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Par
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Étude du microbiote de l'environnement de l'usine agroalimentaire et de son impact sur la qualité et la sécurité des aliments

Application au modèle de production du saumon fumé

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Sommaire

Liste des abréviations (français)

- °C : Degrés Celsius
- ADN : Acide désoxyribonucléique
- ADNr : Acide désoxyribonucléique ribosomique
- AFNOR : Association Française de Normalisation
- AISE : Agence Internationale pour les Savons, Détergents et Produits de Maintenance
- Anses : Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail
- ARNr : Acide ribonucléique ribosomique
- ATP : Adénosine triphosphate
- bp : Paires de bases
- BPH : Bonnes pratiques d'hygiène
- CE : Communauté européenne
- CIFRE : Convention Industrielle de la Formation par la Recherche
- cm^2 : Centimètres carrés
- CO_2 : Dioxyde de carbone
- DLC : Date limite de consommation
- EPS exo-polysaccharides
- g : Grammes
- g/L : Grammes par litre
- Gb : Giga bases
- h : Heure
- IAA : Industries agroalimentaires
- IFREMER : Institut Français de Recherche pour l'Exploitation de la Mer
- kg : Kilogrammes
- mgN : Milligrammes d'azote ammoniacale
- min : Minutes
- mL : Millilitres
- MRS : De Man, Rogosa and Sharpe
- NaCl : Chlorure de sodium
- NaOH : Hydroxyde de sodium
- NEP : Nettoyage en place
- NF : Norme Française
- ng/µL : Nanogrammes par microlitre
- OMS : Organisation Mondiale de la Santé
- pb : Paires de bases
- pH : potentiel hydrogène
- PMA : Propidium monazide
- PMS : Plan de maîtrise sanitaire
- PVC : Chlorure de polyvinyle
- s : Secondes
- TIAC : Toxi-Infections alimentaires collectives
- TMA : Trimethylamine
- TPU : polyuréthane thermoplastique
- UE : Union européenne
- UFC : Unité formant colonie
- UMR : Unité mixte de recherche
- µm : Micromètres
- VNC : Viable non cultivables

Liste des abréviations (anglais)

- ANOVA : Analysis of variance
- AOAC : Association of Official Agricultural Chemists
- a_w : Activity of Water
- BR : Broad Range
- C&D : Cleaning and disinfection
- CCP : Critical Control Point
- CFU : Colony Forming Unit
- CIP : Cleaning In Place
- CSS : Cold-smoked salmon
- DGGE : Denaturing Gradient Gel Electrophoresis
- DNA : Deoxyribonucleic acid
- dsDNA : double stranded DNA
- ECDC : European Centre for Disease Prevention and Control
- EFSA : European Food Safety Authority
- EHEDG : European Hygienic Engineering and Design Group
- EMA : Ethidium monoazide
- EMP : Environment monitoring program
- EU : European union
- EUMOFA : European Market Observatory for Fisheries and Aquaculture Products
- FAO : Food and Agriculture Organisation
- FDA : Food and Drug Administration
- FFS : Maxwell® 16 FFS DNA purification kit
- FPP : Food processing plant
- FROGS : Find Rapidly OTUs with Galaxy Solution
- HACCP : Hazard Analysis Critical Control Point
- HTS : High-throughput sequencing
- ISO : Internation Organisation for Standardization
- ITS : Internal transcript spacer
- LAB : Lactic acid bacteria
- MALDI-TOF MS : Matrix assisted laser desorption ionization-time of flight mass spectrometry
- NGS : Next generation sequencing
- NIH : National Human Genome Research Institute
- ONT : Oxford Nanopore Technologies
- OTU : Operational taxonomic units
- PACBIO : Pacific Biosciences
- PCA : Plate Count Agar
- PCoA : Principal coordinates analysis
- PCR : Polymerase Chain Reaction
- PERMANOVA : Permutational analysis of variance
- Q_2B_2 : Qiagen QIAamp BiOstic Bacteremia
- qPCR : Quantitative PCR
- QPMF : Qiagen DNeasy PowerFood Microbial
- rDNA : Ribosomal DNA
- RDP : Ribosomal Database Project
- RNA : Ribonucleic acid
- rRNA : Ribosomal RNA
- RTE : Ready to eat
- SD : Standard deviation
- SNP : Single nucleotide polymorphism
- spp : Species *plurimae*
- SS : Stainless steel
- SSTH : Smoked salmon tissue homogenate
- STAA : Streptomycin Sulfate thallous Acetate Agar
- T-RFLP : Terminal Restriction Limitation Fragment Length Polymorphism
- TPVC : Total psychrotrophic viable counts
- TTGE : Temporal Temperature Gel Electrophoresis
- TVBN : Total volatile basic nitrogen
- UBD : Use by date
- US : United states
- VBNC : Viable but not culturable
- VRBG : Violet red bile glucose
- wgMLST : whole genome multi locus sequence typing
- WGS : Whole genome sequencing
- ZFS : Zymo Quick-DNA Fecal/Soil Microbe Miniprep Kit

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Introduction Générale

Chaque année, 30% de la production alimentaire mondiale est gaspillée ou jetée entraînant des coûts écologiques et économiques importants (FAO : Food and Agriculture Organization of the United Nations, 2011). Dans un monde où la démographie est en constante augmentation, il apparait important d'être capable de préserver les aliments afin d'assurer la sécurité sanitaire des aliments.

Dans cette problématique globale, l'altération microbiologique des aliments a sa part de responsabilité. En effet, Huis in't Veld (1996) attribue 25% du gaspillage alimentaire aux altérations causées par les microorganismes. Ce gaspillage peut intervenir à chaque étape du processus de fabrication d'un produit alimentaire et notamment au sein des usines agroalimentaires (FAO, 2013).

Les usines agroalimentaires peuvent être considérées comme des réservoirs de microorganismes (Carpentier et Cerf, 2011). En effet, les retours d'expériences des récentes épidémies à toxi-infections alimentaires collectives désignent clairement l'environnement de production comme source de contamination des denrées (Anses, 2020; ECDC-EFSA, 2018; Silva et al., 2018; Thomas et al., 2020). Afin d'assurer la sécurité et la qualité des aliments, les industriels de l'agroalimentaire se doivent de respecter les règlementations en vigueur ainsi que les critères microbiologiques définis (règlement (CE) n°178/2002 et n°2073/2005). Pour cela, ils ont l'obligation de réaliser des autocontrôles sur leurs matrices et leurs environnement de production dans le cadre de plans de maîtrise sanitaire. Ces autocontrôles ciblent essentiellement les flores pathogènes liées au type de produit et quelques indicateurs hygiéniques (*Enterobacteriaceae*, levures et moisissures). Cependant, les contrôles effectués ou les méthodes utilisées ne révèlent qu'une partie de la diversité microbiologique présente au sein des usines et ne s'intéressent pas directement aux flores d'altération (Bokulich et Mills, 2013). Ces contrôles reposent souvent sur des approches culturales aux conditions limitées, et le rôle des microorganismes dénombrés dans la sécurité et la qualité des produits reste non identifié. Certains microorganismes sont capables de s'installer et de résider durablement sur les surfaces de l'environnement de production suivant différentes stratégies de défense. Ces microorganismes sont souvent non pris en compte dans les autocontrôles de l'environnement de production. Bien que la majorité des bactéries résidentes ne soit pas pathogène, elles peuvent être altérantes et donc représenter un risque pour la qualité des denrées (Møretrø et Langsrud, 2017).

Il est donc important de mieux comprendre l'écologie microbienne de ce type d'environnement et de décrire les microorganismes résidents ainsi que leur rôle dans la microbiologie des aliments. Dans cet objectif, il est necessaire de disposer d'approches methodologiques permettant de mieux connaître la diversité du microbiote des usines agroalimentaires.

Le développement rapide des plateformes technologiques de séquençage à haut débit a rendu abordable en termes de coûts et de temps d'analyse les approches de type métaséquençage permettant de caractériser les communautés microbiennes en contexte alimentaire (Ercolini et al., 2013). Le séquençage non ciblé apparaît donc comme une piste privilégiée pour tenter de cartographier les usines agroalimentaires, d'analyser les communautés microbiennes présentes et de décrire leur rôle dans la sécurité et la qualité des aliments (Bokulich et al., 2016).

C'est dans ce contexte que s'inscrit le projet ALTEROBIO dans lequel j'ai réalisé ma thèse de doctorat. Ce projet, financé par la Région des Pays de la Loire, implique deux partenaires académiques et deux industriels :

- L'Unité Mixte de Recherche Secalim 1014 (Oniris - INRAE, Nantes)
- L'Institut Français de Recherche pour l'Exploitation de la Mer (Ifremer, Nantes)
- L'entreprise Mer Alliance Thai Union (Quimper)
- L'entreprise Biofortis Mérieux NutriSciences (Nantes)

L'objectif du projet ALTEROBIO est double. Premièrement, développer et valider une méthode moléculaire innovante (basée sur des approches de séquençage tel que le metabarcoding et la PCR quantitative). Cette méthodologie étant ensuite appliquée pour caractériser les microbiotes des usines et ainsi en réaliser une cartographie, afin d'identifier les points critiques de contamination et finalement de déterminer l'impact de l'environnement de production sur la qualité et la sécurité des denrées alimentaires. Deuxièmement, ce projet sert également de pilote à la mise en place au sein de Biofortis Mérieux NutriSciences d'un service d'analyse des flores environnementales par metabarcoding. C'est dans cet équilibre scientifique et industriel que s'inscrit mon travail de thèse CIFRE (Convention Industrielle de la Formation par la Recherche) que j'ai effectué au sein de l'entreprise Biofortis Mérieux NutriSciences et de l'UMR Secalim.

Ce manuscrit s'articule en quatre chapitres. En premier lieu, une synthèse bibliographique décrit le contexte scientifique de ce travail de thèse.

Cette première partie reprend les principaux enjeux sociétaux liés au gaspillage alimentaire, puis développe l'écologie microbienne des usines agroalimentaires ainsi que les méthodes et outils permettant de l'étudier. Cette synthèse bibliographique détaille également en quoi il est important de mieux connaître l'écologie microbienne des usines de production et ce que cette connaissance peut apporter dans la maîtrise de la sécurité et de la qualité des aliments.

La deuxième partie porte sur l'évaluation de méthodes de prélèvement de microorganismes, d'extraction d'ADN ainsi que de leur impact sur une analyse de metabarcoding. Ces résultats ont permis des choix méthodologiques pour analyser le microbiote de denrées alimentaires et d'échantillons d'environnement d'usine. Pour cela nous avons étudié une matrice : le saumon fumé et mis en place des dispositifs au sein d'un atelier de production favorisant la récupération de biofilms. Cette étude nous a permis d'identifier une méthode d'extraction d'ADN optimale pour ces deux types de matrices, mais également un dispositif de prélèvement des bactéries de surface.

Dans le chapitre III, nous avons décrit les communautés bactériennes de différents lots de saumon fumé, issus de trois usines, au cours de leur durée de vie microbiologique. Pour cela, une approche polyphasique composée de méthodes culturales, de metabarcoding et d'analyses chimiques a été mise en place. Au cours de ce chapitre nous avons mis en évidence une « signature » usine, suggérant que le microbiote d'un lot de saumon fumé est relié à son atelier de production.

La quatrième partie de ce travail a été consacrée à la description de communautés bactériennes au sein d'une usine de production de saumon fumé. Au cours de ce quatrième chapitre nous nous sommes intéressés à la relation microbiote environnemental et microbiote produit. De ce fait, nous avons tenté de mieux comprendre les dynamiques des bactéries environnementales et leur rôle dans la sécurité et la qualité des aliments.

Enfin, pour conclure, ce mémoire se termine par une discussion générale, une ouverture sur des perspectives quant aux problématiques que nous avons pu rencontrer et la proposition de pistes pour aider l'industrie agroalimentaire à mieux connaitre le microbiote des surfaces des ateliers de production en vue de réduire les risques d'altération des produits et ainsi lutter contre le gaspillage alimentaire.

Chapitre I. Synthèse bibliographique

I.1. Le gaspillage alimentaire

Chaque année plus de 30% de la masse des aliments produite dans le monde est perdue ou gaspillée. Le tonnage de perte que ce soit en matières premières ou en produits transformés, est estimé à 2,9 gigatonnes. Ce gaspillage alimentaire a de plus un impact négatif sur l'environnement (impact carbone et sur les ressources hydriques) (FAO, 2011).

Les pertes peuvent survenir à différentes étapes du cycle de vie d'un aliment (**Figure 1**):

- La production primaire (agricole, pêche, etc.) incluant la post-récolte et le stockage
- La transformation
- La distribution
- L'utilisation du produit chez le consommateur

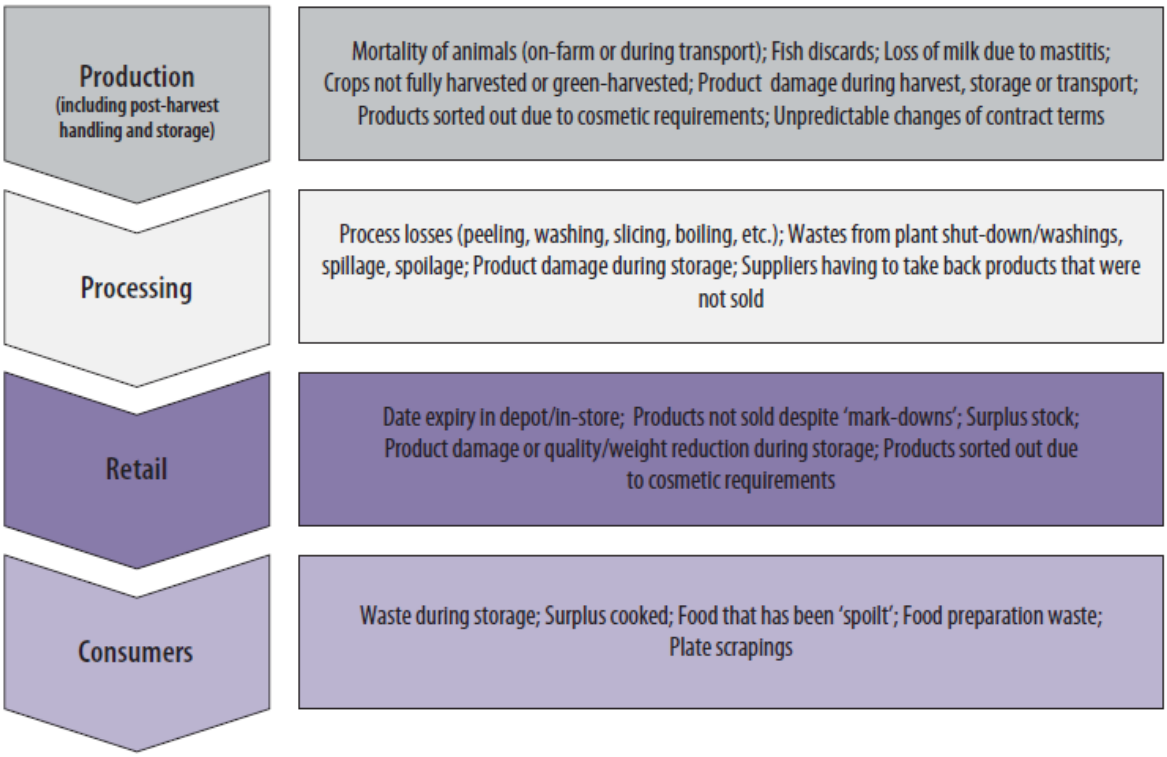

Figure 1. Sources du gaspillage alimentaire à chaque étape du cycle de vie d'un aliment (Food and Agriculture Organization of the United Nations, 2013)

En dépit de l'impact économique ce phénomène a d'énormes conséquences écologiques avec une empreinte carbone estimée à 3,3 gigatonnes de CO_2 (FAO, 2013). Dans le gaspillage des denrées alimentaires, l'altération microbienne joue un rôle important. En effet, Huis in't Veld (1996) postule que l'altération d'origine microbienne est responsable du quart du gaspillage mondial. L'altération est la résultante d'activités métaboliques microbiennes rendant les denrées alimentaires impropres à la consommation par une dégradation de leurs qualités organoleptiques (Remenant et al., 2015).

Chaque année, les phénomènes d'altération microbiologique des aliments sont à l'origine de la perte d'environ 200 millions de tonnes de denrées alimentaires (FAO, 2013). Ceci a pour conséquence pour les industries alimentaires (IAA) un manque à gagner et une perte de compétitivité. Cependant, le coût financier n'est pas le seul impact de ces pertes. En effet, les dépenses énergétiques (eau, électricité, empreinte carbone) constituent un coût environnemental très important. Au total, le coût des pertes et du gaspillage alimentaire annuel mondial est estimé à 2600 milliards de Dollars US, l'équivalent du produit intérieur brut français (Garot, 2015). De plus, une maîtrise moins rigoureuse des processus industriels favorise l'altération et donc le gaspillage auprès des distributeurs ainsi que chez le consommateur, représentant plus de 40% des pertes des denrées alimentaires dans les pays industrialisés (**Figure 2**). Concernant les pays en développement, 40% du gaspillage se fait au niveau industriel (FAO, 2011).

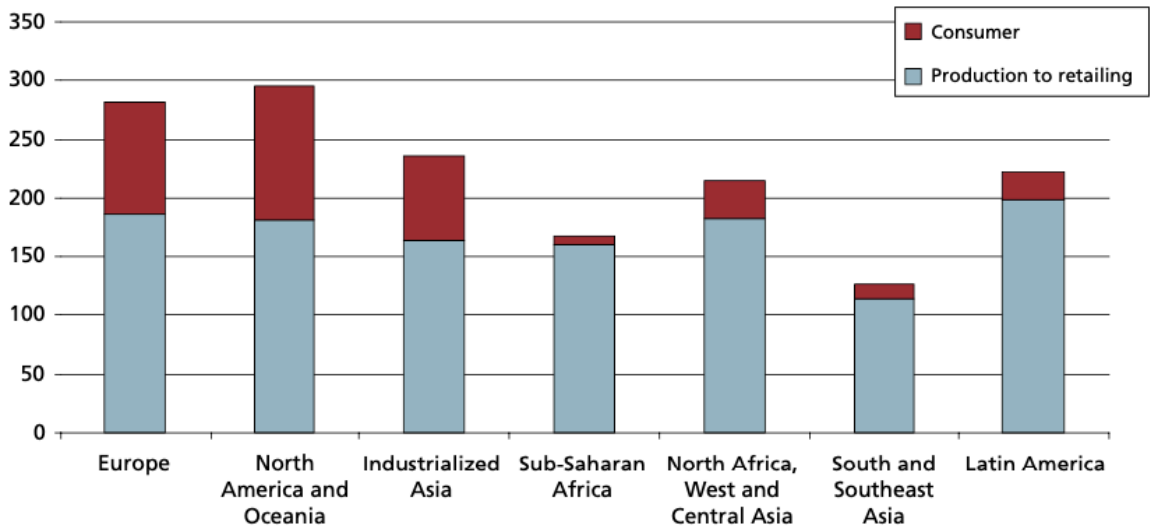

Figure 2. Pertes et gaspillage alimentaires par habitant (kg/an), aux stades de la consommation et de la pré-consommation (production et distribution), dans différentes régions du globe
(Food and Agriculture Organization of the United Nations, 2011).

Les différentes données rapportées par la Food and Agriculture Organization (FAO) caractérisent le rôle important qu'ont les industriels dans la lutte contre l'altération des aliments en vue de réduire le gaspillage. En effet, les usines agroalimentaires sont des réservoirs de microorganismes, pouvant être pathogènes ou altérants, capables de survivre dans ces environnements (Møretrø et Langsrud, 2017). Ceci suggère qu'un des moyens pour réduire le gaspillage alimentaire mondial est une meilleure maîtrise de la microbiologie environnementale, ce qui nécessite des connaissances approfondies en écologie microbienne de ces usines de transformation des aliments.

I.2. L'écologie microbienne

I.2.1. Le concept

La description, l'organisation des communautés microbiennes ont fait l'objet d'un grand nombre d'études notamment en écologie du sol, des océans, de l'air mais également dans le domaine de la santé (étude des flores intestinales, cutanées, etc.). L'écologie microbienne étudie les interactions de l'ensemble des microorganismes (microbiote) entre eux (biocénose) et avec leur environnement et ses constituants physico-chimiques (biotope) (Karl August Möbius, 1877). Cette science permet notamment de décrire la biodiversité des communautés microbiennes au sein d'écosystèmes complexes afin de mieux en comprendre leurs fonctionnements, leurs mécanismes et leurs dynamiques (Tansley, 1935). La biodiversité se définit comme l'étude de la diversité au sein d'un écosystème quel que soit le type d'organismes qui le compose (Bertrand et al., 2015). Ainsi, afin de mieux la décrire il est nécessaire de la qualifier et de la quantifier. Les concepts d'écologie et plus spécifiquement d'écologie microbienne, permettent de déterminer et/ou vérifier des hypothèses quant aux rôles, aux structures ou aux dynamiques des communautés de microorganismes au regard de conditions environnementales. Afin d'étudier le fonctionnement des microbiotes il est primordial, dans un premier temps de les analyser.

I.2.2. Les outils d'analyse en écologie microbienne

I.2.2.1. Évolution des techniques d'analyses

Que ce soit dans le domaine de la santé, de l'environnement ou de l'alimentaire, l'écologie microbienne nécessite d'identifier, de caractériser et de dénombrer l'ensemble des microorganismes associés à un écosystème ou à un individu.

Les premières études visant à décrire les microbiotes ont été réalisées selon des approches culturales consistant à isoler les microorganismes dans différentes conditions de culture. Ces approches, bien qu'ayant permis de mieux comprendre le comportement de microorganismes dans leur environnement sont limitées. En effet, comme le rapporte Amann et al., (1995), ces méthodes culturales entraînent différents biais sur les plans quantitatifs et qualitatifs. Ces auteurs stipulent que la culture de microorganismes ne permettrait d'apprécier que de 0,1 à 1% de leur diversité dans des écosystèmes marins, sols et sédiments. Le stress induit par certaines conditions environnementales, des conditions de cultures inadaptées vont limiter la cultivabilité d'une partie du microbiote (Staley et Konopka, 1985). Ces auteurs relatent qu'il existe une importante différence entre le nombre de colonies bactériennes provenant de milieux aquatiques dénombrées sur milieux de culture gélosés et le nombre de cellules observées par microscopie estimant ainsi à seulement 1% le nombre de cellules cultivables. Cette sous-estimation peut également concerner des microorganismes jamais isolés et cultivés à ce jour. Locey et Lennon (2016) estiment a environ 10^{12} le nombre d'espèces différentes de bactéries et d'archaebactéries alors que seules 12000 ont été identifiées (Vitorino et Bessa, 2018). Ces données suggèrent qu'il existe un champ immense de développement de connaissances sur la diversité microbienne environnementale.

Les approches de microbiologie culturale ne sont pas pertinentes à elles seules pour l'analyse des dynamiques et des interactions microbiennes dans des conditions environnementales non optimales pour la croissance des microorganismes. De plus, l'identification des microorganismes isolés nécessite des méthodes supplémentaires.

Le développement des techniques de biologie moléculaire et des méthodes culture-indépendantes à partir des années 1980 a permis une nouvelle approche dans l'analyse des microbiotes d'écosystèmes complexes (sol, matrice alimentaire, etc.) (Giraffa et Neviani, 2001). Ces approches moléculaires ont vu le jour grâce au développement de l'amplification de l'ADN par réaction en chaîne à la polymérase (PCR). La PCR a permis le développement de nouvelles techniques permettant de détecter et de quantifier spécifiquement les bactéries et donc leur abondance mais également de mieux apprécier leur diversité. Ces approches sont basées sur l'amplification d'un gène ubiquitaire spécifique de l'espèce tel que les gènes de l'ARNr 16S, *gyrB*, *recA*, *rpoB* pour les bactéries, l'ARNr 18S et les séquences ITS (Internal Transcript Spacer) pour les eucaryotes (Poirier et al., 2018).

Parmi ces approches la technique T-RFLP (Terminal Restriction Limitation Fragment Length Polymorphism) consiste en une digestion enzymatique de fragments de PCR d'un gène ubiquitaire suivie d'une comparaison des profils bactériens après électrophorèse. Par l'analyse T-RFLP de la diversité des fragments des gènes codant pour les ARNr 16S et 23S Vihavainen et al. (2007) ont pu identifier, dans un contexte de diversité microbienne au sein des aliments, les bactéries responsables de l'altération de viande de poulet conservée sous atmosphère modifiée. En effet, ces auteurs ont été capables de comparer les groupes de bactéries lactiques identifiés sur produits finis, les carcasses mais également la contamination par l'air de l'usine. Les résultats montrent que les bactéries lactiques altérant les produits finis n'ont pas pour origine la matière première et proviennent d'une contamination aéroportée. Ceci démontre l'importance de l'écologie microbienne de l'usine dans cette problématique.

D'autres techniques électrophorétiques sont également citées dans la littérature pour évaluer la diversité bactérienne au sein de différents types d'écosystèmes environnementaux (Muyzer, 1999; Hernán-Gómez et al., 2000; Ercolini, 2004; Agnelli et al., 2004; Kulhánková et al., 2006). Ces techniques comme la DGGE (Denaturing Gradient Gel Electrophoresis) ou la TTGE (Temporal Temperature Gel Electrophoresis) se basent sur une migration différentielle sous conditions dénaturantes d'amplicons du gène de l'ARNr 16S. Les profils de bandes obtenus, correspondent à des fragments d'ADNr 16S à forte identité de séquence assimilables à une espèce bactérienne. Ces profils sont représentatifs de la diversité bactérienne présente au sein de l'échantillon.

En microbiologie des aliments, la TTGE a pu être utilisée par exemple dans une approche polyphasique pour caractériser la communauté bactérienne de crevettes cuites décortiquées (Jaffrès et al., 2009). Dans ce travail, la TTGE a permis d'identifier des genres bactériens tels que *Psychrobacter* spp., capable d'altérer cette matrice, ces bactéries n'ayant pas pu être identifiées par une approche culturale. Ercolini et al. (2006) ont utilisé la DGGE pour identifier et mieux comprendre la dynamique des bactéries en lien avec l'altération de viande de bœuf conditionnée sous différentes atmosphères, stockée à 5°C durant 14 jours. Ces différents travaux, utilisant la TTGE et la DDGE qui sont des méthodes désormais peu utilisées, ont initié l'apport des approches moléculaires en compléments des approches culturales afin de mieux caractériser les communautés bactériennes dans les aliments.

L'apport de la biologie moléculaire permet également de cibler spécifiquement certaines bactéries préalablement identifiées au sein d'un microbiote.

En effet, la PCR quantitative (qPCR) permet par l'amplification d'un gène d'intérêt (spécifique ou ubiquitaire) de quantifier spécifiquement une bactérie ou plusieurs espèces présentes au sein d'un échantillon. Dans leur revue portant sur l'étude des communautés microbiennes des eaux de stations d'épuration, Kim et al. (2013) montrent l'importance de la qPCR pour dépasser les limites de la microbiologie classique comme dans le cas des bactéries anammox qui réalisent une oxydation anoxique de l'ammoniaque avec une croissance lente (temps de génération de 11 jours). Dans cette communauté bactérienne anammox, la quantification du gène de l'ARNr 16S mais également de marqueurs génétiques spécifiques ont permis de mettre en évidence une forte abondance de *Brocadia anammoxidans* directement à partir d'échantillons d'eaux. L'utilisation de la qPCR dans ce cas spécifique montre clairement l'avantage de la biologie moléculaire pour détecter et quantifier des microorganismes d'intérêt dans des conditions limitantes pour les approches culturales classiques.

Ces approches basées sur l'ADN, apportent donc des informations supplémentaires et montrent un grand intérêt pour éviter la multiplicité des milieux de cultures et des conditions de croissance qui vont réduire la vision du microbiote de matrices et d'environnements complexes. Cependant, ces dernières ne sont pas sans limites. En effet, les bandes des profils électrophorétiques obtenus en TTGE ou DDGE peuvent ne pas être spécifiques à un microorganisme (Al-Mailem et al., 2017). De plus ces méthodes possèdent une faible sensibilité concernant la détection d'organismes rares dans la communauté observée et privilégient les individus les plus abondants (Muyzer, 1999). L'identification des organismes nécessite également une étape supplémentaire, qui implique l'excision des bandes obtenues et un séquençage d'amplicons par méthode Sanger, pouvant s'avérer lourde et chronophage lors d'études à grandes échelles.

Le développement des techniques de séquençage et notamment des plateformes de séquençage de deuxième génération au milieu des années 2000 ont marqué également une certaine révolution dans les pratiques en écologie microbienne (Taïb, 2013).

I.2.2.2. Le séquençage de nouvelle génération

Le développement des technologies de séquençage de l'ADN survenu ces dix dernières années a eu un impact considérable sur l'utilisation de l'information génétique en écologie microbienne.

Que ce soit en santé humaine ou en sécurité sanitaire des aliments, l'utilisation des techniques de séquençage de dernière génération (NGS pour Next Generation Sequencing) s'est généralisée grâce aux développements d'équipements, de réactifs et à la réduction des coûts (**Figure 3**) (NIH, 2021).

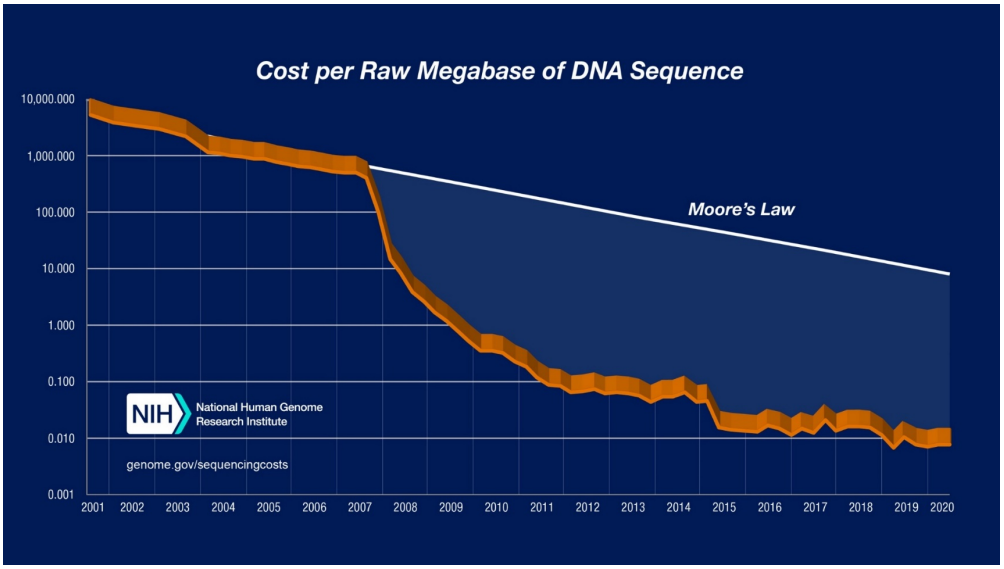

Figure 3. Évolution du coût en US Dollars des données de séquençage entre 2000 et 2020 (NIH, 2021)

On distingue au sein des NGS deux grands types de technologies de séquençage disponibles :

- Les technologies « short-reads »
- Les technologies « long-reads »

Goodwin et al., (2016) dans sa revue portant sur l'évolution des technologies de séquençage décrit de manière très détaillée les chimies de séquençage ainsi que les différentes plateformes. Cette description est résumée dans le **Tableau I**.

Le développement des NGS a donc facilité l'accès de manière plus abordable et plus rapide à un très grand nombre de séquences, en une seule analyse permettant ainsi d'apporter une complémentarité aux méthodes traditionnelles d'écologie microbienne. En effet, les plateformes Illumina sont capables de générer entre 8 Gb et 1000 Gb, permettant ainsi une analyse à haut débit de plusieurs échantillons de microbiotes. Ces plateformes, bien qu'onéreuses, sont les plus précises à ce jour avec un taux d'erreur de séquençage de 1‰ bases.

