 1.0 respectively. Conversely, no significant difference with the control was observed for samples inoculated with *C. maltaromaticum* SF1944 and *V. fluvialis* CD264 (p value > 0.05). No TMA was detected at day 0 and less than 5 mg-N/100g were recorded after 14 days whatever the control or the samples inoculated with the 6 PC.

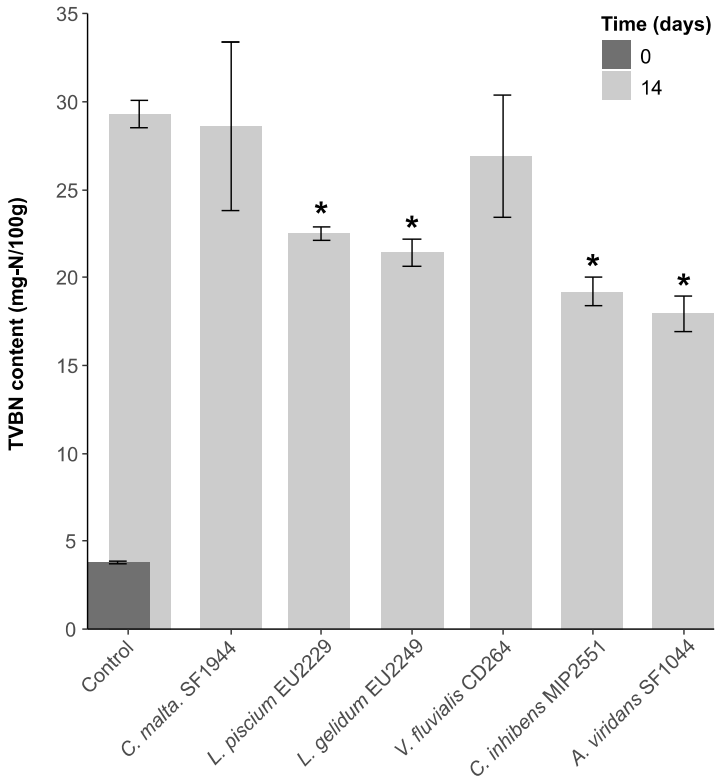

Figure 4.8: Total volatile basic nitrogen (TVBN) production after 14 days of storage in presence of the 6 protective cultures. Values followed by * are significantly different from the control value with a p value of 0.05.

The pH evolution, in presence of the 6 PC is shown in Figure 4.9. The pH in control was stable all along the storage period, with values around 6.0. *L. piscium* EU2229 and *L. gelidum* EU2249 induced a quick acidification of the product. After 1 week of storage, the pH values were equal to 5.3 ± 0.3 and 5.7 ± 0.02 respectively for *L. piscium* EU2229 and *L. gelidum* EU2249. In presence of these two LAB strains, pH reached its minimum values after 14 of storage, with 5.0 ± 0.1 for *L. piscium* and 5.5 ± 0.02 for *L. gelidum* EU2249. The other PC had no incidence on the pH evolution.

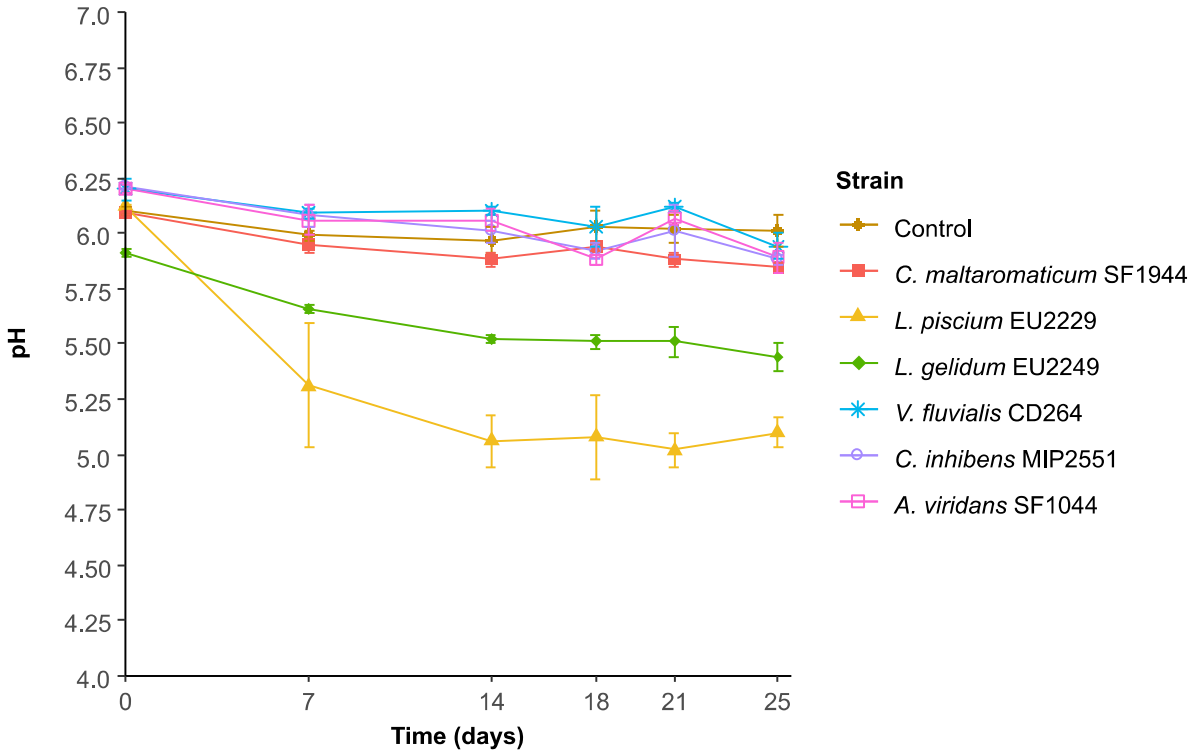

Figure 4.9: Salmon dill gravlax pH evolution in presence of the 6 protective cultures during 25 days of storage at 8 °C.

5.1. Biogenic amines content

Tyramine, cadaverine and putrescine levels at T0 and after 14 and 21 days are shown in Figure 4.10. Variability among samples was important, especially for putrescine and cadaverine. In control, biogenic amines production increased during storage and reached after 21 days 67 ± 108, 125 ± 82, 21 ± 5 mg/kg respectively for putrescine, cadaverine and tyramine. In presence of PC, no significant difference was observed for cadaverine (Tukey multiple comparison test after ANOVA, p value < 0.05). A significant reduction of the cadaverine content was noticed at day 14 and 21 only in presence of *L. gelidum* EU2249. A significantly lower concentration in tyramine was also observed after 14 days only in presence of *L. piscium* EU2229.

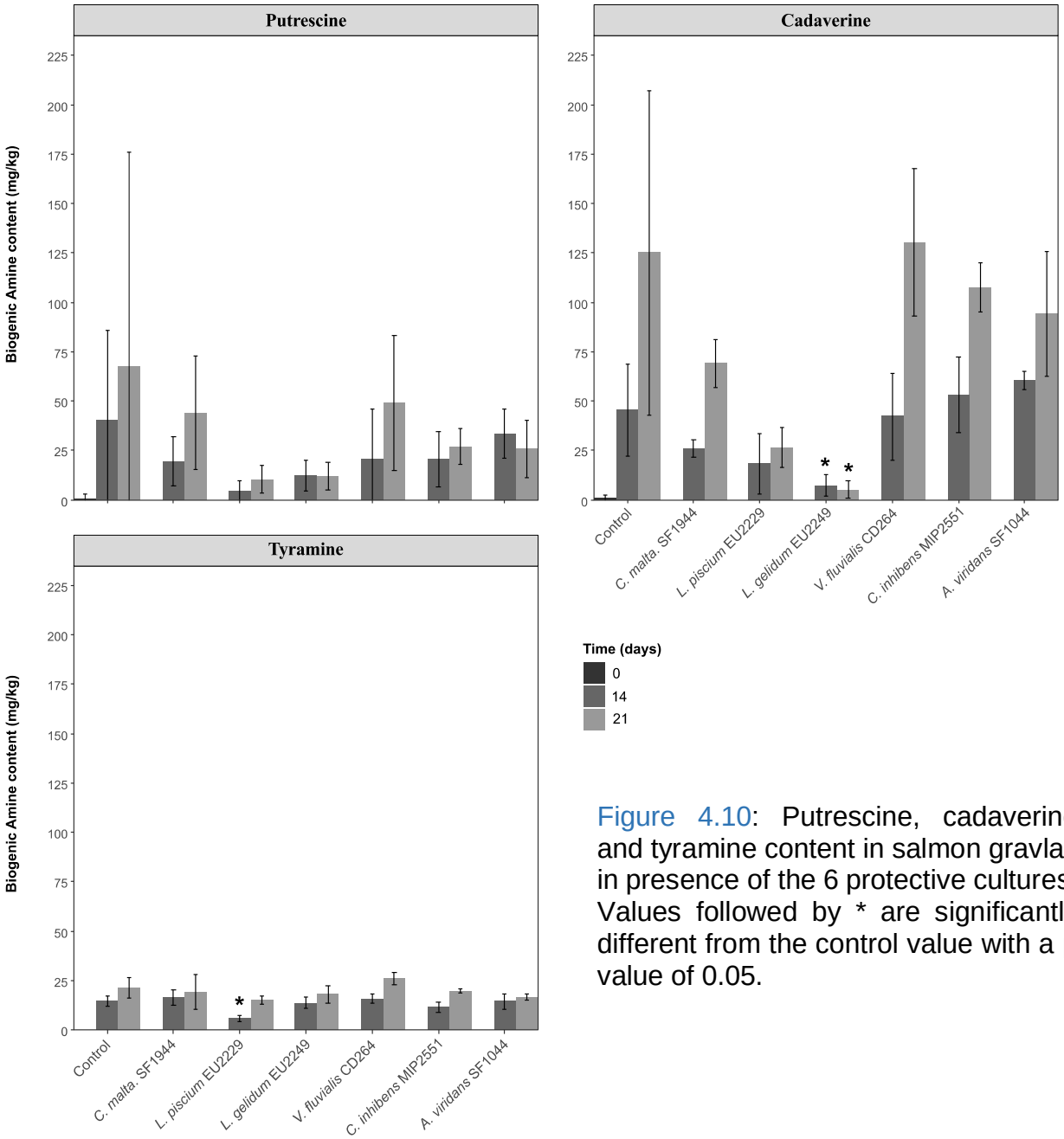

Figure 4.10: Putrescine, cadaverine and tyramine content in salmon gravlax in presence of the 6 protective cultures. Values followed by * are significantly different from the control value with a p value of 0.05.

6. Volatilome composition

VOCs were measured by SPME/GC-MS analysis the first day of experiment for the control and after 14 and 21 days for all samples. In total, 100 VOCs were detected among which 60 were identified, 37 were partially identified and could be related to their chemical category and finally 4 remained unknown (Supplementary Table 3, Table 4.5). Based on log2(n) ratio on VOCs concentration amended by the median, a PCA analysis was performed to visualize potential structures between samples (Figure 4.11) depending on their composition. As the first two dimension expressed only 38.14% of the total variation (Figure 4.11. A), and in the light of the inertia distribution (Figure 1.11. B), no pattern between samples could be drawn regarding their composition in VOCs.

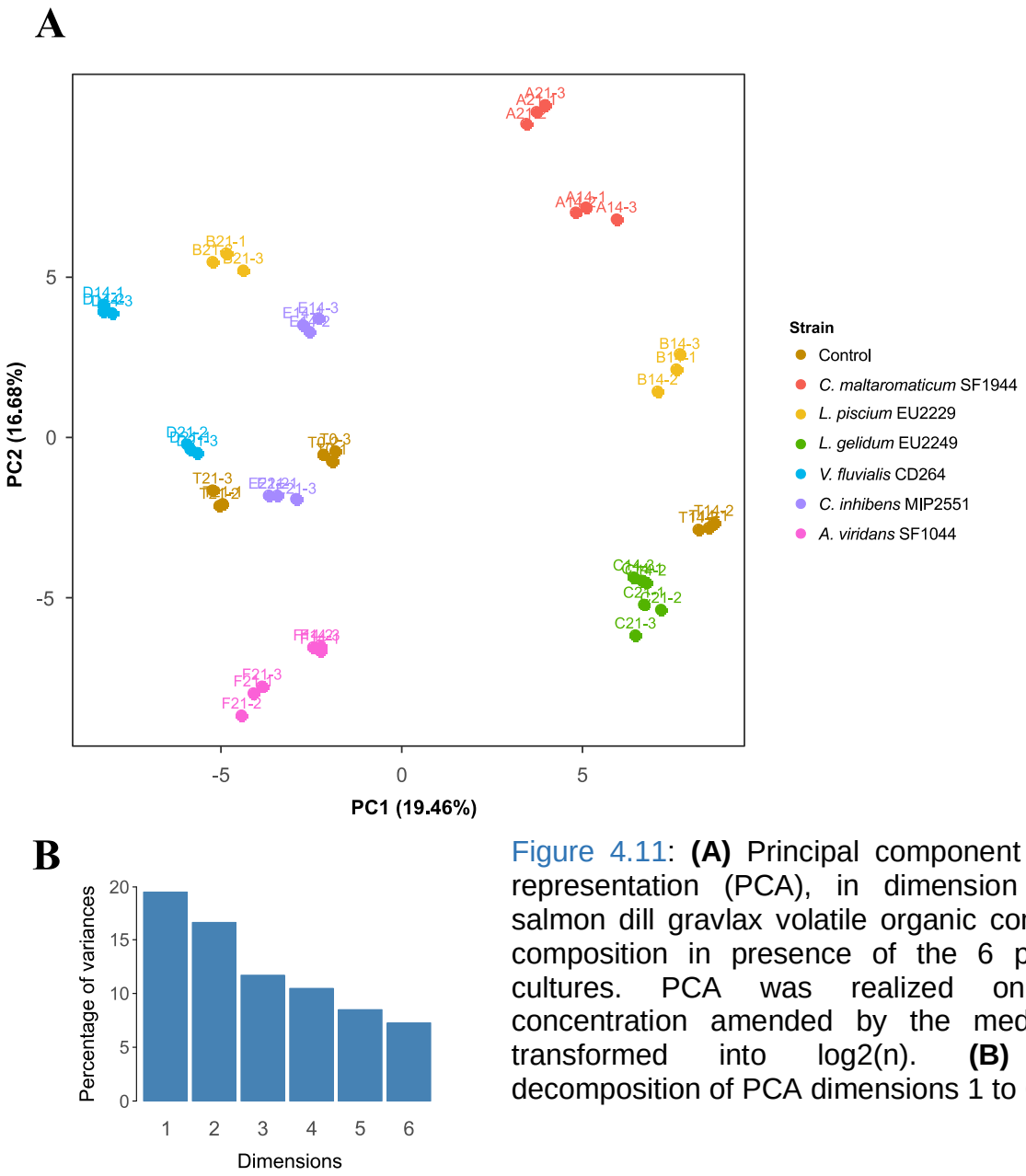

Figure 4.11: **(A)** Principal component analysis representation (PCA), in dimension 1-2, of salmon dill gravlax volatile organic compounds composition in presence of the 6 protective cultures. PCA was realized on VOCs concentration amended by the median and transformed into log2(n). **(B)** Inertia decomposition of PCA dimensions 1 to 6.

Samples volatilome composition and evolution were analyzed further by a HCA analysis coupled with a heatmap visualization (Figure 4.12). At T0 in the control, concentrations were relatively low for almost all compounds, with few exceptions such as cembrene A, hexanal, heptadecane and others unidentified metabolites (alcohol-4/12, alkane-9/15, ester-1, ketones-1 and na-1/2/3). After 14 days of storage, an important increase in acids (acetic acid, acid-2), alcohols (1-penten-3-ol, 3-methyl-1-butanol, 2,3-butanediol, 1-octen-3-ol, ethanol, 1-pentanol and unidentified alcohols-2/35/6/8/14), alkanes (decane, undecane, dodecane, tetradecane and unidentified alkanes-1/2/3/8/6/7/11/13), aldehydes (pentanal, benzenacetaldehyde, hexanal, nonanal, 3-methylbutanal, 2-methylbutanal), amines (dimethylamine, unidentified amine-1) and esters (ethyl acetate, 3-methylbutyl acetate, unidentified ester-3) while a decrease in all terpenes concentration was observed. Conversely, after 21 days, alkanes, alcohols, aldehydes and amines all globally decreased in concentration, with few exception such as 3-methyl-1-butanol, unidentified alcohol-10/13, 2-methylbutanal which slightly increased in comparison to day 14. An important increase in concentration in almost all terpenes, acids (acetic acid, acetic acid derivate-1 and unidentified acid-2), ethyl acetate and 3-methylbutyl acetate was also observed.

In presence of *C. maltaromaticum* SF1944 the volatilome composition was closed between 14 and 21 days (clustered in HCA analysis) (Figure 4.12). Samples were mainly characterized by high concentrations in some alcohols (ethanol, 1-penten-3-ol, 3-methyl-1-butanol, 1-pentanol and unidentified alcohol-9), in aldehydes (2- and 3-methylbutanal, pentanal, hexanal, unidentified aldehyde-2), in dimethylamine and hexadecanoic acid. In addition, compared to day 14, an important increase in acids (acetic acid derivate-1 and -2), in unidentified alcohol-7 and -9, in heptanal, (E,Z)-2,4-heptadienal, (E,E)-2,4-heptadienal, benzenacetaldehyde and in many terpenes (α-pinene, sabinene, β-pinene, β-myercene, 3-carene, ρ-cymene, terpinolene, 4-terpineol, 4-terpinenylacetate, α-copaene, β-cubebene, β-caryophyllene, α-caryophyllene and unidentified monoterpene-1 and sesquiterpene-1) was recorded after 21 days. When compared to all strains and to the control, *C. maltaromaticum* SF1944 was the strongest 3-methybutanal producer during storage with a concentration of 8262 ± 1499 µg/kg at 14 days and 4695 ± 581 µg/kg at 27 days (respectively 9 and 27 times higher than in control) (Supplementary Table 3, Table 4.5).