Les différentes plateformes ont évolué des NGS jusqu'aux séquenceurs de 3ème génération correspondant aux technologies « long reads ». Bien que capables de générer des séquences de très longues tailles, la qualité de séquences des plateformes « long reads » notamment d'Oxford Nanopore Technologies (phred score de Q15) ne sont pas encore optimales pour des études d'écologie microbienne. En effet, elles nécessitent une expertise bioinformatique importante pour corriger les erreurs de séquençage. De plus l'utilisation de ces plateformes reste coûteuse.

Tableau I. Comparaisons des différentes plateformes de séquençage disponibles sur le marché. D'après Goodwin et al., (2016).

Société	**Roche**	**Illumina**			**Thermo Fisher Scientific**		**Pacific Biosciences**	**Oxford Nanopore Technology**
Plateforme	454	HiSeq	Next Seq	MiSeq	Ion PGM	Ion Proton	Sequel	MinIon/ GridIon
Technologie	Short-reads						Long-reads	
Méthode d'amplification	PCR en émulsion	Bridge PCR			PCR en émulsion		Absence d'amplification	
Méthode de séquençage	Synthèse						Séquençage en temps réel	
Capcité de séquençage/run	700 Mb	100-1000 Gb	16-120 Gb	8 Gb	100 Mb-2 Gb	10-32 Gb	3.5-7 Gb	Jusqu'à 1.5 Gb
Taille moyenne des séquences (bases)	700	2x150	2x150	2x250 ou 2x300	400	100 à 200	8-12K	Jusqu'à 200K
Phred Score	Q20	Q30	Q30	Q30	Q20	Q20	Q33	Q15
Taux d'erreur	1%	1‰	1‰	1‰	1%	1%	≈ 1‰	10%
Temps de séquençage	23 h	2 - 11 jours	26 h	26 h	4-7 h	4 h	0.5-6 h	Jusqu'à 48h

Ces technologies innovantes offrent donc de multiples applications qui peuvent être regroupées en deux grandes approches : non-ciblées et ciblées (**Figure 4**). L'approche non ciblée consiste à extraire l'ADN d'un échantillon complexe et de séquencer tout le matériel génétique extrait. On distingue notamment dans cette catégorie le séquençage de génome entier ou « whole genome sequencing » (WGS) ainsi que la métagénomique. L'approche ciblée quant à elle nécessite une étape préalable d'amplification d'un gène spécifique par PCR et correspond au séquençage de ces amplicons (**Figure 4**). On distingue dans cette catégorie d'approche le metabarcoding.

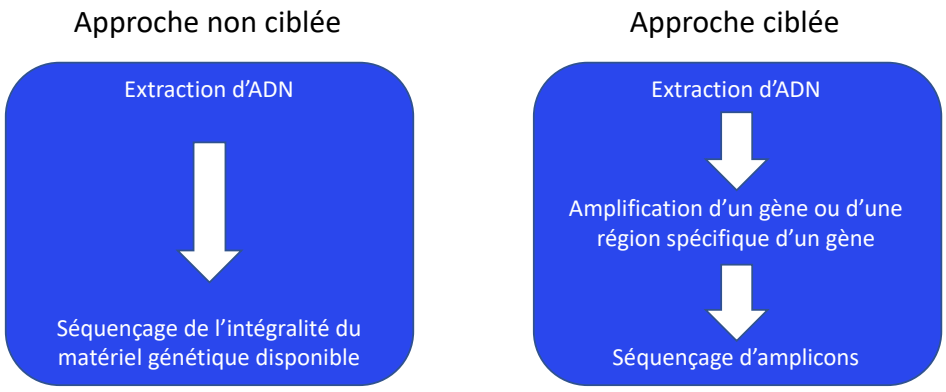

Figure 4. Approches généralisées de séquençage

I.2.2.2.1. Approche de séquençage non ciblée

Cette approche de séquençage correspond au séquençage du matériel génétique extrait disponible. Applicable à de nombreux domaines, en microbiologie, elle peut être utilisée pour séquencer le génome d'isolats microbiens en culture pure (WGS) ou de communautés microbiennes complexes (métagénomique).

Le WGS, en microbiologie des aliments, est aujourd'hui utilisé pour la surveillance des dangers microbiens isolés des produits alimentaires ou des environnements de production (Allard et al., 2018). L'apport du WGS combiné au développement de la bioinformatique a ouvert considérablement la voie à une meilleure maîtrise de la sécurité sanitaire des aliments. Cette approche a notamment permis de développer des schémas ou des méthodes de typage bactérien les plus précis à ce jour et constamment utilisés en épidémiologie pour l'attribution de source de contamination (Gerner-Smidt et al., 2012). En effet, les récentes épidémies à *Listeria monocytogenes* en Afrique du Sud et en Europe témoignent de l'efficacité et de l'importance de ces nouvelles approches dans ce domaine (ECDC-EFSA, 2018; Thomas et al., 2020). En outre, les analyses de whole genome multi locus sequence typing (wgMLST) ou de single nucleotide polymorphism (SNP) permettent d'obtenir un niveau de discrimination optimal entre bactéries de la même espèce, facilitant également les autocontrôles sanitaires au sein des ateliers de production (Jagadeesan et al., 2019).

Une autre approche de séquençage non ciblée est la métagénomique qui en microbiologie a pour objectif d'étudier les génomes d'un ensemble complexe de microorganismes constituant un microbiote au sein d'un environnement spécifique. La quantité de données à traiter est donc très importante et permet d'obtenir beaucoup d'informations sur la composition du microbiote par exemple mais également de prédire des voies métaboliques ou la présence de gènes d'antibiorésistance (Jagadeesan et al., 2019).

Le séquençage des communautés microbiennes complexes peut également être utilisé en microbiologie des aliments pour la détection et la caractérisation des pathogènes. Il permet de déterminer la diversité et l'abondance des microorganismes présents au sein d'un échantillon. Cette approche multi-informative a été notamment éprouvée sur des prélèvements de surface et en contexte aliment tels que des produits laitiers fermentés, produits carnés, végétaux mais également sur des surfaces d'ateliers de brassage, de vinification ou de production de fromage. (Kergourlay et al., 2015; Leonard et al., 2015; Walsh et al., 2017; Yang et al., 2016).

I.2.2.2.2. Approche ciblée : Le metabarcoding

Tout comme les approches DGGE, TTGE le metabarcoding consiste en l'amplification par PCR d'un gène ubiquitaire représentatif de la population microbienne étudiée tel que le gène de l'ARNr 16S. Ce gène est reconnu comme le standard de l'identification des bactéries et l'analyse de leur diversité (Quast et al., 2013). Il s'agit d'un gène de courte taille (environ 1500 pb) et dont la séquence est composée de 9 régions dites hypervariables et espèces dépendantes, encadrées par des régions conservées facilitant le dessin d'amorces spécifiques (Yarza et al., 2014). Les régions hypervariables (V1 à V9) représentées **Figure 5** permettent donc de distinguer les espèces bactériennes.

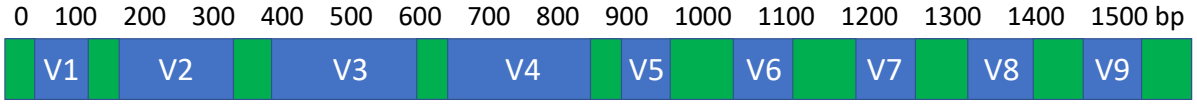

Figure 5. Représentation du gène de l'ARNr 16S composé de 9 régions hypervariables (V1 à V9 représentées en bleu) et de régions conservées (représentées en vert)

Le choix de la région peut induire un biais quant à la diversité bactérienne étudiée (Kim et al., 2011; Zhang et al., 2017). En effet, par l'étude de la variabilité au sein de chaque famille taxonomique, Mysara et al. (2017) mettent en évidence le niveau de conservation d'un taxa en fonction de la région étudiée. Cette donnée a donc une importance au regard de la communauté à analyser. En effet le choix de la région du gène de l'ARNr 16S à séquencer peut-être déterminant dans une analyse comparative du microbiote. L'avantage de cette approche vis-à-vis des précédentes techniques d'écologie microbienne est indéniablement l'analyse à haut débit d'un grand nombre d'échantillons et, par l'apport de la bioinformatique, une identification des différents membres de la communauté microbienne analysée.

L'étape clé de ces approches de séquençage concerne donc l'analyse bioinformatique des données générées. Le metabarcoding a largement été utilisé en microbiologie des aliments, notamment pour mieux comprendre les phénomènes de fermentation ou le comportement d'un produit au cours de sa durée de vie (Rouger, 2017). De plus, cette approche nous paraît être une première étape dans la compréhension de l'écologie microbienne des environnements de production. Pour cette raison, nous avons choisi de développer le metabarcoding du gène de l'ARN 16S au cours de ce travail.

I.2.2.2.3. Analyses des données de séquençage

Le metabarcoding permet donc de générer des séquences brutes d'amplicons. Afin d'en extraire des informations sur les communautés microbiennes étudiées, ces séquences brutes suivent ensuite un traitement et une analyse bioinformatique selon un pipeline dédié (Jagadeesan et al., 2019). Plusieurs pipelines d'analyses sont disponibles dans la littérature et dont le fonctionnement étape par étape est bien documenté tels que : QIIME (Bolyen et al., 2019), Mothur (Schloss et al., 2009), FROGS (Escudié et al., 2017). Malgré quelques variantes, les différents pipelines suivent un cheminement d'étapes que l'on pourrait résumer tel qu'illustré **Figure 6**.

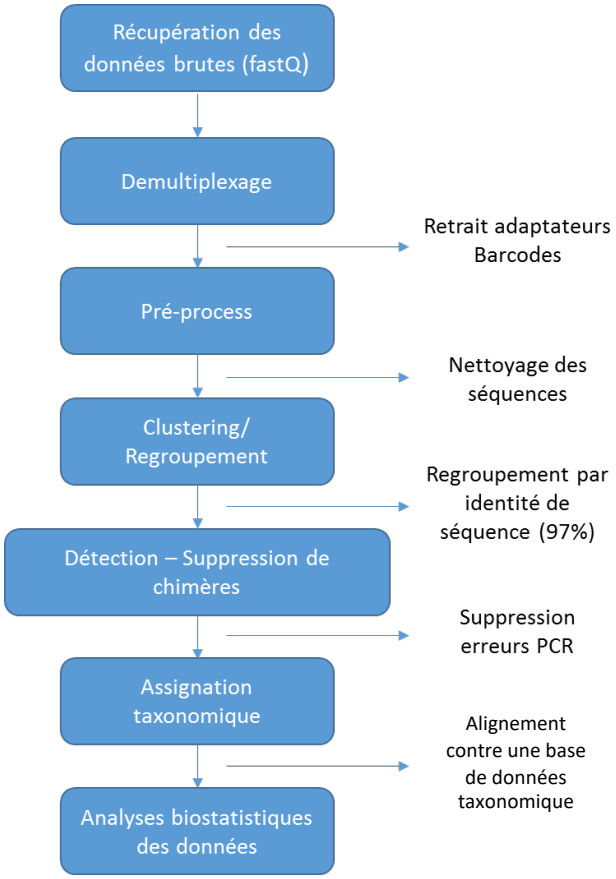

Figure 6. Processus d'analyses bioinformatique de données issues de metabarcoding

En résumé, les traitements bioinformatiques appliqués vont permettre de générer une table de comptage d'organismes permettant d'établir une quantification relative et une table d'assignation taxonomique permettant de proposer une identification des genres ou espèces bactériennes présentes dans les échantillons.

La table de comptage ou table d'OTUs (operation taxonomic units) regroupe l'ensemble des clusters de séquences à 97% d'identité.

Ce concept d'OTUs (Sneath, 2005) se définit comme un groupe d'organismes reliés phylogénétiquement et analysés sans spécification de rang taxonomique (Bertrand et al., 2015). Détecter, identifier les microbiotes est une première étape en écologie microbienne. Il convient ensuite d'analyser et d'interpréter les données obtenues et ainsi d'étudier des concepts d'écologie microbienne pour mieux comprendre les dynamiques microbiennes.

I.2.3. Analyse en écologie microbienne

L'analyse des communautés microbiennes ou de microbiote peut se faire à différentes échelles. Les concepts permettant l'étude d'un écosystème font appels à deux types d'approche : la diversité alpha (α-diversité) et la diversité bêta (β-diversité). L'α-diversité étudie une communauté à petite échelle en décrivant les différents individus qui la composent. La β-diversité quant à elle, permet de comparer les communautés entre elles sur une plus grande échelle.

I.2.3.1. La diversité alpha

La diversité alpha permet la quantification de la biodiversité au sein de communautés microbiennes. Pour cela des indices de biodiversité peuvent être calculés et déterminés. Ces indices sont de deux types : les indices primaires et les indices composites (Bertrand et al., 2015). Il existe de nombreux indices de diversité alpha mais nous détaillerons uniquement les indices utilsés au cours de ce travail et résumés **Tableau II**.

Tableau II. Description des indices de diversité alpha étudiés dans ce travail.

Indices		Formules
Indices primaires	Nombre d'espèces observées (Observed OTUs)	$R = S_0$ S_0 est le nombre de taxa observé au moins une fois dans un échantillon
	Chao1	$S_{Chao1} = S_{obs} + \frac{F_1(F_1 - 1)}{2(F_2 + 1)}$ F_1 et F_2 correspondent à la fréquence des singletons et doubletons (espèces retrouvées 1 ou 2 fois) et Sobs le nombre d'espèces totales observées.
Indices composites	Abondance relative	$p(i) = \frac{N_i}{N}$ $p(i)$ est la proportion d'individu appartenant à l'ième espèce. N_i est le nombre d'individu appartenant à l'*i*ème espèce N est le nombre total d'individus
	Indice de Shannon	$H' = -\sum_{n=1}^{S} p(i)\ln(p(i))$ S = nombre d'espèces i varie entre 1 et S p(i) est la proportion d'individu appartenant à l'ième espèce soit l'abondance relative.

I.2.3.1.1. Les indices primaires

Les indices primaires permettent de dénombrer le nombre d'espèces composant une communauté ; on parle ici d'indice de richesse. Le nombre d'espèces observées (observed OTUs) correspond tout simplement au nombre d'OTUs comptabilisés au sein d'un échantillon. L'indice de Chao1 (Chao, 1984) est un estimateur de richesse en espèce d'une communauté, la construction de cet indice permet de mieux apprécier les espèces peu abondantes. L'utilisation de l'indice de Chao1 peut être donc particulièrement intéressante dans le cadre d'études sur des échantillons paucimicrobiens. En effet, des biais d'estimation liés à la population sous dominante peuvent être ainsi évités (Kim et al., 2017).

Décrire des communautés microbiennes, ne se limite pas au simple fait d'en quantifier le nombre d'espèces qu'elles renferment. En effet, deux communautés peuvent avoir une richesse équivalente mais des compositions ou équilibres de compositions très différents. Il y a donc un besoin de mieux caractériser les représentants d'une communauté à l'aide notamment des indices composites.

I.2.3.1.2. Les indices composites

L'abondance relative permet de décrire la proportion d'une espèce observée au sein d'une communauté par rapport au nombre total d'individus de toutes les espèces confondues.

Cette proportion permet d'observer les taxons majoritaires ou minoritaires et d'observer les dynamiques des populations bactériennes au cours du temps ou selon un traitement particulier. L'indice de Shannon est issu de la théorie de l'information ainsi que des télécommunications (Shannon, 1948). Cet indice permet d'évaluer l'équitabilité des espèces présentes au sein d'une communauté. En outre, cet indice, basé sur l'abondance relative, permet de définir si une communauté bactérienne est composée d'espèces à égales abondances relatives ou de déterminer des populations dominantes ou sous-dominantes. Plus l'indice de Shannon est élevé, plus l'abondance relative des individus qui composent une communauté bactérienne est équitable.

Il existe divers indices permettant d'apprécier la diversité d'une communauté, cependant tous ces indices sont construits sur une même faiblesse: l'identité de l'individu n'est pas prise en compte (Bertrand et al., 2015). Le calcul de cet indice peut donc être biaisé. Ces différents indices d'alpha diversité permettent donc de caractériser la richesse et l'équitabilité décrivant la diversité d'une communauté microbienne.

La richesse, déterminée par les indices primaires permet donc d'apprécier la quantité d'espèces présentes au sein d'une communauté tandis que l'équitabilité permet de déterminer la proportion de chaque espèce (**Figure 7**).

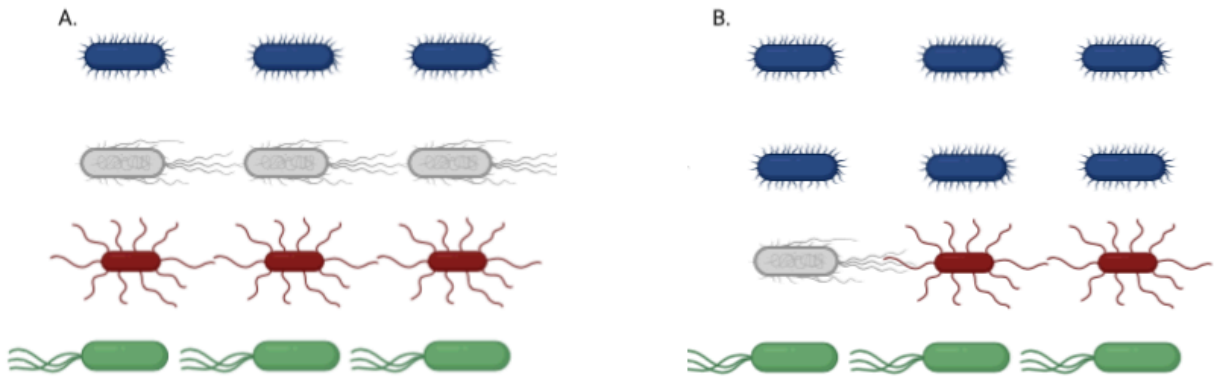

Figure 7. Richesse et équitabilité de deux communautés bactériennes. Les deux communautés A et B possèdent la même richesse (4 espèces chacune). Cependant la communauté A est plus équitablement distribuée que la communauté B. Adapté de Kim et al. (2017).

L'étude statistique de ces différents indices permet donc d'évaluer des effets, des dynamiques sur la structure d'une communauté. Ainsi, en étudiant les effets de la réduction de sel et de l'atmosphère de conditionnement (sous-vide ou modifiée) sur le microbiote de saucisses de porc crues, Fougy et al. (2016) ont pu, par l'analyse d'alpha diversité, mettre en évidence des différences significatives de diversité et de richesse microbienne.

En effet, ces auteurs ont observé que la richesse des communautés des produits était plus élevée en conditionnement sous-vide. De plus, la concentration en sel induisait une chute de l'équitabilité des communautés se traduisant par une abondance relative plus élevée de l'entérobactérie *Serratia proteomaculans*.

Afin de comparer des ensembles de communautés entre eux, l'α-diversité seule ne suffit pas. D'autres paramètres basés sur des distances doivent être utilisées pour répondre à cette question. On parle alors de diversité bêta (β-diversité).

I.2.3.2. La diversité bêta

L'analyse de la diversité bêta (β-diversité) fait appel à des statistiques non paramétriques. Bertrand et al., (2015) indiquent que les données analysées en écologie microbienne, peuvent avoir une structure trop complexe pour être traitées par des statistiques conventionnelles. Des approches multivariées faisant appel à des approches exploratoires donnent une autre perspective de la structure des microbiotes.

Ces approches exploratoires sont basées sur des matrices de distances, reflétant la dissimilarité entre deux communautés prise deux à deux. Il existe plusieurs matrices de distances utilisées en écologie microbienne. Chacune d'entre elles capture des caractéristiques différentes des communautés microbiennes. Les principales matrices utilisées en écologie microbienne sont compositionnelles ou phylogénétiques. Il existe diverses distances permettant de comparer des communautés entre-elles. Cependant, nous avons choisi de ne développer uniquement que les distances utilisées au cours de ce travail et résumées **Tableau III**.

Tableau III. Description des indices et distances de diversité bêta étudiés au cours de ce travail.

Distances		Formules
Indices compositionnels	Distance de Jaccard	$d_{\text{Jac}} = \frac{\sum_s 1_{\{n_s^1 > 0,\, n_s^2 = 0\}} + 1_{\{n_s^2 > 0,\, n_s^1 = 0\}}}{\sum_s 1_{\{n_s^1 + n_s^2 > 0\}}}$ n_s^1 le nombre total d'espèces s (s = 1, …, S) dans la communauté 1. n_s^2 le nombre total d'espèces s (s = 1, …, S) dans la communauté 2.
	Distance de Bray-Curtis	$d_{\text{BC}} = \frac{\sum_s \lvert n_s^1 - n_s^2 \rvert}{\sum_s \lvert n_s^1 + n_s^2 \rvert}$ n_s^1 le total d'espèces s (s = 1, …, S) dans la communauté 1. n_s^2 le total d'espèces s (s = 1, …, S) dans la communauté 2
Indices phylogénétiques	UniFrac pondérée	$d_{\text{WUF}} = \frac{\sum_e l_e \lvert p_e - q_e \rvert}{\sum_e l_e \, (p_e + \, q_e)}$ l_e la longueur de chaque branche e de l'arbre phylogénétique de la communauté 1 ou 2 p_e la fraction de la communauté 1 sous la branche *e*. q_e la fraction de la communauté 2 sous la branche *e*.
	UniFrac (non pondérée)	$d_{\text{UF}} = \frac{\sum_e l_e \left[1_{\{p_e > 0,\, q_e = 0\}} + 1_{\{q_e > 0,\, p_e = 0\}}\right]}{\sum_e l_e \times 1_{\{p_e + q_e > 0\}}}$ l_e la longueur de chaque branche e de l'arbre phylogénétique de la communauté 1 ou 2 p_e la fraction de la communauté 1 sous la branche *e*. q_e la fraction de la communauté 2 sous la branche *e*.

I.2.3.2.1. Les indices compositionnels

I.2.3.2.1.1. Distance/Indice de Jaccard

La distance de Jaccard a été proposée par Paul Jaccard en 1901 pour déterminer les fractions d'espèces spécifiques à une communauté ou à une autre (Jaccard, 1901). Cet indice qualitatif permet d'identifier si deux communautés microbiennes partagent ou non les mêmes espèces. L'utilisation de cet indice binaire ne prend pas en compte la diversité au sein d'une communauté. Il existe donc d'autres indices évaluant également la proportion des espèces. Cette distance qualitative permet donc de comparer les communautés sur la base de l'absence ou de la présence d'OTUs.

I.2.3.2.1.2. Indice de Bray Curtis

L'indice de Bray Curtis (Bray et Curtis, 1957), publié pour la première fois dans le cadre d'une étude portant sur la diversité d'arbres des forêts du Wisconsin, permet d'ordonner les communautés étudiées par espèces ainsi que leur proportion. Cette distance apporte donc une information supplémentaire à la distance de Jaccard. Ceci permet de comparer les communautés, non-seulement sur leurs membres, mais également en tenant compte de leur abondance relative.

I.2.3.2.2. Indices phylogénétiques

Les indices phylogénétiques peuvent être déterminés à partir des séquences d'ADN obtenus. Ces approches se basent sur la construction d'un arbre phylogénétique et permettent de manière plus fine d'étudier les divergences entre OTUs. Ces indices ont été proposés par Lozupone et Knight, (2005) et se basent sur la distance UniFrac. On distingue deux types d'indices :

I.2.3.2.2.1. Distance UniFrac pondérée (weighted uniFrac)

La distance UniFrac pondérée mesure les fractions de l'arbre phylogénétique spécifiques à une communauté ou à une autre et prend également en compte l'abondance relative des taxa. Il s'agit d'un indice quantitatif. Cette distance est le pendant de la distance de Bray-Curtis en prenant une donnée supplémentaire liée aux liens phylogenétiques

I.2.3.2.2.2. Distance UniFrac (non pondérée)

A contrario de la distance UniFrac pondérée, la distance UniFrac s'intéresse uniquement aux fractions de l'arbre phylogénétiques spécifiques à une communauté ou à une autre. Cette distance qualitative est donc le pendant de la distance de Jaccard. La phylogénie apporte donc une profondeur supplémentaire quant à la comparaison des communautés microbiennes.

Ces analyses de β-diversité permettent de calculer des matrices de similarité ou de dissimilarité. Afin de visualiser la structure des communautés par ces matrices, il est nécessaire de les interpréter par des méthodes de clustering ou encore d'analyses en coordonnées principales (PCoA). Ces méthodes permettent de visualiser, dans l'espace, les distances écologiques entre les communautés microbiennes analysées, favorisant ainsi l'observation de l'effet de variables étudiées (impact d'une thérapie, impact d'une atmosphère de conservation des aliments, etc.). Par exemple, Omori et al., (2017) a étudié le microbiote fécal de chiens atteints de lymphomes intestinaux, de maladies intestinales inflammatoires et de chiens sains.

Par le calcul d'une distance UniFrac et une visualisation par PCoA, les auteurs ont pu mettre en évidence des OTUs partagés entre les sujets sains et atteints de maladies inflammatoires ; mais également une communauté spécifique aux chiens atteints de lymphomes intestinaux. Ces approches de β-diversité, bien connues et mises en place dans diverses études d'écologie microbienne permettent facilement de mettre en perspective les données et de mieux comprendre les dynamiques microbiennes.

L'application de ces concepts, combinés à l'apport des données de séquençage apportent donc une perspective supplémentaire notamment dans l'écologie. Cependant, les technologies de séquençage ne sont pas sans défaut et plusieurs paramètres peuvent amener de la variabilité dans les analyses. En effet, comme stipulé dans l'article de Knight et al. (2018) sur les pratiques analytiques, chaque étape du prélèvement au séquençage peut influencer le résultat des analyses de metabarcoding. En effet, en comparant deux protocoles d'extraction de microbiote fécal, Wesolowska-Andersen et al. (2014) ont pu mettre en évidence de fortes différences sur l'abondance relative des différents taxa. En plus de l'extraction d'ADN, le choix du gène ou de la région du gène de l'ARNr 16S peut avoir un fort impact sur l'interprétation de l'analyse de la diversité. En comparant notamment les régions V1-V2 et V4-V5 sur différentes plateformes de séquençage Fougy et al. (2016) ont mis en évidence différents biais suggérant un besoin de standardisation. Ce qui permettrait de comparer plus facilement les différentes études.

Au-delà de l'aspect technique, les analyses de bioinformatique peuvent également influencer les résultats d'analyses de metabarcoding. Cette influence a notamment été montrée par Rouger et al., (2018) en comparant 3 différents échantillons de microbiotes issus de viande de poulet avec différents pipeline et base de données taxonomiques. Selon l'échantillon, les différences entre les approches pouvaient être significatives tant en termes de diversité/d'équitabilité que d'identification de genres bactériens.

Malgré des développements/choix méthodologiques à mettre en place ainsi qu'une standardisation des analyses, les approches de séquençages sont des outils pertinents pouvant apporter une valeur ajoutée dans la compréhension de l'écologie microbienne des surfaces d'usines agroalimentaires. Dans une ambition de mieux comprendre l'écologie des usines, les dynamiques des microbiotes (flores pathogènes ou d'altération), ces technologies de séquençage peuvent apporter de nouvelles connaissances clés pour une meilleure gestion de la qualité et de la sécurité des aliments.

I.3. Écologie microbienne des surfaces dans les industries alimentaires

I.3.1. Introduction

Les professionnels de l'industrie agroalimentaire se doivent de commercialiser des denrées alimentaires répondant aux normes sanitaires en vigueur et relatives à la catégorie du produit fabriqué. En Europe, le paquet hygiène entré en vigueur en 2006, fixe les exigences en matière d'hygiène pour les aliments destinés à la nutrition humaine ou animale (règlement n°178/2002). La règlementation européenne est encadrée par le guide de bonnes pratiques d'hygiène ainsi que la mise en place de démarches HACCP (Hazard Analysis Critical Control Point) (Anses, 2020a). L'application de ce type de référentiel permet de guider les professionnels pour maîtriser la sécurité sanitaire des aliments et respecter la règlementation. De plus, en France, la mise en place de la loi EGAlim n°2018-938, incite les industriels à disposer d'une stratégie de surveillance environnementale. Désormais les industriels doivent prévenir immédiatement les autorités compétentes en cas d'autocontrôle environnemental défavorable et ainsi déclarer tout pathogène détecté dans l'environnement de production. Si la problématique des flores pathogènes est bien encadrée, il n'existe cependant aucun critère microbiologique sur les contaminations de surface dans les ateliers de production. Ce microbiote de surface peut heberger des microorganismes pouvant favoriser le developpement de bactéries pathogènes et/ou des bactéries altérantes jouant ainsi un rôle important dans la maîtrise de la sécurité et de la qualité des denrées alimentaires.

Les bâtiments tels que les ateliers de transformations des aliments constituent des écosystèmes complexes. La nature des matériaux et les différents flux (air, mouvement) conditionnent les communautés microbiennes pouvant coloniser et résider dans ces environnements.

Différentes études portant sur des environnements tels que les hôpitaux, les bureaux et les foyers montrent que le microbiote environnemental peut impacter la santé des occupants (Bokulich et al., 2013a; Kembel et al., 2014; Lax et al., 2014). L'influence de ces communautés microbiennes sur l'Homme ou sur ses activités (production alimentaire) présente un intérêt croissant dans la communauté scientifique. Bien que tout microorganisme puisse avoir un impact sur la qualité et la sécurité des aliments, les travaux de cette thèse se focalisent uniquement sur les communautés bactériennes.

L'usine agroalimentaire est constituée de différentes surfaces soumises à plusieurs contraintes environnementales. Ces conditions peuvent entraîner des stress liés au froid, à l'humidité, à des procédures quotidiennes de nettoyage et de désinfection. Des communautés bactériennes, sont fréquemment retrouvées sur les surfaces de certains ateliers de transformation alimentaire et ce, malgré les procédures d'hygiène, de désinfection et le maintien d'une température basse dans ces environnements (Møretrø et Langsrud, 2017).

La conception hygiénique et la présence de souillures (nutriments) peuvent favoriser l'adhésion et la colonisation des surfaces par les bactéries. Ces dernières, apportées par les opérateurs ou les matières premières peuvent donc s'installer dans ces environnements. Les facteurs permettant l'installation de communautés bactérienne sont : (i) la présence de niches difficiles à nettoyer où se maintient un environnement propice au développement bactérien, (ii) une habileté des microorganismes à s'adapter rapidement à leur environnement grâce à différentes propriétés comme la capacité à élaborer et à s'associer à des biofilms, (iii) une résistance ou une adaptation accrue aux désinfectants et aux variations milieux (température, pH) (Ferreira et al., 2014; Orsi et al., 2011). Ces conditions de stress induisent également la formation de cellules stressées, viables, incapables de croître sur milieux gélosés, mais pouvant recouvrer leur capacité de croissance en conditions favorables. Ces cellules sont dites viables mais non cultivables (VNC) et peuvent également faire partie de biofilms et former des communautés comprenant des bactéries pathogènes et/ou d'altération.

Ces bactéries de surface peuvent par transfert via les équipements ou les surfaces, de manière directe ou indirecte, contaminer les matrices (Ferreira et al., 2014; Midelet et Carpentier, 2002). Les récentes toxi-infections alimentaires collectives (TIAC) européennes à *Salmonella* spp. ou *Listeria monocytogenes* impliquant la persistance de souches dans des sites complexes à nettoyer illustrent tout à fait ce concept de niches et d'adaptation bactérienne aux conditions stringentes que subissent les communautés bactériennes dans les usines (EFSA et ECDC, 2018; Silva et al., 2018). Le retour d'expérience de ces récentes TIAC met clairement en évidence le rôle de l'environnement de production en tant que réservoir de contamination. Fin 2017, la contamination de poudre de lait pour nourrissons par *Salmonella* Agona en France a été associée à une contamination environnementale d'une tour de séchage (Anses, 2020b). Les isolats bactériens identifiés ont également été reliés à un incident de 2005 (Silva et al., 2018).