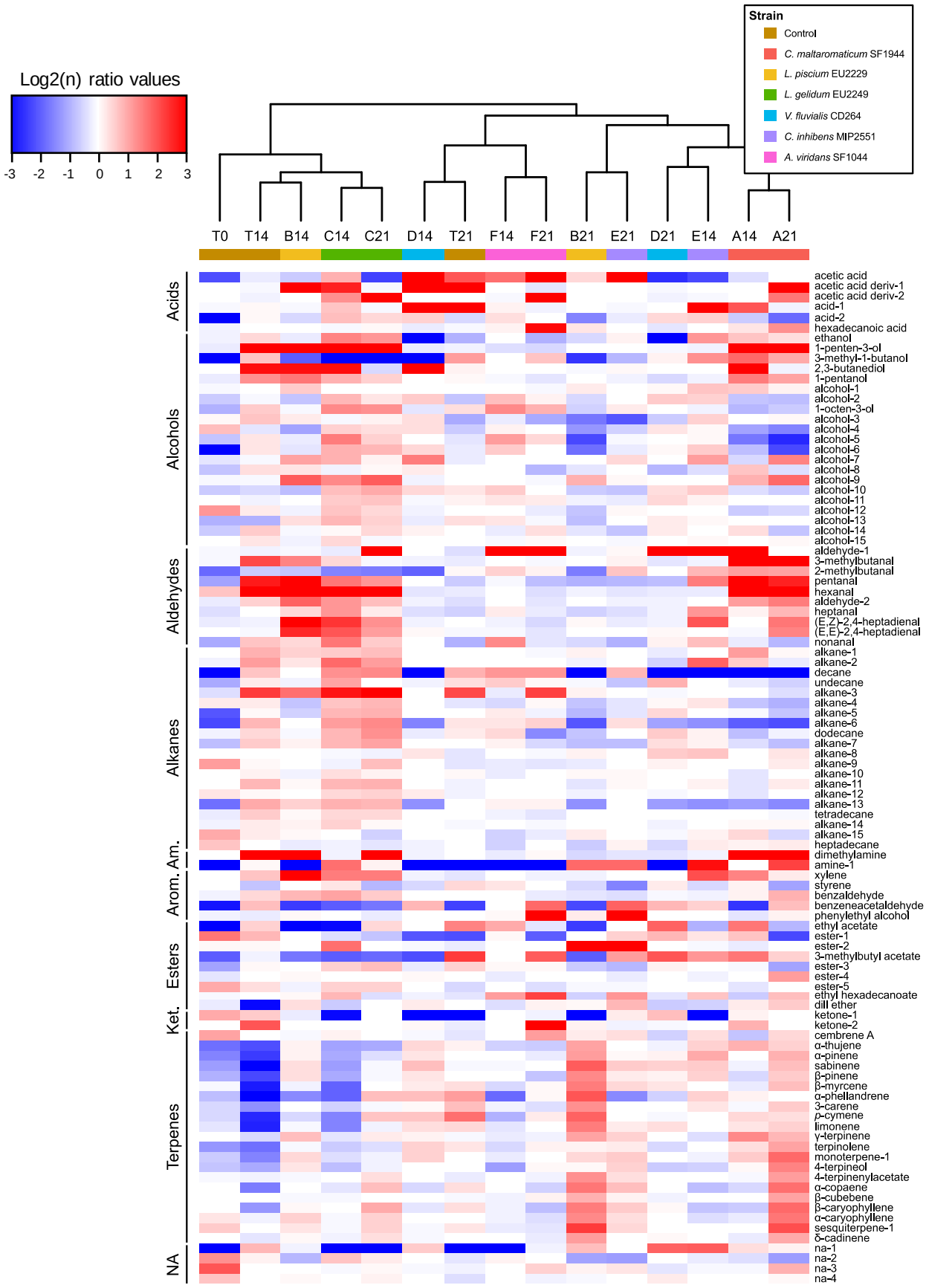

Figure 4.12: Hierarchical Cluster Analysis (HCA) heatmap of control and protective cultures volatilome composition, based on Person correlation calculated from log2(n) transformation of the median based ratio on VOCs mean concentrations (n = 3). Bluish colors indicate lower metabolite concentrations, while reddish colors show higher metabolite level. Am.: Amines, Arom.: Aromatics, Ket.: Ketones., NA: Not identified. See Supplementary Table 3 (Table 4.5).

It also induced the production of the highest concentrations in dimethylamine, 2-methylbutanal, and 3-methylbutan-1-ol (only at T14).

At 14 days, control and samples inoculated with *L. piscium* EU2229 had similar a pattern composition (Figure 4.12) driven by important concentrations in 1-penten-3-ol, 2,3-butanediol, 1-octen-3-ol, 1-pentanol, 3-methylbutanal, pentanal, hexanal, unidentified alkane-3 and dimethylamine. However several differences was recorded. The presence of *L.* piscium EU2229 induced much higher concentrations than control in acetic acid derivate-1, in (E,Z)-2,4-heptadienal, (E,E)-2,4-heptadienal, xylene and in almost all terpenes. After 21 days of storage, all aldehydes, acids, alcohols, alkanes and amines found in high concentration at 14 days, drastically decrease, while an important increase in acetic acid, unidentified amine-1,ester-2 and in many terpenes (α-thujen, α-pinene, sabinene, limonene, β-pinene, β-mycerne, α-phellandrene, 3-carene, γ-terpinene, 4-terpinenylacetate, α-copaen, β-cubebene, α-/ β-caryophyllene, sequiterpene-1 and δ-cadinene) was recorded.

The volatilome of samples inoculated with *L. gelidum* EU2249 was the most complex and was similar between the 2 sampling dates. The composition was characterized by a production of almost all alkanes, alcohols, aldehydes and acids detected by SPME-GC/MS (Figure 4.12). However, compared to 14 days, a decrease in acetic acid, acetic acid derivate-1, unidentified acid acetic-1 and -1, 2,3-butanediol, while an important increase acetic acid derivate-2, unidentified aldehyde-1 and dimethylamine were observed after 21 days.

After 14 days, the volatilome composition in presence of *V. fluvialis* CD264 was mainly characterized by a high production of acids (acetic acid, acetic acid derivate-1 and unidentified acid-1) and 2,3-butanediol. Conversely, at 21 days, all these compounds drastically decrease, while a slight increase in unidentified aldehyde-1, ethyl acetate, 3-methylbutyl acetate and unidentified compound-1 was recorded. In addition, concentrations in ethanol, dimethylamine and amine-1 were very low for both sampling. *V. fluvialis* CD264 was by far the strongest producer of 2,3-butanediol with a concentration of 70855 ± 6671 µg/kg (versus 1475 ± 45 µg/kg in control) (Supplementary Table 3, Table 4.5).

At day 14, samples inoculated with *C. inhibens* MIP2551 has a volatilome mainly characterized by high concentration in acid-1, ethanol, 3-methyl-1-butanol, unidentified

aldehyde-1, pentanal, heptenal, (E,Z)-2,4-heptadienal, alkane-2, amine-1, xylene, 3-methylbutyl acetate and unidentified compounds-1. After 21 days, all acids, alcohols and aldehydes previously found in high concentration decrease drastically, while an important increase in acetic acid, benzenacetaldehyde, phenylethyl alcohol and unidentified ester-2 was observed.

At both sampling date, samples inoculated with *A. viridans* SF1044 had similar volatilome composition characterized by a high production in acetic acid, unidentified aldehyde-1, and in lesser extent in 1-octen-3-ol, unidentified alcohol-5, nonanal, decane, ethyl acetate, ethyl hexadecanoate. In addition, at 21 days an important increase in acetic acid, acetic acid derivate-2, hexadecanoic acid, benzeacetaldehyde, phenylethyl alcohol, 3-methylbutyl acetate, unidentified ketone-2 and cembrene A was found in comparison with day 14. Between 14 and 21 days, concentrations in dimethylamine and especially in unidentified amine-1 remained very low.

7. Discussion

The study of the impact of 6 bioprotective LAB on the microbial ecosystem (cultural method and metabarcoding approach), on physico-chemical parameters, volatilome composition and on the sensory properties allowed to highlight two main strain-dependent scenarios.

7.1. First scenario

The first scenario concerns *C. maltaromaticum* SF1944, *L. piscium* EU2229 and *L. gelidum* EU2249 which grew quickly in the product and remained largely dominant in the microbial ecosystem during the whole storage. These 3 species are known to be well adapted to seafood and meat products, as they are commonly isolated and often found as member of the dominant flora in many matrices at different storage time (Leroi, 2010; Afzal et al., 2010; Ghanbari et al., 2013; Pothakos et al., 2014b; Chaillou et al., 2015; Saraoui et al., 2016b; Zagorec and Champomier-Vergès, 2017a).

C. maltaromaticum SF1944 inhibited the growth of *Enterobacteriaceae* by 1.6 log CFU/g and totally prevented the growth of *L. monocytogenes* during 21 days. *Carnobacterium* species antimicrobial activity against *L. monocytogenes* and more broadly toward Gram-positive bacteria is largely described (Leisner et al., 2007; Afzal et al., 2010). *Carnobacterium* spp. are able to produce a wide range of bacteriocins

(Carnobacteriocin BM1/B2/A, Carnocin CP51/CP52, Piscicocin V1/126/61/CS526, Carnocyclin A) active against many close related Gram-positive bacteria such as *Enterococcus* spp. (*E. faecalis*, *E. faecium*, *E. durans*, *E. hirae*), *Listeria* spp. (*L. monocytogenes*, *L. innocua*, *L. grayi*), *Lactobacillus* spp. (*L. curvatus*, *L. plantarum*), *Lactococcus lactis, Leuconostoc* spp. (*L. mesenteroides*, *L. dextranicum*), *Pediococcus* spp. (*P. acidilactici*, *P. pentosaceus*), *Staphylococcus* spp. (*S. aureus*, *S. thermophilus*), but also against other *Carnobacterium* species (*C. divergens*, *C. maltaromaticum*, *C. gallinarum*) (Leisner et al., 2007; Afzal et al., 2010; Pilet and Leroi, 2011). Thus *Carnobacterium* species, especially *C. maltaromaticum* and *C. divergens* are mainly used in food protection for the control of microbial safety and many studies reported their strong antagonistic activity against *L. monocytogenes* and *L. innocua* in seafood (Duffes et al., 1999b, 1999a; Brillet et al., 2004, 2005; Nilsson et al., 2004; Tahiri et al., 2004, 2009; Vescovo et al., 2006; Saraoui et al., 2017). Conversely, *Carnobacterium* species are not particularly known to be active against Gram-negative bacteria. However, Brillet et al. (2005), Matamoros et al. (2009a) and Saraoui et al. (2017) showed that *C. alterfunditum* EU2257 and *C. divergens* V41 were able to inhibit *Enterobacteriaceae* by 2.0 log CFU/g on cooked and peeled shrimp and cold smoked salmon stored under MAP and vacuum respectively. In cod and salmon juice, 4 *C. maltaromaticum* strains, including *C. maltaromaticum* SF1944, were all strongly active against *Lactobacillus sakei*, *L. monocytogenes*, but also displayed inhibition activity against *S. baltica*, *P. phosphoreum*, and *H. alvei* (Wiernasz et al., 2017).

In presence of *C. maltaromaticum* SF1944, the spoilage was essentially characterized by a strong feet/banana (malty or rhubarb) and light sour odors. This malty sensory note, typical of *C. maltaromaticum* is due to the production of 2-methylbutanal and 3-methylbutanal from leucine and isoleucine catabolism (Leisner et al., 2007a; Afzal et al., 2010). In samples inoculated with *C. maltaromaticum* SF1944, these two compounds were found in high concentrations by SPME-GC/MS analysis. As for many LAB, the spoiler status of *C. maltaromaticum* is ambiguous and seems to be strain and matrix dependent (Leisner et al., 2007; Afzal et al., 2010). *C. maltaromaticum* were indeed found to be responsible for the spoilage of cooked and peeled shrimp and sterile raw salmon (Mejlholm et al., 2005; Jaffrès et al., 2011; Macé et al., 2013a, 2014), while some authors did not find any spoilage evidence (Brillet et al., 2005; Duffes et al., 1999a; Joffraud et al., 2006; Leroi et al., 1996; Matamoros et al., 2009a;

Vescovo et al., 2006). In addition to malty and feet/banana, odors such as burnt, oxidized, sweet/nauseous, nutty, chlorine, grass/hay, plastic, butter, caramel, fruity, bitter and acid were often associated to *C. maltaromaticum* in seafood (Leroi et al., 1998; Paludan-Müller et al., 1998; Truelstrup Hansen and Huss, 1998; Joffraud et al., 2001; Stohr et al., 2001; Brillet et al., 2005; Laursen et al., 2006).

L. piscium EU2229, as *C. maltaromaticum* SF1944, displayed an antimicrobial activity against *Enterobacteriaceae* (2.9 log CFU/g reduction) and *L. monocytogenes* (3.1 log CFU/g reduction). Similar results were obtained with two *L. piscium* strains, including *L. piscium* EU2229, inoculated in peeled and cooked shrimp and cold smoked salmon (Matamoros et al., 2009a; Fall et al., 2010a; Leroi et al., 2015). However, in the two last cited studies, *B. thermosphacta* was also totally inhibited while no such effect was recorded in our experiment. In our study, *L. monocytogenes* was slightly inhibited after 14 days of storage (reduction of 1.0 log CFU/g). After this sampling date, when *L. piscium* EU2229 reached its maximum concentration, *L. monocytogenes* concentration decreased to its initial level. Such behavior was already described for *L. piscium* CNCM I-4031, whose action mechanism seems to be cell contact dependent and induced by quorum sensing (Fall et al., 2010b; Saraoui et al., 2016a, 2018).

On gravlax slices *L. piscium* EU2229 induced a strong production of sulfur, amine and acid smell after 14 days and until the end of storage. Unfortunately the sulfur off-odor could not be related to the volatilome composition. Indeed, sulfur related compounds such as H_2S are not recovered during headspace-SPME extraction. Such strong spoilage profile has never been described in seafood, where *L. piscium* is not considered as a spoiler (Saraoui et al., 2016b). When inoculated on naturally contaminated or sterile matrices, like cooked and peeled shrimp, raw and cold smoked salmon, *L. piscium* did not induce off-odors or only weak sour/fermented, cheese/feet, buttery or floor cloth odors that never led to the rejection by panelists (Matamoros et al., 2009a; Fall et al., 2010a; Macé et al., 2013a; Leroi et al., 2015; Saraoui et al., 2017). When screened for their spoilage potential in fish juice, 15 *L. piscium* strains mainly produced light sour smell and an appreciated acid/lemon in the case of *L. piscium* EU2229. Nevertheless, some isolates were also associated with a very weak sulfur smell production (Wiernasz et al., 2017). In meat products, *L. piscium* can however be associated to spoilage with buttery, sour, feet and wet dog odors (Rahkila et al., 2012; Pothakos et al., 2014a; Andreevskaya et al., 2015). In seafood, sulfur

related off-odors (cabbage, garlic, and boiled egg) are more likely linked to Gram-negative bacteria metabolism such as *H. alvei*, *S. liquefaciens*, *S. putrefaciens,* or some LAB such as *L. sakei* and *L. farciminis* (Stohr et al., 2001; Leroi et al., 1998; Paludan-Müller et al., 1998; Joffraud et al., 2006; Jaffrès et al., 2011; Macé et al., 2013a, 2014). In our case, sulfur odor production induced by the presence of *L. piscium* EU2229 may involve metabolism interactions with the gravlax natural microbiota.

L. gelidum EU2249 displayed the strongest antagonistic activity on gravlax, by inhibiting *B. thermosphacta*, *Enterobacteriaceae* and maintaining *L. monocytogenes* to its initial level all along the storage. *Leuconostoc* species are known to be bacteriocins producers (Woraprayote et al., 2016) and to possess antimicrobial activity toward both Gram-negative and positive bacteria. In *in vitro* conditions several *L. gelidum* strains, including *L. gelidum* EU2249, displayed an antimicrobial activity against, *S. liquefaciens*, *Pseudomonas* sp., *Psychrobacter* sp., *S. putrefaciens*, *P. phosphoreum*, *S. baltica*, *S. proteamaculans*, *C. sporogenes, L. monocytogenes*, *B. thermosphacta*, *L. farciminis*, *C. maltaromaticum*, *L. piscium*, *V. fluvialis*, *C. inhibens* and *A. viridans* (Matamoros et al., 2009b; Wiernasz et al., 2017). When inoculated on naturally contaminated cold-smoked salmon and peeled and cooked shrimp stored under vacuum packaging, *L. gelidum* EU2247 reduced the total flora count by 1 log CFU/g and totally inhibited *Enterobacteriaceae*. In challenge tests, this strain was also able to decrease *L. monocytogenes* population by 2 log CFU/g at the end of storage. (Matamoros et al., 2009a). Furthemore, in our study, *L. gelidum* EU2249, as well as *L. piscium* EU2229, both strongly acidified the product, which may contribute to their antimicrobial activity.

Based on odors, *L. gelidum* EU2249 delayed gravlax spoilage for 21 days. However, after 25 days, slices were strongly spoiled with vinegar and sulfur odors. Usually, *L. gelidum* is not associated with seafood spoilage (Matamoros et al., 2009a; Leroi et al., 2015). In fish juice, among 35 LAB from different species, *L. gelidum* strains were the most neutral, with only a weak acid/lemon smell (Wiernasz et al., 2017). In vacuum-packed cooked and peeled shrimp, *L. gelidum* EU2247 improved the sensory shelf-life, while *L. gelidum* EU2213 produced sulfur related odor (cabbage) after 28 days of storage (Matamoros et al., 2009a). In our experiment, from day 14 *L. gelidum* EU2249 induced from day 14 a gas and slime formation in the package. *Leuconostoc* are heterofermentative LAB that can be important producers of acetic acid, CO_2 and

dextrans from sucrose (Cogan and Jordan, 1994; Björkroth and Holzapfel, 2006). A similar case of exopolysaccharides production by *L. gelidum* and *L. gasicomitatium*, but in much higher quantity, has already been described in acetic-acid marinated herring (Lyhs et al., 2004).

The volatilome composition and evolution during salmon gravlax storage in control was characterized by an increase in concentrations in many alcohols, aldehydes, alkanes, acids and dimethylamine. Such kind of volatiles compounds, resulting mostly from microbial metabolic activity, increases simultaneously with deterioration of seafood organoleptic properties (Jørgensen et al., 2001; Olafsdóttir et al., 2005; Varlet et al., 2006; Jónsdóttir et al., 2008; Parlapani et al., 2014, 2015a). Aldehydes, deriving mainly from lipids microbial oxidation, are especially good indicators of food degradation and actively participate in the rancid, cooked potatoes, fatty, floral, fruity and grassy odors of spoiled fish (Varlet et al., 2007; Jørgensen et al., 2001; Jónsdóttir et al., 2008). Surprisingly after 21 days of storage, none of these usual spoilage markers is found in high concentration. They are replaced by a large production of acetic acid, one of its derivates (acetic acid derivate-1) and an unidentified acid-1. Organic acids also result from microbial activity and can be good spoilage indicators (Jørgensen et al., 2001; Olafsdóttir et al., 2005; Varlet et al., 2006; Jónsdóttir et al., 2008).

In presence of *L. piscium* EU2229 and *C. maltaromaticum* SF1044, the volatilome composition was essentially characterized by an important production of aldehydes (2- and 3-methylbutanal, pentanal, hexanal, (E,Z)-2,4-heptadienal, (E,E)-2,4-heptadienal), alcohols (ethanol, 1-penten-3-ol, 3-methyl-1-butanol, 1-pentanol), dimethylamine, 3-methylbutyl acetate and on derivate from acetic acid. In cooked and peeled tropical shrimp stored under MAP, a cocktail of 5 *C. maltaromaticum* strains were found to produced 3-methylbutanal, 2-methylbutanal, 3-methylbutanol and 2-methylbutanol in higher proportion than control. Moreover, 1-penten-3-ol, cyclopentanol, acetaldehyde, 2-methyl-1-propanal, 2,3-butanedione (diacetyl), 3-methyl-2-butanane, 3-methyl-1-butene, ethyl acetate and thiocarbamide were also found in higher proportion (Jaffrès et al., 2011). Fall et al. (2010a), also on cooked and peeled shrimp, found that *L. piscium* CNCM I-4031 induced the production of 2,3-butanedione, 2-pentanone, 3-hydroxy-2-butanone (acetoin), 2-pentanol and 3-methyl-1-butanol. Acetoin and diacetyl production by *L. piscium*, which were not detected by SPME/GS-MS in our study, was also reported in meat and vegetable products

(Pothakos et al., 2014a). The capacity to produce these two spoilage related compounds, in addition to acetic acid and ethanol, was also confirmed by the presence of four metabolic pathways involved in their production in *L. piscium* genomes (Andreevskaya et al., 2015). However, acetic acid and ethanol were not produced in presence of *L. piscium* EU2229. The strong sulfur off-odor identified during the sensory evaluation could not been related to any specific VOCs compounds

After 21 days of storage, an important increase in terpenes concentrations was observed for *L. piscium* EU2229 and *C. maltaromaticum* SF1944. The presence of terpenes in gravlax products could be attributed to the use of spices (dill and black pepper) involved in their preparation. However, concentrations systematically 2 to 10 times higher than control might suggest that terpenes production could be related to *L. piscium* EU2229 and *C. maltaromaticum* SF1944 metabolism. Terpenes synthesis and degradation by bacteria are not much studied, as the focus is mainly made on fungi or plants as source. However, a recent study demonstrated by genome-based analyses, the presence of predicted terpenes synthases in many bacterial orders (Yamada et al., 2015). In addition, Belviso et al. (2011) were the first authors to described the ability of 5 LAB isolated from cheese (*L. lactis* subsp. *lactis, S. thermophilus*, *S. macedonicus*, *L. paracasei*, *L. lactis* subsp. *cremoris*) to degrade α-campholenal and to produce a monoterpenoid isomer of borneol.