La plus grande épidémie de listériose dans le monde décrite à ce jour s'est déroulée en 2017 en Afrique du sud (OMS, 2018). La consommation d'un produit prêt à consommer, le « French Polony », contaminé par une souche de *Listeria monocytogenes* a entraîné 1060 cas de listériose dont 216 mortels. La souche de *Listeria monocytogenes* a été identifiée dans l'environnement de production d'une seule usine. La source de contamination a été mise en évidence lors du conditionnement des produits. Les pathogènes persistants sur les surfaces étaient donc transférés par contact sur les produits. Ces exemples d'épidémies demontrent le rôle de l'ecologie microbienne de l'environnement de production sur la qualité et la sécurité des denrées alimentaires.

Bien que pouvant présenter un risque pour les denrées, il est aujourd'hui bien connu que l'ensemble d'une communauté microbienne d'un produit alimentaire ou bien isolée de son environnement de production peut également avoir des impacts positifs et recherchés. De fait, il a été montré dans le cadre de produits fermentés, notamment pendant la fabrication de fromages, que le microbiote des surfaces d'ateliers d'affinage, par transfert de consortia microbiens, pouvait influencer la fermentation du produit, conférant des propriétés propres au site de fabrication (Bokulich et Mills, 2013). Les dynamiques des communautés microbiennes issues de l'environnement ou des matières premières peuvent également avoir un effet positif sur la fabrication de produits fermentés tels que des boissons alcoolisées (saké, bières, vins) (Bokulich et al., 2016). Cependant, même si certains microbiotes bénéfiques peuvent induire des effets positifs et désirés, afin de façonner un aspect ou un goût de certains produits, d'autres bactéries peuvent avoir de lourdes conséquences en termes de sécurité sanitaire et de qualité des denrées alimentaires. Que ce soit sous forme de biofilms ou nichées dans des zones difficiles à nettoyer, certaines bactéries pathogènes ou d'altération peuvent re-contaminer un produit lors de sa fabrication est ainsi avoir un effet néfaste (Wagner et al., 2020). Cette collaboration microbienne et la présence de bactéries de surface pouvant résider au sein des usines peuvent apparaître comme un risque pour la sécurité et la qualité des denrées, elles doivent être maîtrisées.

I.3.2. Bactéries résidentes

Il apparaît donc primordial dans la gestion de la qualité et de la sécurité des aliments de pouvoir identifier et caractériser les bactéries de surface. Dans l'industrie agroalimentaire, la surveillance de ces bactéries est effectuée principalement pour contrôler l'efficacité des procédures d'hygiène.

La présence de ces bactéries peut être testée par ATPmétrie ou validée généralement par un dénombrement et un isolement sur des milieux non sélectifs, en condition aérobie. Cependant, leur identité et rôle dans la sécurité sanitaire et qualité des aliments sont, pour la plupart des cas, non identifiés, (Bokulich et Mills, 2013). Møretrø et Langsrud (2017) stipulent que les industriels doivent prendre conscience des risques que peuvent amener ces bactéries de surface. En effet, ces bactéries se développent dans des conditions environnementales similaires à celles retrouvées sur des produits alimentaires au cours de leur stockage. La disponibilité de certains nutriments, les facteurs de stress (température, conservateurs, sel) similaires à l'environnement de production peuvent favoriser la croissance de ces bactéries au cours du stockage chez le consommateur (Møretrø et Langsrud, 2017). Ces bactéries sont donc capables de coloniser les surfaces et d'y résider ou persister.

Les définitions de persistance et de résidence sont des concepts flous variant selon les études. Carpentier et Cerf (2011) ont proposé, dans leur revue bibliographique sur la persistance de *Listeria monocytogenes*, de définir ce phénomène. Nous retenons de cette étude qu'une souche est caractérisée persistante si cette dernière a été isolée d'un même site et reconnue identique par une méthode de typage au moins trois fois en l'espace d'un an. La majorité des études portant sur la persistance de souches bactériennes se focalisent uniquement sur des espèces pathogènes (Broennum Pedersen et al., 2008; Larsen et al., 2014; Ferreira et al., 2014; Bridier et al., 2015). Concernant les bactéries résidentes, seuls Møretrø et Langsrud (2017) tentent de définir ce concept et préconisent des recommandations pour les étudier. Nous retenons donc la définition de ces auteurs pour caractériser des bactéries résidentes : les bactéries retrouvées après nettoyage et désinfection sont considérées comme étant résidentes. La majorité d'entre elles ne sont pas des flores pathogènes mais peuvent être altérantes (Mettler et Carpentier, 1998; Bagge-Ravn et al., 2003).

Les bactéries résidentes, pouvant donc se transférer des surfaces aux produits tout au long du processus de fabrication, peuvent s'avérer être des dangers et peuvent donc représenter un risque pour la qualité et la sécurité des aliments et méritent d'être étudiées et caractérisées (Midelet et Carpentier, 2002; Carpentier et Cerf, 2011). Ces cellules peuvent notamment résider dans un état VNC ou sous la forme de biofilms.

I.3.3. Cellules viables non cultivables (VNC)

Les bactéries VNC évoluent dans un état physiologique particulier. Il s'agit de cellules métaboliquement actives, mais ayant perdu leur capacité de croissance induisant une sous-estimation du nombre de bactéries par méthode culturale.

Ces cellules se distinguent des cellules mortes de par l'intégrité conservée de leur membrane ainsi que de leur matériel génétique, mais surtout par leur activité métabolique conservée (Li et al., 2014). Cette stratégie de défense et/ou d'adaptation améliore sensiblement la survie des bactéries dans des conditions environnementales défavorables. En effet, en étudiant la survie de *Vibrio fluvialis* sous conditions de stress, Amel et al., (2008) ont observé sa survie et le recouvrement de sa capacité de croissance jusqu'à 6 ans. De plus il semble clair que l'état VNC soit un état de latence métabolique sans pour autant impacter la virulence de la bactérie lorsque celle-ci recouvre son état cultivable. Ces cellules adhèrent aux surfaces et peuvent également être issues de biofilms. En effet, en étudiant notamment le comportement de biofilms de *Campylobacter jejuni*, Magajna et Schraft (2015) ont observés une réduction de la viabilité des cellules, accompagnée de la formation d'un état VNC. Ces deux stratégies semblent être augmenter la capacité d'adaptation favorisant la résidence des bactéries.

I.3.4. Les biofilms

I.3.4.1. Introduction

Un biofilm peut se définir par communauté multicellulaire complexe tridimensionnelle de microorganismes adhérant entre eux et à une surface. Ces microorganismes peuvent produire une matrice polymérique extracellulaire protectrice et y être incorporés (Costerton et al., 1999, Carpentier et Cerf, 1993; Azeredo et al., 2017). Une des importantes particularités de ces structures est leur capacité d'adhésion à une surface biotique (cellules) ou une surface abiotique (plancher ou équipement, à l'abattoir ou dans l'usine de transformation). Il est aujourd'hui bien établi que ces structures sont naturelles et très présentes dans la nature sur des surfaces minérales (roche, interfaces air-liquide, etc.), organiques (peau, tube digestif des animaux, plaque dentaire, racines et feuilles des plantes) ou industrielles (canalisations, coques des navires). En médecine, les biofilms se développent également sur les cathéters ou autres dispositifs médicaux invasifs pouvant être source d'infections nosocomiales (Araya et al., 2003; Trautner et Darouiche, 2004; Marsh, 2006).

Les biofilms présentent un challenge quant à leur rôle dans le transfert de bactéries pathogènes ou d'altération sur les denrées alimentaires (Alvarez-Ordóñez et al., 2019). Cette structure confère une résistance accrue à différents agents désinfectant et peut être responsable de nombreux défauts de maîtrise d'hygiène en industrie agroalimentaire (Carpentier et Cerf, 1993; Satpathy et al., 2016). En effet, les biofilms peuvent héberger et favoriser le développement de pathogènes alimentaires tels que *Listeria monocytogenes*.

Ainsi, de nombreuses TIAC se sont révélées liées à ces structures organisées (Chmielewski et Frank, 2003; Zhao et al., 2017). La maîtrise de ce danger paraît essentielle pour assurer la qualité et la sécurité des aliments. Afin de le gérer, il est impératif de comprendre la formation de ces structures.

I.3.4.2. Formations de biofilms

La formation de biofilms, liée à des facteurs génétiques ainsi qu'environnementaux (Marić et Vraneš, 2007) a été largement décrite dans la littérature et suit des étapes présentées **Figure 8**.

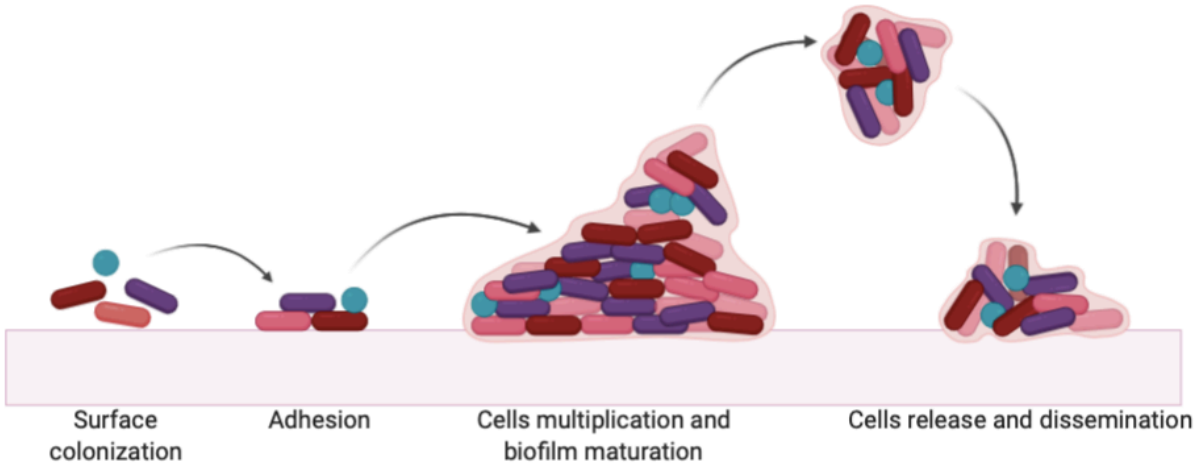

Figure 8. Étapes de la formation d'un biofilm bactérien (adapté de Maunders et Welch, 2017)

I.3.4.2.1. Adhésion

Les cellules bactériennes sont capables d'adhérer aux différentes surfaces retrouvées dans une usine agroalimentaire. L'adhésion et l'attachement des cellules à un support se fait en deux étapes. La première, dite réversible, fait appel à des forces de liaisons physiques telles que des liaisons de Van der Waals, des liaisons électrostatiques et des interactions hydrophobes (Wan Norhana et al., 2010). La deuxième étape, dite irréversible, entraîne la fixation des cellules au support par des propriétés intrinsèques aux bactéries telles que les pilis ou à l'aide d'exopolysaccharides (Marshall et al., 1971).

Dans un premier temps, les cellules vont être apportées et transférées sur les surfaces directement par les matières premières ou indirectement (air, manipulateurs). Dans l'industrie agroalimentaire, les surfaces, notamment pendant les processus de fabrication peuvent être couvertes de salissures (matières premières, déchets organiques) servant de substrat nutritionnel et favorisant l'adhésion des bactéries. La première étape d'adhésion aux surfaces des cellules à l'état planctonique est réversible (Carpentier et Cerf, 1993; Maunders et Welch, 2017).

I.3.4.2.2. Multiplication des cellules et maturation du biofilm

L'adhésion des cellules devient par la suite irréversible. Ces dernières passent alors dans un état dit « sessile ». Les bactéries vont alors croître et se multiplier afin de constituer une architecture de biofilm. Des signaux de communication cellule-cellule appelés quorum sensing ont été décrits comme étant impliqués dans la formation de biofilms (Davies et al., 1998; Sakuragi et Kolter, 2007) et notamment comme un facteur se déclenchant à faible densité cellulaire, induisant la multiplication des bactéries (Kong et al., 2006). Des cellules au sein du biofilm vont produire une matrice extracellulaire composée d'eau, de protéines et d'acides nucléiques ainsi que d'exo-polysaccharides (EPS) (Satpathy et al., 2016). Ces EPS consistent en un assemblage de biopolymères d'origine microbienne (Flemming et al., 2007) et vont apporter au biofilm, de par leur structure et leur propriété visqueuse, une barrière contre les agents antimicrobiens (Bridier et al., 2015; Pinto et al., 2020). Leurs structures leur permettent également d'inclure les bactéries dans les biofilms et de faciliter les interactions entre elles (Flemming et Wingender, 2010).

I.3.4.2.3. Détachement et propagation des cellules

En réponse à des contraintes physiques imputées par les procédures d'hygiène (frottements, pression des jets d'eau) mais également par l'action des cellules en périphérie des biofilms, clivant la matrice, les cellules peuvent être dispersées et recoloniser/recontaminer des surfaces (Maunders et Welch, 2017).

Ces propriétés ainsi que la création de biofilms bactériens favorisent des échanges génétiques par transferts horizontaux. Les usines agroalimentaires, de par leurs caractéristiques environnementales particulières, sont sources de différents stress (thermiques, dessiccation, frottements, biocides) auxquels les bactéries peuvent s'adapter par échanges inter-géniques (Wesche et al., 2009). Ces bactéries capables de s'adapter, de cohabiter sous forme de biofilms ou pouvant survivre sous la forme de cellules viables et non cultivables (VNC) semblent donc posséder des caractéristiques favorisant leur résidence (Møretrø et Langsrud, 2017).

Les biofilms ainsi que les cellules VNC peuvent donc être un réel problème de santé publique ou un risque pour la qualité des produits alimentaires. Et effet, les bactéries résidentes des usines agroalimentaires peuvent utiliser ces stratégies d'adaptation (Iñiguez-Moreno et al., 2019). Leur caractérisation et notamment celle des biofilms paraît donc primordiale dans la compréhension des mécanismes pour une meilleure gestion des risques.

I.3.4.3. Caractérisation des biofilms

Les structures complexes, comme les biofilms, composées de microorganismes ne sont pas propres aux environnements de production alimentaire. Afin de pouvoir lutter contre, il est important de pouvoir les observer et les caractériser.

Il existe plusieurs méthodes permettant d'analyser la force d'attachement des cellules aux surfaces, l'étendue des biofilms, la concentration de cellules qu'ils renferment ainsi que leur viabilité, mais également la structure des matrices EPS (**Figure 9**). Dans le cadre de ce travail de thèse nous nous sommes principalement intéressés aux stratégies facilitant la récupération ou la formation des biofilms.

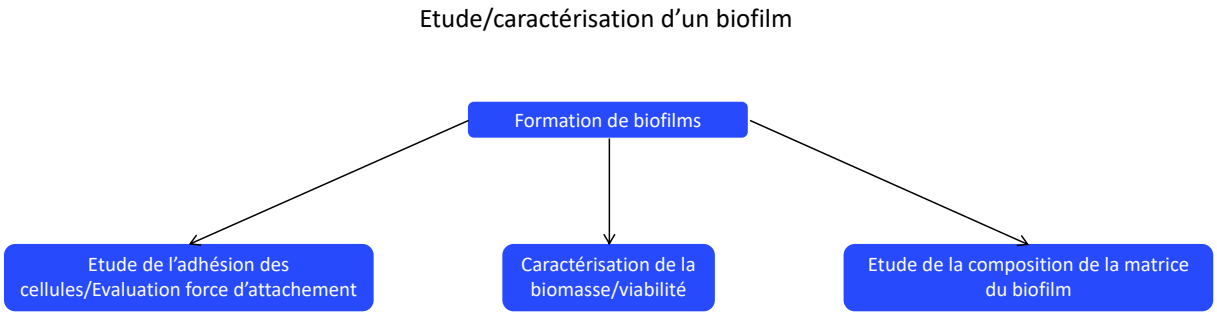

Figure 9. Étude/caractérisation d'un biofilm (adapté de Azeredo et al., 2017)

I.3.4.3.1. Etude de la formation de biofilms

Afin d'observer la formation, la cinétique et la dynamique d'un biofilm, il est primordial de mettre en place des approches favorisant la formation de cette structure. Dans sa revue portant sur les méthodes d'analyses de biofilm, Azeredo (2017) met en évidence un grand nombre de dispositifs de culture de biofilms. Que ce soit en plaques ou en microplaques, le principe est de favoriser une adhésion de cellules. La majorité des dispositifs fait appel à des coupons jouant le rôle de support aux biofilms. La plupart de ces méthodes peuvent être onéreuses et faire appel à un matériel spécifique. Il est également possible de manière simple de récupérer des biofilms produits dans leur milieu naturel.

Dans le cadre d'une étude sur les bactéries retrouvées sur les surfaces industrielles (sessiles ou planctoniques) il semble important d'élaborer des stratégies permettant de reproduire cet environnement particulier.

En vue d'étudier le comportement de *L. monocytogenes* sur des surfaces en inox Overney et al., (2016) ont mis en place des milieux mimant les souillures retrouvées dans les usines agroalimentaires (exsudât de viande et jus de saumon).

En recréant des conditions proches des conditions d'usines (coupons en inox AISI 304 + souillures), les auteurs ont pu observer l'adhésion de *L. monocytogenes* sur ces coupons tests. Cependant le type de souillure pouvait avoir une influence sur l'état viable non cultivable des bactéries.

Dans la perspective de mieux comprendre la persistance de souches d'*Escherichia coli* quinolone-résistantes dans la chaîne de production de poulets en Norvège, Nesse et al., (2020) ont montré que sur 158 souches isolées de poulets ou d'environnements de commerces 157 étaient capables de produire des biofilms en microplaques dans différentes conditions représentatives de l'environnement. Cette capacité peut expliquer la persistance et la dissémination de ces souches quinolone-résistantes.

Ces différentes stratégies *in vitro* ne permettent cependant pas de simuler avec exactitude la réalité de la vie bactérienne en biofilm dans une usine agroalimentaire. Ces études peuvent donc se faire à une autre échelle. Pour contrôler et surveiller la population bactérienne présente dans un atelier traiteur, Firmesse et al., (2012) ont déposé au sein de l'atelier des plaques inox et PVC (chlorure de polyvinyle) durant 7 semaines. Cette étude a permis aux auteurs d'évaluer la quantité de cellules adhérées au cours du temps sur ces plaques et évaluer la force d'attachement des bactéries. Cette stratégie, a permis de démontrer que malgré des procédures de nettoyage, de désinfection et une chute significative de la concentration bactérienne cultivable, une forte concentration de cellules viables évaluée par EMA-qPCR persistait ; suggérant une faille dans les procédures d'hygiène.

L'approche expérimentale utilisée par Firmesse et al., (2012), bien qu'elle soit réalisée dans un atelier traiteur aux procédures d'hygiène peu fréquentes (hebdomadaires), permet de retranscrire de réelles conditions. Répéter ce type de stratégie au sein d'une usine agroalimentaire pourrait être une première étape dans la caractérisation de biofilms et potentiellement de bactéries résidentes.

Une fois le dispositif permettant la formation d'un biofilm choisi, il existe plusieurs approches (microbiologiques, moléculaires, physiques et chimiques) permettant de quantifier les bactéries qu'il renferme.

I.3.4.3.2. Étude de la biomasse bactérienne de biofilms

Afin de déterminer et de quantifier la biomasse bactérienne présente au sein de biofilms, il est possible d'utiliser des approches de cytométrie de flux, culturales ou encore de biologie moléculaire telle que la qPCR.

Cependant comme développé dans le paragraphe I.2.2 consacré aux methodes d'analyse en écologie microbienne, ces approches peuvent être biaisées et ne permettent pas d'identifier de façon globale les membres des communautés bactériennes en biofilm. Les méthodes NGS pourraient être une stratégie efficace permettant d'identifier et de disposer d'une quantification relative des cellules retrouvées sur les surfaces et dans les biofilms bactériens.

En analysant le microbiote de surface par metabarcoding de l'ADNr 16S après nettoyage et désinfection de 4 usines différentes, Caraballo Guzmán et al. (2020) ont pu identifier les genres bactériens majoritaires retrouvés au sein de biofilms de ces environnements. Les auteurs ont notamment identifié un core-microbiote principalement composé des genres *Acinetobacter* et *Pseudomonas*. Cependant, le microbiote de surface est influencé par le type de production ainsi que par les procédures d'hygiène réalisés. D'autre part, en analysant le microbiote de surface de convoyeurs dans une usine de saumon fumé après nettoyage, Langsrud et al. (2016) ont pu observer par metabarcoding de l'ADNr 16S un biofilm dominé par *Pseudomonas*. En reproduisant ce biofilm en condition de laboratoire, les auteurs ont évalué les dynamiques de cette communauté en co-culture avec *Listeria monocytogenes*. Les auteurs ont notamment observé une inhibition de la croissance de ce pathogène mais une persistance au sein du biofilm, démontrant ainsi le risque lié à ces structures et l'intérêt du metabarcoding dans la caractérisation des communautés bactériennes des biofilms.

Il apparait primordial de maîtriser le risque biofilms ou dans un sens plus large les bactéries résidentes dans les ateliers de transformation des aliments. Pour cela des moyens de gestion du risque doivent être développés et mis en œuvre.

I.3.5. Gestion du risque bactérien en industries agroalimentaires

Afin de protéger le consommateur, la règlementation européenne, appelée également « paquet hygiène », impose aux industriels de l'agroalimentaire de mettre en place un Plan de Maîtrise Sanitaire (PMS) pour lutter contre les microorganismes indésirables et les risques de contamination (Règlement (CE) n°2073/2005; ISO, 2018).

Ce PMS est un ensemble de mesures préventives et d'autocontrôles ayant pour but d'assurer la sécurité et la salubrité des aliments au cours de la chaîne de production. Il comporte donc des éléments concernant l'application des Bonnes Pratiques d'Hygiène (BPH) pendant la fabrication des denrées alimentaires, le plan HACCP de l'entreprise, permettant d'identifier les dangers, de mieux les contrôler et de mettre en place des mesures correctives et, enfin, le système de traçabilité des produits et les procédures de retraits-rappels en cas de non-conformité d'un produit mis sur le marché.

L'une des étapes les plus importantes dans ce type de démarche est la mise en application stricte de procédures de nettoyage et de désinfection. Cependant ces procédures seules ne sont pas suffisantes. En effet la conception hygiénique des équipements mais également des bâtiments a aussi une importance capitale dans la maîtrise des bactéries résidentes (Carpentier, 2005).

I.3.5.1. Nettoyage & Désinfection

Les procédures de nettoyage et désinfection sont des pratiques courantes et quotidiennes dans l'industrie agroalimentaire. Partie importante du "paquet hygiène" (CE 178/2002, 2002), ces procédures se déroulent en étapes successives clés et doivent être scrupuleusement respectées (Belloin, 1993):

- Le prélavage : cette étape consiste à préparer l'atelier au nettoyage. Les opérateurs vont donc éliminer les plus gros déchets issus de la production, libérer les surfaces, démonter certains équipements et protéger ceux qui ne peuvent l'être. Ensuite une élimination physique de souillures par brossage, raclage, balayage est effectuée.
- Le nettoyage : cette étape fait appel à l'utilisation de détergents et a pour but d'éliminer toutes les souillures visibles sur les surfaces (déchets minéraux, déchets organiques)
- Le rinçage intermédiaire : ce premier rinçage va permettre d'éliminer toutes traces de souillures décrochées ainsi que de composés chimiques de détergents.

- La désinfection : cette étape clé permet l'élimination des souillures invisibles par l'utilisation de désinfectants (ou biocides). Cette étape vise à la destruction des microorganismes présents sur les surfaces des ateliers de production.
- Le rinçage final : cette dernière étape va permettre d'éliminer toutes traces de résidus chimiques du désinfectant afin d'éviter tout transfert sur les denrées au cours de la production suivante.

Avant toute mise en place de ces procédures d'hygiène, il est important de différencier les surfaces ouvertes et facilement accessibles par les opérateurs des équipements difficilement nettoyables. Dans le cas des équipements non démontables (cuves, tuyauteries, échangeurs thermiques), un Nettoyage En Place (NEP ou CIP) est effectué. L'envoi de solution détergente peut se faire directement et les équipements sont nettoyés par circulation (Overney, 2016). Cette procédure met donc en action deux étapes primordiales : le nettoyage et la désinfection. Le choix des produits utilisés pour ces deux étapes doit être réfléchi et dépend du type de souillure à éliminer, du type de surfaces retrouvées dans l'environnement, de la qualité de l'eau du réseau. De plus, il est important de prendre en compte le temps et la température d'application, la concentration du produit ainsi que les actions mécaniques associées (brossage, balayage) (Overney, 2016).

I.3.5.1.1. La phase de nettoyage

Cette première étape, favorisant l'efficacité de la désinfection, fait appel à des détergents. Un détergent se définit selon l'AISE (Agence Internationale pour les Savons, Détergents et produits de Maintenance) comme une substance ou un mélange contenant des savons ou autres agents de surfaces destinés au nettoyage. Il permet l'élimination de souillures (organiques ou minérales) adhérentes aux surfaces. Il existe plusieurs types de détergents. L'utilisation préférentielle d'un type ou d'un autre dépend essentiellement de la nature de la souillure à éliminer, de la surface à nettoyer et si le produit est facilement rinçable. Dans la majorité des cas, les détergents sont utilisés sous forme de mousse à appliquer au jet d'eau sur l'ensemble des surfaces (Overney, 2016).

I.3.5.1.1.1. Les détergents alcalins :

Les détergents alcalins sont utilisés notamment pour éliminer les souillures de type organique (déchets de productions, matières grasses, protéines). La soude, ou hydroxyde de sodium est le détergent alcalin le plus connu qui va éliminer les souillures organiques par saponification ou solubilisation des composés.

Cependant, les détergents alcalins peuvent être corrosifs pour certains matériaux (aluminium) (Sansebastiano et al., 2007).

I.3.5.1.1.2. Les détergents acides :

Les détergents acides sont utilisés notamment pour éliminer les souillures de type minéral (dépôts de tartre sur les vannes ou différents capteurs des équipements entraînant des dysfonctionnements). Leur action doit donc être à la fois curative (détartrante) et préventive afin d'éviter une nouvelle formation de souillure minérale. Les principaux acides utilisés sont l'acide nitrique, l'acide chlorhydrique et l'acide sulfurique. Cependant les détergents acides peuvent avoir l'inconvénient de décaper les surfaces métalliques (Sansebastiano et al., 2007).

I.3.5.1.1.3. Les détergents neutres :

Les détergents neutres sont essentiellement utilisés pour éliminer les souillures organiques. Cette classe est composée d'agents tensio-actifs dont le rôle est de faciliter la solubilisation et la capture des souillures dans l'eau (Belloin, 1993).

On distingue différents types d'agents tensio-actifs :

- Non ioniques : ces détergents ont un pouvoir émulsifiant et une forte solubilité dans l'eau ce qui facilite leur rinçabilité.
- Anioniques : ces composés ont un fort pouvoir détergent.
- Cationiques : ces composés sont actifs sur une large gamme de pH et possèdent également un pouvoir désinfectant.
- Amphotères : ces composés peuvent se présenter sous une forme anionique ou cationique. De plus leur pouvoir biocide contre les bactéries à Gram positif facilite l'étape de désinfection.

Des substances complémentaires complexantes peuvent également être ajoutées à la solution détergente. Les agents complexant favorisent l'action antitartre du nettoyage.

I.3.5.1.1.4. Les détergents enzymatiques

Afin de disposer de détergents pouvant être plus sains pour l'environnement et pour le manipulateur, de plus en plus de détergents enzymatiques sont disponibles sur le marché. L'utilisation d'enzymes simples ou de mélanges (notamment de lipases, protéases, glucanases et amylases) permettent de réduire ou de remplacer l'utilisation de détergents chimiques.

Leur action va être essentiellement de dégrader la matrice EPS des biofilms, et de faciliter la dispersion des molécules désinfectantes au sein même de ces structures (Kumar et Anand, 1998; Augustin et al., 2004; Simões et al., 2010; Zhao et al., 2017).

L'étape de nettoyage ou détergence à elle seule ne suffit pas à éliminer les microorganismes et donc à réduire les risques de contamination des denrées en cours de production. De la même manière, une désinfection seule n'est pas suffisante. En effet, en étudiant la survie de *Listeria monocytogenes* au sein de biofilms développés *in vitro* sur des coupons inox, Jessen et Lammert (2003) ont mis en évidence que l'effet seul d'un désinfectant était limité. De plus, les auteurs ont également observé que l'élimination de *L. monocytogenes* était effective lorsque le désinfectant était utilisé selon les recommandations du fournisseur et après utilisation préalable d'un détergent.

I.3.5.1.2. La phase de désinfection

La désinfection est une étape primordiale d'une procédure d'hygiène dans l'industrie agroalimentaire, mais ne peut être effective que si l'étape de nettoyage est bien respectée. Cette étape vise à éliminer les microorganismes et donc le danger microbien lors de la production suivante.

Selon la réglementation (CE n°528/2012) un désinfectant est une substance utilisée pour détruire, repousser ou rendre inoffensifs les microorganismes ainsi qu'en prévenir l'action. De la même manière que pour un détergent, il est primordial de bien sélectionner le type de désinfectant utilisé. Ce choix dépend du spectre de microorganismes à éliminer, du support des souillures, du potentiel d'accoutumance voire de résistance des microorganismes à la substance et de sa capacité à être facilement rincé.

Il existe différents principes actifs désinfectants. Les désinfectants principalement utilisés en agroalimentaire sont :

- Les composés chlorés (ex : hypochlorite de sodium, chloramines)
- Les composés iodés (ex : iode, iodophores)
- Les produits oxydants (ex : acide péracétique, peroxyde d'hydrogène)
- Les alcools (ex : éthanol, isopropanol)
- Les ammoniums quaternaires (ex : chlorure de benzalkonium, didécyl diméthyl ammonium)

L'utilisation, le mode d'action ainsi que les avantages et inconvénients du type de molécules désinfectantes sont résumés **Tableau IV**.

Tableau IV. Avantages et inconvénients des composants désinfectants utilisés dans l'industrie agroalimentaire (D'après Belloin, 1993 et McDonnell et Russell, 1999 et Overney, 2016)

Désinfectants	Mode d'action	Avantages	Inconvénients
Composés chlorés (Hypochlorite de sodium, potasse, chloramines)	Oxydation des matières organiques	Large spectre d'activités (bactéries, virus) Peu couteux Peut être utilisé comme détergent Facile à manipuler Rinçage facile Peu toxique	Corrosif pour les surfaces inox Pas d'efficacité biocide en présence de matières organiques Accoutumance des microorganismes
Composés iodés (Iode, iodophores)	Dégradation de groupements protéiques et nucléotidiques des microorganismes	Large spectre d'activités Actif à faible concentration Faible toxicité Détartrant Facile à manipuler	Irritant Corrosif Rinçage difficile Instabilité au stockage et hautes températures (43°C) Sensible à la dureté de l'eau et aux matières organiques
Produits oxydants (Péroxyde d'hydrogène)	Production de radicaux libres oxydés qui vont détruire des composants essentiels aux bactéries (protéines, lipides, ADN)	Large spectre d'activités (notamment bactéries Gram+ et Gram-) Non corrosif Facilement rinçable Ne laisse pas de traces de résidus toxiques	Spectre essentiellement bactéricide Surtout efficace à température élevée (>60°C) Temps de contact et concentration importants pour un effet sporicide Sensible aux matières organiques. Perte d'efficacité au stockage.
Alcools (Éthanol)	Causent des dommages membranaires et dénaturent les protéines essentielles aux bactéries. Inhibent la division cellulaire.	Action rapide Volatile Non corrosif	Faible spectre d'activité (bactéries essentiellement, non sporicide) Inflammable Produits couteux
Ammoniums quaternaires	Influent sur la membrane des microorganismes (cytoplasmique)	Stable Non corrosif Large spectre d'activités (Gram+, levures, moisissures) Sporostatique Peu toxique	Produits couteux Difficile à rincer Sensible à la présence d'agents anioniques et de souillures organiques Risque d'accoutumance des microorganismes

Les exigences sanitaires de la règlementation ont entraîné une augmentation de l'utilisation des désinfectants. La capacité des bactéries à évoluer, à s'adapter à leur environnement et à échanger leur matériel génétique par transfert horizontal, induit une forte recrudescence des phénomènes de résistance aux désinfectants (Langsrud et al., 2003).