The volatilome composition in presence of *L. gelidum* EU2249 was the most complex, but did not differ drastically between day 14 and 21. *L. gelidum* EU2249 induced an higher production than in control of many alkanes (decane, undecane, tetradecane and many unidentified alkanes), alcohols (ethanol, 1-octen-3-ol and unidentified alcohols), aldehydes (hexanal, heptanal, (E,Z)-2,4-heptadienal, (E,E)-2,4-heptadienal, nonanal and benzealdehyde), amines (dimethylamine, amine-1) and in derivatives from acetic acid. In blood sausages, beef, sweet bell peppers and boiled eggs, 4 strains of *L. gelidum* subsp. *gasicomitatum* induced the production of a large amount of acetic acid, ethanol, diacetyl, 2,3-butanediol, acetaldehyde, and acetoin. (Pothakos et al., 2014a). As heterofermentative LAB, *Leuconostoc* are able to perform the phosphoketolase pathway of glucose resulting in a production of CO_2, acetate, ethanol and lactate. The citrate pathway can also be performed by *Leuconostoc*, leading to the production of diacetyl, acetoin, and 2,3-butanediol in addition to acetate and CO_2 (Cogan and Jordan, 1994; Björkroth and Holzapfel, 2006). The absence of differences in VOCs

composition between 14 and 21 days is in accordance with a constant and weak spoilage of the gravlax before 25 days.

7.2. Second scenario

The second scenario concerns *V. fluvialis* CD264, *C. inhibens* MIP2551 and *A. viridans* SF1044 which were not competitive despite an initial inoculum level of 10^6 CFU/g. Very quickly after inoculation and until the end of storage, these strains collapsed in favor of the natural microbiota of the product and remained as minority part of the ecosystem. Furthermore, none demonstrated antimicrobial activity, except a slight inhibition against *L. monocytogenes* after 21 days of storage. However, during their selection, these 3 strains totally inhibited all targets tested (*L. monocytogenes*, *H. alvei*, *P. phosphoreum*, *S. baltica*, *S. proteamaculans* and *L. sakei*) excepted *B. thermosphacta* (Wiernasz et al., 2017). Afterward, we shown that their broad antimicrobial activity probably relied on an important production of H_2O_2 (data not shown), which may not been produced in our case in absence of oxygen due to the storage under vacuum packaging. No data are available concerning *C. inhibens*, *V. fluvialis* or *A. viridans* species as bioprotective strains and this study constitute the first case of their use on seafood.

The sensory profile of the gravlax, in presence of *C. inhibens* MIP2551 and *A. viridans* SF1044, was not very different from the control. The spoilage was mainly characterized by amine and slight acid and sour odors, which are typically associated with raw salmon and CSS spoilage (Cardinal et al., 2004; Macé et al., 2012a). However, their volatilome composition differs a lot in comparison to the control and relatively few metabolites were produced in high concentration for both sampling dates. The *Photobacterium* genera was systematically found largely dominant within the microbial ecosystem of these samples and might be responsible of these off-odors. *P. phosphoreum* was indeed found to produce such spoilage notes in salmon products (Stohr et al., 2001; Macé et al., 2013a). After 21 days of storage *C. inhibens* MIP2551 was also associated with a strong sulfur odor, also found for *A. viridans* SF1044, in addition to a light cheese/feet smell. Interestingly, *V. fluvialis* CD264 reduced significantly the global spoilage score and extended the salmon gravlax sensory shelf-life. As for the two others PC strains, *V. fluvialis* CD264 did not induced the production of many metabolites, especially at day 21. It is worthy to notice that the important production of acetic acid, acetic acid derivate-1, unidentified acid-1 and 2,3-butanediol

at 14 days was not found after 21 days. It might coincide and explain the quick spoilage between 7 and 14 days and its low decrease after 18 days. In literature, none of these 3 species was found to be involved in food spoilage. On the contrary, during their spoilage potential assessment in cod and salmon juice, they did not produce off-odors and were quite neutral from a sensory point of view (Wiernasz et al., 2017).

7.3. Quality parameters

Biogenic amines in seafood, especially cadaverine and putrescine, may be spoilage indicators. Significant concentrations are often correlated with product degradation (Jørgensen et al., 2000a; Biji et al., 2016; de la Torre and Conte-Junior, 2018). However, in our study, despite a quick and strong spoilage, putrescine and cadaverine level were quiet low even after 21 days of storage, with average values never exceeding 125 mg/kg. A significant decrease in cadaverine amount was found for *L. gelidum* EU2249, in accordance with the fact that spoilage was lowered in its presence until 21 days of storage. During their screening, *C. maltaromaticum* SF1944 and *V. fluvialis* CD264 were found to be important tyramine producers (Wiernasz et al., 2017). If ingested in high concentration (100-800 mg/kg), tyramine may cause headaches and hypertensive effects in individual deficient in monoamine oxidase (ten Brink et al., 1990; Halász et al., 1994). In consequence salmon gravlax tyramine content was also measured. Concentrations never exceeded 25 mg/kg after 21 days and no overproduction was recorded in biopreserved samples.

TVBN is also a criterion commonly used in seafood for spoilage assessment. In our study, as the biogenic amines content, TVBN was not a reliable spoilage indicator. After 14 days of storage, altough the spoilage was higher than control, TVBN was significantly lower in presence of *L. piscum* EU2229, *C. inhibens* MIP2551 and *A. viridans* SF1044. In the case of *L. piscum* EU2229, at that sampling date, the spoilage was not driven by amine related odor, but more by sulfur, sour and acid smells. Conversely, *C. inhibens* MIP2551 and *A. viridans* SF1044 spoilage profile was mainly characterized by amine off-odor. Nevertheless as for biogenic amines, TVBN content was also significantly lower in presence of *L. gelidum* EU2249, which is in accordance with its low spoilage score at 14 days.

8. Conclusion

The effect of the 6 PC on salmon dill gravlax quality could be classified in two main scenarios. The first scenario includes *C. maltaromaticum* SF1944, *L. piscium* EU2229, *L. gelidum* EU2249, which were competitive in the product and dominated the ecosystem until the end of the experiment. They displayed antimicrobial activity against common spoilage bacteria and against *L. monocytogenes*. They also expressed their own sensory signature and produced many volatiles compounds. *L. piscium* EU2229 and *L. gelidum* EU2249 do not seem suitable for gravlax biopreservation. They both strongly acidified the product, leading to its visual aspect deterioration. In addition, the first one induced a strong sulfur spoilage note and the later produced slime and gas in the package. The presence of residual sugar in the gravlax may explain such metabolic activity. Conversely, *C. maltaromaticum* SF1944, as its spoilage score was not different from the control, seems suitable for preserving salmon gravlax microbial safety.

The second scenario includes *V. fluvialis* CD264, *C. inhibens* MIP2551, and *A. viridans* SF1044, which were not competitive and quickly collapsed in favor of the gravlax natural microbiota. They did not demonstrated strong antimicrobial activity and did not produced many volatile compounds. In addition, *C. inhibens* MIP2551 and *A. viridans* SF1044 sensory profile were relatively closed to the control. However, among these 3 strains, *V. fluvialis* CD264 maintained the salmon gravlax sensory quality below the rejection threshold even after 25 days.

This work suggests that biopreservation of naturally contaminated products remains a complex matter to apprehend and may further relies on metabolic interactions between microorganisms from an ecosystem more than on antimicrobial activity.

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Table 4.3: **Supplementary Table 1 – OTUs relative bundance tables**
OTU relative abundance (%) in gravlax **control** samples at D0, D7, D14, D21 (in grey OTUs > 0,1%).

OTUs Affiliation	T1D0	T2D0*	T3D0*	T1D7	T2D7	T3D7	T1D14	T2D14	T3D14	T1D21	T2D21	T3D21
Photobacterium	49,14	25,79	32,41	68,04	92,12	84,30	60,32	55,05	46,46	48,59	40,93	38,80
Lactococcus	0,20	0,25	0,49	0,54	0,02	0,29	10,62	19,99	7,44	29,66	22,11	11,83
Carnobacterium	0,50	0,98	0,93	1,33	0,17	0,68	3,14	5,02	4,68	1,80	3,62	2,21
Serratia/Yersinia	1,21	3,14	1,28	8,17	0,86	2,89	19,88	16,08	22,80	15,92	19,50	23,51
Vibrio	1,66	1,37	1,02	20,99	5,99	10,30	4,44	1,08	14,02	1,33	5,38	3,85
Leuconostoc	0,31	0,27	0,28	0,00	0,00	0,00	0,00	0,00	0,00	0,06	0,11	0,08
Aerococcus	0,15	0,22	0,07	0,00	0,01	0,02	0,00	0,00	0,00	0,39	0,00	0,01
Vagococcus	0,01	0,09	0,13	0,00	0,00	0,00	0,01	0,00	0,00	0,02	0,01	0,00
Pseudomonas	13,01	25,41	10,78	0,08	0,18	0,56	0,11	0,01	0,16	0,06	0,03	0,00
Lactococcus 2	0,00	0,01	0,01	0,03	0,00	0,00	0,74	1,91	0,81	1,67	2,05	0,61
Lactobacillus	0,09	0,00	0,00	0,23	0,00	0,07	0,00	0,13	2,91	0,06	5,65	18,67
Enterobacter/Klebsiella	3,95	7,03	8,83	0,01	0,01	0,01	0,10	0,01	0,06	0,06	0,04	0,01
Pseudomonas 2	2,67	7,51	2,03	0,06	0,03	0,08	0,00	0,01	0,03	0,01	0,00	0,00
Morganella	0,06	0,19	0,13	0,08	0,00	0,04	0,16	0,35	0,39	0,19	0,44	0,30
Brochothrix	0,32	0,12	0,13	0,22	0,22	0,18	0,05	0,28	0,15	0,10	0,04	0,11
Psychrobacter	2,77	1,31	0,78	0,06	0,08	0,10	0,00	0,00	0,01	0,01	0,00	0,00
Bacillus	2,80	3,08	5,32	0,01	0,03	0,10	0,00	0,00	0,01	0,00	0,00	0,01
Acinetobacter	1,34	2,89	2,20	0,00	0,01	0,01	0,00	0,00	0,00	0,00	0,00	0,00
Pseudoalteromonas	1,88	0,40	0,48	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Brachybacterium	1,50	0,71	1,13	0,01	0,01	0,07	0,01	0,01	0,01	0,00	0,00	0,00
Duganella	0,83	0,25	14,72	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Acinetobacter 2	0,83	2,60	1,03	0,00	0,01	0,00	0,01	0,00	0,00	0,00	0,00	0,00
Bacillus 2	0,54	1,09	1,17	0,00	0,01	0,02	0,00	0,00	0,00	0,00	0,00	0,00
Shewanella	0,32	0,87	0,33	0,07	0,00	0,05	0,04	0,02	0,00	0,04	0,03	0,01
Pantoea	1,20	1,13	2,07	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00
Enterococcus	2,44	1,43	1,45	0,00	0,00	0,01	0,00	0,00	0,01	0,00	0,00	0,00
Weissella	0,80	0,89	1,58	0,01	0,04	0,02	0,00	0,00	0,01	0,00	0,00	0,00
Corynebacterium	0,98	1,52	1,26	0,01	0,03	0,03	0,01	0,00	0,01	0,01	0,00	0,00
Pseudomonas 3	0,45	0,27	0,26	0,01	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00
Cobetia	1,09	0,07	0,30	0,00	0,06	0,05	0,01	0,00	0,01	0,00	0,00	0,00
Psychrobacter 2	0,49	0,23	0,07	0,00	0,00	0,02	0,00	0,00	0,01	0,00	0,00	0,00
Psychrobacter 3	0,56	0,56	0,00	0,00	0,00	0,01	0,00	0,00	0,01	0,00	0,00	0,00
Comamonas	0,22	2,42	0,81	0,01	0,00	0,01	0,01	0,00	0,01	0,00	0,00	0,00
Halorubrum	0,31	0,17	0,35	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00
Hafnia/Obesumbacterium	0,01	0,00	0,00	0,00	0,00	0,01	0,33	0,03	0,01	0,03	0,04	0,01
Spelaeicoccus	0,44	0,60	0,44	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Escherichia/Shigella	0,20	0,67	0,74	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Acinetobacter 3	0,40	0,25	0,73	0,01	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00
Sphingobacterium	0,14	0,52	0,26	0,00	0,01	0,01	0,00	0,00	0,00	0,00	0,00	0,00
Cellulosimicrobium	0,18	0,66	0,19	0,01	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Acinetobacter 4	0,14	0,18	0,28	0,00	0,01	0,01	0,00	0,00	0,00	0,00	0,00	0,00
Halorubrum 2	0,22	0,11	0,57	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Microbacterium	0,15	0,17	0,39	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,01	0,00
Staphylococcus	0,77	0,56	0,68	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00
Brevibacterium	0,15	0,05	0,25	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00
Bacillus 3	0,74	0,29	0,04	0,00	0,01	0,01	0,00	0,00	0,00	0,00	0,00	0,00
Pseudomonas 4	0,24	0,11	0,22	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Stenotrophomonas	0,12	0,50	0,19	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Brevibacterium 2	0,11	0,19	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,01
Pelomonas	0,34	0,19	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Stenotrophomonas 2	0,39	0,04	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,02
Terribacillus	0,09	0,38	0,00	0,00	0,01	0,00	0,00	0,01	0,00	0,00	0,00	0,01
Haloplanus	0,07	0,26	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,08
Psychrobacter 4	0,05	0,12	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01

OTU relative abundance (%) in gravlax samples inoculated with ***C. maltaromaticum* SF1944** at D0, D7, D14, D21 (in grey OTUs > 0,1%).

OTUs Affiliation	A1D0	A2D0	A3D0	A1D7	A2D7	A3D7	A1D14	A2D14	A3D14	A1D21	A2D21	A3D21
Photobacterium	1,94	3,23	8,52	49,54	54,14	50,57	46,10	45,47	33,38	39,87	40,76	36,79
Lactococcus	0,03	0,15	0,15	0,26	0,13	0,22	0,71	0,52	1,53	10,87	6,08	2,53
Carnobacterium	86,86	86,41	81,48	47,38	44,70	48,49	50,00	48,80	55,11	42,83	44,05	52,16
Serratia/Yersinia	0,33	0,42	0,48	1,28	0,33	0,32	1,75	2,71	7,25	1,22	4,51	5,54
Vibrio	0,29	0,09	0,18	1,26	0,56	0,25	0,86	1,85	1,25	0,06	0,86	1,20
Leuconostoc	0,04	0,08	0,06	0,10	0,07	0,10	0,00	0,00	0,00	0,10	0,06	0,10
Aerococcus	0,00	0,01	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01
Vagococcus	0,01	0,01	0,01	0,01	0,03	0,01	0,00	0,00	0,01	0,02	0,00	0,01
Pseudomonas	4,02	2,46	2,46	0,00	0,01	0,00	0,01	0,02	0,00	0,00	0,01	0,00
Lactococcus 2	0,00	0,01	0,00	0,08	0,02	0,01	0,46	0,27	1,11	4,94	3,51	1,38
Lactobacillus	0,00	0,01	0,01	0,01	0,00	0,00	0,01	0,16	0,18	0,01	0,06	0,01
Enterobacter/Klebsiella	0,87	1,29	0,81	0,01	0,00	0,00	0,01	0,01	0,01	0,01	0,00	0,00
Pseudomonas 2	0,91	0,45	0,55	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01
Morganella	0,01	0,01	0,00	0,04	0,00	0,01	0,03	0,05	0,10	0,03	0,05	0,19
Brochothrix	0,01	0,00	0,01	0,01	0,00	0,00	0,01	0,04	0,01	0,00	0,02	0,01
Psychrobacter	0,49	0,11	0,19	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00
Bacillus	0,13	0,43	0,38	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00
Acinetobacter	0,78	0,62	0,93	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00
Pseudoalteromonas	0,22	0,08	0,18	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Brachybacterium	0,29	0,67	0,17	0,00	0,00	0,00	0,01	0,02	0,01	0,00	0,00	0,01
Duganella	0,13	0,10	0,08	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Acinetobacter 2	0,18	0,26	0,21	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Bacillus 2	0,15	0,24	0,12	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00
Shewanella	0,05	0,04	0,03	0,01	0,00	0,00	0,03	0,00	0,00	0,00	0,00	0,01
Pantoea	0,27	0,70	0,79	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Enterococcus	0,06	0,06	0,06	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00
Weissella	0,07	0,23	0,10	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00
Corynebacterium	0,33	0,28	0,36	0,00	0,01	0,01	0,01	0,01	0,01	0,00	0,00	0,00
Pseudomonas 3	0,08	0,01	0,05	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Cobetia	0,10	0,01	0,07	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Psychrobacter 2	0,06	0,01	0,06	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Psychrobacter 3	0,17	0,01	0,13	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Comamonas	0,07	0,04	0,23	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00
Halorubrum	0,10	0,17	0,12	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Hafnia/Obesumbacterium	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,04	0,03	0,02	0,03
Spelaeicoccus	0,16	0,03	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Escherichia/Shigella	0,01	0,13	0,06	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Acinetobacter 3	0,03	0,15	0,09	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00
Sphingobacterium	0,11	0,04	0,07	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Cellulosimicrobium	0,06	0,07	0,10	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00
Acinetobacter 4	0,10	0,07	0,03	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Halorubrum 2	0,08	0,10	0,11	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Microbacterium	0,03	0,08	0,06	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00
Staphylococcus	0,06	0,29	0,07	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Brevibacterium	0,03	0,03	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Bacillus 3	0,01	0,05	0,06	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Pseudomonas 4	0,08	0,04	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Stenotrophomonas	0,04	0,01	0,15	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00
Brevibacterium 2	0,03	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,04
Pelomonas	0,06	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,13
Stenotrophomonas 2	0,02	0,02	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,09
Terribacillus	0,03	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,06
Haloplanus	0,07	0,03	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,05
Psychrobacter 4	0,00	0,03	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00

OTU relative abundance (%) in gravlax samples inoculated with ***L. piscium*** **EU2229** at D0, D7, D14, D21 (in grey OTUs > 0,1%).

OTUs Affiliation	B1D0	B2D0	B3D0	B1D7	B2D7	B3D7	B1D14	B2D14	B3D14	B1D21	B2D21	B3D21
Photobacterium	1,22	1,01	33,20	10,11	24,82	30,49	34,61	24,64	15,36	16,01	12,40	18,55
Lactococcus	82,62	76,61	47,87	57,49	68,48	65,41	53,67	60,34	72,01	77,29	76,61	77,75
Carnobacterium	0,33	0,18	0,15	0,08	0,10	0,13	0,94	0,06	0,06	0,25	0,13	0,24
Serratia/Yersinia	0,74	0,96	0,60	0,56	1,19	2,95	4,26	14,18	1,84	3,35	3,99	2,28
Vibrio	0,33	1,04	0,95	31,53	5,14	0,81	2,93	0,59	9,20	2,56	2,55	0,54
Leuconostoc	0,14	0,03	0,08	0,01	0,08	0,10	0,10	0,06	0,08	0,01	0,02	0,00
Aerococcus	0,00	0,01	0,03	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Vagococcus	0,01	0,00	0,01	0,01	0,01	0,01	0,02	0,01	0,01	0,01	0,03	0,02
Pseudomonas	5,20	5,36	7,76	0,03	0,00	0,01	0,02	0,01	0,01	0,04	0,01	0,05
Lactococcus 2	0,35	0,24	0,13	0,05	0,03	0,01	0,31	0,01	0,05	0,07	0,08	0,11
Lactobacillus	0,01	0,03	0,00	0,00	0,04	0,02	2,91	0,01	0,97	0,06	3,94	0,18
Enterobacter/Klebsiella	1,86	2,29	1,04	0,01	0,01	0,00	0,00	0,01	0,00	0,01	0,01	0,01
Pseudomonas 2	1,18	2,87	1,85	0,00	0,01	0,00	0,02	0,00	0,01	0,00	0,01	0,02
Morganella	0,00	0,01	0,02	0,01	0,01	0,03	0,10	0,06	0,04	0,02	0,06	0,01
Brochothrix	0,01	0,07	0,06	0,06	0,00	0,00	0,01	0,01	0,17	0,13	0,03	0,07
Psychrobacter	0,40	1,41	1,11	0,01	0,00	0,00	0,00	0,00	0,07	0,04	0,02	0,00
Bacillus	0,72	1,27	0,40	0,01	0,00	0,01	0,00	0,00	0,00	0,01	0,00	0,01
Acinetobacter	0,27	0,39	0,17	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00
Pseudoalteromonas	0,26	0,38	0,68	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,01	0,00
Brachybacterium	0,10	0,14	0,14	0,00	0,01	0,00	0,01	0,01	0,00	0,01	0,00	0,01
Duganella	0,32	0,17	0,08	0,00	0,00	0,00	0,01	0,00	0,01	0,01	0,02	0,00
Acinetobacter 2	0,18	0,29	0,26	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01
Bacillus 2	0,19	0,17	0,12	0,01	0,00	0,01	0,00	0,00	0,01	0,00	0,00	0,00
Shewanella	0,06	0,08	0,05	0,00	0,00	0,00	0,04	0,00	0,01	0,00	0,01	0,00
Pantoea	0,51	0,83	0,54	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00
Enterococcus	0,31	0,18	0,20	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01
Weissella	0,17	0,27	0,17	0,01	0,01	0,00	0,00	0,01	0,00	0,00	0,00	0,01
Corynebacterium	0,31	0,29	0,23	0,01	0,00	0,01	0,01	0,01	0,03	0,01	0,00	0,01
Pseudomonas 3	0,28	0,19	0,20	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,01
Cobetia	0,03	0,07	0,20	0,01	0,01	0,00	0,00	0,00	0,02	0,02	0,03	0,02
Psychrobacter 2	0,09	0,13	0,17	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00
Psychrobacter 3	0,11	0,08	0,24	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Comamonas	0,12	0,29	0,01	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00
Halorubrum	0,17	0,13	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Hafnia/Obesumbacterium	0,03	0,03	0,03	0,00	0,01	0,00	0,00	0,01	0,00	0,01	0,00	0,00
Spelaeicoccus	0,13	0,07	0,07	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00
Escherichia/Shigella	0,31	0,26	0,17	0,00	0,01	0,00	0,00	0,00	0,00	0,02	0,01	0,06
Acinetobacter 3	0,08	0,50	0,04	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Sphingobacterium	0,05	0,09	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Cellulosimicrobium	0,11	0,19	0,09	0,00	0,00	0,00	0,01	0,00	0,01	0,00	0,00	0,00
Acinetobacter 4	0,03	0,04	0,06	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Halorubrum 2	0,06	0,08	0,01	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00
Microbacterium	0,04	0,04	0,05	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,01	0,00
Staphylococcus	0,10	0,13	0,12	0,00	0,01	0,00	0,01	0,00	0,00	0,06	0,01	0,01
Brevibacterium	0,03	0,07	0,07	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Bacillus 3	0,03	0,03	0,04	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Pseudomonas 4	0,02	0,08	0,03	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Stenotrophomonas	0,00	0,20	0,03	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01
Brevibacterium 2	0,05	0,08	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,10
Pelomonas	0,09	0,06	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,17
Stenotrophomonas 2	0,10	0,06	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,26
Terribacillus	0,29	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,10
Haloplanus	0,10	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,27
Psychrobacter 4	0,13	0,19	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,42

OTU relative abundance (%) in gravlax samples inoculated with ***L. gelidum*** **EU2249** at D0, D7, D14, D21 (in grey OTUs > 0,1%).

OTUs Affiliation	C1D0	C2D0	C3D0	C1D7	C2D7	C3D7	C1D14	C2D14	C3D14	C1D21	C2D21	C3D21
Photobacterium	11,97	51,13	70,89	49,04	51,01	53,48	41,37	36,67	35,43	37,94	35,44	39,53
Lactococcus	0,45	0,31	0,19	0,06	0,12	0,09	0,29	0,26	0,26	0,29	0,21	0,12
Carnobacterium	0,29	0,15	0,13	0,11	0,17	0,15	0,13	0,16	0,13	0,14	0,06	0,15
Serratia/Yersinia	1,18	1,31	0,86	1,16	1,43	0,88	3,69	5,02	7,37	5,82	5,95	6,01
Vibrio	1,04	0,86	0,48	2,44	3,33	1,33	1,65	2,58	1,50	0,80	0,86	0,35
Leuconostoc	44,97	17,55	6,66	47,03	43,78	43,94	52,52	54,82	54,81	54,50	56,82	53,51
Aerococcus	0,00	0,04	0,03	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,01	0,00
Vagococcus	0,03	0,01	0,01	0,00	0,01	0,01	0,00	0,00	0,00	0,00	0,03	0,00
Pseudomonas	9,26	7,85	7,32	0,01	0,01	0,01	0,03	0,03	0,04	0,03	0,10	0,03
Lactococcus 2	0,00	0,01	0,00	0,01	0,00	0,01	0,00	0,00	0,01	0,00	0,00	0,00
Lactobacillus	0,03	0,02	0,03	0,02	0,00	0,01	0,08	0,01	0,06	0,01	0,00	0,04
Enterobacter/Klebsiella	3,83	2,76	0,79	0,02	0,01	0,00	0,01	0,00	0,03	0,03	0,08	0,04
Pseudomonas 2	3,17	1,98	4,60	0,01	0,01	0,00	0,01	0,00	0,01	0,02	0,05	0,01
Morganella	0,06	0,03	0,01	0,01	0,01	0,02	0,07	0,29	0,20	0,20	0,12	0,13
Brochothrix	0,13	0,13	0,10	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,02	0,01
Psychrobacter	2,03	1,38	1,04	0,01	0,00	0,00	0,01	0,03	0,01	0,01	0,00	0,01
Bacillus	4,77	1,54	0,56	0,00	0,00	0,01	0,01	0,01	0,02	0,03	0,01	0,01
Acinetobacter	0,83	1,65	0,40	0,00	0,00	0,00	0,00	0,00	0,01	0,01	0,00	0,00
Pseudoalteromonas	1,36	0,93	0,52	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00
Brachybacterium	0,43	0,65	0,36	0,00	0,00	0,01	0,00	0,02	0,01	0,01	0,02	0,00
Duganella	0,70	1,12	0,38	0,00	0,00	0,01	0,01	0,00	0,00	0,01	0,01	0,00
Acinetobacter 2	0,36	0,42	0,30	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00
Bacillus 2	1,56	0,45	0,29	0,01	0,01	0,00	0,00	0,00	0,00	0,01	0,00	0,00
Shewanella	0,11	0,09	0,03	0,01	0,01	0,00	0,05	0,01	0,02	0,01	0,02	0,01
Pantoea	1,89	0,63	0,27	0,00	0,01	0,00	0,00	0,01	0,00	0,00	0,00	0,00
Enterococcus	0,70	0,78	0,21	0,00	0,00	0,02	0,02	0,00	0,00	0,01	0,02	0,00
Weissella	1,00	0,79	0,26	0,01	0,01	0,00	0,01	0,01	0,01	0,01	0,02	0,01
Corynebacterium	0,95	0,94	0,40	0,01	0,01	0,00	0,01	0,02	0,02	0,03	0,08	0,01
Pseudomonas 3	0,19	0,40	0,31	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Cobetia	0,47	0,28	0,23	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Psychrobacter 2	0,17	0,20	0,41	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Psychrobacter 3	0,49	0,31	0,11	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Comamonas	0,23	0,39	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00
Halorubrum	0,40	0,13	0,15	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Hafnia/Obesumbacterium	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,03	0,01	0,00	0,00
Spelaeicoccus	0,40	0,24	0,15	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,02	0,01
Escherichia/Shigella	1,02	0,10	0,09	0,00	0,01	0,00	0,01	0,01	0,00	0,01	0,01	0,00
Acinetobacter 3	0,36	0,28	0,09	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Sphingobacterium	0,07	0,33	0,13	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00
Cellulosimicrobium	0,33	0,30	0,07	0,00	0,00	0,01	0,00	0,00	0,01	0,01	0,01	0,01
Acinetobacter 4	0,18	0,17	0,08	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Halorubrum 2	0,17	0,13	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Microbacterium	0,20	0,10	0,08	0,01	0,00	0,02	0,00	0,00	0,00	0,01	0,01	0,00
Staphylococcus	0,31	0,28	0,15	0,00	0,02	0,00	0,01	0,00	0,00	0,01	0,00	0,01
Brevibacterium	0,07	0,06	0,11	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00
Bacillus 3	0,25	0,05	0,02	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00
Pseudomonas 4	0,15	0,07	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Stenotrophomonas	0,12	0,15	0,03	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00
Brevibacterium 2	0,17	0,04	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,02
Pelomonas	0,07	0,08	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,02
Stenotrophomonas 2	0,08	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01
Terribacillus	0,08	0,01	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,02
Haloplanus	0,11	0,14	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,03
Psychrobacter 4	0,02	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00

OTU relative abundance (%) in gravlax samples inoculated with ***V. fluvialis* CD264** at D0, D7, D14, D21 (in grey OTUs > 0,1%).

OTUs Affiliation	D1D0	D2D0	D3D0	D1D7	D2D7	D3D7	D1D14	D2D14	D3D14	D1D21	D2D21	D3D21
Photobacterium	61,57	1,77	3,53	33,31	59,65	47,19	57,30	21,62	9,39	28,10	25,18	42,95
Lactococcus	0,02	0,04	0,07	0,83	0,35	0,72	9,27	6,86	8,76	14,21	14,89	32,74
Carnobacterium	0,35	0,52	0,74	1,11	0,61	0,56	0,84	2,87	2,61	3,08	1,94	0,88
Serratia/Yersinia	0,15	0,85	0,70	8,45	3,69	7,32	8,59	35,11	35,66	32,50	32,58	14,49
Vibrio	0,18	6,24	2,68	40,10	24,72	29,97	5,03	17,68	30,29	12,87	16,00	3,05
Leuconostoc	0,03	0,10	0,05	0,02	0,01	0,06	0,29	0,09	0,06	2,09	0,09	0,07
Aerococcus	0,01	0,00	0,02	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00
Vagococcus	32,96	69,42	73,07	15,27	10,37	13,45	4,99	10,40	8,00	3,92	4,78	2,57
Pseudomonas	1,83	6,21	5,80	0,03	0,16	0,17	0,03	0,05	0,06	0,11	0,12	0,03
Lactococcus 2	0,00	0,00	0,01	0,25	0,03	0,14	1,25	1,58	2,01	1,91	2,25	2,49
Lactobacillus	0,03	0,01	0,01	0,17	0,01	0,12	11,76	2,27	1,34	0,05	0,83	0,21
Enterobacter/Klebsiella	0,31	2,27	2,44	0,05	0,01	0,01	0,01	0,01	0,08	0,03	0,03	0,01
Pseudomonas 2	0,47	1,37	1,80	0,02	0,05	0,02	0,01	0,01	0,00	0,01	0,00	0,01
Morganella	0,00	0,01	0,01	0,10	0,05	0,04	0,10	1,05	1,02	0,74	0,93	0,28
Brochothrix	0,00	0,03	0,02	0,15	0,07	0,03	0,46	0,24	0,56	0,24	0,25	0,12
Psychrobacter	0,15	0,89	1,23	0,01	0,03	0,03	0,01	0,01	0,01	0,00	0,01	0,00
Bacillus	0,23	0,54	1,06	0,01	0,03	0,01	0,00	0,00	0,02	0,01	0,01	0,00
Acinetobacter	0,29	0,58	0,62	0,00	0,01	0,01	0,00	0,00	0,00	0,01	0,00	0,01
Pseudoalteromonas	0,06	0,72	0,58	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01
Brachybacterium	0,12	0,79	0,61	0,01	0,00	0,01	0,00	0,01	0,01	0,03	0,01	0,01
Duganella	0,07	1,32	0,12	0,00	0,00	0,00	0,00	0,01	0,01	0,02	0,00	0,00
Acinetobacter 2	0,04	0,30	0,24	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01
Bacillus 2	0,06	0,26	0,24	0,00	0,01	0,01	0,00	0,01	0,00	0,00	0,00	0,00
Shewanella	0,01	0,06	0,17	0,03	0,01	0,01	0,00	0,03	0,01	0,05	0,08	0,02
Pantoea	0,13	0,86	0,73	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Enterococcus	0,06	0,19	0,21	0,01	0,01	0,00	0,01	0,00	0,01	0,00	0,01	0,01
Weissella	0,07	0,27	0,17	0,02	0,01	0,00	0,00	0,00	0,00	0,01	0,00	0,00
Corynebacterium	0,13	0,68	0,70	0,01	0,03	0,01	0,03	0,01	0,03	0,00	0,01	0,00
Pseudomonas 3	0,08	0,25	0,21	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00
Cobetia	0,02	0,17	0,15	0,01	0,02	0,02	0,00	0,03	0,00	0,00	0,00	0,00
Psychrobacter 2	0,01	0,12	0,22	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00
Psychrobacter 3	0,03	0,18	0,29	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00
Comamonas	0,04	0,17	0,06	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00
Halorubrum	0,05	0,13	0,10	0,00	0,00	0,01	0,00	0,01	0,00	0,00	0,00	0,00
Hafnia/Obesumbacterium	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,02	0,05	0,00	0,00	0,03
Spelaeicoccus	0,06	0,17	0,07	0,00	0,00	0,00	0,01	0,00	0,00	0,01	0,00	0,00
Escherichia/Shigella	0,03	0,10	0,06	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Acinetobacter 3	0,03	0,09	0,11	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Sphingobacterium	0,03	0,07	0,11	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Cellulosimicrobium	0,02	0,13	0,10	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00
Acinetobacter 4	0,01	0,09	0,07	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Halorubrum 2	0,02	0,13	0,08	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Microbacterium	0,03	0,08	0,12	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Staphylococcus	0,07	0,22	0,10	0,01	0,03	0,01	0,00	0,00	0,01	0,01	0,00	0,00
Brevibacterium	0,02	0,14	0,08	0,00	0,01	0,01	0,00	0,00	0,00	0,00	0,01	0,00
Bacillus 3	0,01	0,10	0,05	0,00	0,01	0,01	0,00	0,00	0,00	0,00	0,00	0,00
Pseudomonas 4	0,00	0,97	0,02	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00
Stenotrophomonas	0,01	0,06	0,03	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00
Brevibacterium 2	0,09	0,08	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,08
Pelomonas	0,03	0,01	0,00	0,01	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,06
Stenotrophomonas 2	0,07	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,08
Terribacillus	0,03	0,06	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,06
Haloplanus	0,07	0,04	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,06
Psychrobacter 4	0,03	0,14	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,04

OTU relative abundance (%) in gravlax samples inoculated with ***C. inhibens*** **MIP2551** at D0, D7, D14, D21 (in grey OTUs > 0,1%).

OTUs Affiliation	E1D0	E2D0	E3D0	E1D7	E2D7	E3D7	E1D14	E2D14	E3D14	E1D21	E2D21	E3D21
Photobacterium	12,87	10,85	8,58	68,60	57,07	69,02	45,93	35,35	38,80	16,08	17,73	26,19
Lactococcus	0,13	0,17	0,02	0,71	2,16	1,20	19,74	12,61	12,63	16,70	9,83	18,32
Carnobacterium	77,39	77,11	82,46	8,01	8,91	9,03	3,38	5,93	6,55	5,96	6,45	5,82
Serratia/Yersinia	0,56	0,44	0,40	11,39	21,16	13,95	24,10	33,89	24,82	41,73	34,05	35,36
Vibrio	0,56	0,51	1,02	10,21	7,92	5,54	1,05	3,74	9,81	9,74	6,78	4,19
Leuconostoc	0,10	0,06	0,02	0,10	1,11	0,00	2,48	1,89	3,78	4,02	22,84	6,14
Aerococcus	0,00	0,01	0,01	0,01	0,00	0,01	0,01	0,01	0,00	0,01	0,00	0,01
Vagococcus	0,03	0,01	0,04	0,00	0,00	0,00	0,01	0,01	0,01	0,01	0,01	0,00
Pseudomonas	1,68	2,97	2,01	0,07	0,15	0,13	0,07	0,03	0,02	0,20	0,08	0,08
Lactococcus 2	0,00	0,01	0,00	0,14	0,56	0,25	2,25	1,89	2,21	2,30	1,43	2,71
Lactobacillus	0,02	0,01	0,00	0,10	0,01	0,04	0,22	3,83	0,29	1,31	0,00	0,25
Enterobacter/Klebsiella	1,39	2,50	0,83	0,03	0,06	0,06	0,07	0,05	0,08	0,20	0,03	0,02
Pseudomonas 2	0,52	0,56	0,34	0,01	0,01	0,01	0,01	0,07	0,00	0,03	0,01	0,00
Morganella	0,02	0,01	0,00	0,31	0,37	0,37	0,51	0,49	0,34	0,76	0,62	0,55
Brochothrix	0,01	0,01	0,01	0,03	0,05	0,12	0,08	0,06	0,47	0,44	0,07	0,20
Psychrobacter	0,17	0,42	0,54	0,01	0,03	0,05	0,00	0,01	0,00	0,03	0,00	0,01
Bacillus	0,24	0,37	0,15	0,01	0,01	0,01	0,00	0,00	0,03	0,00	0,00	0,01
Acinetobacter	0,28	0,33	0,17	0,00	0,01	0,01	0,00	0,00	0,00	0,00	0,00	0,00
Pseudoalteromonas	0,06	0,13	0,36	0,01	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00
Brachybacterium	0,63	0,17	0,59	0,01	0,00	0,00	0,00	0,01	0,01	0,02	0,01	0,01
Duganella	0,09	0,10	0,06	0,01	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00
Acinetobacter 2	0,16	0,11	0,16	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00
Bacillus 2	0,23	0,33	0,10	0,01	0,01	0,01	0,00	0,00	0,02	0,01	0,00	0,00
Shewanella	0,03	0,03	0,01	0,19	0,31	0,17	0,07	0,06	0,06	0,13	0,03	0,08
Pantoea	0,79	0,64	0,26	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Enterococcus	0,13	0,03	0,07	0,01	0,00	0,00	0,01	0,00	0,01	0,00	0,00	0,00
Weissella	0,12	0,26	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Corynebacterium	0,28	0,43	0,20	0,01	0,02	0,00	0,00	0,01	0,01	0,06	0,00	0,01
Pseudomonas 3	0,03	0,06	0,08	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Cobetia	0,04	0,08	0,12	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Psychrobacter 2	0,02	0,02	0,01	0,00	0,00	0,00	0,00	0,01	0,01	0,00	0,00	0,00
Psychrobacter 3	0,02	0,04	0,11	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00
Comamonas	0,03	0,02	0,03	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00
Halorubrum	0,13	0,17	0,13	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Hafnia/Obesumbacterium	0,00	0,01	0,00	0,00	0,06	0,01	0,00	0,06	0,01	0,07	0,04	0,03
Spelaeicoccus	0,08	0,10	0,03	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00
Escherichia/Shigella	0,11	0,13	0,06	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Acinetobacter 3	0,11	0,04	0,05	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Sphingobacterium	0,02	0,03	0,06	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00
Cellulosimicrobium	0,14	0,04	0,04	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,01
Acinetobacter 4	0,04	0,05	0,19	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Halorubrum 2	0,02	0,10	0,12	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Microbacterium	0,02	0,06	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00
Staphylococcus	0,04	0,10	0,09	0,01	0,00	0,01	0,01	0,01	0,01	0,01	0,00	0,00
Brevibacterium	0,01	0,05	0,01	0,00	0,00	0,00	0,01	0,00	0,01	0,13	0,00	0,00
Bacillus 3	0,02	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00
Pseudomonas 4	0,05	0,03	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Stenotrophomonas	0,19	0,04	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Brevibacterium 2	0,03	0,03	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,01
Pelomonas	0,04	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,02
Stenotrophomonas 2	0,03	0,03	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01
Terribacillus	0,01	0,17	0,00	0,02	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,01
Haloplanus	0,08	0,03	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01
Psychrobacter 4	0,06	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00

OTU relative abundance (%) in gravlax samples inoculated with ***A. viridans* SF1044** at D0, D7, D14, D21 (in grey OTUs > 0,1%).