La dissémination de plasmides ou de cassettes de résistance notamment aux ammoniums quaternaires est favorisée par l'apport de matières premières contaminées et les différents flux vecteurs au sein des usines. Les communautés de type biofilm favorisent la dissémination des résistances aux désinfectants. (Møretrø et Langsrud, 2017). Le contact des cellules bactériennes avec certains désinfectants peut induire une croissance des bactéries sous forme de biofilm et ainsi les protéger (Chapman, 2003). Ces phénomènes d'adaptation ou d'acquisitions de résistance vont dans un premier temps orienter l'industriel à augmenter la dose de désinfectant utilisé (Belloin, 1993) rendant petit à petit les procédures moins efficaces. Pour éviter ou réduire les phénomènes d'adaptation, il est conseillé de varier les désinfectants dans les plans de nettoyage et désinfection. Une stratégie complémentaire pour tenter de maîtriser la résidence bactérienne concerne la conception hygiénique des bâtiments ainsi que des équipements.

I.3.5.2. Conception hygiénique

Les ateliers de production (surfaces, murs, sols) ainsi que les équipements doivent être conçus dans le but de disposer d'une excellente nettoyabilité.

Ce qui permet ainsi de réduire l'existence de niches rendant difficile ou impossible l'accès des produits de nettoyage et de désinfection et favorisant la persistance de biofilms (Carpentier, 2005).

Les bâtiments et les surfaces doivent répondre à des règles de conception limitant tout transfert de toxicité (chimique ou microbiologique) aux denrées alimentaires. La conception, l'installation des équipements ainsi que les surfaces en support doivent être réalisées dans le but d'éviter tout risque de condensation, d'humidité supplémentaires et d'éviter la création de niches physiques propices à la résidence bactérienne (EHEDG, 2018). De plus, le choix du matériau des surfaces peut être critique. En effet, sa porosité, son usure peuvent avoir un impact important sur l'adhésion des cellules. Dans ce sens, la rugosité, la présence de rainures et l'aspect non lisse de certains matériaux peut faciliter l'attachement de cellules aux surfaces. Les surfaces les plus représentées en usine agroalimentaire sont :

- L'acier inoxydable : retrouvé sur la majorité des équipements
- La céramique : sols, murs
- Les surfaces de type plastique (chlorure de polyvinyle (PVC), polyuréthane thermoplastique (TPU) : tapis de convoyage.

Les surfaces lisses telles que l'inox et la céramique sont plus facilement nettoyables. *A contrario* les matières plastiques s'usent plus rapidement et peuvent présenter des aspérités pouvant se transformer en nouvelle niche (Verran et al., 2008). L'acier inoxydable est incontournable dans les usines agroalimentaires. Ce matériau possède une forte résistance aux chocs (thermiques, chimiques), une usure faible et sa surface lisse facilite la désinfection (Midelet et Carpentier, 2002). Cependant, la finition de l'inox utilisée peut avoir une porosité variable et une nettoyabilité ainsi réduite (Leclercq-Perlat et Lalande, 1994). De nombreux biofilms ont déjà pu être observés sur ce type de surface (Carpentier et Cerf, 1993; Firmesse et al., 2012; Bridier et al., 2015; Overney et al., 2017)

Les matériaux plastiques tels que les TPU sont utilisés pour répondre à certaines contraintes techniques comme le convoyage des matières. Cependant, ces matériaux possèdent une porosité pouvant faciliter l'adhésion des cellules ainsi que la formation de biofilms. Le nettoyage de ces surfaces est donc rendu plus difficile sans action mécanique (Midelet et Carpentier, 2002). La surface de ces matériaux plastiques est plus facilement rayable favorisant ainsi l'installation des microorganismes. La **Figure 10** présente la colonisation d'une surface en PVC par une souche bactérienne contenue dans un défaut de la surface. En effet, après incubation en condition favorable, cette souche a été capable de se développer hors de sa niche et de coloniser les surfaces environnantes (Carpentier et Cerf, 2011; Midelet, 2002).

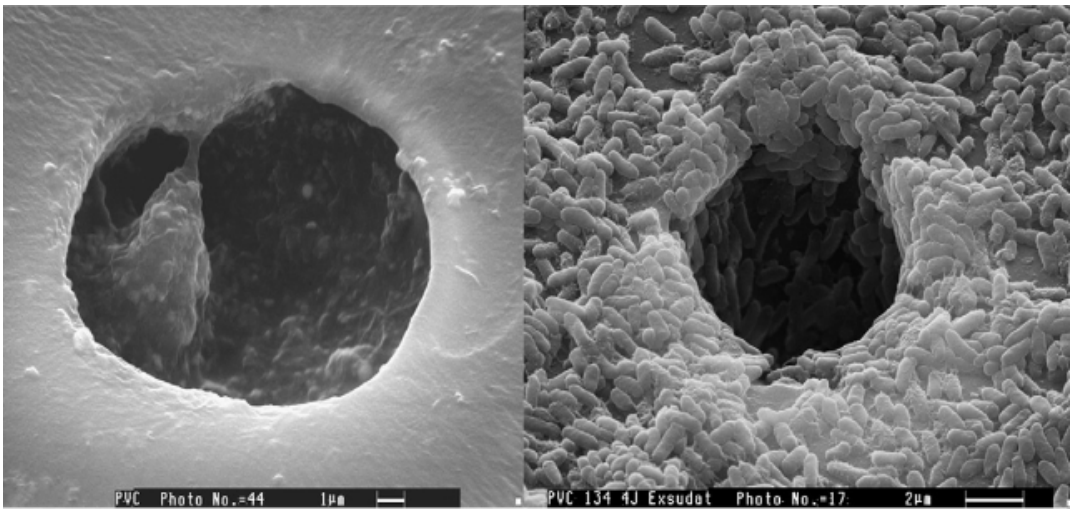

Figure 10. Colonisation par *Pseudomonas fluorescens* CCL 134 après 4 jours d'incubation en exsudât de viande d'une cavité liée à la fabrication d'une plaque de PVC (Carpentier et Cerf, 2011)

Toutes les aspérités et défauts retrouvés sur les surfaces peuvent donc retenir des cellules ainsi que des souillures liées aux productions et être un catalyseur pour la formation de biofilms. Il est donc impératif de prendre en compte les aspects nettoyabilité des surfaces et équipements et également de mettre en place des plans de contrôle et de surveillance au sein des usines.

I.3.5.3. Programmes de surveillance environnementale des usines agroalimentaires

La contamination d'un produit en cours de fabrication peut être liée à de nombreux vecteurs : matières premières, surfaces en contact avec les denrées, poussières, aérosols ou manipulations humaines (Doyle et al., 2017a). Il apparait donc primordial dans la gestion de la qualité et de la sécurité des aliments de pouvoir détecter et quantifier les bactéries présentes à la surface des ateliers de production. Afin de protéger le consommateur, les industriels réalisent de nombreux autocontrôles. Dans l'industrie agroalimentaire, la surveillance de ces microorganismes de surface est effectuée afin de contrôler et valider les procédures d'hygiène. Cependant seul un faible nombre d'espèces est ciblé et l'identification des micro-organismes isolés reste dans la plupart des cas inconnue. Par ces approches classiques, les industriels ne trouvent donc que ce qu'ils cherchent (Doyle et al., 2017a; Møretrø et Langsrud, 2017).
Il est connu aujourd'hui que les méthodes de contrôles microbiologiques utilisés en routine peuvent avoir un spectre de recherche ainsi qu'une efficacité insuffisantes pour permettre la détection et la quantification de pathogènes en très faible concentration (Habraken et al., 1986; Brouard et al., 2007). De plus, les récentes TIAC françaises et européennes liées à *L. monocytogenes* et à *Salmonella* spp. ont permis de mettre en évidence des lacunes dans les stratégie de contrôles microbiologiques environnementaux (ECDC-EFSA, 2018).

Afin de disposer d'une meilleure prévention des risques, les industriels mettent en place des programmes de contrôles environnementaux (Environment Monitoring Program/EMP). Ces programmes sont basés sur une approche d'évaluation des risques et sont de plus recommandés par les autorités, gestionnaires des risques, compétentes (Canada, Europe, Etats-Unis, Nouvelle-Zélande) (IDF/FIL, 2020). La pratique de ces EMP se renforce également en France avec notamment la mise en place de la loi EGALIM n°2018-938, obligeant les industriels à déclarer la présence de pathogènes au sein des usines agroalimentaires. La problématique de ces plans de surveillance repose notamment dans leur conception :

- Où prélever ?
- Comment prélever ?
- Combien d'échantillons à prélever ?
- Comment analyser les échantillons prélevés ?
- Quelle gestion des résultats ?

Selon la Food and Drug Administration (FDA) et notamment dans le cadre du contrôle de *L. monocytogenes*, une usine peut être cloisonnée (zoning) en 4 grandes zones résumées **Tableau V** (FDA, 2017). Cette sectorisation permet de conceptualiser les environnements de production et d'identifier ainsi les surfaces ou équipements les plus difficiles à nettoyer, et donc les zones au potentiel de contamination fort.

Le nombre d'échantillons à prélever peut déterminer la robustesse du plan de surveillance. Bien que difficile à déterminer précisément et dépendant plus de l'expertise des industriels, Zoellner et al. (2018) proposent dans une revue portant sur les plans de surveillances de *Listeria monocytogenes* différentes méthodes de calculs basées sur l'historique des environnements.

Tableau V. Découpage des environnements de production en zones permettant d'identifier les points de prélèvements microbiologiques adéquats (FDA, 2017).

Zones	Définition	Exemple
Zone 1	Surfaces ou équipements en contact avec les denrées alimentaires	Trancheurs, tables, trémies, couteaux, etc.
Zone 2	Surfaces ou équipements qui n'entretiennent aucun contact avec les denrées alimentaires mais qui peuvent être proches de la Zone 1	Boîtiers des équipements, murs, sols, drains à proximité immédiate de surfaces Zone 1
Zone 3	Surfaces ou équipements qui n'entretiennent aucun contact avec les denrées alimentaires mais qui peuvent amener à une contamination des Zones 1&2	Chariots élévateurs, chariots circulant dans l'usine, murs, sols, drains à proximité non immédiate de surfaces Zone 1
Zone 4	Surfaces ou équipements qui n'entretiennent aucun contact avec les denrées alimentaires et qui se trouvent en dehors de la zone de production. Ces surfaces peuvent être le point de départ de l'introduction de pathogènes dans l'atelier.	Vestiaires, cafétérias, couloirs en dehors de la zone de production ou de la zone de stockage des denrées (matières premières, produits finis)

Les prélèvements sont effectués suivant la norme ISO18593 (International Organisation for Standardization) (ISO, 2018) et le sont souvent après les procédures d'hygiène pour évaluer leur efficacité. Cette norme fixe des recommandations claires quant à la manière de prélever ainsi que le dispositif à utiliser. En effet, selon la surface à prélever ainsi que son accessibilité, le choix de la méthode de prélèvement est primordial et doit garantir un décrochage de cellules optimal pour une analyse microbiologique. Il existe plusieurs dispositifs de prélèvements : éponges, écouvillons, chiffonnettes et lames contact. Le choix du système dépend essentiellement de la surface à prélever.

Par exemple, les lames contacts et écouvillons vont être utiles sur des zones de faibles surfaces quand les éponges ou chiffonnettes sont à privilégier sur des zones de taille supérieures ou égales à 100cm^2. Le choix de la méthodologie de prélèvement est important et peut avoir un impact sur la viabilité et la quantité de microorganismes échantillonnés. La force d'attachement des cellules ainsi que les conditions environnementales (température, humidité relative) doivent être prises en compte car peuvent avoir un impact sur le décrochement des microorganismes (Møretrø et Langsrud, 2017). Les conditions de transport des échantillons avant analyse sont également importantes et peuvent mener à un résultat d'analyses biaisé. Il est courant d'utiliser des liquides de transport neutralisants pour inhiber les traces de désinfectants, susceptibles de rester sur les surfaces et de détruire la population microbienne prélevée (**Tableau VI**). Le choix du milieu de transport mais également des conditions de transport est primordial. En effet, en étudiant la survie de bactéries après procédures d'hygiène, Langsrud et al. (2016) ont effectué divers prélèvements de surfaces industrielles. Les échantillons ont alors été stockés et transportés en froid positif puis analysés dans les 24h. Les dénombrements effectués ont alors montré une surestimation du dénombrement des cellules d'un facteur 10. Un milieu de transport trop riche pourrait également faciliter la croissance des microorganismes prélevés et fausser le résultat d'analyses.

Tableau VI. Exemple de molécules neutralisant les agents désinfectants (ISO, 2008).

Agent antimicrobien	**Composés chimiques capables de neutraliser l'activité anti-microbienne résiduelle**
Composés à base d'ammonium quaternaire et amines grasses	Lécithine, saponine, polysor-bate 80, dodécyl sulfate de sodium, condensat d'oxyde d'éthylène d'alcool gras (tensioactifs non ioniques)
Biguanides et composés similaires	Lécithinec, saponine, polysorbate 80
Composés oxydants (chlore, iode, peroxyde d'hydrogène, acide peracétique, hypochlorites, etc.)	Thiosulfate de sodium
Aldéhydes	L-histidine ou glycine

L'analyse des résultats et la gestion des données est également un point clé de ces programmes. Les analyses effectuées vont être de deux types : qualitatif (présence/absence) ou quantitatif et donner une concentration en unité formant colonie (UFC) par centimètre carré de surface. Dans la plupart des cas, les plans d'analyses sont focalisés sur les principaux pathogènes identifiés comme des dangers dans le produit ainsi que des indicateurs hygiéniques. Ainsi, les résultats obtenus ne concernent qu'une partie de la communauté bactérienne des surfaces, et ne permettent pas de quantifier les cellules à l'état VNC (Doyle et al., 2017a).

Ces données ne permettent aucune évaluation de l'impact des bactéries résidentes de surface sur la qualité et la sécurité des produits. L'utilisation d'autres techniques s'affranchissant d'une culture microbienne, comme notamment le séquençage de l'ADNr 16S par metabarcoding, peut être un point clé pour une meilleure compréhension de l'écologie microbienne de ces écosystèmes d'environnements de production, et une meilleure maîtrise des risques sanitaires.

I.3.6. Impact des environnements de production sur la qualité des produits

Dans la littérature, différentes équipes ont étudié le lien entre microbiote environnemental caractérisé par metabarcoding de l'ADNr 16S et la qualité du produit (Bokulich et Mills, 2013; Doyle et al., 2017b).

Ces travaux ont pour but d'améliorer la maîtrise de l'environnement de production et d'améliorer la qualité et la durée de vie du produit, par une meilleure compréhension des transferts des microorganismes aux produits et de leur dynamique.

Comme précisé par Møretrø et Langsrud (2017), la majorité des flores résidentes retrouvées sur les surfaces (après nettoyage et désinfection) ne sont pas pathogènes mais peuvent être responsables d'altération. Dans ce sens, diverses études ont mis en évidence l'importance des communautés bactériennes de l'environnement de production dans l'altération de produits carnés, laitiers ou plats cuisinés. Les principales flores résidentes identifiées dans la littérature sont répertoriées **Tableau VII**.

Tableau VII. Principales bactéries résidentes classées par type d'industrie (adapté de Møretrø et Langsrud (2017)).

Vinification	Brasserie	Produits laitiers/ Fromages	Viande et volaille	Produits de la mer
Pseudomonas *Flavobacterium*	*Acinetobacter* *Staphylococcus*	*Pseudomonas* *Acinetobacter* *Psychrobacter* *Serratia*	*Pseudomonas* *Acinetobacter* *Psychrobacter* *Carnobacterium* *Brochothrix* *Pantoea* *Staphylococcus* *Sphingomonas*	*Pseudomonas* *Acinetobacter* *Aeromonas* *Psychrobacter* *Serratia* *Carnobacterium* *Brochothrix* *Staphylococcus* *Flavobacterium* *Micrococcus*
(Bokulich et Mills, 2013)	(Bokulich et al., 2013a)	(Bokulich et Mills, 2013, Stellato et al., 2015)	(Fillipis et al., 2013, Stellato et al., 2016)	(Møretrø et al., 2016, Langsrud et al., 2016)

En étudiant le microbiote de produits carnés au cours du stockage, le microbiote environnemental et celui des carcasses à chaque étape du processus, De Filippis *et al*. (2013) ont pu retracer l'origine de l'altération des produits finis. En effet, les flores majoritaires retrouvées sur la viande bovine (*Pseudomonas, Brochothrix thermosphacta* mais également *Acinetobacter, Psychrobacter, Enterobacteriaceae*) ont également été retrouvées sur les carcasses correspondantes. L'étude environnementale menée en parallèle a permis de mettre en évidence la présence de ces mêmes bactéries dans l'atelier de transformation.

Cependant, l'abondance de ces genres étant plus élevée dans l'environnement, les auteurs suggèrent une colonisation de l'environnement par les microorganismes présents sur les carcasses, et qui lors des découpes et transformations auraient contaminé le produit. Ces travaux identifient l'abattoir comme point de contrôle critique (CCP) dans l'altération de ces steaks, et soulignent l'importance des pratiques d'hygiène et de maîtrise de l'environnement à chaque étape d'un processus de transformation.

Par l'analyse du microbiote des matières premières (saucisses) à chaque étape du processus et les surfaces correspondantes, Hultman et al. (2015) ont mis en place un système de surveillance dans un environnement de production de saucisses cuites emballées sous atmosphère modifiée. Cette étude montre que le genre majoritaire retrouvé sur le produit altéré, *Leuconostoc*, s'avère être présent à une très faible prévalence dans l'environnement (<5%). *A contrario*, *Yersinia spp.* a été identifiée en forte prévalence sur les surfaces (jusqu'à 50%) mais très faiblement sur le produit fini (<1%). Cette étude montre la capacité de certains genres à survivre à différents traitements thermiques et désinfectants, à contaminer le produit fini et à l'altérer ensuite au cours de la conservation.

De la même manière, Pothakos et al. (2015) soulèvent l'importance du genre *Leuconostoc* dans l'altération de plats cuisinés. Ce genre, retrouvé parmi les genres majoritaires sur produit fini, est apporté par toutes les matières premières et est retrouvé dans la quasi-totalité des prélèvements environnementaux. *Leuconostoc* contamine facilement le produit et peut croître après conditionnement et altérer l'aliment. Plus récemment, Stellato et al. (2016) ont mis en évidence un microbiote partagé entre matrices et environnement dans une vingtaine de boucheries, composé de *Pseudomonas* spp., *Brochothrix* spp., *Psychrobacter* spp., *Streprococcus* spp. et *Acinetobacter* spp. Si aucune différence de richesse n'a été observée entre les différents ateliers, un impact sur la diversité a été mis en évidence sur différents équipements (planche à découper en bois, matériel de découpe en inox).

Ceci, pouvant suggérer un impact de la surface sur la diversité des communautés bactériennes. Le type de surface doit donc être un élément pris en compte dans ce type d'études.

Dans la filière des produits laitiers, Stellato et al., (2015) montrent l'utilité du metabarcoding dans la caractérisation des flores bactériennes. Les auteurs ont étudié le microbiote de différents types de fromages issus d'un même atelier de fabrication ainsi que des prélèvements environnementaux. Ces derniers ont mis en évidence que la plupart des microorganismes retrouvés sur les fromages provenaient des surfaces.

De plus, la présence de ces mêmes flores après les procédures d'hygiène montre l'importance des bactéries résidentes dans la qualité du produit. Cette étude suggère que la résidence et persistance de certaines bactéries de surface, telles que les bactéries lactiques ou *Pseudomonas,* peut entraîner de futurs problèmes d'altération. Cette méthodologie a également permis d'analyser l'effet de la période de production (début/fin) sur la diversité des communautés bactériennes. En effet, l'accumulation de microorganismes sur les surfaces pendant la production peut entraîner un transfert bactérien par contact direct. Ce transfert peut modifier de la diversité bactérienne retrouvée sur le produit fini.

Par l'analyse des flores de différents fromages au cours de leur affinage, et issus de deux périodes de production distinctes (début de production/fin de production), O'Sullivan et al. (2015) ont montré des différences significatives dans la composition et la richesse des microbiotes. En effet, les auteurs ont observé le développement de genres non attendus tels que *Thermus* ou *Psychrobacter* en fin de production. Ces résultats suggèrent que ces genres peuvent provenir d'un apport exogène tel que le sel, servant à l'affinage des fromages, ou une flore provenant de l'environnement de production.

Les pratiques industrielles, les processus de fabrication ainsi que la saisonnalité d'une production peuvent façonner le microbiote d'un environnement industriel. Dans une étude portant sur un atelier de production de vin, Bokulich et al. (2013) ont analysé le microbiote environnemental de l'atelier à trois périodes distinctes du processus : avant, pendant ainsi qu'après vendange. Les auteurs ont observé des différences significatives dans la composition du microbiote environnemental au cours des différentes étapes du processus. Les flores caractérisées au sein de l'environnement semblent transitoires et ne s'installent pas sur les surfaces. Cependant, même si la résidence des microorganismes n'est pas mise en évidence, la dynamique de certaines flores peut influencer le procédé de fabrication. En effet, dans cette étude les levures *Saccharomyces cerevisiae* identifiées dans l'environnement sont suspectées de contribuer à la fermentation alcoolique et finalement à la typicité du vin.

En analysant le lait d'un troupeau de vache en stabulation et le comparant au lait d'un même troupeau nourri en pâturage, Doyle et al., (2017b), ont mis en évidence l'influence de la saisonnalité, la nutrition et les pratiques d'élevage sur la qualité du lait et la composition de son microbiote. Le lait issu de vaches élevées en pâturages est composé de flores environnementales telles que *Pseudomonas* ou *Acinetobacter,* alors que le lait du troupeau en stabulation est plus riche en flores de type intestinal retrouvées dans les fèces. Ces études soulignent l'importance de la prise en compte de variables de saisonnalité et de périodicité de production, dans l'analyse de l'influence de bactéries environnementales sur une matrice alimentaire.

Plus récemment, Zwirzitz et al. (2020) ont étudié par metabarcoding de l'ADNr 16S les sources de contaminations au sein d'un atelier de transformation de viande. Par l'utilisation de cette méthode, les auteurs ont pu caractériser les bactéries des produits finis qui ne provenaient pas des matières premières mais principalement de l'environnement de production. Cette approche a permis de réaliser une cartographie de l'usine dans le but de prédire des zones potentielles de contamination et vecteurs de flores résidentes.

La relation entre la qualité des matrices alimentaires (aliments fermentés, viande, lait) et le microbiote environnemental par metabarcoding de l'ADNr 16S a donc pu être établie dans la littérature et a montré la puissance de ce type de méthodologie. Les enjeux de la compréhension des flux bactériens dans les usines de transformation sont de pouvoir caractériser finement les bactéries résidentes et de pouvoir mieux comprendre leur impact sur la durée de vie des produits. Une surveillance dans les ateliers agroalimentaires des bactéries de surface, basée sur une étude de metabarcoding, permettrait de mieux comprendre les flux microbiens dans cet environnement particulier et leurs influences sur le produit. Cette analyse permettrait de mieux comprendre le fonctionnement de cet écosystème et d'optimiser les différents processus (transformation, nettoyage), afin d'assurer une meilleure qualité et une meilleure sécurité sanitaire des aliments en accompagnant les industriels pour une meilleure gestion de la qualité des produits (Huang et al., 2017). Une meilleure compréhension de l'écologie microbienne des usines à l'aide du metabarcoding, pourrait en effet être une piste pour mieux maîtriser les bactéries d'altération des produits au sein des usines, des réseaux de distribution et chez le consommateur et ainsi réduire le gaspillage alimentaire. C'est dans ce contexte que s'inscrit ce travail de thèse.

I.4. Les objectifs de la thèse

Le principal objectif de ce travail est d'approfondir les connaissances sur les communautés bactériennes des surfaces dans les ateliers de production des aliments et de caractériser leur dynamique dans cet environnement. Ces connaissances seront utilisées pour mieux comprendre l'impact de bactéries résidentes sur la qualité microbiologique des produits Il s'agit aussi pour l'entreprise Mérieux NutriSciences de développer un savoir-faire technique utilisant le metabarcoding valorisable dans un service d'analyse et de conseil pour les industries alimentaires. Le saumon fumé a été choisi comme matrice alimentaire modèle.

Un developpement spécifique consacré à la microbiologie du saumon fumé sera présenté et discuté dans le chapitre III. Cependant, il est présenté ci-dessous de manière synthétique l'état des connaissances sur la microbiologie du saumon fumé necessaire à la justification du choix de cette matrice alimentaire comme modèle pour l'étude.

Ce produit est du poisson prêt à consommer, faiblement préservé (salage, fumage), conservé au froid et sous-vide pendant une conservation longue (3 à 4 semaines) et dont la qualité microbiologique dépend essentiellement des évènements de contaminations rencontrés dans les ateliers de production.

Au début de ce travail, la communauté bactérienne du saumon fumé a largement et uniquement été décrite dans la littérature par l'utilisation d'approches culturales (Løvdal, 2015). Les bactéries à Gram négatif telles que *Shewanella putrefaciens, Aeromonas* spp. et *Photobacterium phosphoreum* ont été décrites comme dominant le microbiote de cette matrice dans les premiers stades du stockage (Leroi et al., 1998). En fin de durée de vie microbiologique (3 à 4 semaines), les bactéries lactiques à Gram positif (*Lactobacillus*, *Carnobacterium maltaromaticum*) semblent dominer la microflore avec une concentration de 10^7- 10^8 CFU/g. (Paludan-Müller et al., 1998). Ces bactéries lactiques semblent co-dominer le microbiote avec des entérobactéries psychrotrophes (Joffraud et al., 2006; Løvdal, 2015). Le danger microbiologique majeur du saumon fumé est *Listeria monocytogenes* (Løvdal, 2015). Le processus de fabrication de cette matrice ne comporte pas de point critique de contrôle (CPP). De ce fait, le saumon fumé est un donc un modèle d'étude facilitant le suivi de potentiels transferts bactériens au cours de sa fabrication.

Afin de décrire les communautés bactériennes d'ateliers de production de saumon fumé et d'en évaluer leur impact, nous nous sommes fixé trois objectifs spécifiques principaux.

Dans un premier temps, il est apparu primordial de pouvoir mettre en place une méthodologie adéquate et standardisée, pour caractériser des microbiotes de surfaces industrielles mais également de matrices alimentaires (saumon fumé) par metabarcoding de l'ADNr 16S. Ce premier chapitre de résultat a donc visé à évaluer l'efficacité de différentes méthodes d'extraction d'ADN et de prélèvements de surfaces.

Dans le second chapitre consacré aux résultats, nous avons développé une stratégie expérimentale visant à évaluer une corrélation entre l'environnement de production et le microbiote de produits finis. Pour cela, nous avons caractérisé la diversité des communautés bactériennes de plusieurs lots de saumon fumé du commerce, produits sous différentes marques, dans des ateliers de production identiques.

Enfin, dans la troisième partie des résultats, une campagne de prélèvement au sein d'une usine de production de saumon fumé a permis la comparaison du microbiote de l'environnement avec celui des produits. Ceci dans l'objectif d'évaluer *in situ* l'impact de l'environnement d'usine sur la qualité microbiologique des produits finis et d'identifier les zones principales de contamination. Ces informations ont été utilisées pour la mise en place d'actions correctives ciblées proposées à l'industriel pour réduire les risques de contamination et d'altération des aliments.

Chapitre II. Impact de l'extraction de l'ADN et des méthodes d'échantillonnage sur les communautés bactériennes monitorées par métabarcoding de l'ADNr 16S dans le saumon fumé et les surfaces des usines de transformation

II.1. Préambule

Dans l'industrie, les bactéries de surface et leur impact sur la qualité et la sécurité des produits apparaissent peu étudiés. En effet, les industriels ne recherchant que les principaux dangers pathogènes liés à leur production ainsi que quelques indicateurs d'hygiène, ne réalisent que peu d'études sur l'identité et le rôle des bactéries retrouvées sur les surfaces de leurs ateliers. Cependant, de nombreux articles scientifiques démontrent que ces bactéries jouent un rôle important dans la sécurité et la qualité des produits.

A cause des biais techniques relatifs aux méthodes culturales classiquement utilisées, la caractérisation de bactéries de surface nécessite le développement d'une approche polyphasique basée sur des méthodes culturales et des méthodes basées sur l'ADN. Dans l'objectif de caractériser et d'évaluer l'impact du microbiote environnemental d'un atelier de production alimentaire sur la qualité des produits, il apparaît primordial de disposer d'une méthodologie robuste et reproductible sur nos matrices d'intérêt : surface et matrice alimentaire.

Les principaux livrables de ce chapitre ont été de :

- Disposer d'une méthode d'extraction d'ADN optimale pour réaliser du metabarcoding de l'ADNr 16S sur des échantillons de prélèvements de surface ainsi que de matrice alimentaire (saumon fumé).
- Disposer d'une méthode de prélèvement de surface permettant un décrochage efficace des microorganismes présents et une analyse optimale par metabarcoding de l'ADNr 16S.

Afin de produire ces livrables, nous avons décidé de comparer différentes méthodes d'extraction d'ADN identifiées dans la littérature en se basant sur plusieurs indicateurs :

- Quantité/concentration de l'ADN extrait (ng/µL)
- Richesse de la communauté bactérienne (indice de Chao1/OTUs observés)
- Equitabilité de la communauté bactérienne (indice Shannon)

Notre but était de disposer d'une méthode permettant d'observer une communauté bactérienne avec la meilleure richesse et diversité. La comparaison de ces méthodes devant se faire sur des échantillons standardisés, nous avons décidé d'effectuer cette première étape sur une matrice saumon fumé Label Rouge issu du commerce stocké à 4°C et analysé à DLC (21 jours après conditionnement).

Concernant les matrices environnementales, nous avons fixé au sein de l'usine, sous des équipements nécessaires au filetage, des plaques inox représentant une des surfaces majoritaires de cet environnement (**Figure 11**). Ces plaques ont été installées plusieurs semaines avant prélèvement et écouvillonnées avant et après nettoyage et désinfection.