OTUs Affiliation	F1D0	F2D0	F3D0	F1D7	F2D7	F3D7	F1D14	F2D14	F3D14	F1D21	F2D21	F3D21
Photobacterium	55,6	37,0	23,5	75,6	80,2	50,9	74,1	52,4	52,7	11,3	22,4	28,4
Lactococcus	0,0	0,0	0,0	0,7	1,7	1,0	11,9	15,1	18,7	18,6	25,5	17,6
Carnobacterium	0,4	0,4	0,4	1,5	1,5	2,9	0,9	2,6	2,8	5,1	3,6	2,2
Serratia/Yersinia	0,4	0,4	0,8	6,7	8,2	16,9	4,8	19,1	20,8	47,7	34,8	40,6
Vibrio	0,0	0,4	4,8	9,5	1,0	22,5	3,7	6,2	1,7	10,2	8,1	2,8
Leuconostoc	0,0	0,0	0,0	0,0	0,0	0,0	0,1	0,1	0,1	0,0	0,0	0,0
Aerococcus	36,8	49,9	53,2	5,4	6,2	3,4	3,5	2,7	1,2	0,7	1,3	2,0
Vagococcus	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,1	0,0
Pseudomonas	3,4	5,0	9,1	0,1	0,1	0,1	0,0	0,0	0,0	0,1	0,0	0,0
Lactococcus 2	0,0	0,0	0,0	0,1	0,1	0,1	0,4	1,2	1,3	2,9	2,8	1,1
Lactobacillus	0,0	0,0	0,0	0,1	0,3	0,0	0,2	0,1	0,1	1,4	0,4	4,5
Enterobacter/Klebsiella	0,3	1,0	0,7	0,0	0,0	0,0	0,0	0,0	0,0	0,1	0,1	0,0
Pseudomonas 2	0,5	0,8	1,5	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Morganella	0,0	0,0	0,0	0,1	0,1	0,3	0,1	0,3	0,3	0,8	0,4	0,4
Brochothrix	0,0	0,0	0,0	0,1	0,0	1,5	0,2	0,2	0,0	0,6	0,3	0,1
Psychrobacter	0,1	0,4	0,9	0,0	0,0	0,1	0,0	0,0	0,0	0,0	0,0	0,0
Bacillus	0,4	0,3	0,3	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Acinetobacter	0,1	0,7	0,4	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Pseudoalteromonas	0,0	0,1	0,9	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Brachybacterium	0,2	0,4	0,2	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Duganella	0,0	0,0	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Acinetobacter 2	0,5	0,5	0,4	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Bacillus 2	0,1	0,3	0,2	0,0	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Shewanella	0,1	0,1	0,2	0,0	0,0	0,1	0,0	0,0	0,1	0,1	0,1	0,1
Pantoea	0,1	0,3	0,3	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Enterococcus	0,1	0,2	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Weissella	0,1	0,1	0,2	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Corynebacterium	0,1	0,2	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Pseudomonas 3	0,0	0,1	0,2	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Cobetia	0,0	0,1	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Psychrobacter 2	0,0	0,1	0,4	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Psychrobacter 3	0,0	0,1	0,2	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Comamonas	0,0	0,0	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Halorubrum	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Hafnia/Obesumbacterium	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,2	0,0	0,0
Spelaeicoccus	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Escherichia/Shigella	0,0	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Acinetobacter 3	0,0	0,1	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Sphingobacterium	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Cellulosimicrobium	0,0	0,2	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Acinetobacter 4	0,0	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Halorubrum 2	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Microbacterium	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Staphylococcus	0,0	0,0	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Brevibacterium	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Bacillus 3	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Pseudomonas 4	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Stenotrophomonas	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Brevibacterium 2	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Pelomonas	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Stenotrophomonas 2	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Terribacillus	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Haloplanus	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Psychrobacter 4	0,0	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0

Table 4.4: **Supplementary Table 2 – DESeq2 differential abundance analysis**

***C. maltaromaticum* SF1944 versus Control**				
baseMean	**Log2fc**	**pvalue**	**padj**	**OTUs**
T0				
18599	9,27	1,27E-77	3,18E-76	*Carnobacterium*
Day 7				
21	-4,00	1,94E-03	9,71E-03	*Psychrobacter*
59	6,62	6,92E-10	8,65E-09	*Leuconostoc*
18	6,59	1,12E-06	9,34E-06	*Vagococcus*
35900	8,03	2,09E-17	5,22E-16	*Carnobacterium*
54	-6,94	1,20E-05	7,53E-05	*Brochothrix*
Day 14				
4695	-2,52	1,85E-05	2,23E-04	*Lactococcus*
44276	5,20	6,52E-27	1,57E-25	*Carnobacterium*
Day 21				
1335	-7,08	3,50E-07	3,85E-06	*Lactobacillus*
12505	5,22	9,08E-17	2,00E-15	*Carnobacterium*

***L. piscium* EU2229 versus Control**				
baseMean	**Log2fc**	**pvalue**	**padj**	**OTUs**
at T0				
9964	9,69	7,18E-57	1,80E-55	*Lactococcus*
37	7,14	8,01E-09	1,00E-07	*Lactococcus 2*
Day 7				
71	-3,72	4,40E-04	2,75E-03	*Pseudomonas*
29361	9,43	2,07E-27	5,17E-26	*Lactococcus*
34	5,91	1,82E-08	2,28E-07	*Leuconostoc*
7	5,30	1,13E-04	9,46E-04	*Vagococcus*
Day 14				
53001	3,59	4,23E-04	5,28E-03	*Lactococcus*
56	8,65	1,34E-09	3,36E-08	*Leuconostoc*
Day 21				
34093	3,66	5,99E-18	1,50E-16	*Lactococcus*
206	-1,91	4,46E-04	3,72E-03	*Lactococcus 2*
362	-2,09	6,42E-06	8,02E-05	*Carnobacterium*

L. gelidum EU2249 versus Control				
baseMean	**Log2fc**	**pvalue**	**padj**	**OTUs**
at T0				
2152,63	↑ 7,01	5,49E-32	1,37E-30	*Leuconostoc*
Day 7				
25527,49	↑ 15,78	1,44E-71	3,61E-70	*Leuconostoc*
6,78	↑ 5,53	3,24E-05	2,70E-04	*Vagococcus*
41,61	↓ -7,55	2,44E-07	3,05E-06	*Brochothrix*
Day 14				
9733,28	↓ -0,99	1,33E-04	5,55E-04	*Serratia/Yersinia*
12,41	↑ 2,61	2,37E-03	7,40E-03	*Psychrobacter*
12,28	↑ 2,36	6,23E-04	2,23E-03	*Corynebacterium*
4915,05	↓ -4,94	1,12E-20	1,39E-19	*Lactococcus*
432,92	↓ -7,60	1,02E-15	6,39E-15	*Lactococcus 2*
33467,99	↑ 17,56	1,18E-63	2,96E-62	*Leuconostoc*
1510,03	↓ -4,31	3,81E-16	3,17E-15	*Carnobacterium*
55,33	↓ -3,83	9,95E-06	4,98E-05	*Brochothrix*
Day 21				
19162,37	↑ 1,12	3,18E-03	8,48E-03	*Photobacterium*
3,94	↑ 5,73	7,77E-04	2,66E-03	*Duganella*
10,39	↑ 4,67	6,67E-05	2,67E-04	*Corynebacterium*
2994,11	↓ -5,61	3,11E-46	3,73E-45	*Lactococcus*
188,99	↓ -8,18	3,74E-18	2,99E-17	*Lactococcus 2*
18726,82	↑ 10,72	2,60E-170	6,24E-169	*Leuconostoc*
1334,40	↓ -7,26	5,05E-05	2,43E-04	*Lactobacillus*
384,32	↓ -3,16	6,05E-14	3,63E-13	*Carnobacterium*
14,79	↓ -3,10	9,43E-04	2,83E-03	*Brochothrix*

V. fluvialis CD264 versus Control				
baseMean	**Log2fc**	**pvalue**	**padj**	**OTUs**
T0				
343	↑ 2,46	1,77E-04	2,21E-03	*Vibrio*
8618	↑ 11,57	1,35E-36	3,37E-35	*Vagococcus*
Day 7				
15	↑ 4,11	2,83E-08	3,53E-07	*Leuconostoc*
4746	↑ 14,12	7,86E-40	1,96E-38	*Vagococcus*
Day 14				
55	↑ 8,02	6,91E-10	8,30E-09	*Leuconostoc*
2654	↑ 10,03	2,91E-39	6,99E-38	*Vagococcus*
Day 21				
601	↑ 7,39	9,08E-58	2,27E-56	*Vagococcus*

C. inhibens MIP2551 versus Control				
baseMean	**Log2fc**	**pvalue**	**padj**	**OTUs**
T0				
14981	↑ 9,05	4,24E-74	1,06E-72	*Carnobacterium*
Day 7				
164	↑ 3,17	1,57E-03	7,83E-03	*Shewanella*
304	↑ 3,08	1,55E-03	7,83E-03	*Morganella*
267	↑ 4,72	2,04E-05	2,55E-04	*Lactococcus 2*
312	↑ 7,78	1,08E-07	2,71E-06	*Leuconostoc*
7154	↑ 3,92	4,22E-05	3,52E-04	*Carnobacterium*
Day 14				
881	↑ 11,92	2,02E-24	5,05E-23	*Leuconostoc*
Day 21				
11168	↓ -1,65	8,54E-05	9,82E-04	*Photobacterium*
1826	↑ 6,84	1,48E-14	3,41E-13	*Leuconostoc*

A. viridans SF1044 vs Control à J0				
baseMean	**Log2fc**	**pvalue**	**padj**	**OTUs**
T0				
21821	↑ 2,57	9,70E-04	7,90E-03	*Photobacterium*
567	↓ -4,33	1,26E-03	7,90E-03	*Duganella*
29	↓ -5,38	7,37E-05	9,22E-04	*Lactococcus*
18701	↑ 10,62	6,82E-108	1,70E-106	*Aerococcus*
Day 7				
2438	↑ 8,16	1,28E-41	3,19E-40	*Aerococcus*
Day 14				
24	↑ 7,40	2,95E-08	3,69E-07	*Leuconostoc*
852	↑ 11,86	3,54E-23	8,86E-22	*Aerococcus*
Day 21				
No statistical differences				

Table 4.5: **Supplementary Table 3 - VOCs composition in presence of the different PC.**

		Average concentration (µg/kg)															Standard deviation (µg/kg)													
		Control			*C. maltar* . SF1944		*L. piscium* EU2229		*L. gelidum* EU2249		*V. fluvialis* CD264		*C. inhibens* MIP2551		*A. viridans* SF1044		Control			*C. maltar* . SF1944		*L. piscium* EU2229		*L. gelidum* EU2249		*V. fluvialis* CD264		*C. inhibens* MIP2551		*A. viridans* SF1044	
Compound	**Category**	**T0**	**T14**	**T21**	**A14**	**A21**	**B14**	**B21**	**C14**	**C21**	**D14**	**D21**	**E14**	**E21**	**F14**	**F21**	**T0**	**T14**	**T21**	**A14**	**A21**	**B14**	**B21**	**C14**	**C21**	**D14**	**D21**	**E14**	**E21**	**F14**	**F21**
acetic acid	acid	215	751	3578	697	905	584	1247	1696	198	14353	167	204	12909	2858	16750	19	11	1088	260	205	39	410	212	47	2801	43	5	1761	148	3814
acetic acid deriv.-1	acid	204	199	22875	217	29192	1932	222	1238	191	27470	199	211	207	205	167	12	24	8464	49	1258	273	66	95	45	4958	26	20	27	22	33
acetic acid deriv.-2	acid	209	190	214	215	662	213	215	530	47036	222	189	202	221	203	33533	50	12	14	46	153	60	54	19	7233	30	33	7	19	6	8337
acid-1	acid	182	216	5642	811	173	183	201	322	189	14562	191	7632	183	229	173	25	31	756	352	25	15	91	22	34	2748	47	267	52	27	30
acid-2	acid	190	4386	2953	2640	1197	2891	1469	7304	5920	6221	5715	6142	3495	5471	4126	46	711	726	982	147	345	297	1424	1797	994	941	1169	649	508	224
hexadecanoic acid	acid	182	208	194	259	530	203	261	194	231	172	164	210	209	238	1511	19	7	27	55	141	53	54	35	39	27	42	23	50	35	499
ethanol	alcohol	10710	16635	6218	19878	16522	9947	8600	33024	28209	199	220	28179	14094	12124	6337	2677	1724	467	7302	3340	2645	3587	3284	6525	38	26	1987	1522	1475	468
1-penten-3-ol	alcohol	207	8999	211	14074	2367	14962	247	3352	2187	199	224	213	243	184	175	55	895	15	4228	580	2214	51	204	321	40	43	13	37	17	31
3-methyl-1-butanol	alcohol	204	6239	8511	12752	7338	1000	721	283	218	220	4199	9087	2011	3722	6141	32	1329	1087	3058	1355	173	160	56	55	45	1163	1308	95	501	1291
2,3-butanediol	alcohol	208	1475	241	2504	191	1488	200	1473	150	70855	213	204	227	209	206	16	58	37	1270	27	324	66	214	43	6671	12	42	36	16	47
1-pentanol	alcohol	199	575	248	686	504	705	233	448	336	227	163	206	202	221	186	22	91	70	214	25	163	46	55	12	27	35	9	34	31	52
alcohol-1	alcohol	189	196	197	320	229	314	245	211	205	198	223	345	206	202	195	13	9	43	70	26	46	39	52	4	29	26	20	51	23	19
alcohol-2	alcohol	213	309	211	205	198	204	239	625	423	520	516	510	339	577	384	22	43	50	83	63	28	47	87	138	115	98	30	50	144	96
1-octen-3-ol	alcohol	184	556	467	197	227	321	241	942	887	289	356	312	437	913	690	14	73	147	3	28	23	39	199	88	20	43	48	30	77	193
alcohol-3	alcohol	967	1464	436	880	963	1073	284	825	1021	1171	574	1281	220	716	428	98	157	30	287	211	82	35	13	209	164	131	128	18	66	58
alcohol-4	alcohol	1886	847	678	506	390	499	452	1557	1401	1401	1376	1175	712	1245	1066	353	21	138	161	54	65	24	347	146	200	319	225	47	121	118
alcohol-5	alcohol	785	1612	1008	403	225	1066	282	3676	1821	1289	1263	1357	1199	2834	1781	66	48	167	150	26	358	45	519	586	201	130	201	213	498	283
alcohol-6	alcohol	187	3863	2495	1756	679	2357	960	5942	5568	4250	3287	4696	2565	4805	3048	38	508	499	624	140	289	253	907	659	365	691	457	282	555	617
alcohol-7	alcohol	167	189	177	152	589	440	205	395	233	606	201	461	296	210	204	29	22	20	42	34	69	10	26	36	88	51	33	58	28	52
alcohol-8	alcohol	189	421	319	504	234	399	243	274	340	369	171	233	314	329	176	39	66	98	131	16	7	59	40	77	77	26	29	50	39	36
alcohol-9	alcohol	191	194	205	393	709	773	392	541	784	181	199	200	217	197	189	8	41	57	63	124	91	93	100	155	20	1	38	12	9	10
alcohol-10	alcohol	190	207	376	224	191	171	191	522	588	419	393	457	182	451	296	11	44	22	97	8	29	56	46	56	67	82	103	17	47	74
alcohol-11	alcohol	218	201	251	225	223	215	201	359	373	213	311	249	185	277	268	20	52	42	74	52	55	8	62	60	30	93	28	17	58	65
alcohol-12	alcohol	1589	815	693	439	491	591	427	1139	968	599	704	666	735	687	585	269	95	84	178	53	156	70	210	251	38	171	87	133	148	176
alcohol-13	alcohol	203	207	530	390	377	532	234	714	594	322	480	424	395	491	372	53	6	80	86	55	86	37	105	175	45	89	25	84	61	76
alcohol-14	alcohol	223	532	340	457	205	312	270	372	484	383	400	341	205	290	447	22	141	21	78	53	46	100	67	66	55	46	14	27	27	66
alcohol-15	alcohol	204	254	197	199	218	179	213	293	188	213	203	219	189	189	220	3	21	17	68	33	34	80	24	49	47	71	36	12	26	30
aldehyde-1	aldehyde	210	200	171	4132	224	210	197	183	1803	223	3431	8547	245	4893	3983	32	44	41	729	45	42	66	29	535	29	482	1175	39	708	793
3-methylbutanal	aldehyde	192	891	171	8262	4695	570	207	297	195	183	167	180	234	214	196	7	10	30	1499	581	81	57	65	67	17	39	20	32	12	26
2-methylbutanal	aldehyde	189	424	685	1438	1402	387	219	238	229	186	649	1151	1051	940	748	10	83	193	312	217	84	37	42	40	12	133	122	196	188	116
pentanal	aldehyde	169	2280	238	3262	2192	3700	190	1125	867	363	210	1077	198	331	207	27	253	34	1128	441	455	16	118	173	20	58	124	77	49	6
hexanal	aldehyde	416	3071	215	3399	3036	6606	228	6062	2744	205	209	208	201	253	191	73	260	27	550	419	390	28	656	402	29	37	29	56	33	19
aldehyde-2	aldehyde	193	338	167	521	657	823	225	551	395	201	199	228	197	228	177	8	38	36	45	186	169	21	17	98	5	6	24	36	31	21
heptanal	aldehyde	203	266	175	302	474	381	177	617	316	199	231	637	197	324	223	44	22	31	23	75	74	36	61	59	23	33	39	33	12	59
(E,Z)-2,4-heptadienal	aldehyde	192	200	233	229	705	1816	253	1200	560	199	200	912	191	205	167	23	14	35	70	73	414	49	86	205	15	22	87	25	52	36
(E,E)-2,4-heptadienal	aldehyde	202	191	187	189	551	1182	197	805	455	221	217	209	195	188	163	5	40	23	28	110	139	14	134	152	21	40	8	39	11	36
nonanal	aldehyde	193	626	199	318	212	549	282	1203	616	389	451	673	201	996	302	52	59	52	46	43	151	117	59	109	34	51	106	12	145	13
alkane-1	alkane	206	395	203	464	231	304	241	325	373	207	174	202	205	211	196	43	43	29	46	40	68	48	52	74	16	38	13	47	29	38
alkane-2	alkane	204	506	225	362	187	285	201	757	518	228	154	804	223	235	227	38	60	36	67	22	41	39	38	136	24	41	154	50	33	39

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		Average concentration (µg/kg)															Standard deviation (µg/kg)													
		Control			*C. maltar* . SF1944		*L. piscium* EU2229		*L. gelidum* EU2249		*V. fluvialis* CD264		*C. inhibens* MIP2551		*A. viridans* SF1044		Control			*C. maltar* . SF1944		*L. piscium* EU2229		*L. gelidum* EU2249		*V. fluvialis* CD264		*C. inhibens* MIP2551		*A. viridans* SF1044	
Compound	**Category**	**T0**	**T14**	**T21**	**A14**	**A21**	**B14**	**B21**	**C14**	**C21**	**D14**	**D21**	**E14**	**E21**	**F14**	**F21**	**T0**	**T14**	**T21**	**A14**	**A21**	**B14**	**B21**	**C14**	**C21**	**D14**	**D21**	**E14**	**E21**	**F14**	**F21**
decane	alkane	678	16458	18953	232	209	9868	287	24403	26107	221	627	203	17710	23496	22726	103	3149	5615	35	33	1965	55	3960	4485	22	72	30	4399	1439	6566
undecane	alkane	173	424	473	281	229	306	329	745	350	300	670	346	215	542	375	26	67	150	80	30	42	38	52	97	19	102	32	13	79	51
alkane-3	alkane	173	1080	993	216	170	713	262	1479	2133	223	210	217	225	187	1022	27	49	0	33	26	54	54	102	400	34	50	27	38	35	171
alkane-4	alkane	1675	1683	1102	888	748	892	864	2315	2595	1424	1560	1793	1110	1032	1567	248	392	179	18	99	146	114	47	582	105	325	211	177	97	431
alkane-5	alkane	195	825	783	712	476	543	431	1430	1490	818	1088	805	609	922	672	9	62	113	150	78	123	80	175	212	84	217	86	33	80	228
alkane-6	alkane	204	1687	1151	237	223	912	233	1994	2407	325	439	368	1196	1174	1349	24	274	337	53	34	118	60	579	441	41	12	13	255	118	229
dodecane	alkane	442	676	581	290	425	456	307	919	1211	421	749	565	493	708	193	33	88	48	116	112	79	37	123	246	29	139	39	139	77	52
alkane-7	alkane	225	616	395	300	230	450	224	709	833	382	520	438	227	453	280	25	74	99	9	35	60	54	28	201	50	68	22	24	47	101
alkane-8	alkane	191	200	158	201	248	194	224	185	175	254	306	338	207	210	190	32	6	37	65	42	55	81	15	33	26	32	47	10	23	58
alkane-9	alkane	461	233	164	216	260	209	175	188	358	219	243	211	203	173	169	34	39	38	87	25	37	22	19	82	33	38	25	20	24	36
alkane-10	alkane	215	256	241	180	222	196	235	296	251	211	216	219	243	186	222	38	13	36	70	36	16	45	32	85	34	54	39	39	21	21
alkane-11	alkane	195	369	181	165	247	221	162	363	396	204	203	229	221	203	199	28	48	41	54	43	38	33	73	101	32	23	31	51	9	71
alkane-12	alkane	257	217	183	161	203	209	176	291	295	239	192	214	197	204	200	25	28	35	61	3	10	54	69	22	36	27	16	59	58	44
alkane-13	alkane	163	1055	515	231	199	743	211	960	946	205	228	202	513	576	576	33	96	111	32	42	117	38	113	254	4	25	21	60	142	64
tetradecane	alkane	175	323	212	205	195	228	201	304	304	217	222	209	222	183	166	22	30	59	85	5	40	68	53	39	19	51	17	19	17	33
alkane-14	alkane	187	251	190	223	225	232	209	313	227	207	187	199	206	217	198	12	7	48	59	23	28	30	53	54	27	12	17	59	21	65
alkane-15	alkane	9559	5901	4857	6735	3445	5136	5864	4650	3232	4700	4574	5467	4924	3343	4026	1241	804	1014	1464	812	1046	1115	333	825	404	412	451	294	325	242
heptadecane	alkane	9542	5972	6117	6880	7263	4933	3800	5119	4719	5255	4243	7053	6698	3957	6290	1278	1309	911	2238	1406	1068	972	796	292	1017	1141	1000	443	590	1002
dimethylamine	amine	232	6816	235	15035	14017	8585	168	205	4826	207	185	209	183	193	257	28	1202	31	1902	382	1441	28	28	1524	38	33	36	43	17	50
amine-1	amine	205	5087	172	4727	22402	178	16271	15611	5246	205	207	33018	15950	193	181	4	425	36	1108	3216	27	3747	3332	1627	51	48	2304	468	22	27
xylene	aromatic	215	355	173	609	261	2615	187	631	623	193	217	903	207	214	193	20	45	30	94	48	568	18	86	158	34	45	93	30	18	38
styrene	aromatic	424	252	603	483	199	423	301	543	227	306	509	298	156	522	430	6	22	49	168	37	47	53	116	36	28	83	24	39	30	101
benzaldehyde	aromatic	221	338	245	278	480	441	198	521	389	240	224	219	181	251	207	19	74	39	148	37	39	15	66	57	38	25	24	50	24	8
benzeneacetaldehyde	aromatic	171	2020	244	224	2034	237	255	301	371	2089	2142	1688	4102	1149	3893	26	209	39	29	226	38	50	27	99	298	357	229	401	183	632
phenylethyl alcohol	aromatic	185	176	209	167	241	220	245	205	172	205	199	209	1591	220	2504	14	21	12	59	38	19	43	5	39	46	30	22	262	38	342
dill ether	ester	1243	199	1458	1719	2311	1978	1553	928	1405	1712	946	969	2616	1079	1641	78	37	186	374	365	542	379	112	337	99	162	175	270	260	349
ethyl acetate	ester	206	4725	9147	9807	1774	204	673	436	4429	3240	11390	1711	3280	6597	2620	24	401	2694	1988	133	28	142	22	750	246	1265	123	945	553	606
ester-1	ester	2439	1435	196	1300	183	778	804	600	487	205	1439	991	1173	862	209	649	212	36	305	19	50	63	41	122	16	247	106	71	99	12
ester-2	ester	225	217	173	169	209	206	6957	673	197	188	201	188	9448	205	208	37	18	24	46	11	27	1090	103	8	23	36	23	2314	22	7
3-methylbutyl acetate	ester	205	649	3686	2313	1091	244	205	206	219	179	3054	1771	1715	756	2796	43	94	635	205	181	38	43	53	67	19	473	120	441	99	498
ester-3	ester	189	444	590	382	195	405	268	606	695	344	466	409	227	436	452	25	32	100	66	40	37	45	22	70	8	31	80	27	54	122
ester-4	ester	168	212	211	205	477	231	221	203	218	227	216	190	175	177	210	28	13	18	65	64	41	17	12	42	23	14	17	31	20	28
ester-5	ester	432	281	156	175	219	190	197	324	312	225	219	205	203	215	165	99	35	39	39	50	25	53	48	87	31	41	46	62	20	33
ethyl hexadecanoate	ester	204	222	172	143	379	240	153	410	165	194	172	367	526	502	1013	40	26	25	59	50	38	41	41	41	20	25	20	24	51	329
ketone-1	ketone	3290	2528	193	1858	1722	1399	189	221	1720	199	2811	205	1956	1637	1442	219	312	32	90	538	215	17	34	338	41	438	29	264	178	255
ketone-2	ketone	202	841	173	403	203	204	241	199	215	218	188	209	183	207	13125	33	79	24	65	11	66	51	24	72	16	36	33	17	12	2583
a-thujene	terpene	407	321	1484	2459	1952	1465	2816	632	653	1865	1130	1991	1315	869	850	69	41	218	739	120	353	828	41	202	290	118	156	173	149	163
a-pinene	terpene	939	528	2493	2598	4960	3028	5814	1288	1906	3341	3015	5251	2761	2516	2138	220	42	458	278	695	281	1397	115	315	511	494	245	336	260	167
sabinene	terpene	3742	1096	7703	7415	14740	11002	31088	3664	7913	14576	12331	13086	12913	7602	8386	622	122	1462	1107	1258	1450	9357	364	470	1408	2755	1832	928	524	667

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		Average concentration (µg/kg)															Standard deviation (µg/kg)													
		Control			*C. maltar* . SF1944		*L. piscium* EU2229		*L. gelidum* EU2249		*V. fluvialis* CD264		*C. inhibens* MIP2551		*A. viridans* SF1044		Control			*C. maltar* . SF1944		*L. piscium* EU2229		*L. gelidum* EU2249		*V. fluvialis* CD264		*C. inhibens* MIP2551		*A. viridans* SF1044	
Compound	**Category**	**T0**	**T14**	**T21**	**A14**	**A21**	**B14**	**B21**	**C14**	**C21**	**D14**	**D21**	**E14**	**E21**	**F14**	**F21**	**T0**	**T14**	**T21**	**A14**	**A21**	**B14**	**B21**	**C14**	**C21**	**D14**	**D21**	**E14**	**E21**	**F14**	**F21**
b-pinene	terpene	2819	1212	5410	3867	8347	7173	14468	2665	4941	7014	6086	7973	6471	5350	3368	740	203	83	1309	1298	2269	2956	533	1767	1448	1437	1187	1627	294	746
b-myrcene	terpene	1231	214	1823	1157	2361	1212	3892	386	1354	1652	1733	1305	1914	981	1445	95	14	251	12	397	395	295	50	164	129	519	167	478	43	8
a-phellandrene	terpene	3288	466	12923	6650	5713	2149	22214	1611	9697	9916	3927	8467	2235	1672	6114	668	74	2061	1213	1507	213	2351	135	1804	932	374	913	387	139	1216
3-carene	terpene	326	180	780	408	693	449	1126	300	419	474	466	580	466	403	341	46	18	114	143	204	52	160	59	66	37	115	27	111	66	36
p-cymene	terpene	787	195	3604	1528	1487	779	3858	389	1762	1789	1025	1092	1039	565	1280	47	16	572	443	227	125	479	60	534	340	147	79	295	118	183
limonene	terpene	8116	1725	14063	12759	15351	9314	29610	3680	8981	13896	13766	9728	12333	7975	10219	611	212	2291	2331	3386	924	7821	392	3383	1736	1659	951	1883	532	1788
g-terpinene	terpene	257	186	239	663	471	432	538	204	305	291	256	189	372	219	227	6	13	17	273	70	77	61	14	62	18	22	18	60	19	18
terpinolene	terpene	291	216	871	912	1473	663	779	456	535	1032	513	702	830	502	780	29	35	138	443	298	124	72	39	77	192	109	46	141	51	146
monoterpene-1	terpene	374	213	577	900	1958	805	792	438	499	851	561	496	880	486	590	45	17	136	138	308	296	149	129	30	60	43	81	138	91	142
4-terpineol	terpene	281	226	483	506	1316	600	505	273	412	467	299	316	703	205	497	42	31	23	102	229	50	122	7	80	10	70	13	127	32	66
cembrene A	terpene	1037	489	396	301	680	463	600	548	486	449	330	663	658	487	1085	217	15	107	63	36	65	119	53	34	44	96	73	22	39	285
4-terpinenylacetate	terpene	187	185	173	186	452	189	515	206	278	207	198	205	274	213	179	17	16	24	16	71	15	148	26	59	46	57	5	47	24	45
a-copaene	terpene	949	339	1282	684	3107	975	2951	781	1668	681	990	558	1647	988	663	234	45	463	75	917	212	450	211	26	68	144	15	172	144	148
b-cubebene	terpene	218	213	191	210	444	193	395	196	208	201	179	200	237	186	233	40	30	48	52	72	57	42	12	32	21	21	30	33	23	30
b-caryophyllene	terpene	1937	830	2584	1218	6650	2042	5652	1873	3867	1560	2074	1172	2943	1735	1124	173	118	271	244	646	448	1265	425	884	66	431	129	616	193	81
a-caryophyllene	terpene	341	229	269	219	829	381	700	240	397	262	264	199	349	235	197	50	25	74	42	190	58	121	23	136	32	90	38	91	31	34
sesquiterpene-1	terpene	353	221	280	211	924	279	1062	205	323	206	222	219	319	181	180	31	37	12	18	150	65	218	25	50	41	1	18	36	20	28
d-cadinene	terpene	207	173	215	176	444	227	489	193	285	196	229	205	221	222	175	8	24	34	22	78	27	125	21	64	26	31	29	38	22	23
na-1	na	227	7489	187	5296	4583	3574	6952	197	214	6191	14317	15327	4015	209	3610	25	500	30	2508	177	702	871	8	60	875	450	1824	374	40	734
na-1	na	1174	548	375	357	215	260	225	754	557	487	484	386	206	536	470	128	64	27	58	37	41	30	93	150	32	76	32	55	77	60
na-2	na	868	221	191	201	431	230	255	203	233	204	179	205	273	214	322	78	18	49	78	77	36	50	3	24	38	31	26	34	35	42
na-3	na	341	208	156	215	228	227	196	201	235	208	247	214	210	206	183	42	37	38	17	40	26	80	34	37	33	41	43	38	5	18

General discussion

General discussion

Metabarcoding approach, a new way for microbial community profiling

Through the quick advances in high-throughput sequencing technologies and bioinformatics tools development, these recent years have brought extraordinary new perspectives for the research in the field of microbiology. Based on multi-omics approaches we are now able to better understand microbial communities and their interaction with the surrounding environment (Aguiar-Pulido et al., 2016; Cocolin et al., 2018; Mataragas et al., 2018). Depending on the strategy adopted, microbiologists can addressed the following scientific questions: "*Who is there?*" (metabarcoding and metagenomic), "*What can they potentially do?*" (metagenomic) and finally "*what are they doing*?" (metatranscriptomic and metabolomic).

During this thesis work, we endeavored to give an answer to the first question by characterizing the microbial ecosystem of 3 salmon based products (salmon gravlax, VP and MAP CSS) and to investigate the impact of the manufacturing process (smoking and atmosphere packaging) on their composition. By the use of a metabarcoding approach targeting the hypervariable region V4 of the 16S rRNA gene, we demonstrated that MAP favored the selection of LAB genera, mainly *Lactobacillus* and *Leuconostoc*. Conversely, VP CSS microbiota was totally dominated by *Photobacterium* and in a lesser extent by OTUs corresponding to *Lactobacillus* and *Lactococcus* genera. Salmon gravlax microbiota was more diverse, mainly dominated by *Serratia/Yersinia*, *Photobacterium*, *Lactococcus*, *Lactobacillus* and in a lesser extent by *Carnobacterium* and *Brochothrix*. In addition we showed that the main shift in microbial composition (population dynamic) occurred between 14 and 28 days of storage for the 3 products. The metabarcoding approach also revealed itself to be an adequate tool to monitor the implantation of the 6 biopreservation strains and to observe their impact on the microbial diversity. On the 6 protective cultures, *C. maltaromaticum* SF1944, *L. piscium* EU2229 and *L. gelidum* EU2249 were well implanted on the salmon gravlax and remained the main bacteria within the whole storage time. *V. fluvialis* CD264, *C. inhibens* MIP2551 and *A. viridans* SF1044 were

much less competitive and totally collapse in favor of the gravlax natural microbiota after 7 days of storage.

In seafood and fishery products, the main protagonists involved in spoilage were usually studied via isolation on selective or non-selective media and were subsenquently identified by phenotypic tests and/or by partial (or entire) 16S rRNA gene sequencing (Gram and Huss, 1996; Gram et al., 2002; Leroi, 2014; Boziaris and Parlapani, 2016). Comparing to this classical approach, metabarcoding offers the possibility to get a whole picture of the main bacteria present in a product, and may evidence genera or species that would not have been detected otherwise. As an example, an OTU belonging to the Fusobacteriales order, was identified as the most dominant bacteria in spoiled cod, but was never isolated by cultural methods (Chaillou et al., 2015)

Despite all the advantages described above, the metabarcoding approach has nevertheless some limitations. In this work, although we portrayed the microbial ecosystem composition and identified the taxa present in our products, the taxonomic affiliation was always limited to the genus level. Considering that the spoilage potential of a microorganism is often species or even strain dependent, further identification may be required. Therefore, in a food microbial ecosystem study, an affiliation only limited to the genus represent an important lock for microbiologists to tentatively predict the product spoilage or the microorganisms' behavior within the microbiota.

Currently, this lock is essentially technical. Introduced by Woese and Fox (1977), the 16S rRNA gene is still the target of reference for phylogenetic comparison of prokaryotes microorganisms (in particular within the bacteria reign). However, restricted by the current technologies, sequencing the full 16S rRNA gene length is not yet feasible for metabarcoding. For several years, with the 454-pyrosequencing technology (Roche) it was possible to generate long sequencing reads (>550) (Liu et al., 2012; Ghanbari et al., 2015) and many studies in food microbiota used the V1-V3 regions of the 16S rRNA as phylogenetic marker. This portion offered enough resolution to discriminate many bacterial taxa to the species level (Chaillou et al., 2015). Unfortunately, mainly due to its high cost and its relatively low throughput (1 million reads per plate), the use pyrosequencing is not available anymore (Liu et al., 2012). The scientific community rapidly switch to cheaper technologies such as Ion Torrent and Illumina (HiSeq, MiSeq). However, for instance, the MiSeq platform, the

most popular technology due to its accuracy and high sequencing depth, only allows to sequence 2 x 300 bp (Liu et al., 2012; Ghanbari et al., 2015). This short sequencing reads length seriously limit the choice of the gene portion to be sequenced. Most of the recent studies dealing with microbial ecosystem in food are focusing on the V4 (~290 pb) or V3-V4 (~450 pb) hypervariable regions of 16S rRNA gene (Poirier et al., 2018). However, these 2 hypervariable regions only offer a poor resolution. The Pacific Biosciences technology (PacBio), if improved, could become an interesting alternative, allowing to sequence the 16S rRNA gene entirely. Indeed, it does not require a PCR amplification step, can generate very long reads (up to 8000 pb) and is quite fast. However, this technology also has a very high error rate (11-15%), is relatively expensive and only generate a small amount of data per run (0.05 million reads), which make it not suitable yet for metabarcoding approach (Ghanbari et al., 2015; Rodrigues et al., 2018).

Although often minimized or omitted in studies, the ribosomal RNA operon copy number represents a significant biological bias that might distort our vision of the real microbial composition of an ecosystem (Větrovský and Baldrian, 2013; Angly et al., 2014; Stoddard et al., 2015; Louca et al., 2018). Regarding the bacterial reign, 16S rRNA gene copy numbers is highly variable and spans over an order of magnitude from 1 to up to 15, while the average number is around 4.8 (Stoddard et al., 2015). In addition, the sequence diversity within copies increases concomitantly with increasing copy numbers (Větrovský and Baldrian, 2013; Angly et al., 2014). Our results might be affected as we systematically detected *Photobacterium* in large proportion within all our samples. *Photobacterium* and especially *P. phosphoreum*, the most encountered species in seafood (Leroi, 2014; Chaillou et al., 2015), possess according to the rrnDB database, 15 copies in its genome (Stoddard et al., 2015). By metabarcoding approach its detection was probably largely overestimated. Three recent tools can be used to correct sequence count based on phylogenetic methods and sequenced genomes: PICRUSt (Langille et al., 2013), CopyRighter (Angly et al., 2014) and PAPRICA (Bowman and Ducklow, 2015). However, according to a recent study, none of these tools demonstrated sufficient accuracy to properly predict the right copy number, which could bring additional noise to the community profiles interpretation (Louca et al., 2018). Moreover, all these corrections are made *a posteriori*. If a microorganism with a high copy number is largely present in an ecosystem, as it was the case in our

samples with *Photobacterium*, it will be favorably amplified during the PCR step to the detriment of the subdominant microbiota. This small fraction of the microbial population might not be recovered during sequencing, representing a loss of information in term of diversity. In addition, these corrections consist basically in dividing the sequence number obtained for an OTU by the 16S rRNA gene copy number of the corresponding affiliated microorganisms. This might greatly affect the samples sequencing depth and in consequence the statistical downstream analyses.