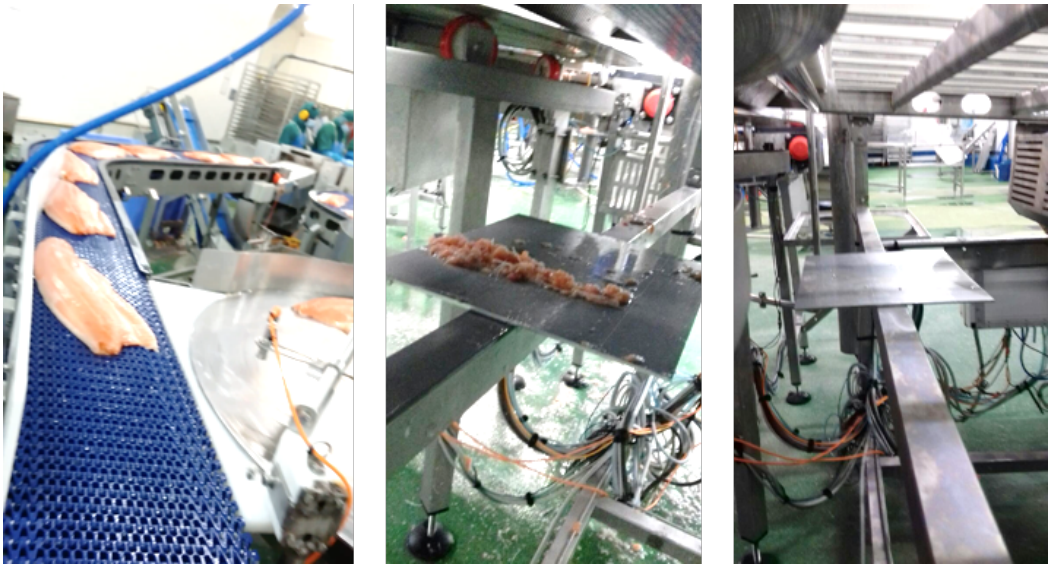

Figure 11. Plaques inox installées au sein de l'usine pour étudier les méthodes d'extraction d'ADN sur échantillon surface

Après avoir identifié une méthode d'extraction optimale pour notre étude, deux méthodes de prélèvement classiquement utilisées dans l'industrie agroalimentaire : écouvillon et éponge ont été comparées. Ces méthodes de prélèvements ont été évaluées sur la base des indicateurs suivants :

- Quantité de cellules dénombrées (UFC/cm^2)
- Richesse de la communauté bactérienne (indice de Chao1/OTUs observés)
- Equitabilité de la communauté bactérienne (indice Shannon)

Notre but était de disposer d'une méthode de prélèvement permettant un décrochage important des bactéries de surface en termes de cellules cultivables, ainsi que de prélever des communautés à richesse et diversité fortes.

Pour cela les plaques utilisées précédemment ont été remplacées afin de disposer de coupons tests représentatifs des deux surfaces majoritairement retrouvées dans l'usine : inox et TPU. Ces coupons ont donc été prélevées avant et après nettoyage et désinfection durant 5 jours consécutifs à l'aide d'éponges ou d'écouvillons (**Figure 12**).

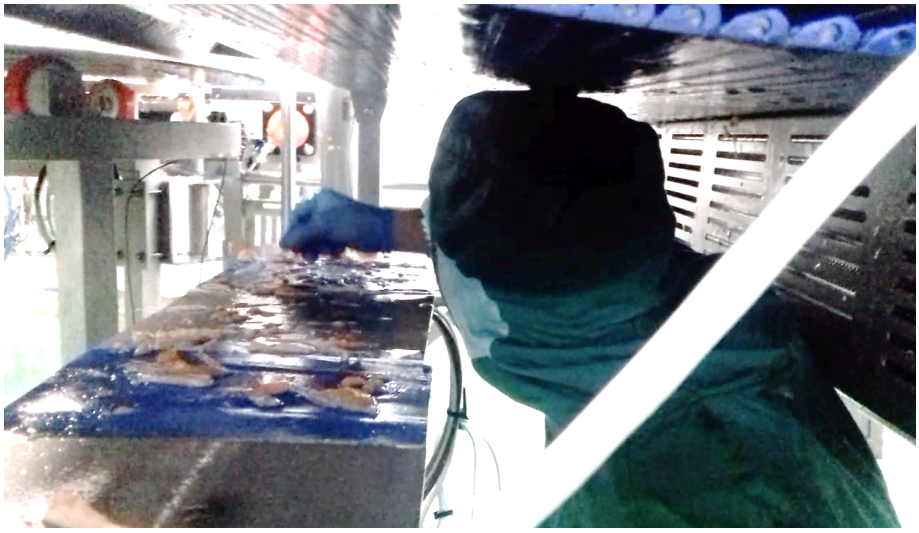

Figure 12. Plaques inox et TPU installées dans l'usine pour étudier les méthodes de prélèvement

Grace à cette approche méthodologique nous avons pu caractériser des communautés bactériennes sur matrice saumon fumé et sur les surfaces par metabarcoding de l'ADNr 16S.
Ces travaux ont fait l'objet d'un article accepté et publié en décembre 2020 dans *Food Microbiology.*

Maillet, A., Bouju-Albert, A., Roblin, S., Vaissié, P., Leuillet, S., Dousset, X., Jaffrès, E., Combrisson, J. & Prévost, H. (2021). Impact of DNA extraction and sampling methods on bacterial communities monitored by 16S rDNA metabarcoding in cold-smoked salmon and processing plant surfaces. Food Microbiology, 95, 103705.
https://doi.org/10.1016/j.fm.2020.103705.

Afin d'assurer une homogénéité dans la présentation de ce document, les données publiées dans les articles acceptés ou en cours de préparation sont présentées en conservant le style de présentation adopté pour l'ensemble du mémoire de thèse, assurant ainsi la continuité dans la numérotation des pages, des paragraphes et des figures. Les références bibliographiques sont regroupées et consignées dans la liste finale du document. Toutefois si le lecteur le préfère il pourra retrouver l'article en fac-similé de la version publiée ainsi que les « supplementary data » en annexe 1.

II.2. Impact of DNA extraction and sampling methods on bacterial communities monitored by 16S rDNA metabarcoding in cold-smoked salmon and processing plant surfaces

II.2.1. Introduction

Microorganisms from food and food processing environments can negatively impact food safety and quality. As part of quality management systems, food processing plants are regularly cleaned and sanitized, and routine monitoring is conducted to detect surviving bacteria (Doyle et al., 2017a; Møretrø and Langsrud, 2017). This monitoring is mainly based on culture-dependent methods. However, these techniques can lead to several analytical biases, such as non-specificity for some selective media, and difficulties to appreciate subdominant populations (Bokulich and Mills, 2012; Giraffa and Neviani, 2001). Furthermore, processing environments are ecosystems which are submitted to harsh conditions (temperature, moisture and disinfectants). These different stresses could induce viable but not cultivable cells (VBNC) (Li et al., 2014) that viable count methods could fail to detect. Moreover, bacteria adapted to a processing environment do often grow on products during storage (Møretrø and Langsrud, 2017).

The growing field of high-throughput DNA sequencing (HTS) has provided new perspectives on the overall dynamics of bacterial communities in food products and food processing plants (Hultman et al., 2015). These methods allow surface bacterial communities within processing environments, to be identified and better understood. Moreover, in food microbial ecology, 16S rDNA amplicon sequencing techniques have been used to obtain more information on the evolution of bacterial communities during the shelf life of a product (Chaillou et al., 2015; De Filippis et al., 2013; Fougy et al., 2016; Macé et al., 2013). However, these methods are not without drawbacks. For example, dead bacteria might be revealed by 16S rDNA metabarcoding. There are no standardized procedures for sequencing-based analysis methods. From sampling to sequencing, several workflows could lead to different interpretations of results. 16S rDNA metabarcoding results can be dependent on the DNA extraction methods (Keisam et al., 2016; Witte et al., 2018). Bacterial DNA extraction from food matrices can be difficult and needs several lytic agents (chemical, mechanical, enzymatic, thermal or combinations). Taxa do not share the same lysis sensibility, indeed Firmicutes and other Gram-positive bacteria are more difficult to lyse (Ercolini et al., 2013).

In addition to the DNA extraction method, the sampling device and sampling methodology used can have a major impact on the recovery of surface bacteria and the observed diversity of the bacterial community (Møretrø and Langsrud, 2017). The microorganisms' surface attachment strength, surface sampling, sampling medium, and environment conditions should be considered when choosing a sampling method.

This study focused on the bacterial communities of a product, cold-smoked salmon (CSS), and its processing plant using 16S rDNA metabarcoding. CSS is a lightly preserved product with no thermal treatment and is mainly consumed as a ready-to-eat food (RTE). CSS microbial communities have been widely described in the scientific literature, with most studies focusing on CSS microbial shift during storage and the impact of packaging on CSS microbial communities (Joffraud et al., 2001, 2006; Leroi et al., 1998, 2000; Løvdal, 2015; Paludan-Müller et al., 1998). This product has been described as being dominated by Gram-positive bacteria, such as lactic acid bacteria (LAB: *Lactobacillus*, *Carnobacterium maltaromaticum*), and Gram-negative bacteria, such as marine *Vibrionaceae* including *Photobacterium phosphoreum* and psychrotrophic *Enterobacteriaceae* (*Serratia liquefaciens, Hafnia alvei*) (Leroi et al., 2000). In addition, *Aeromonas* spp., *Shewanella putrefaciens* and *Brochothrix thermosphacta* have also been isolated (Løvdal, 2015). This microflora could be involved in the CSS spoilage process. CSS products also face pathogenic bacteria such as *Listeria monocytogenes* and *Clostridium botulinum* (Løvdal, 2015). This study aimed to assess whether the DNA extraction methods implemented and the sampling strategy used influence the identification by 16S rDNA metabarcoding of the bacterial communities on CSS products and processing plant surfaces.

II.2.2. Materials and methods

II.2.2.1. Smoked salmon tissue homogenate preparation

The CSS samples and surface samples came from the same smokehouse processing plant located in Brittany, France. One batch of cold-smoked salmon (Salmo salar) in a slide package that was preserved under vacuum at 4 ◦C, was collected from a local supermarket six days after packaging with no cold chain rupture. It then was analyzed at the Use by Date (UBD = 21 days). A 10-g portion with an approximative size of 6.5 × 6.5 × 0.25 cm was cut off from the slide and aseptically weighed in a sterile stomacher plastic bag provided with a 63 µm porosity filter (Interscience, Saint-Nom-la-Breteche, France).

To obtain a 10-fold dilution, 90 mL of sterile buffered peptone water (25.5 g/L) (Biokar Diagnostics, Allonne, France) was added to the bag. This sample was then homogenized for 2 min using a stomacher 400 device (Intersciences, Saint-Nom-la-Breteche, France). Smoked salmon tissue homogenate (SSTH) was either used immediately to enumerate the total viable counts or stored at − 20 °C for later DNA extraction.

II.2.2.2. Stainless steel and TPU coupons preparation and installation

Stainless steel (SS, 2 RB finish, AISI 304L) and tri-polyurethane (TPU, Ropanyl DM 8/2) were used in this study. SS and TPU coupons (30 × 25 cm, 750 cm^2) were purchased respectively from Laser 53 (Bazougers, France) and Ammeraal Beltech (Seclin, France). Before installation in the CSS processing plant, the SS coupons were degreased and sterilized with a mixture of ethanol at 95% and acetone (3v/v) (Overney et al., 2017). The TPU coupons were cleaned and sterilized with ethanol (70% v/v). The sterilized SS and TPU coupons were transferred into the food processing plant (FPP) in the same area where filleting and salting steps are performed before the smoking step. Coupons were placed near food contact surfaces, under a conveyor between the salting step and the filleting machine. Before surface samples were collected, the SS and TPU coupons were kept in the FPP for four weeks and were exposed to the routine daily cleaning and disinfection (C&D) program used by the processing plant. The C&D program consisted of applying 5% of ARVO CLM300 (Quaron, Rennes, France) during 5–15 min. This detergent is composed of sodium hypochlorite (with 5% of active Cl). After rinsing, the sanitizer ARVO 21 SR (Quaron, Rennes, France) was used. This sanitizer is composed by Ethanol 60%, sprayed pure on surfaces and not rinsed.

II.2.2.3. Sampling devices and surface sampling procedure

Surface bacterial communities were collected by swabbing or sponging, both procedures performed according to the ISO 18593:2018 (ISO, 2018). Swabs (Copan SRK Letheen Broth, Brescia, Italy) and sponges soaked with 10 mL Letheen Broth (3M™ Hydrated Sponge HS10LET, Cergy, France) were used. The SS and TPU coupons, which had been kept in the FPP for four weeks, were sampled before and after C&D at the end of a production run. These samples were used to compare the different DNA extraction methods and the efficiency of swabbing and sponging.

To compare DNA extraction methods, samples from SS surface were used. Fifteen different 50 cm^2 areas of one SS coupon were swabbed using a new swab for each. The 15 swabs samples then were pooled, resulting in a 60 mL sample from a 750 cm^2 SS surface.

The two samples from SS surface swabbed before and after C&D were used for the comparison of the DNA extraction methods. To compare swabbing and sponging methods, five different areas of 100 cm^2 of SS or TPU coupons were sampled using a new swab or sponge for each, before and after C&D during five consecutive days. The five swabs samples were pooled resulting in a 20 mL sample from a 500 cm^2 SS or TPU surface. The sponge samples were supplemented with 20 mL of buffered peptone water (25.5 g/L) (Biokar Diagnostics, Allonne, France), homogenized 2 min with a stomacher 400 (Intersciences, SaintNom-la-Breteche, France), and then the five samples were pooled. This ` resulted in 150 mL of sponged samples from a 500 cm^2 SS or TPU surface. The forty samples from the SS or TPU 500 cm^2 surfaces that were swabbed or sponged before and after C&D were used for the comparison of the sampling methods and were extracted using a single DNA extraction method.

II.2.2.4. Colony counts

The smoked salmon tissue homogenate and the sponged and swabbed samples prepared as described above were used to enumerate the total viable counts. Colony-forming units (CFU) were counted on Plate Count Agar (PCA, Oxoid, Thermo Fisher Diagnostics, Dardilly, France), supplemented with 1% NaCl (Sigma-Aldrich, Merck, Saint Quentin Fallavier, France), with 100 µL of appropriate dilution in buffered peptone water spread over the agar. CFU counts were performed in triplicate after the incubation of PCA plates in aerobic conditions at 25 °C for five days. The results were expressed in CFU per gram CSS (CFU/g) or CFU per cm^2 suface area (CFU/cm^2).

II.2.2.5. DNA extraction

DNA was extracted by using four commercial DNA extraction kits. The first was the Maxwell® 16 FFS DNA Purification kit referenced as Maxwell (Promega, Charbonniere-les-Bains, ` France). This method required the Maxwell® 16 Instruments. This automat purifies samples using paramagnetic particles providing a mobile solid phase that optimizes DNA capture, washing and elution through purification reagents in prefilled cartridges. This purification system allows an automatic, standardized and simultaneous extraction of 16 samples at the same time.

To our knowledge, no metabarcoding studies focused on CSS and FPP surface bacteria have been performed using this DNA Maxwell® 16 system extraction method. Two kits were purchased from Qiagen company (Qiagen, Courtaboeuf, France): Qiagen DNeasy PowerFood Microbial (formerly known as Mobio Powerfood Microbial) and Qiagen QIAamp BiOstic Bacteremia, referenced as QPFM and Q_2B_2, respectively. The fourth kit used, Zymo Quick-DNA Fecal/Soil Microbe Miniprep Kit referenced as ZFS, was purchased from Zymo (Ozyme, SaintCyr-l'Ecole, France). Qiagen and Zymo kits have already been used in previous surface and food microbial ecology studies (Bokulich et al., 2015; Stellato et al., 2016). The DNA purification technology is based on DNA selective binding to a spin column containing silica-based membrane, followed by several washing steps and a DNA elution. Both Qiagen kits use the MoBio PCR inhibitors removal technology. A first step of mechanical cell lysis using glass beads was achieved using a FastPrep (MPbiomedicals, Illkirch, France) for 30 s at a frequency of 6 m/s. When the Qiagen kits were used, the mechanical lysis was performed using glass beads provided by the kits. For the other DNA extraction methods, Maxwell and Zymo, 0.3 g of zirconium beads (100 µm diameter) were used (Scientific Industries, New-York, USA). DNA was extracted from three biological replicates from each batch. A Qubit® 2.0 fluorometer using the Qubit® dsDNA BR Assay Kit (Life technologies, Thermo Fisher Scientific, Villebon-sur-Yvette, France) was used to quantify DNA. Additional blank negative controls with no sample were used to exclude DNA contamination during extraction and from reagent DNA traces.

II.2.2.6. The 16S rRNA gene sequencing

II.2.2.6.1. Library preparation and sequencing via the Illumina® MiSeq platform

Extracted DNA was amplified by PCR to construct a sequencing library targeting the V3–V4 region of the bacterial 16S rRNA gene. PCR reactions were performed using 5 µL of DNA template, 12.5 µL of 2X Kapa HiFi Hotstart ready mix (Roche, Boulogne-Billancourt, France), 5 µL of 1 µM primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 785R (5′GACTACHVGGGTATCTAATCC-3′) (Klindworth et al., 2013). PCR grade water was added to reach a final volume of 25 µL. The amplification was performed with first a denaturation step at 95 °C for 5 min followed by 30 cycles of [denaturation (98 °C, 30 s) annealing (57 °C, 30 s) extension (72 °C, 30 s)] and a final extension step (72 °C, 5 min) then held at 4 °C. The resulting PCR products were purified using the Agencourt AMPure kit (Beckman Coulter, Villepinte, France).

Concentration and size were checked on a 2100 Bioanalyzer platform, using the DNA 7500 kit (Agilent Technologies, Les Ulis, France) and indexed using the Nextera XT DNA Library Prep kit (Illumina®, Paris, France) following Illumina® recommendations. Samples were then pooled in an equimolar concentration (4 nM). As described by the Illumina® protocol, the pool was denatured with 0.2N NaOH, diluted to 6 pM and mixed with 20% of 6 pM denatured phiX DNA, then sequenced on the Illumina® MiSeq using reagent kit v2 500 cycles.

II.2.2.6.2. Sequencing data processing and analysis

Both demultiplexed sequencing reads (approximately 250 bp in length) files were acquired under FASTQ format. The data processing was performed using the FROGS bioinformatic pipeline (Escudié et al., 2017). Paired-end raw reads were contiged using FLASh 1.2.11 (Magoč and Salzberg, 2011) with a maximum of 10% mismatch in the overlapped region and then filtered on their length (between 410 bp and 485 bp) with non-ambiguous nucleotides. Primers were removed using Cutadapt 1.18. Denoising and clustering of reads into Operational Taxonomic Unit (OTU) (97% identity) were performed using Swarm 2.2.2 (Mahé et al., 2015). Chimera detection and removing was then conducted using VSearch 1.3.0 (Rognes et al., 2016). OTUs with less than 5/100,000 of the total number of sequences in the entire dataset were removed (Bokulich et al., 2013b). Taxonomy assignments were performed using RDP classifier 2.11 (Wang et al., 2007; Cole et al., 2009) and the Silva 16S rRNA gene database (SSURef_128_SILVA), (Quast et al., 2013). OTUs with a genus affiliation bootstrap threshold < 0.8 were removed.

II.2.2.7. Statistical analysis

Statistical analyses and plots were performed in the R environment (R Core Team, 2018). Means were compared using *t*-test for paired observations to compare CFU numbers. ANOVA was used to assess each condition effect on CFU counts and alpha-diversity metrics. The Tukey test was also used as an ANOVA *post hoc* test.

For metabarcoding, alpha-diversity indices (number of observed OTUs, Chao1 and Shannon indices) were calculated in the R environment by using the Phyloseq 1.30.0 package and its dependencies (McMurdie and Holmes, 2013). For the three different experiments, samples' library sizes (number of reads) were rarefied to an even depth (smallest number of reads per sample) to be normalized.

Permutational multivariate analysis of variances (PERMANOVA) based on Jaccard and Bray-Curtis distance matrices were carried out by using 9,999 permutations to detect significant effects in the bacterial community analyzed. For DNA extraction efficacy estimation, a ratio $\frac{Observed\ OTUs\ index}{Chao1\ index}$ was calculated.

II.2.3. Results

II.2.3.1. Comparison of DNA extraction methods to characterize CSS bacterial communities

II.2.3.1.1. Bacteria counts and quantification of DNA extracted

Bacterial communities were collected from a vacuum-packed CSS sample stored at 4 °C. Total DNA was extracted in triplicate from 5 mL of smoked salmon tissue homogenate (SSTH), corresponding to 0.5 g CSS (1/10 dilution). The total viable counts of the CSS sample were 5.36 ± 0.08 Log CFU/g. DNA concentrations ranged between 7.3 ± 1.4 ng/µL to 129.5 ± 21.9 ng/µL (**Table I**) depending on the DNA extraction method.

Table I. DNA concentration and overall 16S rDNA sequencing output parameters of samples from cold-smoked salmon using different DNA extraction methods

	Maxwell	**QPMF**	**Q_2B_2**	**ZFS**
DNA (ng/µL)	129.5 ± 21.9^{a}	24.75 ± 3.96^{b}	80.6 ± 55.9^{ab}	7.32 ± 1.41^{b}
Observed OTUs	32.3 ± 4.51^{ab}	26 ± 2.65^{a}	33.3 ± 3.21^{ab}	45.7 ± 10.5^{b}
Chao1	34.4 ± 6.75^{a}	28.8 ± 2.41^{a}	40.2 ± 5.69^{a}	46.8 ± 11.5^{a}
Observed OTUs/Chao1	0.95 ± 0.05	0.90 ± 0.03	0.84 ± 0.08	0.98 ± 0.02
Shannon index	1.81 ± 0.062^{a}	1.57 ± 0.106^{b}	1.17 ± 0.022^{c}	2.40 ± 0.104^{d}

OTU: Operational Taxonomic Unit. The DNA extraction methods were Maxwell® 16 FFS DNA Purification kit (Maxwell), Qiagen DNeasy PowerFood Microbial kit (QPFM), Qiagen QIAamp BiOstic Bacteremia kit (Q_2B_2), Zymo Quick-DNA Fecal/Soil Microbe Miniprep Kit (ZFS). ^{a,b,c,d} Means sharing the same letter superscript do not differ significantly, $p < 0.05$.

II.2.3.1.2. Effect of DNA extraction methods on CSS bacterial diversity

DNA extracted by the four methods were used as the template for the V3–V4 regions of the 16S rRNA gene sequencing. A total of 544,921 reads passed filters applied through the FROGS pipeline workflow with an average of 45,411 reads per sample ± 19,869 reads. Samples were rarefied to an even depth of 10,521 reads. Observed richness (number of observed OTUs), estimated richness (Chao1) and evenness diversity index (Shannon) were calculated for each DNA extraction method (**Table I**). The DNA extraction methods had a significant effect on observed OTUs richness ($p = 0.02$).

The main difference was due to the ZFS and QPMF methods with 45.7 ± 10.5 observed OTUs for ZFS and 26 ± 2.6 for QPMF. There was no impact on the Chao1 index ($p = 0.07$). DNA extraction influenced the community evenness ($p < 0.0001$). Based on the Shannon index, the ZFS method provided the most diverse bacterial community.

Based on the observed OTUs/Chao1 ratio, the Maxwell, QPMF and ZFS methods showed a high OTUs recovery rate. To evaluate richness and diversity differences, metabarcoding data of each sample were examined at the genus level (**Figure 13**). A total of 90 OTUs, which were agglomerated in 61 genera, were identified. Dominant genera were Firmicutes *Staphylococcus* (28.45% ± 18.5), *Brochothrix* (24.73% ± 9.68), *Carnobacterium* (0.54% ± 0.51), β-Proteobacteria *Photobacterium* (21.28% ± 29.88), *Vibrio* (10.02% ± 6.18), *Serratia* (3.15% ± 2.79), γ-Proteobacteria *Psychrobacter* (1.66% ± 1.07) and α-Proteobacteria *Sphingomonas* (0.37% ± 0.69). However, in samples extracted using ZFS, other dominant bacteria were observed, such as α-Proteobacteria *Rhizobium* (3.16% ± 5.8), β-Proteobacteria *Delftia* (2.02% ± 4.44) and Firmicutes *Anaerobacillus* (1.57% ± 2.84). The Bray-Curtis (**Figure 14**) and Jaccard Principal Coordinates Analysis (PCoA) (**Supplementary Figure S1**) highlighted a potential effect of DNA extraction methods on both richness and evenness. These observations were confirmed by PERMANOVA analysis on both Jaccard ($p < 0.0001$) and Bray-Curtis distance matrices ($p < 0.0001$) explaining respectively 62.6% and 88.7% of the microbiota differences. The ZFS method was removed from our comparison and the three other methods were kept to evaluate their impact on surface bacterial communities.

II.2.3.2. Comparison of DNA extraction methods to characterize surface bacterial communities

II.2.3.2.1. Bacteria counts and quantification of DNA extracted

Surface bacterial communities were collected by swabbing SS coupons after or before C&D. Fifteen swabs samples were pooled, resulting in a 60 mL surface sample corresponding to a 750 cm^2 SS surface. Total DNA was extracted in triplicate from 2 mL of SS surface samples using the different DNA extraction methods (18 DNA extracted samples). Total CFU counts varied from 3.29 ± 0.21 Log CFU/cm^2 to 4.39 Log CFU/cm^2 ± 0.04 after and before C&D respectively. C&D had a significant effect on total viable bacteria counts ($p < 0.0001$). DNA concentrations ranged between 0.35 ± 0.04 ng/µL to 1.06 ± 0.62 ng/µL before C&D and between 0.05 ± 0.02 ng/µL to 0.150 ± 0.003 ng/µL after C&D (**Table II**). The results showed that the range of DNA concentrations before C&D were ten times higher than after C&D.

This highlighted a significant effect of C&D on DNA concentrations ($p = 0.0004$). No differences in the DNA concentration extracted using the three extraction methods were observed ($p = 0.059$).

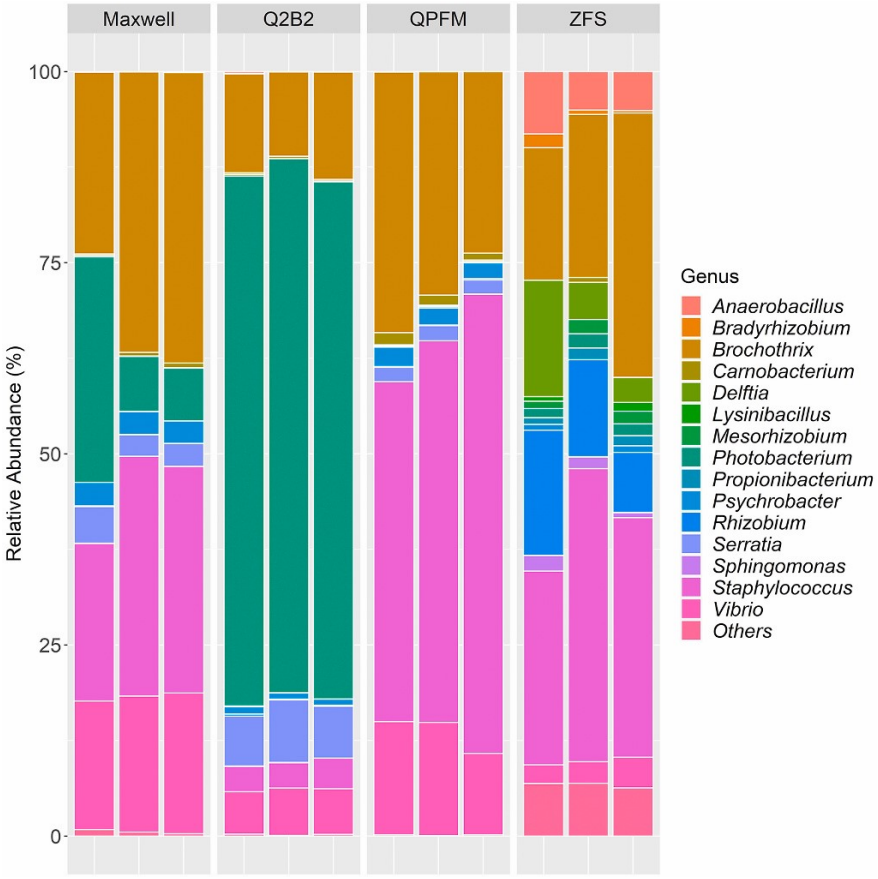

Figure 13. Relative abundance of bacterial genera in cold-smoked salmon sample extracted with four different DNA extraction methods in triplicate. Taxa present on average in all samples at a threshold ≥ 0.5% or having a 90th percentile ≥ 0.5% are individually represented. In other cases, taxa are grouped and labeled "Others".

II.2.3.2.2. Effect of DNA extraction methods on the 16S rDNA analysis of surface bacterial communities

DNA extracted by the three remaining methods were used as the template for the V3–V4 regions of the 16S rRNA gene sequencing. A total of 1,495,580 reads passed filters applied through the FROGS pipeline workflow with an average of 83,088 reads/sample ± 33,177 reads. Samples were rarefied to an even depth of 34,345 reads. Observed OTUs, Shannon and Chao1 alpha-diversity indices were calculated for each DNA extraction method and cleaning conditions (**Table II**). DNA extraction methods had no effect on the population richness: observed OTUs ($p = 0.15$) and Chao1 index ($p = 0.84$) and no influence on Shannon diversity ($p = 0.2$). These methods had a high observed OTUs/Chao1 ratio and seemed to share a high OTUs recovery rate.

Indeed, no differences on richness and evenness were observed between the methods. Among the different methods, C&D had an effect on both observed OTUs richness and evenness ($p = 0.0001$ and $p = 0.002$ respectively) but no effect on the Chao1 index ($p = 0.11$). Overall, samples after C&D appeared to be richer (with an average of 154 observed OTUs ± 3.63) and more diverse (Shannon index average of 3.14 ± 0.06) than before C&D. Interestingly, no cleaning-linked differences on observed OTUs were identified on samples extracted by Maxwell (148 OTUs ± 3.61 before C&D and 154 OTUs ± 1.53 after C&D, $p = 0.85$) and Q_2B_2 (143 OTUs ± 8.5 before C&D and 155 OTUs ± 2.52 after C&D, $p = 0.22$). Furthermore, no cleaning-linked differences on the Shannon index were observed on samples extracted by Maxwell (2.99 ± 0.02 before C&D and 3.08 ± 0.03 after C&D, $p = 0.40$) and QPMF (3.07 ± 0.11 before C&D and 3.12 ± 0.036 after C&D, $p = 0.94$).

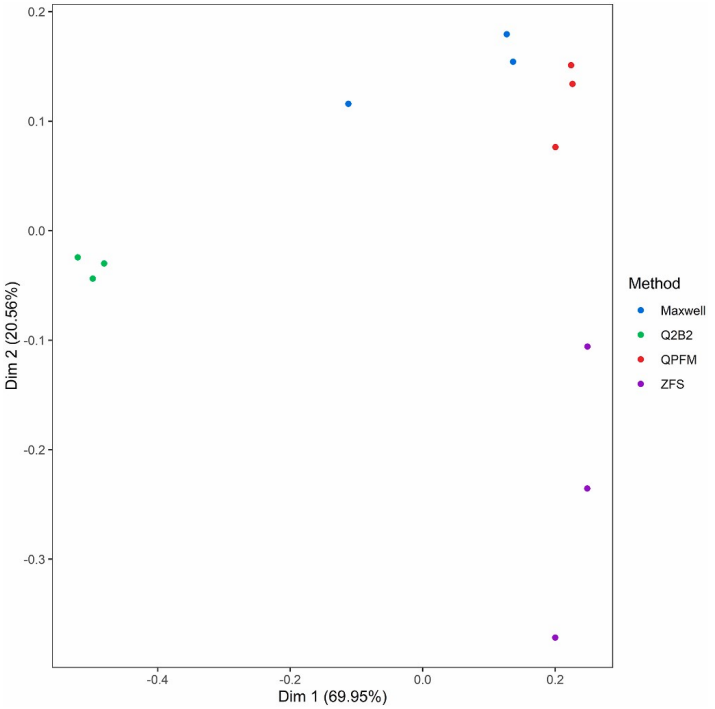

Figure 14. Bray-Curtis Principal Coordinates Analysis plot of CSS samples extracted with four different DNA extraction methods.

Table II. DNA concentrations, observed diversity richness (OTUs), estimated OTU richness (Chao 1), Shannon, observed species before and after cleaning and disinfection step and the DNA extraction methods

	Before C&D			After C&D		
	Maxwell	**QPFM**	**Q_2B_2**	**Maxwell**	**QPFM**	**Q_2B_2**
DNA (ng/µL)	0.354 ± 0.04^{ab}	0.612 ± 0.13^{ab}	1.06 ± 0.617^{b}	0.074 ± 0.01^{a}	0.054 ± 0.02^{a}	0.15 ± 0.003^{a}
Observed OTUs	148 ± 3.61^{ab}	135.3 ± 9.29^{a}	143 ± 8.50^{ab}	154 ± 1.53^{b}	152 ± 6.11^{b}	155 ± 2.52^{b}
Chao1	153 ± 10.1^{a}	146 ± 15.7^{a}	153 ± 11.1^{a}	157 ± 0.525^{a}	159 ± 6.37^{a}	159 ± 2.56^{a}
Observed OTUs/Chao1	0.97 ± 0.04	0.93 ± 0.04	0.94 ± 0.01	0.98 ± 0.01	0.96 ± 0.02	0.98 0.00
Shannon index	2.99 ± 0.02^{ab}	3.07 ± 0.11^{bc}	2.89 ± 0.06^{a}	3.08 ± 0.03^{bc}	3.12 ± 0.036^{bc}	3.21 ± 0.04^{c}

OTU: Operational Taxonomic Unit. C&D: cleaning and disinfection step. The DNA extraction methods were Maxwell® 16 FFS DNA Purification kit (Maxwell), Qiagen DNeasy PowerFood Microbial kit (QPFM), Qiagen QIAamp BiOstic Bacteremia kit (Q2B2), Zymo Quick-DNA Fecal/Soil Microbe Miniprep Kit (ZFS).

[a,b,c] Means sharing the same letter superscript do not differ significantly, $p < 0.05$.

To evaluate differences in the bacterial communities, the metabarcoding data of each sample were examined at the genus level (**Figure 15**). A total of 167 OTUs, which were agglomerated into 49 genera, were identified. The most abundant genera were *Flavobacteriaceae Chryseobacterium* (37.02% ± 5.8), *Flavobacterium* (2.72% ± 1.23), γ-Proteobacteria *Acinetobacter* (40.77% ± 3.82) and *Pseudomonas* (9.2% ± 4.1). All of these genera were found before and after sanitization procedures. Both Bray-Curtis (**Figure 16**) and Jaccard (**Supplementary Figure S2**) PCoA showed a clear separation between samples according to the C&D. PERMANOVA analysis based on Bray-Curtis and Jaccard dissimilarity index showed that cleaning procedures influenced the bacterial community ($p < 0.0001$) and explained 75.9% and 33.5% of the microbiota differences. No effect from DNA extraction methods was observed based on Bray-Curtis ($p = 0.76$) and Jaccard dissimilarity index ($p = 0.16$). DNA extraction methods explained only respectively 5.9% and 15.7% of the microbiota differences.

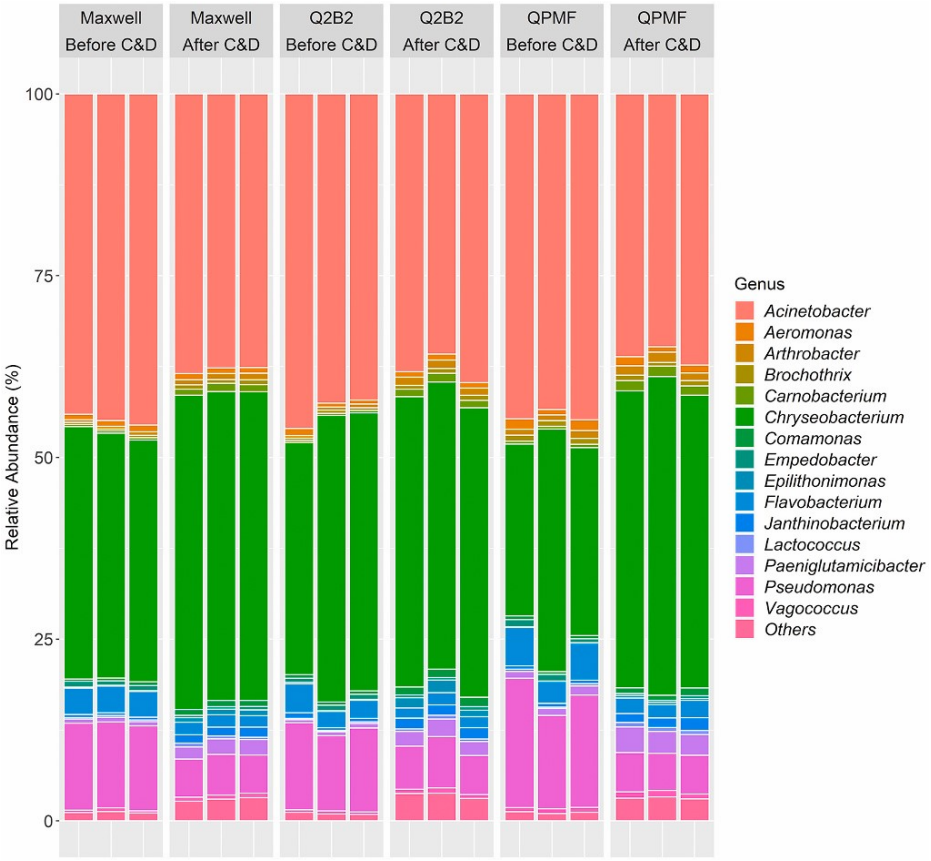

Figure 15. Relative abundance of bacterial genera on stainless steel coupon placed within the food processing plant extracted by three different DNA extraction method in triplicate before and after cleaning and disinfection. Taxa present on average in all samples at a threshold ≥ 0.5% or having a 90th percentile ≥ 0.5% are individually represented. In other cases, taxa are grouped and labeled "Others".

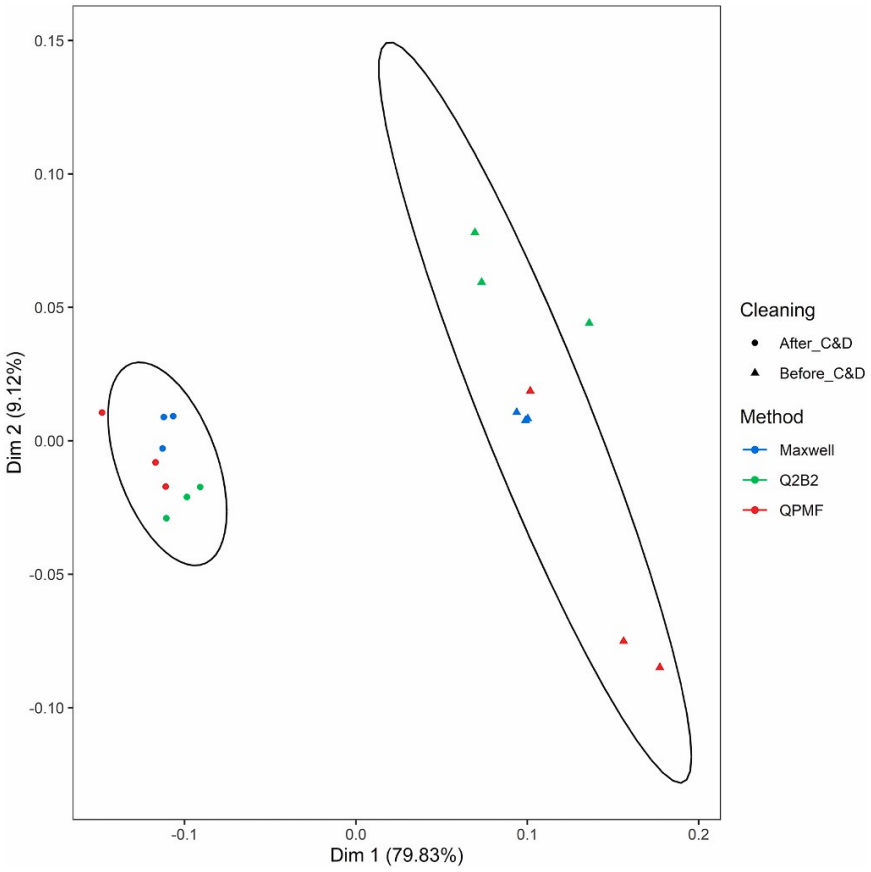

Figure 16. Bray-Curtis Principal Coordinates Analysis plot of environment samples according to cleaning and disinfection procedures and DNA extraction methods.

II.2.3.3. Effect of surface bacterial sampling method on 16S rDNA metabarcoding alpha-diversity data analysis

SS and TPU coupons (30 × 25 cm, 750 cm2) were installed in the FPP near food contact surfaces and under a conveyor between the salting step and the filleting machine for four weeks before surface sampling. The SS and TPU coupons then were sampled by swabbing and sponging each day before and after C&D over five consecutive days. This generated 40 samples which were analyzed for total viable counts and 16S rDNA metabarcoding. Enumerations were variable among the sampling days and methods. (**Table III**). The sampling methods (swabbing or sponging), the surface (SS or TPU) and the C&D step (before, after) had a significant effect on the viable counts with no interaction effect ($p < 0.0001$, $p = 0.04$, $p = 0.04$ respectively). Indeed, on both SS and TPU, before C&D, sponging showed a higher CFU count (4.68 ± 0.84 Log CFU/cm^2 on SS and 5.58 ± 0.72 Log CFU/cm^2 on TPU) than swabbing (2.52 ± 0.85 Log CFU/cm^2 on SS and 3.07 ± 0.49 Log CFU/cm^2 on TPU).

The same trend was observed after C&D: 3.45 ± 1.70 Log CFU/cm^2 on SS and 4.84 ± 0.55 Log CFU/cm^2 on TPU for sponging and 1.68 ± 2.06 Log CFU/cm^2 on SS and 2.36 ± 1.79 Log CFU/cm^2 on TPU for swabbing. Whatever the conditions, swabbing provided a lower colony recovery.

In addition, whatever the sampling method or the cleaning step, more CFU were enumerated on TPU samples ($p = 0.04$), with an average of 3.96 ± 1.64 Log CFU/cm^2, than on SS samples, whose average was of 3.08 ± 1.76 Log CFU/cm^2.

To avoid a DNA extraction bias, the extraction was performed with a single method. Extraction using QPFM were performed in triplicate. One hundred and twenty samples were sequenced. A total of 5,483,544 reads passed filters applied through the FROGS pipeline workflow with an average of 35,648 reads/sample ± 20,209 reads. Due to a low number of reads, five samples were removed. The relative abundance at the genus level was represented (**Figure 17**). The other 115 samples were rarefied to an even depth of 8,455 reads. For each sample, richness and evenness represented by observed OTUs, Chao1 and Shannon indexes were evaluated (**Table III**). Richness was impacted significantly by the sampling method and interactions between C&D procedures/surfaces and sampling methods/surfaces ($p < 0.0001$, $p = 0.011$, $p = 0.007$ respectively for observed OTUs and $p < 0.0001$, $p = 0.003$, $p = 0.007$ respectively for Chao1). C&D have an effect on Chao1 richness ($p = 0.04$) but not on observed OTUs index ($p = 0.06$). The surface material type (SS or TPU) did not modify the richness ($p = 0.17$ and $p = 0.52$ respectively for observed OTUs and Chao1 index). The Chao1 richness was higher within the population recovered before C&D and using the sponge as the sampling method. The Shannon index (**Table III**) showed that diversity was dependent on the sampling method and C&D ($p < 0.0001$ and $p = 0.04$ respectively). Bacterial communities appeared to be richer and more diverse when sampled by sponging. Interestingly, no differences on richness and evenness were observed on swab samples ($p > 0.05$)

Table III. CFU count, DNA concentrations observed diversity richness (OTUs), estimated OTU richness (Chao 1), Shannon, observed species before and after cleaning and disinfection step and the sampling method.

	Before C&D				After C&D			
	Stainless Steel		TPU		Stainless Steel		TPU	
	Swab	Sponge	Swab	Sponge	Swab	Sponge	Swab	Sponge
Log(CFU)/cm²	2.52 ± 0.85^{ab}	4.68 ± 0.84^{bc}	3.07 ± 0.49^{abc}	5.58 ± 0.716^{c}	1.68 ± 2.06^{a}	3.45 ± 1.7^{abc}	2.36 ± 1.79^{ab}	4.84 ± 0.545^{bc}
[ADN]	0.39 ± 0.4^{ab}	3.32 ± 1.83^{bc}	1.52 ± 1.05^{ab}	5.97 ± 7.7^{c}	0.3 ± 0.55^{ab}	0.11 ± 0.09^{a}	0.13 ± 0.22^{ab}	0.4 ± 0.36^{ab}
Observed OTUs	111.7 ± 13.8^{a}	159.9 ± 13.8^{b}	109 ± 8.37^{a}	135 ± 19.6^{c}	100 ± 24.6^{a}	141 ± 22^{bc}	111 ± 16.9^{a}	138 ± 16.9^{c}
Chao1	121 ± 24.8^{a}	190 ± 18.5^{b}	120 ± 13^{a}	159 ± 26^{c}	108 ± 28.4^{a}	159 ± 26.7^{c}	126 ± 23.7^{a}	161 ± 19.7^{c}
Shannon index	2.67 ± 0.24^{a}	2.91 ± 0.29^{ab}	2.64 ± 0.33^{a}	3.09 ± 0.17^{bc}	2.65 ± 0.43^{a}	3.11 ± 0.47^{bc}	2.69 ± 0.34^{a}	3.32 ± 0.15^{c}

OTU: Operational Taxonomic Unit. C&D: cleaning and disinfection step.

[a,b,c,] Means sharing the same letter superscript do not differ significantly, $p < 0.05$.

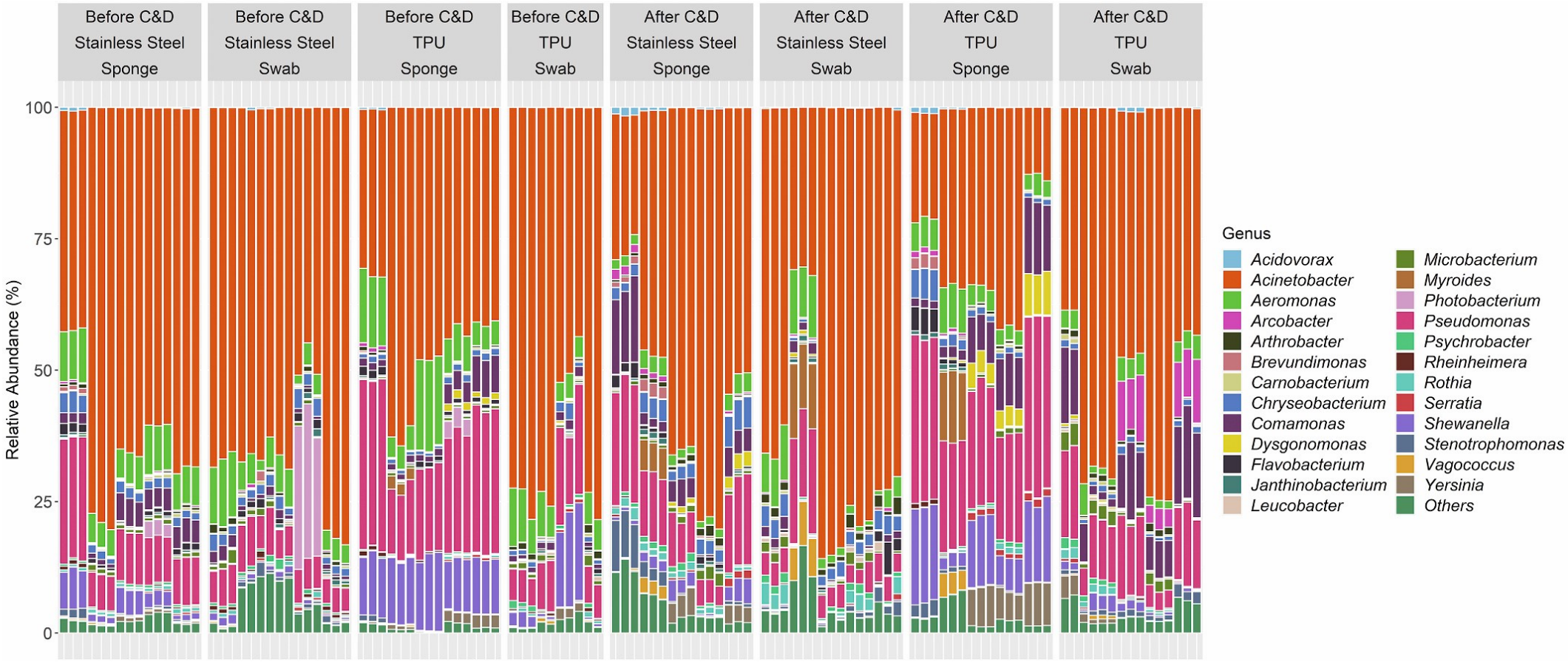

Figure 17. Relative abundance of bacterial genera on stainless steel and tri-polyurethane coupons placed within the food processing plant in triplicate. Sampling was performed by swabbing and sponging, before and after cleaning and disinfection procedures during 5 consecutive days. Only 115 samples are represented: 5 outliers were removed for statistical purposes. Taxa present on average in all samples at a threshold ≥ 0.5% or having a 90th percentile ≥ 0.5% are individually represented. In other cases, taxa are grouped and labeled "Others".

A total of 279 OTUs, which were agglomerated into 101 genera, were identified. The dominant populations in each condition of the study were γ-Proteobacteria *Acinetobacter* (55.23% ± 18.71), *Pseudomonas* (13.63% ± 8.61), *Aeromonas* (5.56% ± 3.73), *Shewanella* (4.52% ± 5.46) and β-Proteobacteria *Comamonas* (4.09% ± 4.41). PERMANOVA analysis based on Bray-Curtis and Jaccard dissimilarity index showed that the cleaning procedures, surface and sampling method influenced the bacterial community ($p < 0.0001$). Based on the Bray-Curtis dissimilarity index, cleaning procedures, sampling surfaces and sampling devices explained respectively 6.13%, 9.85% and 10.51% of microbiota differences. Based on the Jaccard dissimilarity index, cleaning procedures, sampling surfaces and sampling devices explained respectively 5.65%, 3.36% and 8.9% of microbiota differences.

II.2.4. Discussion

This study sought to evaluate the impact of DNA extraction and surface sampling methods on 16S rDNA metabarcoding analysis results. The efficiency of four available commercial kits with different lytic and purification technologies were compared to extract bacterial DNA from cold-smoked salmon and SS coupons installed in a CSS processing plant. In addition, the efficiency of two sampling devices were evaluated on SS and TPU coupons installed in the same FPP. β-Proteobacteria *Photobacterium* and *Serratia* and the Firmicutes *Brochothrix*, *Carnobacterium,* and *Staphylococcus* were identified in this study as the dominant genera in the CSS bacterial community. With the exception of *Staphylococcus,* these genera are known as CSS spoilers. *Staphylococcus* is more rarely described as a CSS dominant bacterium. This result confirms already published data on CSS microbiota studied by viable and cultivable methods (Leroi et al., 1998; Løvdal, 2015; Olofsson et al., 2007). Because of its halophilic property, the salt additive used in the CSS process could be a source and a root cause for *Staphylococcus* predominance (Marino et al., 2017). The *Psychrobacter* genus is widely represented in our study. *Psychrobacter* are psychrothrophic and halotolerant bacteria which already have been isolated from fresh salmon fillets and the processing plant involved (Møretrø et al., 2016). Raw salmon might be a source for *Psychrobacter* residence in FPPs (Møretrø and Langsrud, 2017). *Sphingomonas* was identified as a low abundant bacterium. This genus was previously identified as a fish pathogen and could be part of the aquacultured rainbow trout intestinal microbiota (Pękala-Safińska, 2018; White et al., 1996; Wong et al., 2013).

The dominant surface microbiota identified was mainly composed by *Flavobacteriaceae Flavobacterium*, *Chryseobacterium*, γ-Proteobacteria *Acinetobacter*, *Pseudomonas*, *Aeromonas, Shewanella* and β-Proteobacteria *Comamonas*. *Acinetobacter* and *Pseudomonas* already have been described to dominate the bacterial population in processing plants of many foods such as vegetables, meat, poultry, dairy products, seafood and seafood products (Møretrø and Langsrud, 2017). Both *Acinetobacter* and *Pseudomonas* can be responsible for CSS spoilage (Langsrud et al., 2016; Møretrø et al., 2016) and are able to persist in these environments due to their biofilm production and psychrotrophic properties (Møretrø and Langsrud, 2017). *Flavobacterium* was described to promote *Listeria monocytogenes* growth in biofilms (Bremer et al., 2001). *Chryseobacterium* have been highlighted in a previous study on Atlantic salmon fillet microbiota. This genus was found in the dominant population (Wang et al., 2019) and could be brought into the FPP by raw material. In addition, *Chryseobacterium* was also identified in the microbial composition of drain water and drain biofilms (Dzieciol et al., 2016). *Shewanella* and *Aeromona*s have been described as fish and seafood product spoilers (Gram and Dalgaard, 2002; Gram and Huss, 1996). These genera have been characterized within the residential microbiota of fish and seafood FPP (Møretrø and Langsrud, 2017).

The first part of this work was dedicated to CSS samples. Significant differences were observed in the DNA concentrations obtained using four different DNA extraction methods. The four methods were used on the same sample, highlighting the different efficiency of each method to extract DNA from CSS. Three of the four methods tested were spin column method-based. These methods shared the same lysis principle, unlike the Maxwell kit which is based on paramagnetic beads system technology. Moreover, the Maxwell kit recovered the highest DNA quantity. The lysis and purification principles of the different methods could explain the difference in the DNA extraction efficiency. Keisam et al. (2016) tested eight different DNA extraction methods with different lysis principles on several fermented products (milk, fish, bamboo shoot, soybean). In this study, the authors observed that the method used had a strong effect on the DNA concentration. Their observation was mostly explained by the different foods tested. Even when the bacteria concentration was homogenous (between 7.54 ± 0.23 to 10.79 ± 0.10 Log of the 16S rRNA gene copies/g of food), strong differences were observed. Communities were different, dominated by Gram-positive bacteria in bamboo shoot and fish samples and Gram-negative bacteria in milk and soybean. Gram-negative bacteria are easier to extract, which could explain this difference.

In addition, the authors did not discuss sample chemical composition, which could lead to different DNA extraction yields or community assessments (lipids, PCR inhibitors). Hart et al. (2015) compared methods used to extract DNA from the feces of different animal species. These authors tested five different DNA extraction methods, including spin columns types such as the Mobio and Qiagen kits, as well as isopropanol precipitation, and found a statistically significant difference. These results showed that the Qiagen and Mobio methods lead to consistent DNA concentrations.

In this study, the DNA methods used to extract DNA from CSS samples had an effect on the alpha-diversity observed OTUs index but not on the Chao1 index. Chao1 is a species richness estimator (Kim et al., 2017). The difference between the observed OTUs and Chao1 indices, especially the Q2B2 method, could be explained by a lower DNA extraction quality or yield. The four methods tested also had an effect on the diversity and relative abundance of the CSS sample microbiota. Keisam et al. (2016) showed that different DNA extraction methods induce a variability in the diversity of fermented food microbiota. In addition, a high DNA concentration did not necessarily lead to the highest community diversity. In our study, we confirmed this observation as ZFS, which seemed to recover the lowest DNA yield, provided the highest bacterial diversity. Regarding our results, the following genera were found only on ZFS samples: *Massilia, Pseudoxanthomonas* and *Deinococcus*. It is likely that we were not able to optimize the ZFS method, most probably due to the low bacterial concentration in the CSS sample, leading to a weak amount of bacterial DNA extracted. Since the above-mentioned genera including *Delftia, Anaerobacillus* and *Rhizobium* were already described as present in molecular biology kits reagents (Salter et al., 2014), we assume that most probably the Taq DNA polymerase during the V3–V4 16S rDNA PCR also could have amplified DNA from reagents or exogenous contamination. This also could explain the high diversity within the ZFS samples. Keisam et al. (2016) suggested that weak diversity in the case of high DNA concentrations also may be explained by an easier access to dominant bacterial DNA, limiting the purification of low abundant taxa. Several studies have demonstrated strong differences in dominant bacteria of human feces microbial communities depending on the DNA extraction and purification methods used (Kennedy et al., 2014; Wesolowska-Andersen et al., 2014). Our data confirmed that DNA extraction methods could influence the analysis of bacterial community profiles.

To compare the three remaining DNA extraction methods on surface samples, bacterial communities were collected on SS coupons installed in the FPP by swabbing before and after C&D.

As described with the CSS samples, the method used did not have an effect on the DNA concentration. However, a decrease in DNA concentrations and total viable counts was observed after C&D. Alpha-diversity metrics highlighted that contrary to CSS samples, the DNA extraction methods showed no effect on community richness or evenness. These observations were also confirmed by PERMANOVA analysis. This suggests that the capacity of these methods to extract DNA from surface samples is similar in terms of quality and yield. Cleaning procedures strongly affect the microbiota and alpha-diversity indicators. According to Bray-Curtis and Jaccard PCoA, the cleaning and disinfection procedures have a strong effect on bacterial community relative abundances, and a lighter effect on its richness. Interestingly, the observed OTUs richness and Shannon diversity were higher after cleaning and disinfection. Water flow and friction may have destabilized some biofilms structure and allowed us to capture more bacteria or different taxa.

However, C&D had no impact on the number of OTUs of samples extracted by Q_2B_2 and Maxwell, nor on the microbiota evenness on Maxwell and QPMF samples. Total CFU counts varied from 3.29 ± 0.21 Log CFU/cm^2 to 4.39 Log CFU/cm^2 ± 0.04 after and before C&D respectively. Bacterial concentration was impacted by sanitation but not OTUs number. 16S rDNA metabarcoding targets all bacterial DNA (living cells, dead cells, stressed cells). It is likely, even if water flow may have physically dispatched cells, that we also could have sampled dead or stressed bacteria. QPMF and Q_2B_2 share the same lytic and purification system; however, Maxwell uses paramagnetic particles system technology. Vesty et al. (2017) evaluated the efficiency of different commercial kits (including MoBio PowerSoil® DNA Isolation Kit, QIAamp® DNA Mini Kit, Zymo Bacterial/Fungal DNA Mini Prep™) for DNA extraction from oral human samples. The authors also did not observe any differences on alpha-diversity data or observed microbiota. In contrast, in a study comparing four DNA extraction methods on skin microbiota (Nucleospin® Soil (Macherey Nagel), Nucleospin® Tissue (Macherey Nagel), FastDNA™ SPIN Kit for Soil (MP biomedicals) and DNeasy Blood & Tissue Kit (Qiagen), Boulesnane et al. (2020) found that the richness identified by each method varied. The authors concluded that the differences mainly were due to the co-extraction of PCR inhibitors as well as the method's DNA binding system. DNA extraction protocols and lysis principles seem to be key factors in metabarcoding analysis.

As previously suggest by Hart et al. (2015), our results showed that DNA extraction and standardized experimental protocols in microbiome analysis are critical steps and should be subject to validation procedures. This work showed the clear importance to test the DNA extraction method before 16S rDNA metabarcoding studies to ensure that the method fits the intended purpose. By studying the impact of DNA extraction methods on fecal samples from five different species (zebrafish, mouse, cat, dog and horse), Hart et al. (2015) highlighted host species differences on DNA yield and NGS output. As we could see, DNA extraction methods are matrix dependent. Most of the differences were observed on CSS samples and not on surface samples. Even if surface samples can contain disinfectant traces, food matrices are more complex to process. QPMF and Maxwell used on product samples shared a high OTUs recovery rate and a high evenness. Based on this work and our observations, Maxwell and QPMF seemed to be the best DNA extraction methods in the context of our study. Because Maxwell is an automatic approach and is equipment dependent, QPMF was chosen for further analysis.

Attachment strength, exopolysaccharides, and biofilm production are key factors that should be considered carefully when recovering surface bacteria. A critical stage in the analysis of surface microbiota is the choice of the sampling method. In this study, we evaluated cell recovery by swabbing and sponging methods used to sample SS and TPU surfaces. Viable cell counts showed that sponging provided a better recovery strength on SS and TPU. These observations could be explained by the pressure variation on the device and its sampling surface (Yamaguchi et al., 2003). In addition, a swab tip would be saturated faster than a device such as a sponge and could lead to a reduced recovery capacity (Pérez-Rodríguez et al., 2008). Friction and pressure on devices should enhance and facilitate the bacterial transfer from the surface to the sampling system. Even when the sampling is made by a single operator, sponges are clearly easier devices to press on surfaces and to facilitate the transfer of cells (Knobben et al., 2007; Pérez-Rodríguez et al., 2008). We observed that whatever the sampling device, more CFU could be recovered on TPU than SS coupon surfaces, suggesting a higher bacterial surface population on TPU. Firmesse et al. (2012), when studying bacterial detachment in a delicatessen environment, described no significant differences between cultivable and viable but non-cultivable cells recovered from PVC or SS. Bacterial communities and biofilms should be more difficult to remove from polymer surfaces. Moreover, a porous surface such as TPU could facilitate cell attachment and biofilm formation (Midelet and Carpentier, 2002).

This could explain the differences in bacterial surface populations from SS and TPU surfaces observed in this work.

16S rDNA metabarcoding data, and especially alpha-diversity evaluation of bacterial communities, gave another perspective on sampling method effects. The bacterial concentration on FPP surfaces, especially after C&D, is very low. In our study, total counts varied from 1.68 ± 2.06 to 5.58 ± 0.72 Log CFU/cm2.

According to several standards, C&D procedures can decrease the level of contamination to 2.5 CFU/cm2 (Møretrø and Langsrud, 2017). Coupons were placed under food contact surface samples but were easy to clean. Surfaces were visually soiled before C&D, cleaned after procedures and mimicked well the FPP surfaces. A common challenge in the analysis of low bacterial numbers is to extract DNA and to dispose of enough material for sequencing purposes. Indeed, a maximum of 5.97 ± 7.70 ng/µL of DNA were extracted in total and a maximum of 0.40 ± 0.36 ng/µL were extracted after C&D. The limit for sequencing application on a low bacterial environment has been evaluated between 10^3 and 10^5 CFU/mL in drinking water samples (Pinto et al., 2012). However, in their study on detection limits, DNA extraction and primer choice influences on drinking water bacterial communities, Brandt and Albertsen (2018), were able to sequence samples at a limit of 10^1 CFU/mL. This was achieved by spiking samples with different concentrations of an *E. coli* culture. However, background noise OTUs were identified at this low concentration. Due to the limit of the method, we had to remove five samples from our study.

To our knowledge, no previous publications reporting a sampling effect on the alpha-diversity indices of bacterial surface communities evaluated from 16S rDNA metabarcoding data are available. We observed that both richness and evenness were impacted by sampling devices and cleaning and disinfection. However, the surface material (SS or TPU) had no influence on richness and evenness. The analysis of sponged samples showed a higher bacterial community richness and evenness, suggesting that this method was more efficient than swabbing in recovering surface bacteria. The microbiota composition was affected by cleaning and disinfection, surface material and sampling devices. The dominant populations were not affected; however, the subdominant population may have been sampled. As for DNA extraction comparison on surface samples, we did not assess the viability of recovered cells. Indeed, using HTS after C&D cannot determine whether or not the bacteria are viable. 16S rDNA amplicons allow the targeting of one or more microorganisms within samples. However, the induced PCR reaction will target all free DNA in the reaction medium. The DNA of dead cells is thus amplified and identified (Klein et al., 2012). One way to counteract this bias is the use of a DNA intercalating molecule: propidium monoazide (PMA).

In their study to characterize the total and the viable bacterial and fungi populations in the international space station, Checinska Sielaff et al. (2019) processed samples with PMA. Their results highlighted no significant differences on microbiome richness and diversity, suggesting that the 16S rDNA amplified was extracted from a viable population. Moreover, an average of 45% of the microorganisms were cultured suggesting that an average of 55% of the microorganims were in a VBNC state. The use of PMA in our study could be a future relevant approach to assess C&D surviving populations.