Since few years some alternative tracks are studied in order to overcome, among other things, this biological bias and to find markers with better phylogenetic resolution. This concerns particularly mono-copy protein-coding genes such as DNA gyrase subunit B (*gyrB)*, RNA polymerase subunit B (*rpoB*), TU elongation factor (*tuf*), DNA recombinase protein (*recA*) and the protein synthesis elongation factor-G (*fusA*). Although some studies have shown promising results (Roux et al., 2011; Vos et al., 2012; Poirier et al., 2018), many technical boundaries, such as the size and the quality of the reference database, still need to be crossed.

In seafood, but also more broadly in other type of food commodities, the quantification of the SSO is important to predict or explain the spoilage (see chapter 1 part 3). In this work, through metabarcoding we detected in salmon gravlax, MAP and VP CSS potential SSOs, such as *Photobacterium*, *Serratia/Yersinia* in high proportions and in a lesser extent *Brochothrix*. However, as a qualitative tool, the sequencing does not provide any concentrations magnitude. In addition, no specific culture medium currently exists to quantify selectively *Photobacterium* or *Serratia*. As a quantitative tool, q-PCR can be used to detect and enumerate such spoilage related bacterial genera or species. We tentatively tried to enumerate especially *Photobacterium* (potential overestimation due to 16s rRNA copy number) by targeting the Luciferase subunit A gene (*LuxA*). However, in our case, the calibration curves obtained from DNA extraction from artificially contaminated sterile salmon gravlax (and other matrices) with *P. phosphoreum,* using the methodology described in chapter 2 and 3, did not allow to obtain a good correlation between cycle threshold values (C_T) and the viable counts on LH medium. In consequence, we were not able to precisely quantify *P. phosphoreum*. PCR inhibitors might have been co-extracted with the DNA.

Salmon gravlax safety and quality improvement through biopreservation

During this thesis, we also studied the use of 6 LAB to improve the salmon dill gravlax quality and microbial safety. This work revealed that 2 strains, *C. maltaromaticum* SF1944 and *V. fluvialis* CD264, demonstrated a promising potential as bioprotective cultures.

Without inducing any sensory adverse effects, *C. maltaromaticum* SF1944 totally prevent the growth of *L. monocytogenes* in the product during 21 of storage. As we saw in chapter 1, part 7.3., the biopreservation use to improve or control seafood (but more broadly for all food commodities) safety aspect is well documented. Numerous studies demonstrated its effectiveness in inhibiting *L. monocytogenes* or closed related bacteria (*L. innocua*) growth. Inhibition mechanisms are also often well-known and described, since they actually involve bacteriocins producing LAB, such as *C. maltaromaticum*, *C. divergens* and *L. sakei.* Nevetherless some exception remained like the *L. piscium* antilisterial activity, which seems to be cell contact-dependent and induced by quorum sensing (Saraoui et al., 2018).

The selection of an anti-listerial strain showing a good tolerance to chitosans and freezing was of particular interest to study the use of combined preservatives technologies. During the SAFEFISHDISH project, a work package was dedicated to the use of chitosan treatment, superchilling and biopreservation (*C. maltaromaticum* SF1944) in combination to control *L. innocua* growth on whole fresh salmon fishes and CSS fillets stored under vacuum. Briefly, chitosan in association with PC was the most effective treatment to inhibit *L. innocua* in whole fishes (2.0 log CFU/g inhibition). In the case of the smoked fillets, the use of superchilling combined with PC showed the best results (1.2 log CFU/g of inhibition). In addition, PC inoculation just after fish filleting, upstream of all transformation, had higher significant effect compared to the inoculation only in the finished smoked product.

Compared to microbial safety control, which only necessitate to monitor a limited number of responses (e. g. growth of the target to inhibit), the biopreservation applied for shelf-life improvement is far less documented. Spoilage is a complex multifactorial matter which can be very hard to characterize. In addition, no universal spoilage marker currently exists. Their response differs from a fish species to another, but also

from a fresh fish to a transformed product. Moreover, as we demonstrated in this work, the improvement of a spoilage indicator such as TVBN and biogenic amines content, or the inhibition of a certain microbiota, did not necessarily lead to a better sensory quality. For attempting to understand the effect of a biopreservation strain on product spoilage it is necessary to apply a polyphasic approach combining sensory and advanced biochemical analyses (ABVT, TMA, VOCs, biogenic amines etc…), as well as techniques to monitor and characterize the microbial ecosystem (metabarcoding or metagenomic). Within such approach, we demonstrated on a pool of 6 PC, that *V. fluvialis* CD264 was the only strain able to maintain the salmon gravlax quality to an acceptable level even after 25 days of storage. This represented more than 4 days in comparison to the control. Compared to *C. maltaromaticum* SF1944, *L. piscium* EU2229, and *L. gelidum* EU2249, this strain was not very competitive, totally collapse in favor of the natural salmon gravlax microbiota, and did not displayed specific inhibitory activity toward spoilage bacteria (based on cultural and metabarcoding approach). However, its presence at a subdominant level seemed to prevent the formation of strong spoiling off-odors and did not lead to the production of many VOCs in high concentration. This suggests that *V. fluvialis* CD264 biopreservation activity relied mainly on metabolic interactions with the surrounding microbial ecosystem. In addition, this work refutes the fact that a strain must necessarily possess antimicrobial activity and be well implanted within a product to exert a biopreservation activity.

Often referred under the term metabiosis, such bacterial interactions, especially involved in spoilage, have already been described in the literature (Jørgensen et al., 2000b; Mejlholm et al., 2005; Joffraud et al., 2006; Laursen et al., 2006; Macé et al., 2014). For instance, Joffraud et al. (2006), demonstrated that *Vibrio* sp., *B. thermosphacta* and *C. maltaromaticum* spoilage activity in sterile CSS was potentiated when they were co-cultured. Mejlholm et al. (2005) and Laursen et al. (2006) showed that only a co-culture of *B. thermosphacta* and *C. maltaromaticum* was responsible of the particular wet dog off-flavor of cooked and peeled shrimp. However, all aforementioned works were limited to co-cultures between 2 species. The study of interactions within a microbial ecosystem is much more difficult and remains a complex challenge to grasp. Metagenomic and metatranscriptomic could have been used to further identify and characterized the metabolic pathways performed by each microorganisms within the microbial ecosystem. In addition, based on 16S rRNA

amplicon sequencing, some bioinformatics tools such as PAPRICA (Bowman and Ducklow, 2015) and PICRUSt (Langille et al., 2013) or costumed pipelines as the one described by Mataragas et al. (2018), are able to predict the functional potential of an identified operational taxonomic unit. An additional difficulty in studying interactions within a microbiota lies in the fact that microbial ecosystem can vary a lot from a batch of a product to another (Chaillou et al., 2015). To minimize this biological bias and to performed reproducible experiments, different approaches are possible:

- Analyse larger batches of products may help to get more significant results, but it is time and cost consuming. Such approach is usually performed for environmental samples sequencing but are not really applicable when complementary analyses such as sensory evaluation or VOCs composition are required.
- Bacterial consortia can be recreated *de novo* based on metabarcoding microbial profiles and be used to inoculate food matrices. However, this implies that all bacteria species have been already isolated and are cultivable. In addition, the question of the strain representativeness within a species and the quantitative proportion therefore arises.
- Natural bacterial consortia can be directly extracted from the matrix itself and stored as aliquots at -80 °C prior to inoculation, as performed by Rouger et al. (2017).

In the two last cases, microbial communities are used to recreate reproductible ecosystems in sterile matrices or broths, and their interactions with an added protective culture can be more easily studied using transcriptomic and/or metabolomics approaches.

Towards a statistical multiblock analysis

Within this thesis work, for the matrices we studied, we generated a large number of different data types: classical cultural microbial analyses, metabarcoding profile, biochemical analyses (ABVT, pH, TMA, biogenic amines), sensorial and volatilome profiles. In order to get out of a purely descriptive scheme, some predictive statistical tools such as the Canonical Analysis (CA), the Partial least squares regression (PLS regression) and Random forests can be used to try to model relationships between different variables within a data set. However, in our situation these techniques are not

really appropriate. Our data blocks are indeed too disparate, with some possessing a very high number of variables with too few observations (Metabarcoding, volatilome). Through a partnership with the Oniris statistical unit StatSC, we are currently studying the applications of a more flexible multiblock analysis tool: the ComDim (Common Dimensions) analysis. Briefly, the ComDim analysis, allows to find a common space (or dimension) of representation between blocks of variables. In addition, each dimension of this common space may be differentially weighted for each block. For a review see Ghaziri et al. (2016). However, with such disparity among the data blocks, a difficulty remains and can drastically change the outputs: Should we standardize the data within a block and if so, what kind of standardization? In addition, this type of statistical analyses has never been applied to metabarcoding data sets, we therefore have a limited hindsight regarding the outputs.

Going further in protective cultures safety assessment

Since the publication in 2017 of the work on the LAB screening and selection for biopreservation purpose, the genomes of *C. maltaromaticum* SF1944, *L. piscium* EU2229 and *L. gelidum* EU2249, *V. fluvialis* CD264, *C. inhibens* MIP2551 and *A. viridans* SF1044 have been sequenced. Unfortunately we did not had the time to fully investigate further their safety status. It would be worth it to do so in a near future, at least for the two strains with a promising potential as PC (*C. maltaromaticum* SF1944 and *V. fluvialis* CD264). Based on the current databases described in Chapter 1, part 7.6. for virulence factors (MvirDb, VFDB, VirulenceFinder) and antibiotic resistance screening (ARDB, ResFinder and CARD database), we can deepen some observations raised during the screening step, such as:

- The presence of the gene coding for the tyrosine decarboxylase, the enzyme responsible for the production of tyramine from tyrosine. Both strains were strong tyramine producers.
- The presence of genes coding for hemolysins and other virulence factors for *V. fluvialis* CD264, as it displayed a β-hemolytic activity on blood agar medium.
- The presence of genes responsible for the resistance toward clindamycin for both strains and ampicillin for *C. maltaromaticum* SF1944. and if so, determine whereas these resistances are intrinsic or acquired.

Moreover, the use of some genome-wide annotation tools for secondary metabolites biosynthesis gene clusters, like antiSMASH (Blin et al., 2017), can provide further additional information regarding PC antimicrobial activity (e. g. prediction of bacteriocins or nonribosomal peptides gene clusters).

Synthèse détaillée

Synthèse détaillée

Introduction

En tant que source de nutriments précieux (protéines, vitamines, minéraux, omega-3 etc…), les produits de la mer et de la pêche contribuent à une part importante de notre alimentation. Avec une consommation moyenne mondiale de 20.5 kg/habitant/an en 2017, ces produits représentent un marché conséquent, estimé à 171 millions de tonnes pour une valeur marchande de 362 milliards de dollar US (FAO, 2018).

Cependant, les produits de la mer sont des aliments fragiles dont la durée de conservation se limite à plusieurs jours pour les produits frais, et jusqu'à 3 à 4 semaines pour les produits à haute valeur ajoutée, peu transformés, tels que les poissons fumés, les gravlax de poissons, les salades de la mer et les produits marinés. Cette faible durée de vie s'explique principalement par un pH *post-mortem* élevé, ainsi qu'une fraction importante en azote non protéique et acides aminés libres, qui font de ces produits un environnement de croissance idéal pour les microorganismes (Gram and Dalgaard, 2002). Parmi ces microorganismes, des bactéries altérantes telles que *Photobacterium phosphoreum*, *Brochothrix thermosphacta*, *Shewanella* spp., *Pseudomonas* spp., *Serratia* spp., *Hafnia* spp., *Psychrobacter* spp. Et *Pseudoalteromonas* spp. sont capables de croître, et d'induire de par leur métabolisme une dégradation sensorielle (apparition de mauvaises odeurs et saveurs, décoloration des produits etc…) (Gram and Dalgaard, 2002; Leroi, 2014; Boziaris and Parlapani, 2016). Par ailleurs, les produits de la mer peuvent également être d'important vecteur d'intoxication alimentaire et d'infections humaines. En effet, 10 à 20% de toutes les maladies d'origine alimentaire sont attribuées à la consommation de poisson et de coquillages (Le Fur et al., 2013). Dans le secteur industriel des produits de la mer, en particulier dans les produits légèrement préservés ou prêt-à-consommer, *Listeria monocytogenes* représente actuellement le principal risque (Jami et al., 2014).

Ayant été évalués comme responsables de la perte de 20 à 25% de la production alimentaire post-récolte, toutes denrées confondues, la limitation ou le control de l'altération microbienne, ainsi que la croissance des agents pathogènes, constituent actuellement un enjeux crucial (Bondi et al., 2014; Bevilacqua et al., 2016). En complément des technologies de conservation traditionnelles, telles que le

refroidissement, le fumage, le salage et le séchage, la biopréservation apparait comme une méthode complémentaire prometteuse. Elle permet de prolonger la durée de conservation et de réduire le risque microbien tout en répondant à la demande des consommateurs souhaitant des produits peu transformés et dépourvu de conservateurs chimiques (Ghanbari et al., 2013).

La biopréservation consiste à ajouter des microorganismes (sans danger pour la santé) et/ou leurs métabolites dans les aliments afin d'inhiber la croissance de microorganismes indésirables, et ainsi prolonger leur durée de vie et améliorer leur sécurité microbienne (Stiles, 1996). A cet effet, les bactéries lactiques (LAB) sont de loin les microorganismes les plus utilisés et les plus étudiés. En effet, elles font partie de la flore naturelle de nombreux aliments et sont capables de produire une vaste gamme de composés antimicrobiens comme les bactériocines, les acides organiques, le peroxyde d'hydrogène, le diacétyle, etc…(Caplice and Fitzgerald, 1999; Singh, 2018). De plus, en raison de leur implication dans de nombreux processus de fermentation ancestraux (laits fermentés, fromages, saucisson sec, choucroute, etc…), la plus part des LAB sont reconnues comme ne représentant aucun risque pour la santé humaine, et bénéficient de plus d'une image saine et naturelle auprès consommateurs (Rodgers, 2001; Ghanbari et al., 2013).

Dans ce contexte, l'objectif du projet européen « SAFEFISHDISH » était d'améliorer la sécurité et la qualité microbiologique du cabillaud et du saumon frais, ainsi que des produits à base de saumon (saumon fumé et gravlax), de la capture jusqu'à la consommation, via l'utilisation combinée de technologies de conservation. Afin de répondre à cet objectif, ce projet, soutenu par le programme ERA-NET du COFASP et financé en France par l'Agence Nationale de la Recherche (ANR-14-COFA-0001), a impliqué 11 partenaires de France, de Norvège et d'Islande :

- 5 universitaires ou instituts de recherche : NTNU (Trondheim, Norvège), NOFIMA (Tromsø, Norvège), Matís (Reykjavík, Islande), Ifremer (Nantes, France) et Oniris (Nantes, France).
- 4 industriels ou plateformes de recherche et développement : Primex (Siglufjordur, Islande), Arnarlax (Bíldudalur, Islande), Samherji (Akureyri, Islande), PFI Nouvelles Vagues (Boulogne-sur-Mer, France).
- 1 consortium d'industriels : CITPPM (Paris, France).

Au cours du projet, les partenaires ont étudié l'application de différentes technologies telles que la super-réfrigération (superchilling), le traitement au chitosan, la biopréservation et le conditionnement sous atmosphère protectrice, et ce à différentes étapes du processus de transformation du poisson. L'effet de ces techniques sur des poissons entiers et/ou transformés a été évalué sur qualité microbiologique, physico-chimique et sensorielle. Ce travail de thèse était inclus dans le projet SAFEFISHDISH, et portait plus particulièrement sur l'étude de la biopréservation appliquée au gravlax de saumon.

Effet du procédé de fabrication sur l'écosystème microbien, les propriétés organoleptiques et le volatilome de 3 produits à base de saumon

Afin d'ajuster au mieux notre stratégie de biopréservation du gravlax de saumon, nous avons tout d'abord dû caractériser ce produit finalement peu connu d'un point de vue microbiologique, physico-chimique et sensoriel. En effet, les travaux effectués par Leisner et al., (1994) et Lyhs et al (2001) constituent les seules études menées sur la flore microbienne du gravlax de poisson. Par ailleurs, avant ce travail de thèse, le panel sensoriel interne de l'Ifremer n'avait jamais analysé sur ce type de produits. Par conséquent, ce premier travail constituait, pour une part, une étude préliminaire visant à identifier des marqueurs pertinents de dégradation sensorielle ou de fraîcheur (odeurs, saveurs, aspect visuel), des potentielles bactéries altérantes, mais également des marqueurs biochimiques d'altération (composés organiques volatils, ABVT, amines biogènes).

Nous avons donc cherché à caractériser le gravlax de saumon à l'aneth par une approche polyphasique combinant des analyses microbiennes (culturales et aculturales via metabarcoding du gène codant pour l'ARNr 16S), des analyses biochimiques (ABVT, TMA, amines biogènes et volatilome), ainsi qu'une description sensorielle (test de profil). Cette expérience a également été étendue au saumon fumé à froid stocké sous vide ou en atmosphère protectrice, afin de comparer l'effet du processus de fabrication et du conditionnement sur la flore microbienne et la conservation des produits. A partir d'un même lot de poissons, ces 3 produits à ont été transformés à la demande par une entreprise locale, puis stockés 49 jours pour le saumon fumé sous vide (VP CSS) et 35 jours pour le gravlax et le saumon fumé sous atmosphère protectrice (MAP CSS) (1 semaine à 4 °C, puis le reste du temps à 8 °C).

Chaque semaine, toutes les analyses décrites précédemment ont été réalisées, excepté l'analyse d'écosystème microbien réalisée à J0, J14 et J28 uniquement.

A la fin de l'expérience, aucun produit n'a atteint le rejet sensoriel, en particulier le MAP CSS avec un score final d'altération globale de 0.5 sur une échelle de 10, même après 35 jours. Malgré une faible altération, des changements au niveau organoleptique ont tout de même été observés pour le VP CSS et le gravlax de saumon, comme une augmentation de la perception des odeurs et saveurs « aminée » et « acide », une augmentation de l'aspect gras, une légère décoloration et une perte de fermeté en bouche. Il a également été observé une augmentation de la concentration de certains composés organiques volatils (COVs), notamment associés à l'altération du poisson, comme le décanal, le nonanal, l'hexadécanal, le benzaldéhyde, le benzenacétaldéhyde, l'éthanol, le 3-méthyl-1-butanaol, le 2,3-butanediol, le 1-octène-3-ol, le 2-butanone et le 1-octène-3-one. Par ailleurs, le microbiote initial des 3 produits était presque identique, principalement dominé par les genres *Photobacterium*, *Lactococcus* et *Lactobacillus*. Le principal changement de composition de flore s'est déroulé entre 14 et 28 jours. L'écosystème microbien du VP CSS était alors principalement composé du genre *Photobacterium* (> 60%), ainsi qu'en fonction du réplicat biologique, des genres *Lactococcus* et *Lactobacillus*. Dans le cas du MAP CSS, l'atmosphère enrichie en CO_2 a favorisé la sélection du genre *Lactobacillus* uniquement, dont l'OTU était largement majoritaire (> 95 % du nombre de séquences). L'écosystème du gravlax de saumon était plus divers en étant constitué *d'Enterobacteriaceae* (OTU multi-affiliée aux genres *Serratia* ou *Yersinia*), de *Photobacterium*, *Lactobacillus* et *Lactococcus*.