II.2.5. Conclusion

Due to their important effects on food product quality and safety, residential bacteria in food processing plants are an important concern. Thanks to high-throughput sequencing technologies and the development of 16S rDNA metabarcoding approaches, it is now possible to estimate the taxonomic composition of bacteria communities without needing to use cell cultures. However, these techniques require strict standardization to avoid downstream analytical biases. Although similar comparative research on the impact of different DNA extraction methods has been performed on other ecological niches, this is the first investigation on cold-smoked salmon product microbiota and the product's processing plants using Illumina® MiSeq amplicon sequencing. The bias generated due to the differential recovery of OTUs by different DNA extraction methods is demonstrated here for CSS bacterial communities but not for surface microbiota. We report that both richness and evenness were impacted by sampling devices and cleaning and disinfection. Higher bacterial community richness and evenness were observed when sponge was used as the sampling method.

The Maxwell and QPMF DNA extraction methods and the sponge device appeared to be efficient and reliable methods to study the CSS and surface microbiota. This study highlighted the importance of sample preparation and laboratory practices on the results of 16S rDNA metabarcoding studies. The sequencing platforms and bioinformatic pipelines also could be critical choices for these kinds of analyses. This study only represents a survey of one CSS processing plant and provides basic insights on the surface bacterial community profile. Future investigations would be necessary to investigate potential contamination/cross contamination to CSS products deriving from the processing plant environment. Surface bacterial community monitoring by 16 rDNA metabarcoding may become a valuable approach in food processing plants to identify critical control points, thereby allowing the improvement of process and sanitation management.

II.3. Ce qu'il faut retenir du Chapitre II

Les approches de metabarcoding ont été largement utilisées en écologie microbienne alimentaire. Cependant, les choix méthodologiques peuvent biaiser les résultats. Dans cette étude, les communautés bactériennes associées aux produits de saumon fumé et aux surfaces des usines de transformation ont été analysées par metabarcoding du gène de l'ARNr 16S. L'impact des méthodes d'extraction d'ADN, des méthodes d'échantillonnage (écouvillonnage ou épongeage) et des matériaux de surface sur les communautés bactériennes a été étudié.

Les analyses de diversité α et β ont révélé que les méthodes d'extraction d'ADN influencent principalement la composition du microbiote du saumon fumé observé. De plus, les différentes méthodes d'extraction d'ADN ont révélé des différences significatives dans la richesse et la régularité des communautés observées. Les β-Proteobactéries *Photobacterium, Serratia* et les Firmicutes *Brochothrix, Carnobacterium* et *Staphylococcus* ont été identifiés comme population dominante sur le saumon fumé à DLC.

La richesse, la diversité et la composition du microbiote de surface ont été principalement affectées par les procédures de nettoyage et de désinfection, mais ne l'ont pas été par les méthodes d'extraction d'ADN. La richesse et l'équitabilité des communautés de surface sont apparues plus élevées lors de l'échantillonnage par éponge que par écouvillonnage.

Les analyses de diversité β ont pu mettre en évidence que la typologie des surfaces, les procédures d'hygiène ainsi que le type de prélèvement affectaient la composition des communautés bactériennes étudiées. Les principales bactéries de surface identifiées étaient : *Flavobacteriaceae*, ainsi que des membres des familles des β- Proteobactéries et γ-Proteobactéries, décrites comme des bactéries d'altération du poisson, telles que *Acinetobacter*, *Pseudomonas* et *Shewanella*. Les méthodes d'extraction et d'échantillonnage de l'ADN peuvent avoir un impact sur les résultats du séquençage et l'analyse écologique des structures des communautés bactériennes. Cette étude a confirmé l'importance de la standardisation des méthodes et la nécessité d'une validation analytique avant les études de metabarcoding de l'ADNr 16S.

Dans le cadre de ce premier chapitre, nous avons comparé diverses méthodes d'extraction d'ADN ainsi que des méthodes de prélèvements de surface. Ceci dans le but de disposer d'une approche méthodologique pour effectuer du metabarcoding de l'ADNr 16S au sein d'atelier de production alimentaire et d'évaluer l'impact des bactéries de surfaces sur la qualité des produits.

Deux méthodes nous sont apparues comme optimales pour notre étude sur le microbiote des usines agroalimentaires : Maxwell® 16 FFS DNA Purification (Promega, Charbonnière-les-Bains, France) ainsi que Qiagen DNeasy PowerFood Microbial (Qiagen, Courtaboeuf, France). De par sa facilité de manipulation et sa disponibilité nous avons choisi la méthode Qiagen DNeasy PowerFood Microbial pour l'intégralité des travaux de cette thèse. Concernant le prélèvement des microorganismes, la quantité de cellules dénombrées, la richesse ainsi que la diversité des échantillons issus d'éponges étaient significativement supérieures à celles des échantillons issus d'écouvillons quelque ce soit la condition testée (nettoyage, typologie de surface). Ces résultats nous ont donc confortés dans le choix des éponges dans la suite des travaux de cette thèse.

Une fois ces aspects méthodologiques étudiés et standardisés, nous avons cherchés dans la suite de ce travail à mettre en évidence par metabarcoding de l'ADNr 16S, un lien entre un environnement de production et son produit (saumon fumé).

Chapitre III. Evaluation de l'impact d'un atelier de fabrication sur le microbiote d'un produit : le saumon fumé

III.1. Préambule

Afin de déterminer si un processus de fabrication et son environnement avaient un rôle dans la communauté microbienne isolée sur des produits finis, nous avons dans un premier temps souhaité décrire la diversité bactérienne isolée sur des lots de saumon fumé au cours du stockage. Pour cela, nous nous sommes procuré dans le commerce 3 lots de saumon fumé de marques différentes issus de 3 usines de production différentes (A, B, C) pour un total de 9 lots à analyser. Ces produits ont été analysés pendant 28 jours de stockage avec une alternance de température (1/3 du temps à 4°C et 2/3 du temps à 8°C).

Chacun des produits a été analysé suivant une approche polyphasique composée de méthodes culturales, de metabarcoding de l'ADNr 16S ainsi que d'analyses chimiques. Nous souhaitions par cette approche explorer le microbiote du saumon fumé et tenter de mettre en évidence des spécificités propres à l'usine de production.

Cette étude a fait l'objet d'un article accepté et publié dans la revue *Foods* en février 2021.

Maillet, A.; Denojean, P.; Bouju-Albert, A.; Scaon, E.; Leuillet, S.; Dousset, X.; Jaffrès, E.; Combrisson, J.; Prévost, H. Characterization of Bacterial Communities of Cold-Smoked Salmon during Storage. Foods 2021, 10, 362. https://doi.org/10.3390/foods10020362.

Une version fac-similé de cet article se trouve en annexe 2.

III.2. Characterization of bacterial communities of cold-smoked salmon during storage

III.2.1. Introduction

With 175,000 tons produced in the European Union in 2019, cold-smoked salmon (CSS) is a leading fish product with an important trade value (€2.77 billion) (EUMOFA, 2020, 2021). CSS is a lightly preserved product with no thermic treatment and is mainly consumed as a ready-to-eat (RTE) food. Due to a large number of intrinsic and extrinsic factors, such as pH, water activity (a_w), temperature, environmental origins and processing practices, such commodities are highly fragile (Gram and Huss, 1996; Wiernasz et al., 2017). Salting and smoking are mandatory steps in CSS processing to decrease foodborne pathogens and spoilage risks (Løvdal, 2015). As described by Leroi et al. (2000), the purpose of salting and smoking is to decrease the a_w through dehydration. The chloride ions from salt additives are also toxic for some microorganisms, and the phenolic compounds produced during the smoking step have a bacteriostatic effect. Smoking is furthermore used to bring out specific tastes and aromas (Leroi et al., 1998).

The CSS bacterial community has been widely studied in the scientific literature aiming to describe spoilage and pathogenic microbiota (Løvdal, 2015). Gram-negative bacteria such as *Shewanella putrefaciens, Aeromonas* spp. and marine *Vibrionaceae Photobacterium phosphoreum* have been described as dominating CSS microbiota in the early stages of storage (Leroi et al., 1998).

Gram-positive lactic acid bacteria (LAB: *Lactobacillus, Carnobacterium maltaromaticum*) seem to dominate CSS microbiota at the end of the product's shelf-life. Paludan-Müller et al. (1998) reported a high number of LAB (10^7–10^8 CFU/g). Gram-negative psychrotrophic bacteria *Enterobacteriaceae Serratia liquefaciens* were also reported in some cases to co-dominate the microbiota at the end of the shelf-life (Paludan-Müller et al., 1998; Joffraud et al., 2006; Løvdal, 2015). In addition, *Brochothrix thermosphacta* has already been described as dominating CSS microbiota (Illikoud et al., 2019). As an RTE food product, CSS are often faced with the foodborne pathogenic bacteria *Listeria monocytogenes* (Løvdal, 2015).

The majority of the studies mentioned were based on culturable approaches. Traditional methods can be time-consuming and lead to technical biases (viable but non-culturable cells, non-specific media and culture conditions) (Cambon-Bonavita et al., 2001).

Due to the challenging storage conditions of a product like CSS (temperature, phenolic compounds due to the smoking step, salt), culturable approaches might be insufficient for studying the entire CSS bacterial community. Culture-independent methods such as fingerprinting (Denaturing Gradient Gel Electrophoresis, Temperature Gradient Gel Electrophoresis) are DNA-based methods which offer tools to monitor the bacterial community on food products and food-associated microbial ecosystems (Giraffa and Neviani, 2001; Jaffrès et al., 2009; Postollec et al., 2011). More recently, next-generation sequencing (NGS) has offered new ways to explore food microbial ecology (Bokulich et al., 2016). Bacterial diversity can now be assessed through high throughput sequencing approaches which facilitate the identification of microbes and the relative abundance of taxa for a high number of samples in a single analysis (Ercolini, 2013).

A few studies have sought to assess the CSS bacterial community using DNA-based methods (Cambon-Bonavita et al., 2001; Rachman et al., 2004; Olofsson et al., 2007). Although NGS was previously used to determine contamination of fresh salmon filets, to our knowledge no study of the evolution of the CSS microbial ecology during shelf-life has used this type of approach (Møretrø et al., 2016; Jääskeläinen et al., 2019). Yet, NGS could provide an increasingly deeper insight into the microbial diversity of seafood and seafood products (Parlapani, 2020).

This study used 16S rRNA gene metabarcoding to assess the evolution of bacteria on 45 CSS products from three different factories that were stored for 28 days at two different temperatures (4 °C first week, 8 °C remaining weeks).

A polyphasic approach was implemented in this study; culture-dependent and independent methods associated with chemical analyses were used.

III.2.2. Materials and Methods

III.2.2.1. Cold-smoked Salmon Sampling

Forty-five vacuum-packed CSS, originating from nine different batches and three different French processing factories (referred to henceforth as A, B and C) with a similar use-by date, were collected from local supermarkets. The CSS packs were stored for seven days at 4 °C then 21 days at 8 °C as described by Chaillou et al. (2015), in accordance with the French food aging test standard AFNOR NF V01-003 (Afnor, 2018). Details on the samples are summarized in **Table IV**.

Table IV. Cold-smoked salmon samples description (processing factory, production batch, origin and use-by date).

Factory	Production	Origin/Label	Use-by-Date
A	A1	Scotland	13 March 2019
	A2	Norway	13 March 2019
	A3	Norway	07 March 2019
B	B1	Scotland	09 March 2019
	B2	Norway	09 March 2019
	B3	Scotland/Label Rouge	04 March 2019
C	C1	Scotland	15 March 2019
	C2	Norway	15 March 2019
	C3	Ireland/Organic	09 March 2019

III.2.2.2. Bacterial Enumeration

From each sample, a 10-g portion of CSS was added to 90 mL of sterile buffered peptone water (25.5 g/L) (Biokar Diagnostics, Allonne, France) to obtain a 10-fold dilution. Samples were homogenized for 2 min in a sterile stomacher plastic bag provided with a 63 µm porosity filter (Interscience, Saint-Nom-la-Bretèche, France) using a stomacher 400 device (Intersciences, Saint-Nom-la-Bretèche, France).

Total psychrotrophic viable counts (TPVC) were enumerated on plate count agar (PCA) medium (Oxoid, Thermo Fisher Diagnostics, Dardilly, France) supplemented with 2% NaCl. The PCA plates were incubated at 15 °C for five to seven days. Lactic acid bacteria (LAB) were enumerated on de Man, Rogosa and Sharpe (MRS) agar plates (bioMérieux, Crapone, France) incubated for two days at 30 °C. *Brochothrix thermosphacta* were investigated on streptomycin sulfate thallous acetate agar (STAA) (Oxoid, Thermo Fisher Diagnostics, Dardilly, France) incubated for two days at 25 °C (Gardner, 1966). *Enterobacteriaceae* were enumerated after two days at 30 °C on violet red bile glucose agar (VRBG) (Biokar Diagnostics, Allonne, France) and marine *Vibrio* were enumerated on marine agar (five days at 25 °C) (Becton Dickinson, Rungis, France). To enumerate bacterial colonies, 100 µL of appropriate dilution in buffered peptone water were spread over the agar. Results were expressed in colony forming unit per gram CSS (CFU/g). Detections limits were 1 and 2 Log CFU/g, respectively, for *Enterobacteriaceae* and other counts.

III.2.2.3. Chemical Analyses

Total fat, dry matter content, salt content and total phenol were measured as described by Leroi et al. (2015). Total volatile basic nitrogen (TVBN) and trimethylamine (TMA) were determined in duplicate from 100 g of CSS using the Conway micro-diffusion method (Conway and Byrne, 1933).

III.2.2.4. DNA Extraction

DNA were extracted using Qiagen DNeasy PowerFood Microbial (Qiagen, Courtaboeuf, France). A first step of mechanical cell lysis was performed using the glass beads provided and a FastPrep (MPbiomedicals, Illkirch, France) for 30 s at a frequency of 6 m/s. DNA were extracted from three technical replicates from each sample. A Qubit® 2.0 fluorometer using a Qubit® dsDNA BR Assay Kit (Life technologies, Thermo Fisher Scientific, Villebon-sur-Yvette, France) was used to quantify DNA. Additional blank negative controls with no samples were used to exclude DNA contamination during extraction.

III.2.2.5. 16S rRNA Gene Sequencing

III.2.2.5.1. Library Preparation and Sequencing Using Illumina® MiSeq Platform

Briefly, the extracted DNA were PCR amplified to construct a sequencing library targeting the V3—V4 region of the bacterial 16S rRNA gene. PCR reactions were performed using 5 µL of DNA template, 12.5 µL of 2 × Kapa HiFi Hotstart ready mix (Roche, Boulogne-Billancourt, France) and 5 µL of 1 µM primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 785R (5′-GACTACHVGGGTATCTAATCC-3′) (Klindworth et al., 2013). Amplicons were purified using an Agencourt AMPure kit (Beckman Coulter, Villepinte, France). PCR product concentration and size were checked on a 2100 Bioanalyzer platform using the DNA 7500 kit (Agilent Technologies, Les Ulis, France) and indexed using a Nextera XT DNA Library Prep kit (Illumina, Paris, France) following Illumina recommendations. Samples were then pooled in an equimolar concentration (4 nM) and sequenced through the Illumina® MiSeq platform using a 2 × 250 V2 chemistry kit (Illumina, Paris, France) according to the Illumina® standard operating procedures.

III.2.2.5.2. Sequencing Data Processing and Analyses

The count table and taxonomy of the operational taxonomic units (OTUs) were obtained using the FROGS bioinformatic pipeline (Escudié et al., 2017).

Paired-end raw reads were merged using FLASh 1.2.11 with a maximum of 10% mismatch in the overlapped region (Magoč and Salzberg, 2011). Primers were removed using Cutadapt 1.18. Clustering of reads into OTUs (97% identity) was performed using Swarm 2.2.2 (Mahé et al., 2015), and a denoising step was performed. Chimera were then detected and removed using VSearch 1.3.0 (Rognes et al., 2016). OTUs with less than 5/100,000 of the total number of sequences from the whole dataset were removed (Bokulich et al., 2013b). Taxonomy assignments were performed using RDP classifier 2.11 and the Silva 16S rRNA gene database (SSURef_128_SILVA) (Cole et al., 2009; Quast et al., 2013; Wang et al., 2007). OTUs with a genus affiliation bootstrap threshold < 0.8 were removed.

III.2.2.6. Statistical Analyses

Statistical analyses and plots were performed in the R environment (v. 3.6.2) (R Core Team, 2018). For metabarcoding data, alpha and beta diversity analyses were conducted and relative abundances were determined using the Phyloseq package (1.30.0) and its dependencies (McMurdie and Holmes, 2013). Samples read libraries were rarefied to an even depth (10,000 reads per sample) to be normalized. Permutational multivariate analysis of variance (PERMANOVA) based on a weighted UniFrac distance matrix was carried out using 9999 permutations to detect significant effects/differences in the bacterial community analyzed (Lozupone and Knight, 2005). UpSet plots were used to assess OTU intersections according to the processing factories and storage date (Lex et al., 2014). These plots were generated using the UpSetR package (1.4.0) (Conway et al., 2017).

The chemical parameters, the relative abundance of each taxon at the genus level, and the alpha diversity metrics were studied using linear mixed models considering the factory, the storage time and their interaction as fixed effects, and the production batch as a random effect. For all endpoints, the *p*-values were adjusted using Tukey's method for pairwise comparisons between factories at each time point and between time points for each factory. A *p*-value < 0.05 was considered statistically significant.

III.2.3. Results

III.2.3.1. Microbiological Analyses

Bacterial growth of the nine CSS batches during the 28-day storage period are presented in **Figure 18** and summarized in **Table S1**.

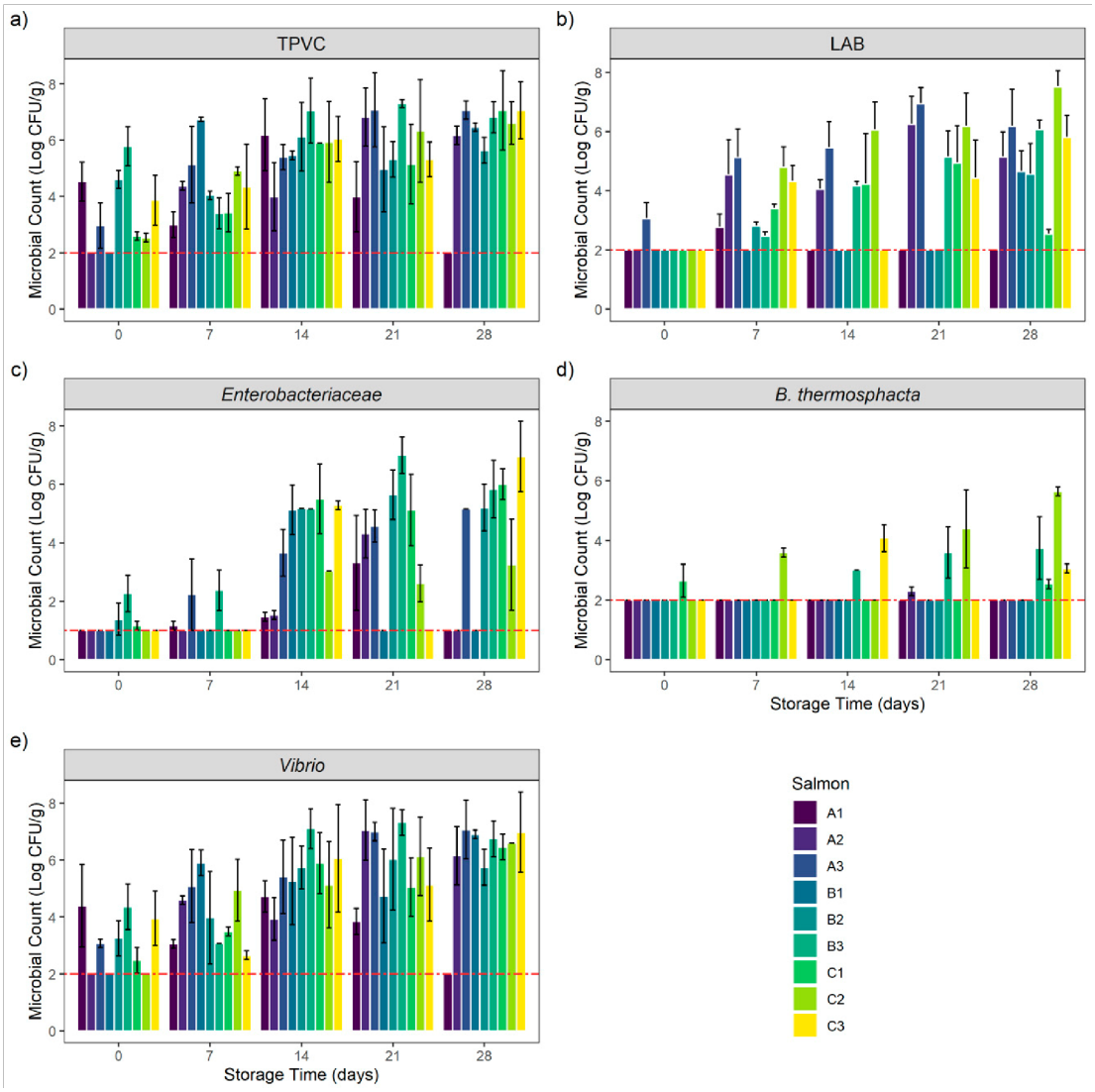

Figure 18. Bacterial growth evolution of (**a**) total psychrotrophic viable count (TPVC), (**b**) lactic acid bacteria (LAB), (**c**) *Enterobacteriaceae*, (**d**) *Brochothrix thermosphacta* and (**e**) *Vibrio* in vacuum-packed cold-smoked salmon (CSS) products during 28 days of storage. Results are expressed in Mean ± SD Log CFU/g of CSS products. The red-dashed line represents the limits of detection: 1 and 2 Log CFU/g, respectively, for *Enterobacteriaceae* and other counts.

At the beginning of the bacterial kinetic, total psychrotrophic viable counts (TPVC) were heterogenous among the different samples. Except for products A1, B2 and B3, TPVC increased during the storage period to reach D28 counts between 5.64 ± 0.45 and 7.07 ± 0.32 Log CFU/g.

Interestingly, TPVC on products A1, B2 and B3 were high at the beginning of the experiment (D0) (between 4.53 ± 0.69 and 5.78 ± 0.69 Log CFU/g) and remained stable during the storage period. The A1 sample count at D28 was below the enumeration limit (<2 Log CFU/g). Lactic acid bacteria (LAB) counts were low at the beginning of the experiment (D0). Except for product A3, which had an enumeration of 3.06 ± 0.55 Log CFU/g, all counts were below the enumeration limit. This microbial group quickly grew and reached its maximum after 21 days of storage. Interestingly, product A1's count was low or below the enumeration limit during the entire storage period with a maximum at D7 (2.77 ± 0.45 Log CFU/g). We observed the same situation on B1 and B2 products. However, these two samples reached respectively 4.66 ± 0.69 and 4.55 ± 1.05 Log CFU/g after 28 days of storage.

Enterobacteriaceae initial enumerations (D0) were low or below the enumeration limit (<1 Log CFU/g). Between 1.17 ± 0.15 and 2.27 ± 0.62 Log CFU/g were counted on products B2, B3 and C1 at the beginning of the storage period. *Enterobacteriaceae* counts then increased during storage on products A3, B2, B3 and C1 to reach a maximum at D28 (between 5.21 ± 0.8 Log CFU/g for B2 and 6.96 ± 1.21 Log CFU/g for C1). Product C3 counts after 7 and 21 days of storage were below the enumeration limit, whereas 5.29 ± 0.15 Log CFU/g and 6.96 ± 1.21 Log CFU/g were enumerated at D14 and D28. The same situation was observed on product B1: all counts were below the enumeration limit except for D14 with a count of 5.13 ± 0.85 Log CFU/g. In addition, this trend was observed on product A2: all counts were low except for D21 with a count of 4.32 ± 0.84 Log CFU/g. *Enterobacteriaceae* counts on products A1 and C2 increased slowly to reach a maximum of 3.32 ± 1.62 Log CFU/g at D21 for A1 and 3.26 ± 1.56 Log CFU/g at D28 for C2. The A1 sample count at D28 was below the enumeration limit.

For *Brochothrix thermosphacta*, the initial enumerations (D0) were below the enumeration limit on all samples except for product C1, with an enumeration of 2.65 ± 0.55 Log CFU/g. *B. thermosphacta* counts were below the enumeration limit during the entire storage period on products A1, A3, B1 and B2. The same situation also was initially observed on product A2; however, 2.3 ± 0.15 Log CFU/g were enumerated on this product at D21. *B. thermosphacta* was then counted on products B3 and C2, with an increase during the storage period to reach a maximum of respectively 3.74 ± 1.05 and 5.64 ± 0.15 Log CFU/g at D28. Product C1 counts remained stable during the storage period. The count was below the enumeration limit from D7 to D21 to reach 2.54 ± 0.15 Log CFU/g at D28. The same situation was encountered on product C3. Counts were below the enumeration limit at D0, D7 and D21 but 4.08 ± 0.45 and 3.06 ± 0.15 Log CFU/g were enumerated respectively at D14 and D28.

Vibrio initial counts (D0) were high (between 3.07 ± 0.15 Log CFU/g on product A3 and 4.39 ± 1.45 Log CFU/g on product A1) on all products except for A2, B2 and C2, where the counts were below the enumeration limit. Except for product A1, *Vibrio* counts increased during the storage period to reach a maximum count after 21 and 28 days of storage (between 6.03 ± 1.79 Log CFU/g at D21 on product B2 and 7.07 ± 1.03 Log CFU/g at D28 on product A3). Globally, *Vibrio* counts followed the same trend as TPVC. Product A1 *Vibrio* counts were stable during 21 days of storage with counts between 3.06 ± 0.15 and 4.72 ± 0.55 Log CFU/g. The A1 sample count at D28 was below the enumeration limit.

III.2.3.2. Chemical Analyses

The evolution of the chemical components of each CSS sample during the 28 days of storage is represented in **Figure 19**.

Dry matter content among all of the CSS samples significantly increased during storage ($p < 0.0001$) from 63.32 ± 1.86% to 66.25 ± 1.55%. Interestingly, no significant differences in the dry matter content between the three factories' samples were observed ($p = 0.07$). Contrary to dry matter, total fat among all CSS samples significantly decreased during storage ($p < 0.0001$) from 9.47 ± 1.65 g/100 g to 6.29 ± 1.62 g/100 g. No significant differences in total fat content were observed for the three factories' samples ($p = 0.08$).

Total phenols, issued from the cold-smoking step, were homogeneous among the different factories' samples ($p = 0.46$). The total phenols rate among the 45 samples decreased from 0.71 ± 0.24 mg/100 g at D0 to 0.57 ± 0.15 mg/100 g at D28. This difference was only due to the significant decrease ($p < 0.0001$) of A samples' total phenols from 0.94 ± 0.24 mg/100 g at D0 to 0.55 ± 0.12 mg/100 g at D28.

As far as salt content was concerned, no significant differences were observed among the different factories' samples ($p = 0.55$) or during storage ($p = 0.18$). Indeed, this parameter was stable throughout the storage period, from 2.89 ± 0.41 g/100 g at D0 to 2.91 ± 0.67 g/100 g at D28.

Spoilage markers TVBN and TMA were also measured at each storage date. TVBN globally increased during the storage period from 13.17 ± 5.81 mgN/100 g at D0 to 24.09 ± 4.19 mgN/100 g at D28 ($p < 0.0001$). TVBN concentrations were also homogeneous among the different factories ($p = 0.61$).

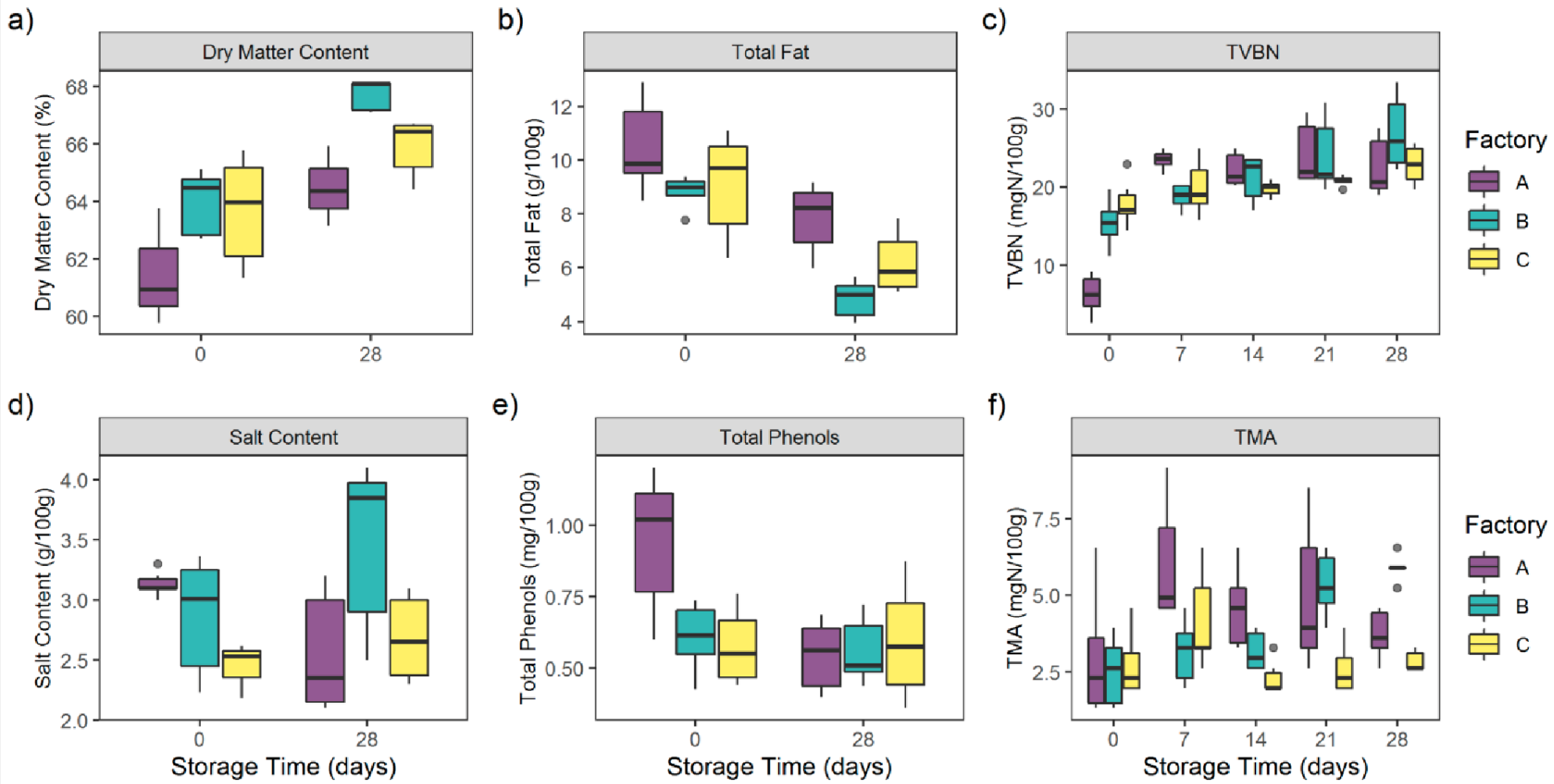

Figure 19. Evolution of (**a**) dry matter content (%), (**b**) total fat (g/100 g), (**c**) total volatile basic nitrogen (TVBN) (mgN/100 g), (**d**) salt content (g/100 g), (**e**) total phenols (mg/100 g), (**f**) trimethylamine (TMA) (mgN/100 g) of 45 CSS products during 28 days of storage.