Sélection de souches de bactéries lactiques pour la biopréservation de produits de la mer

Lors de ce premier travail, nous avons pu générer suffisamment de données sur le gravlax (écosystème microbien, évolution sensorielle et biochimique) pour appréhender sa biopréservation. Pour ce faire, nous avons opté pour une stratégie de criblage afin de sélectionner des souches de LAB prometteuses en tant que cultures bioprotectrices dans les produits de la mer.

Basé sur les précédentes études menées au sein des laboratoires EM^{3}B (Ifremer) et Secalim (Oniris), portant sur les propriétés antimicrobiennes des LAB et leur utilisation

comme cultures protectrices (PC) dans les produits de la mer (Leroi et al., 1996, 2015; Duffes et al., 1999a, 1999b; Brillet et al., 2004, 2005; Matamoros et al., 2009b, 2009a; Fall et al., 2010b, 2010a, 2012; Saraoui et al., 2016a, 2017), nous avons constitué une pré-sélection de 35 souches présentant le potentiel de biopréservation le plus intéressant. Nous leur avons ensuite appliqué une stratégie de sélection basée sur 7 critères, dont 5 technologiques comme l'étude de leur activité antimicrobienne vis-à-vis de 6 bactéries d'altération communes des produits de la mer (*S. baltica*, *P. phosphoreum, B. thermosphacta, L. sakei, H. alvei*, *S. proteamaculans*) et contre une bactérie pathogène majeure (*L. monocytogenes)*, leur potentiel d'altération, leur tolérance au traitement au chitosan et à la congélation, leur activité d'inhibition croisée ; ainsi que 2 critères relatifs à l'évaluation de leur innocuité : étude de leur capacité à produire des amines biogènes (notamment tyramine et histamine), et de leur profil de résistance aux principaux antibiotiques. Ce travail de criblage, nous a permis de sélectionner 6 souches de LAB (*C. maltaromaticum* SF1944, *L. piscium* EU2229, *L. gelidum* EU2249, *V. fluvialis* CD264, *C. inhibens* MIP2551 et *A. viridans* SF1044). Ces souches ont en effet démontré une très forte activité antimicrobienne en co-culture, un très faible potentiel d'altération, une tolérance élevée vis-à-vis de la congélation et du traitement au chitosan. Aucun profil atypique de résistance aux antibiotiques n'a par ailleurs été relevé, ainsi qu'aucune capacité à produire de l'histamine. Ces résultats ont fait l'objet d'un article publié dans *Frontiers in Marine Science*.

Biopréservation du gravlax de saumon par six souches de bactéries lactiques

Ces 6 souches précédemment sélectionnées, ont par la suite été testées sur le gravlax de saumon afin d'évaluer leur potentiel bioprotecteur. Des tranches de gravlax de saumon à l'aneth conditionnées sous vide, d'une durée de vie commerciale de 21 jours, ont été achetées auprès d'un industriel local. Elles ont ensuite été inoculées par spray (aérographe) en laboratoire avec les PC afin d'atteindre une concentration initiale de 10^6 log d'UFC/g dans le produit. L'impact des PC sur l'écosystème microbien (méthodes culturales, metabarcoding), sur les propriétés sensorielles (test de profil), sur les paramètres biochimiques (pH, TMA, ABVT, amines biogènes), ainsi que sur le volatilome du gravlax ont été mesuré pendant 25 jours de stockage à 8 °C. Leur activité antimicrobienne contre *L. monocytogenes* a également été évaluée *in situ*.

Cette approche polyphasique a permis de mettre en exergue deux scénarios de réponses en fonction des souches. Dans le premier scénario, *C. maltaromaticum* SF1944, *L. piscium* EU2229 et *L. gelidum* EU2249 ont été très compétitives par rapport à la flore endogène du gravlax. Elles ont dominé très largement l'écosystème microbien du produit pendant toute la durée de l'expérience, et ont également démontré une forte activité inhibitrice contre les flores d'altération et vis-à-vis de *L. monocytogenes*. Par ailleurs, elles ont induit une signature sensorielle qui leur était propre, ainsi que la production de nombreux COVs. Parmi ces 3 souches, *L. piscium* EU2229 et *L. gelidum* EU2249 ne semblaient pas adaptées à la biopréservation du gravlax. Elles ont en effet toutes deux très fortement acidifié le produit, entraînant une détérioration de son aspect visuel. De plus, *L. piscium* EU2229 a été associée à la production d'une très forte note soufrée, et *L. gelidum* EU2249 a produit des exopolysaccharides et du gaz dans l'emballage. La présence de sucres résiduels dans le gravlax pourrait expliquer cette activité métabolique. A l'inverse, *C. maltaromaticum* SF1944, dans la mesure où son score d'altération globale n'était pas différent de celui du témoin, semble approprié pour préserver la sécurité microbienne du gravlax de saumon. Dans le deuxième scénario, *V. fluvialis* CD264, *C. inhibens* MIP2551 et *A. viridans* SF1044 n'étaient pas compétitives et se sont rapidement effacées au profit de la flore microbienne naturelle du produit. Elles n'ont pas démontré de forte activité antimicrobienne et n'ont pas induit la production de nombreux COVs. De plus, les profils sensoriels en présence de *C. inhibens* MIP2551 et de *A. viridans* SF1044 étaient relativement proches du témoin. Cependant, parmi ces 3 souches, *V. fluvialis* CD264 a maintenu la qualité sensorielle du gravlax à un niveau acceptable même après 25 jours (témoin ayant rejeté par le panel sensoriel après 21 jours). Ces résultats suggèrent que la biopréservation de produits naturellement contaminés reste une question complexe à appréhender et qu'elle repose davantage sur les interactions métaboliques entre les microorganismes au sein d'un même écosystème que sur l'activité antimicrobienne.

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List of scientific publications and communications

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Papers published in peer reviewed journals

- **Wiernasz, N.**, Cornet, J., Cardinal, M., Pilet, M.-F., Passerini, D., and Leroi, F. (2017). Lactic Acid Bacteria Selection for Biopreservation as a Part of Hurdle Technology Approach Applied on Seafood. *Front. Mar. Sci.* 4. doi:10.3389/fmars.2017.00119.

- **Wiernasz, N.**, Chevalier, F., Cornet, J., Cardinal, M., Rohloff, J., Courcoux, P., Vigneau, E., Passerini, D., Skírnisdóttir, S., Pilet, M. -F., and Leroi. F. Effect of the manufacturing process on the microbial ecosystem, organoleptic properties and volatilome of 3 salmon based products: salmon gravlax and cold-smoked salmon packed under vacuum or modified atmosphere. **In preparation.**

- **Wiernasz, N.**, Chevalier, F., Cornet, J., Cardinal, M., Rohloff, J., Passerini, D., Skírnisdóttir S., Leroi. F and Pilet, M. -F. Salmon gravlax biopreservation with six lactic acid bacteria: a polyphasic approach to assess the impact on organoleptic properties, microbial ecosystem and volatilome composition. **In preparation.**

Communications at international conferences

- Oral communications

  - **Wiernasz, N.**, Cornet, J., Cardinal, M., Passerini, D., Pilet, M.-F., and Leroi, F. Lactic acid bacteria selection for biopreservation application on fishery products, 46th WEFTA Conference, Oct 2016, Split, Croatia.

  - **Wiernasz, N.**, Cornet, J., Cardinal, M., Passerini, D., Pilet, M.-F., and Leroi, F. Lactic acid bacteria selection for biopreservation application on fishery products, 8th FoodFactory Conference, Oct 2016, Laval, France.

- Poster

  - **Wiernasz, N.**, Chevalier, F., Cardinal, M., Cornet, J., Rohloff, J., Jérôme, M., Chopin, C., Donnay-Moreno, C., Skirnisdóttir, S., Pilet, M. –F., Passerini, D., and Leroi, F. Salmon Gravlax biopreservation: impact on organoleptic properties, microbial ecosystem and volatilome, FoodMicro Conference, Sept 2018, Berlin, Germany.

Salmon Gravlax Biopreservation: Impact on organoleptic properties, microbial ecosystem and volatilome

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1. Introduction

Seafood and fishery products are very fragile commodities with a short shelf-life as the consequence of organoleptic and microbiological qualities quick deterioration. Spoilage, resulting from microbial growth and activity, is responsible for up to 25% of food losses in post-harvest and industry[1]. In this context and to meet the consumer's demand for minimally processed food, developing mild preserving technologies such as biopreservation, represents a crucial challenge. In this work, we studied the impact of 6 lactic acid bacteria (LAB), selected by Wiernasz *et al*. (2017)[2], used as bioprotective agents on dill Gravlax microbial ecosystem, organoleptic properties and volatilome.

2. Methods

6 LAB strains[2]:
Carnobacterium maltaromaticum SF1944
Lactococcus piscium EU2229
Leuconostoc gelidum EU2249
Vagococcus fluvialis CD264
Carnobacterium inhibens MIP2551
Aerococcus viridans SF1044

Inoculated at 10^8 CFU/g

Gravlax characteristics:
Shelf-life of 21 days
Vacuum packed
Stored at 8 °C from day 0 to 25

3. Results

3.1. Microbial enumeration

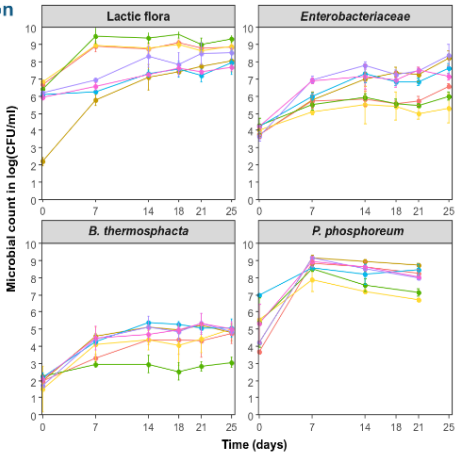


Figure 1. Evolution of different microbial flora during storage, in presence of the 6 LAB. *B. thermosphacta* was enumerated on STAA, lactic flora on NAP, *Enterobacteriaceae* on VRBG and *P. phosphoreum* by q-PCR targeting the luxA gene.

Slight inhibition exerted by *C. maltaromaticum*, *L. piscium* and *L. gelidum* on main spoilage bacteria.

3.2. Biochemical analyses

- pH: quick acidification for *L. piscum* and *L. gelidum* (cooked aspect).
- No significant differences with the control for the 6 biogenic amines quantified (histamine, tyramine, cadaverine, putrescine, spermine, spermidine).
- Significant decrease in gravlax TVBN content in presence of *L. piscium*, *L. gelidum*, *C. inhibens* and *A. viridans*.
- No Trimethylamine production during storage.

3.3. Impacts on sensory quality

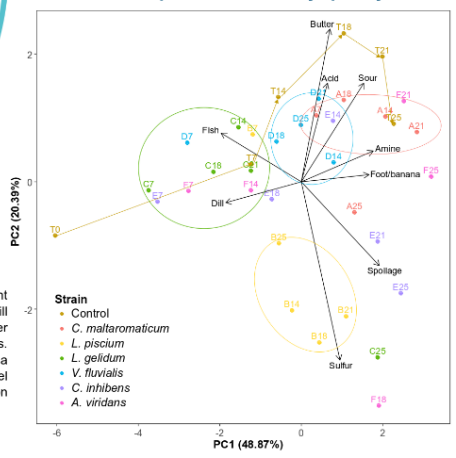
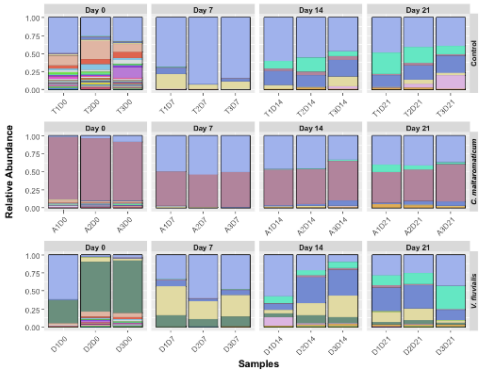


Figure 2. Principal component analysis (PCA) representation of dill Gravlax slices sensory profiles after inoculation with the 6 LAB strains. Odor descriptors were scored on a scale from 0 to 10 by a trained panel of 20 juges. PCA was performed on descriptors scores mean.

- Control was strongly spoiled after 21 days with mainly amine and acid odors.
- Although very close to the control, *V. fluvialis*, was the only strain able to improve and maintain the sensory quality to an acceptable level after 25 days.
- *C. maltaromaticum* was associated with foot/banana and sour odor.
- *L. piscium* related to a strong sulfur odor production after only 7 days.
- *L. gelidum* was a good canditate with dill and fishy odor until 21 days, but a production of gaz and slime was visible after only 14 days.

3.4. Gravlax ecosystem monitoring through metabarcoding

Figure 3. Ecosystem evolution during storage. The hypervariable region V4 of the 16S rRNA gene was targeted[3]. Amplicons were sequenced with Illumina MiSeq technology and output data were treated with FROGS pipeline[4]. On bar plots of ecosystem composition, only figure results obtained for the control and samples inoculated with *C. maltaromaticum* and *V. fluvialis*.



Two patterns were observable:
- *C. maltaromaticum*, *L. piscium*, *L. gelidum* were well implanted in the product and remain predominant during storage.
- *V. fluvialis*, *C. inhibens*, *A. viridans* were not competitive and became a minority after 1 week of storage (ecosystem closed to the control).

3.5. Gravlax volatilome

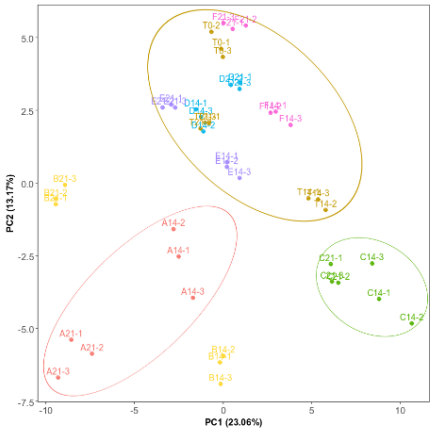


Figure 4. Principal component analysis (PCA) representation of dill Gravlax volatilome after inoculation with the 6 LAB. Volatile organic compounds (VOCs) were identified and quantified by HS-SPME/GC-MS.

Strain
Control
C. maltaromaticum
L. piscium
L. gelidum
V. fluvialis
C. inhibens
A. viridans

V. fluvialis, *C. inhibens*, *A. viridans* were closed to the control with a volatilome mainly composed by ethanol, ethyl acetate, acetic acid, hexanal, decane and dimethylamine.

- *C. maltaromaticum* was associated with VOCs such as, 3 and 2-methylbutanal (malt odor), 3-methyl-1-butanol and dimethylamine.
- In presence of *L. gelidum*, ethanol, acetic acid and many derivates were strongly produced.
- After 14 days *L. piscium* were associated with the production of hexanal, pentanal, 1-penten-3-ol and dimethylamine; while after 21 days, volatilome was driven by high concentration in terpenes (limonene, sabinene)

4. Conclusion

The 6 LAB effect on dill gravlax quality could be classified in two main scenarios:

I) *C. maltaromaticum*, *L. piscium*, *L. gelidum* were competitive in the product by dominating the ecosystem till the end of the experiment, expressed antimicrobial activity against spoilage bacteria but also against *Listeria monocytogenes* (data not shown), and possessed their own sensory signature and volatilome specificity.

II) *V. fluvialis*, *C. inhibens*, *A. viridans* were not well implanted at the gravlax surface, with a sensory and volatilome profil very closed to the control, and did not demonstrate antimicrobial activity. Nevertheless, *V. fluvialis* maintained the gravlax sensory quality below the rejection threshold after 25 days (control was rejected after 21 days).

Biopreservation of naturally contaminated products remains complex to apprehend and may further relies on metabolic interactions between microorganisms from an ecosystem more than antimicrobial activity.

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Titre : **Amélioration de la qualité et de la sécurité des produits de la mer par la technologie des barrières, incluant la biopréservation. Impact sur les écosystèmes microbiens.**

Mots clés : biopréservation, metabarcoding, écosystème microbien, volatilome, analyse sensorielle, gravlax

Résumé : Les produits de la mer représentent une part importante de notre alimentation. Ils sont cependant fragiles et possèdent une durée de vie limitée du fait de l'altération par des microorganismes. L'objectif de cette thèse était de développer une stratégie de biopréservation appliquée à un produit à base de saumon, le gravlax, afin d'en améliorer la qualité et la sécurité microbiologique. Nous avons d'abord caractérisé ce produit peu connu sur le plan microbiologique (analyse culturale et metabarcoding du gène codant pour l'ARNr 16S), physico-chimique (amines biogènes et composés organiques volatils…) et sensoriel. En comparaison avec le saumon fumé conditionné sous vide ou sous atmosphère protectrice, le gravlax possède une flore microbienne plus diverse, dominée par les entérobactéries, en lien avec un volatilome et un profil sensoriel spécifiques. Dans un second temps, six souches de bactéries lactiques, ont été sélectionnées sur des critères technologiques comme leur activité antimicrobienne, leur potentiel d'altération, la tolérance à la congélation et au chitosan, ainsi que sur des critères d'inocuité comme la production d'amines biogènes et la résistance aux antibiotiques. Ces 6 souches ont ensuite été testées sur le gravlax pour leur potentiel bioprotecteur. Elles se distinguent par leur capacité d'implantation ainsi que par leur impact sur l'écosystème microbien, le volatilome, et les propriétés sensorielles du produit. Une souche de *Vagococcus fluvialis* et de *Carnobacterium maltaromaticum* ont montré leur efficacité, respectivement sur la maîtrise de l'altération sensorielle et l'inhibition de *Listeria monocytogenes*, lors du stockage réfrigéré du gravlax.

Title: **Seafood quality and safety improvement through hurdle technology, including biopreservation. Impact on microbial ecosystems.**

Keywords: biopreservation, metabarcoding, microbial ecosystem, volatilome, sensory analysis, gravlax

Abstract: Seafood products count for an important part of our diet. However, they are fragile and have a limited shelf-life due to microbial spoilage. The objective of this thesis was to develop a biopreservation strategy applied to a salmon-based product, the gravlax, in order to improve its microbiological quality and safety. We firstly characterized this poorly known product on a microbiological (cultural analysis and metabarcoding of the 16S rRNA encoding gene), physico-chemical (biogenic amines and volatile organic compounds...) and sensorial point of view. Compared to smoked salmon packed under vacuum or modified atmosphere, the gravlax shows a higher microbial diversity, dominated by enterobacteria and linked to a specific volatilome and sensory profile. In a second time, six lactic acid bacteria strains were selected on technological criteria such as antimicrobial activity, spoilage potential, tolerance to freezing and chitosan, as well as safety criteria such as biogenic amines production and antibiotic resistance. These 6 strains were then applied on gravlax to evaluate their bioprotective potential. They are distinguished by their implantation capacity and their impact on the product microbial ecosystem, volatilome and sensory properties. A strain of *Vagococcus fluvialis* and *Carnobacterium maltaromaticum* were able respectively to control the sensorial spoilage and to inhibit *Listeria monocytogenes*, during gravlax refrigerated storage.