TVBN increased significantly within A samples, from 6.22 ± 2.51 mgN/100 g at D0 to 23.47 ± 1.2 mgN/100 g at D7 ($p < 0.0001$). Concentrations were then homogeneous from D7 to D28 ($p > 0.05$). For B samples, TVBN were stable at D0 and D7 ($p = 0.68$), with respectively 15.39 ± 2.95 mgN/100 g and 18.78 ± 1.58 mgN/100 g. The concentrations then significantly increased to reach a maximum of 26.96 ± 4.78 mgN/100 g at D28 ($p < 0.05$). TVBN concentrations of C samples were homogeneous during the storage period ($p > 0.05$), from 17.9 ± 2.98 mgN/100 g at D0 to 22.81 ± 2.49 mgN/100 g at D28. TMA followed the TVBN trend with a significant increase from 2.73 ± 1.39 mgN/100 g at D0 to 4.15 ± 1.42 mgN/100 g at D28 ($p < 0.0001$). Interestingly, TMA concentrations differed significantly among the different factories' samples ($p < 0.0001$). TMA increased significantly within A samples, from 2.95 ± 2.02 mgN/100 g at D0 to 6.0 ± 2.0 mgN/100 g at D7 ($p = 0.011$). Concentrations were then homogeneous from D7 to D28 ($p > 0.05$). For B samples, TMA were stable at D0 and D7 ($p = 0.68$), with respectively 2.51 ± 0.12 mgN/100 g and 3.16 ± 1.04 mgN/100 g. The concentrations then significantly increased to reach a maximum of 5.89 ± 0.41 mgN/100 g at D28 ($p < 0.05$). The TMA concentrations of C samples were homogeneous during the storage period ($p > 0.05$), from 2.73 ± 1.03 mgN/100 g at D0 to 2.83 ± 0.33 mgN/100 g at D28.

III.2.3.3. Metabarcoding Analyses

Out of over 135 samples, six DNA samples could not be amplified and sequenced: A1 at D28 and B2 at D28. A total of 3,584,463 reads passed filters applied through the FROGS pipeline workflow with an average of 27,787 reads/sample ± 27,189 reads.

The sizes of the libraries were highly heterogenous. Interestingly, library size increased simultaneously with storage time (**Figure 20**). Bacterial growth during storage impacted the number of reads ($p < 0.05$). Library sizes were higher at D28 with an average of 57,552 reads ± 22,263. The library sizes of some D0, D7 and D14 samples were too low, and were considered as not being representative of the microbiota of interest. Thus, due to a low number of reads (<10,000) over 15 triplicates (45 samples) were removed for statistical purposes and were not taken into account in any microbial ecology analyses.

The other 84 samples were rarefied to an even depth of 10,000 reads and used for microbial ecology analyses.

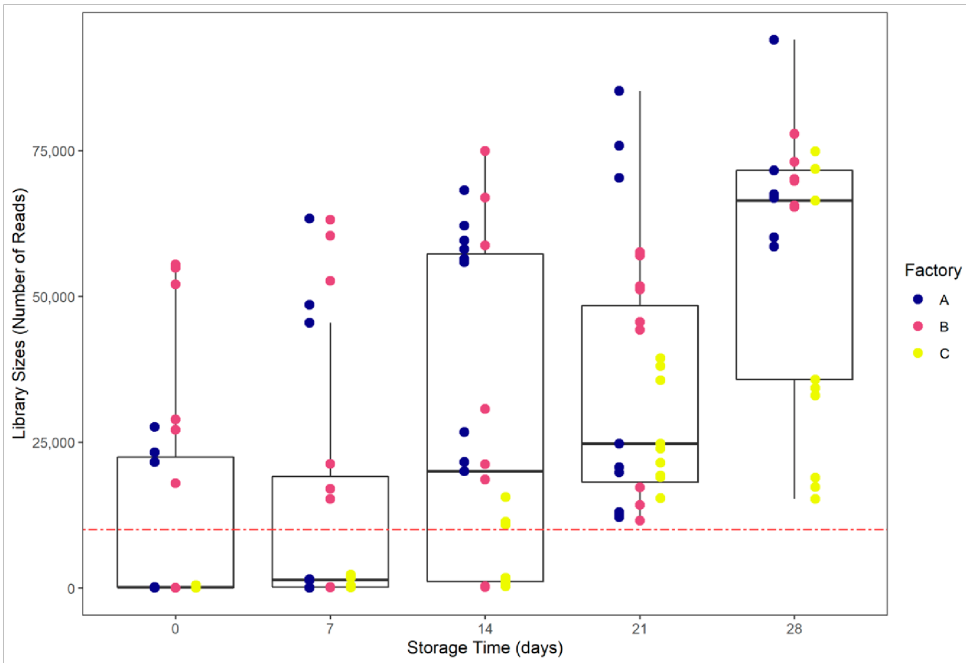

Figure 20. Library sizes distribution according to storage date. The red-dashed line represents a 10,000 reads threshold.

A total of 56 OTUs were identified and agglomerated in 19 genera including 12 dominants. Dominant populations among all samples were represented by Firmicutes *Staphylococcus* (5.48 ± 10.8%), *Carnobacterium* (18.9 ± 32.3%), *Lactobacillus* (5.24 ± 17.2%), β-Proteobacteria *Photobacterium* (30.4 ± 43.5%), *Vibrio* (6.79 ± 24.6%), *Aliivibrio* (2.55 ± 13.3%), *Salinivibrio* (5.71 ± 20.7%), *Enterobacteriaceae Serratia* (6.8 ± 18.8%), *Pantoea* (3.6 ± 11.7%), γ-Proteobacteria *Psychrobacter* (6.43 ± 18.2%), *Shewanella* (4.75 ± 17.5%) and *Pseudomonas* (2.92 ± 10.4%). The relative abundances at the genus level are represented in **Figure 21**.

Genera initially shared a homogeneous repartition among the CSS originating from the three different processing environments ($p > 0.05$). However, the relative abundances significantly differed during the storage period ($p < 0.001$). Indeed, the samples had different dominant populations. *Photobacterium* and *Aliivibrio* dominated all D0 microbiotas (with respectively 75.66 ± 36.44% and 23.82 ± 35.72%).

After seven days of storage, the bacterial communities were dominated by *Photobacterium* (63.95 ± 47.96%), *Vibrio* (33.35 ± 49.92%) and *Carnobacterium* (1.89 ± 2.87%).

After 14 days of storage, dominant genera were *Photobacterium* (31.27 ± 45.51%) and *Carnobacterium* (16.55 ± 38.05%). Five other genera emerged: *Staphylococcus* (4.94 ± 8.76%), *Lactobacillus* (12.77 ± 29.37%), *Serratia* (24.23 ± 34.40%), *Shewanella* (6.96 ± 15.58%) and *Psychrobacter* (2.81 ± 6.44%).

After 21 days of storage, the microbiotas were dominated by *Photobacterium* (27.20 ± 41.25%), *Psychrobacter* (13.79 ± 29.19%), *Shewanella* (9.73 ± 27.64%) and *Staphylococcus* (7.62 ± 14.73%). Two genera also emerged at D21: *Pantoea* (6.31 ± 18.21%) and *Salinivibrio* (8.95 ± 25.79%).

At the end of the storage period (28 days) *Carnobacterium* (28.32 ± 30.68%), *Lactobacillus* (9.89 ± 18.79%), *Pantoea* (5.99 ± 9.76%), *Pseudomonas* (10.92 ± 18.88%), *Salinivibrio* (11.33 ± 28.38%), *Serratia* (5.77 ± 9.82%), *Vibrio* (12.85 ± 32.23%), *Staphylococcus* (7.88 ± 10.01%) and *Psychrobacter* (5.35 ± 10.61%) dominated the microbiotas.

Except for *Salinivibrio*, the *Vibrionaceae* ratio decreased during storage: *Photobacterium* relative abundance was reduced on A samples between D7 and D28 ($p < 0.0001$). The *Aliivibrio* proportion significantly changed on B samples ($p < 0.05$) and decreased during the storage period ($p < 0.0001$). *Vibrio* relative abundance decreased from D14 to D28 on A products ($p < 0.0001$) and from D7 to D28 on B samples ($p < 0.05$). As far as *Salinivibrio* is concerned, the relative abundance increased from D14 to D28 on A products ($p < 0.05$) and from D7 to D28 on B samples ($p < 0.0001$).

Firmicutes did not share the same evolution: the *Carnobacterium* ratio increased during storage, especially from D0 to D21 on B samples ($p < 0.0001$), whereas the *Lactobacillus* relative abundance increased between D0 and D14 and then decreased from D14 to D28 on B samples ($p < 0.0001$). Moreover, the *Lactobacillus* proportion increased from D7 to D28 on A products ($p < 0.05$). In addition, the *Staphylococcus* ratio increased between D7 to D21 on A samples ($p < 0.0001$) and from D21 to D28 on C salmons.

As far as the *Enterobacteriaceae* family is concerned, the *Serratia* relative abundance increased from D0 to D14 on A CSS ($p < 0.05$) and then was reduced during the remaining period ($p < 0.0001$). The *Pantoea* ratio increased between D7 and D28 on A products ($p < 0.05$). Globally, γ-Proteobacteria increased during the storage: the *Shewanella* proportion increased between D0 and D21 on B products ($p < 0.001$).

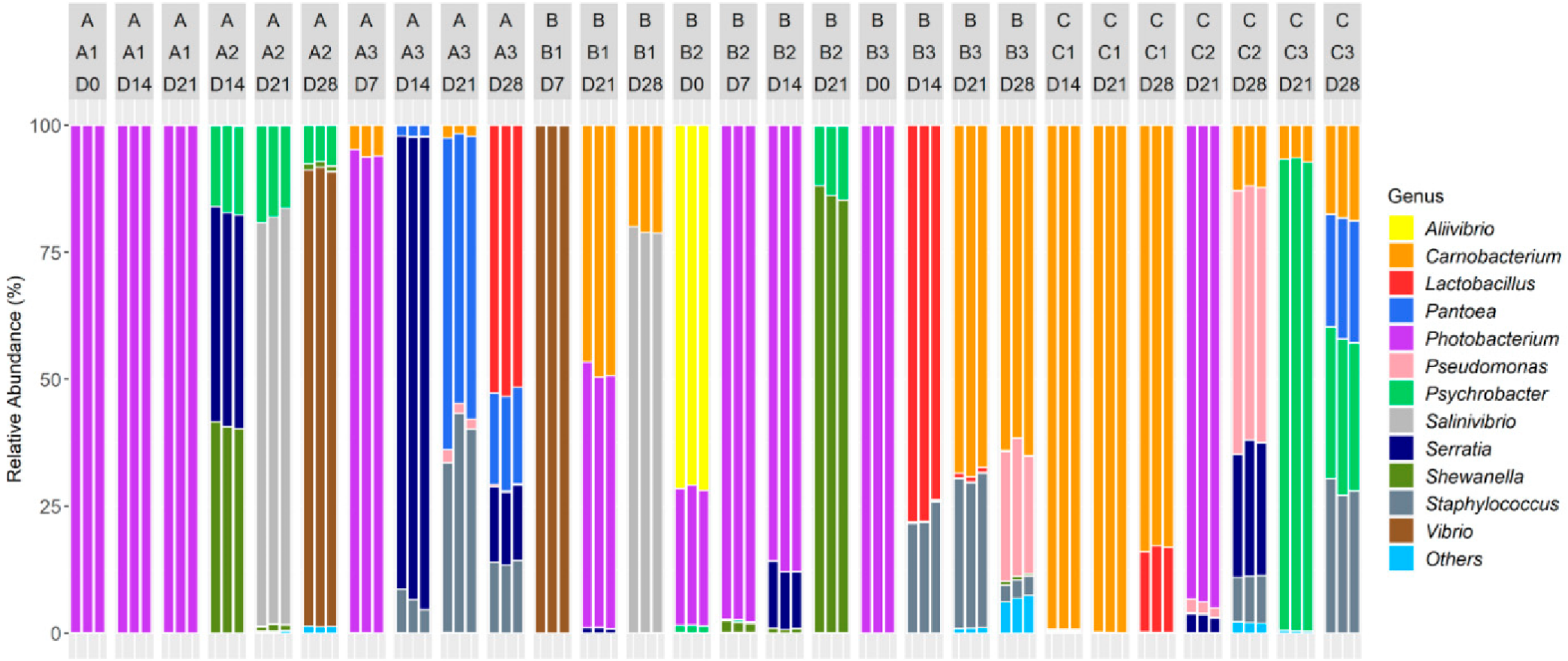

Figure 21. Relative abundance of bacterial genera of vacuum-packed cold-smoked salmon products stored during 28 days in triplicate (D0, D7, D14, D21, D28). Three different production batches (e.g., A1, A2, A3) were processed in three different processing factories (A, B, C). Only 84 samples are represented: 6 DNA could not be amplified and 45 outliers were removed due to a low number of reads (<10,000). The removed samples were identified in some D0, D7, D14 and D28 samples. Taxa present on average in all samples at a threshold ≥0.5% or having a 90th percentile ≥0.5% are individually represented. In other cases, taxa are grouped and labeled "Others".

The *Psychrobacter* ratio increased significantly from D21 to D28 on C salmons ($p < 0.0001$). The *Pseudomonas* relative abundance increased between D21 and D28 C samples ($p < 0.0001$) and from D0 to D28 on B products ($p < 0.05$).

Among the 56 OTUs, 29 core OTUs were identified in the three different factories (**Figure 22**), which were agglomerated in 12 genera composed by *Carnobacterium*, *Lactobacillus*, *Staphylococcus*, *Pantoea*, *Serratia*, *Proteus*, *Salinivibrio*, *Vibrio*, *Photobacterium*, *Shewanella*, *Psychrobacter* and *Pseudomonas*.

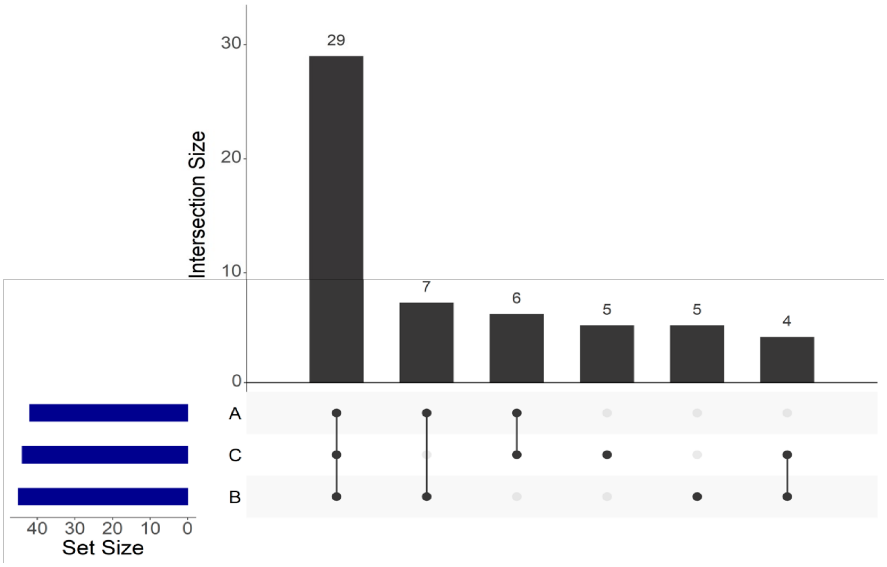

Figure 22. UpSet plot of shared operational taxonomic units (OTUs) identified within cold-smoked salmon vacuum-packed products according to the food processing factory.

Seven core OTUs were identified between A and B samples. These seven OTUs were agglomerated in seven genera composed by *Brochothrix*, *Lactobacillus*, *Staphylococcus*, *Enhydrobacter*, *Psychrobacter*, *Marinimonas* and *Arcobacter*. Six core OTUs between A and C samples were identified and agglomerated in six genera: *Carnobacterium*, *Lactobacillus*, *Staphylococcus*, *Serratia*, *Psychrobacter* and *Brevibacterium*. Four core OTUs between B and C samples were identified and agglomerated in four genera composed by *Carnobacterium*, *Aliivibrio*, *Photobacterium* and *Psychrobacter*. Five OTUs were only identified within B samples, which were agglomerated in three genera composed by *Carnobacterium*, *Serratia* and *Shewanella*. Five unique OTUs were also only identified within C samples, which were agglomerated in four genera composed by *Carnobacterium*, *Lactobacillus*, *Aerococcus* and *Pseudomonas*.

Among the 56 OTUs, seven core OTUs were identified among the CSS products at each storage analysis date (**Figure 23**), which were agglomerated in six genera composed by *Staphylococcus*, *Vibrio*, *Photobacterium*, *Shewanella*, *Psychrobacter* and *Pseudomonas*. Twelve core OTUs were identified only at D14, D21 and D28, which were agglomerated in five genera composed by *Carnobacterium*, *Arcobacter*, *Enhydrobacter*, *Psychrobacter* and *Pseudomonas*. Eleven OTUs were unique to D21 and D28, which were agglomerated in seven genera composed by *Carnobacterium*, *Staphylococcus*, *Pantoea*, *Salinivibrio*, *Psychrobacter*, *Brevibacterium* and *Pseudomonas*. Finally, eight OTUs were unique to D28, which were agglomerated in six genera composed by *Carnobacterium*, *Lactobacillus*, *Aerococcus*, *Shewanella*, *Marinomonas* and *Pseudomonas*.

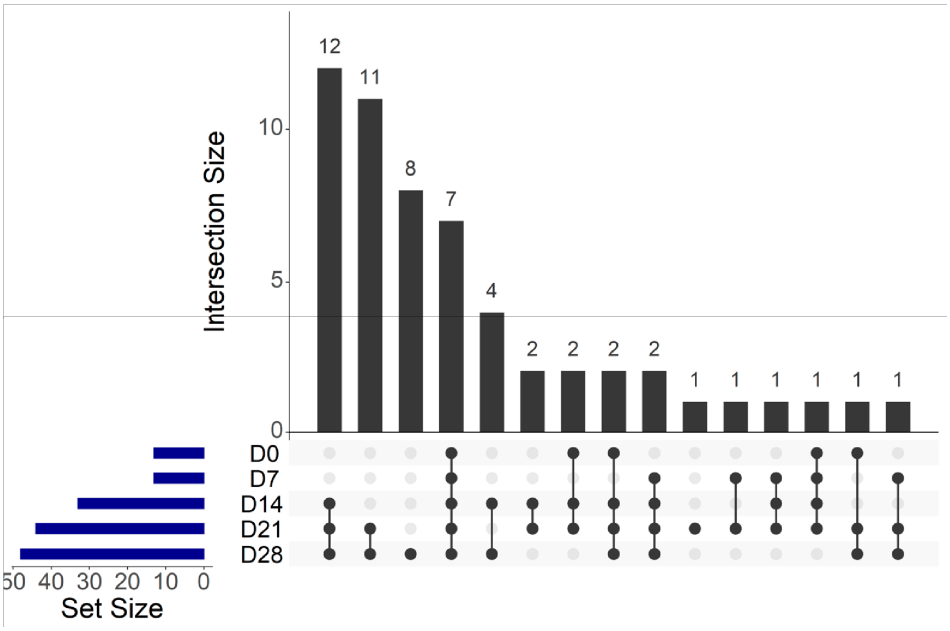

Figure 23. UpSet plot of shared operational taxonomic units (OTUs) identified within cold-smoked salmon vacuum-packed products during 28 days of storage.

The genera *Brevibacterium*, *Marinomonas*, *Enhydrobacter* and *Arcobacter* belonged for their part to the subdominant population with a relative abundance below 0.05%. Communities' richness (observed OTUs) and evenness (Shannon diversity index) were assessed for all 84 samples and are summarized in **Table V**. The storage time had an effect on both richness ($p < 0.0001$) and evenness ($p < 0.0001$). Communities were richer after 28 days of storage (with an average of 15.43 ± 4.95 OTUs). No richness differences were observed between D0 and D7 (respectively with an average of 4.56 ± 2.35 and 4.89 ± 1.83 OTUs). With regard to the evenness of communities, this was higher after 28 days of storage (with an average of 1.07 ± 0.54). Interestingly, the processing environment appeared to have no impact on either richness or evenness (respectively $p = 0.60$ and $p = 0.83$).

Table V. Observed richness and evenness for 16S rRNA amplicons analyzed in this study. Data are expressed in Mean ± SD.

Factory	Salmon	Date	Observed OTUs	Shannon Index
A	A1	D0	4.000 ± 0.000	0.006 ± 0.001
A	A1	D14	1.333 ± 0.577	0.000 ± 0.001
A	A1	D21	2.667 ± 1.155	0.004 ± 0.002
A	A2	D14	12.667 ± 0.577	1.130 ± 0.018
A	A2	D21	12.000 ± 1.000	0.576 ± 0.020
A	A2	D28	18.667 ± 1.528	0.501 ± 0.016
A	A3	D7	4.000 ± 1.000	0.221 ± 0.023
A	A3	D14	8.000 ± 1.000	0.400 ± 0.065
A	A3	D21	9.000 ± 0.000	0.902 ± 0.010
A	A3	D28	16.333 ± 1.155	1.333 ± 0.018
B	B1	D7	3.667 ± 1.155	0.008 ± 0.001
B	B1	D21	11.000 ± 1.000	0.807 ± 0.006
B	B1	D28	5.667 ± 1.155	0.518 ± 0.012
B	B2	D0	7.333 ± 1.528	0.664 ± 0.006
B	B2	D7	7.000 ± 1.000	0.135 ± 0.013
B	B2	D14	7.333 ± 0.577	0.428 ± 0.026
B	B2	D21	13.000 ± 1.732	0.537 ± 0.040
B	B3	D0	2.333 ± 0.577	0.002 ± 0.002
B	B3	D14	12.000 ± 0.000	0.678 ± 0.054
B	B3	D21	14.000 ± 1.000	1.074 ± 0.022
B	B3	D28	17.333 ± 0.577	1.080 ± 0.027
C	C1	D14	10.333 ± 0.577	0.060 ± 0.002
C	C1	D21	7.000 ± 1.000	0.054 ± 0.005
C	C1	D28	11.667 ± 1.155	0.539 ± 0.002
C	C2	D21	13.667 ± 1.528	0.337 ± 0.062
C	C2	D28	17.667 ± 1.528	1.744 ± 0.007
C	C3	D21	8.333 ± 2.309	0.816 ± 0.014
C	C3	D28	20.667 ± 0.577	1.800 ± 0.004

Weighted UniFrac principal coordinates analysis (PCoA) was generated to visualize samples (**Figure 24**). This PCoA highlighted shared taxa between samples, especially between factories A and B, but also differences according to the processing environment. PERMANOVA analysis based on weighted UniFrac distance showed that the processing environment, the storage date and the production batch influenced the bacterial community (respectively $p < 0.0001$) and explained respectively 17.6%, 14.2% and 45.7% of the sample microbiota differences.

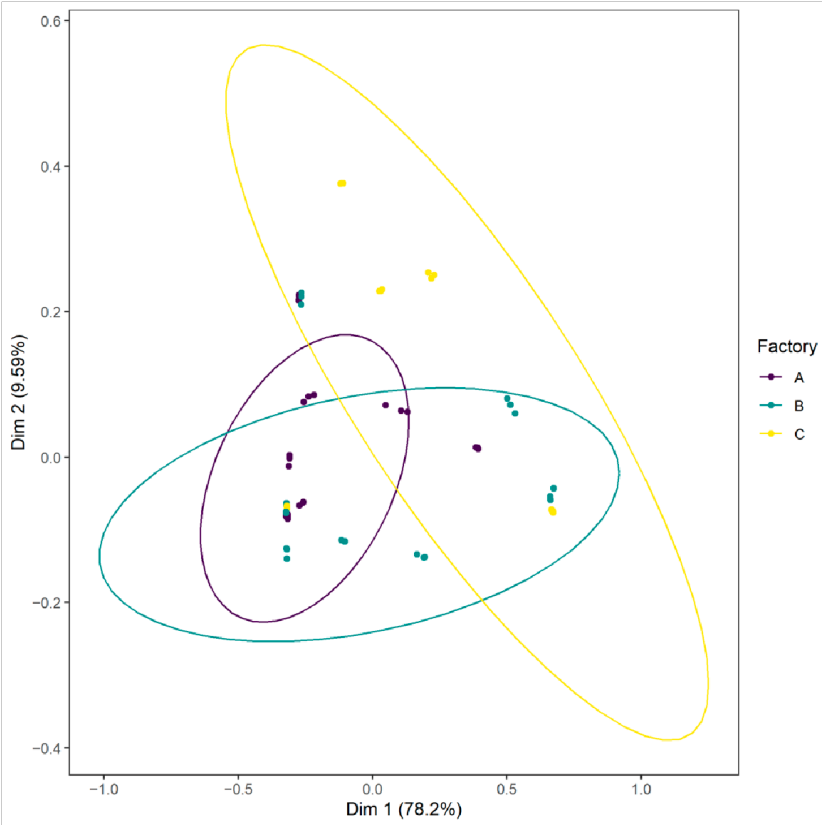

Figure 24. Weighted UniFrac principal coordinates analysis (PCoA) plot of CSS samples according to the food processing factory.

III.2.4. Discussion

The first part of this study aimed to evaluate the culturable bacterial population of several CSS products processed in three different factories. The microbial load was high after one week of storage. The dominant population on D0 products consisted of Gram-negative *Vibrio*, *Enterobacteriaceae*, and Gram-positive LAB. The microbial load reached an average of 10^7 CFU/g at the end of the experiment. These observations and the bacterial concentration were consistent with already published data. Indeed, Leroi et al. (1998) studied the microbial ecology of CSS during 35 days of storage at 8 °C. The authors enumerated aerobic viable counts at a maximum of 10^6 to 10^7 CFU/g after 6 days of storage. In addition, Paludan-Müller et al. (1998) studied the role of LAB in vacuum-packed CSS spoilage. The authors evaluated the total psychrotrophic viable counts during 7 weeks at 5 °C. Counts reached 10^6 to 10^7 CFU/g in two weeks and remained stable during the storage. Moreover, LAB growth did not seem to compete with Gram-negative bacteria as described by Leroi et al. (1998).

Marine *Vibrio* such as *Photobacterium phosphoreum* were dominant among the bacterial populations of the different samples.

This bacterium has already been described as a potential spoiler due to its ability to produce TMA from trimethylamine N-oxide (TMAO), which is known to be responsible for the typical strong fishy, urine and ammonia-like off-odors (Gram and Dalgaard, 2002; Gram and Huss, 1996; Remenant et al., 2015).

Enterobacteriaceae were dominant within B and C product communities. Psychrotrophic *Enterobacteriaceae* have been already identified on spoiled CSS, and particularly reported as dominant within injection brined products (Løvdal, 2015).

Lactic acid bacteria (LAB) such as *Carnobacterium maltaromaticum* and *Lactobacillus curvatus* have been widely described as dominant at a high level (10^7–10^8 CFU/g) on CSS products and could be involved in spoilage processes (Jørgensen et al., 2000).

Interestingly, *Brochothrix thermosphacta* was enumerated on only one B product, yet on all C products. Several studies have reported the spoilage potential of this bacterium (Løvdal, 2015; Odeyemi et al., 2018), notably able to produce butter/plastic/rancid, blue-cheese, sour/pungent off-odors, due to the high release of chemical compounds such as 2-heptanone and 2-hexanone (Joffraud et al., 2001; Stohr et al., 2001). More broadly, Stohr et al. (2001), by studying the inoculation of different spoilage bacteria on CSS (*Shewanella putrefaciens*, LAB, *Brochothrix thermosphacta*, *Aeromonas* spp., *Serratia liquefaciens*), were able to design a sensory and spoilage profile to better understand the CSS spoilage process and its major actors.

As described by Joffraud et al. (2006) in a study to evaluate CSS spoilage following different microbiota interaction, CSS spoilage due to metabolites production is often strain-dependent, which can explain the intraspecies diversity in terms of spoilage potential. Furthermore, spoilage is also related to interactions, either between bacterial species, such as antagonistic or cooperative behavior, or between bacterial species and food matrices and the food processing environment. Indications of bacterial species interaction have been found in other food matrices, for example by Jaffrès et al. (2009), who studied the bacterial community in tropical cooked and peeled shrimps using a polyphasic approach (cultivable, non-cultivable and sensory analyses). These authors hypothesized that the spoilage process might be the result of interactions between *Brochothrix thermosphacta* and *Carnobacterium divergens*.

Chemical parameters (dry matter content, total fat, salt content and total phenols) were similar among the different samples and fluctuated during the experiment. These parameters were aligned with the NF V45-065 standard (Afnor, 2012) on CSS properties.

Total fat significantly decreased after 28 days of storage. It is known that bacteria are able to degrade lipids. Notably, it has been reported that *Serratia*, *Staphylococcus* and *Pseudomonas* have the ability to degrade vegetable oil (Ibrahim et al., 2020). These genera are known to be part of the CSS microbiota. Their metabolic activities could explain this significant decrease of total fat.

Salt content was stable from 2.89 ± 0.41 g/100 g to 2.91 ± 0.67 g/100 g. It has been reported that despite its bacteriostatic effect, a low salt concentration could reduce the product sensory rejection limit and could not be sufficient to inhibit *Listeria monocytogenes* growth (Løvdal, 2015; Peterson et al., 1993).

Total phenols were also stable during the storage period. In addition, no growth was observed at D28 on product A1. The total phenols on products from A at the beginning of the storage were higher than those on other products. The bacteriostatic effect of the smoking process may impact microbial growth or induce viable but non-culturable cells. Indeed, liquid smoke strongly affected growth and survival of *Listeria monocytogenes* (Guilbaud et al., 2008). Moreover, Neunlist et al. (2005), by assessing the impact of salting and cold-smoking processes on the cultivability of *Listeria monocytogenes*, showed a reduction of 2 Log CFU/g for inoculated processed salmon compared with raw salmon during 28 days of storage. The authors also tested inoculation after the cold-smoked process and observed a 0.9 Log CFU/g reduction of the *Listeria monocytogenes* concentration on processed samples compared with unprocessed salmon within the first two weeks of storage. The concentrations of the control and processed samples were similar at the end of the storage period. Even if the authors did not highlight a viable but non-culturable state, the reduced concentration of *Listeria monocytogenes* in the processed samples during the first two weeks of storage, and the subsequent increase to reach the same concentration as the control, may indicate that the phenols compounds most probably stress bacterial cells but these cells later regain the ability to grow.

Total volatile basic nitrogen (TVBN) and trimethylamine (TMA) are considered as spoilage indicators. Their production increased significantly during the storage period. High TVBN concentrations suggest that CSS spoilage occurred after 14 days of storage. No strong differences in TVBN concentrations were observed across the different factories. However, as previously described in several studies, the use of TVBN alone as a relevant spoilage indicator must be put in perspective.

In their study on CSS spoilage, Joffraud et al. (2006) found that *Vibrio* spp. produced a significant amount of TVBN although no off-odor was detected by a trained sensory panelist. Furthermore, Brillet et al. (2005) showed that *Carnobacterium maltaromaticum* strains did not produce TVBN when inoculated in pure culture in sterile CSS, whereas when inoculated in naturally contaminated products, TVBN production was significantly enhanced. Contrary to TVBN, TMA concentrations mostly increased on the products of A and B, suggesting that *Photobacterium phosphoreum* might be implicated in the spoilage process of these products (Leroi et al., 1998; Macé et al., 2013).

Cold-smoked salmon can be re-contaminated during the manufacturing process through contact with contaminated surfaces (such as slicers, conveyors, etc.) (Løvdal, 2015). The microbiota during storage may be different according to how and where products are processed. Metabarcoding analyses could help to explore this hypothesis.

Metabarcoding on 45 samples stored for 28 days and analyzed every seven days in triplicate allowed us to identify and to analyze the bacterial communities of nine CSS batches produced in three different factories. Out of over 129 sequenced samples, a total of 45 samples displayed low library sizes (<10,000 reads).

Bukin et al. (2019), by studying the effect of different 16S rRNA regions on bacterial communities monitored by metabarcoding, highlighted that the major bacterial diversity (covered by 95% of reads) could be achieved at a library size of 10,000 reads. Thus, we decided to remove the 45 outliers for statistical purposes.

The dominant population identified confirmed already published data on CSS microbiota studied using cultivable methods, but also data from a few studies using culture-independent methods (Chaillou et al., 2015; Leroi et al., 1998; Løvdal, 2015; Olofsson et al., 2007; Wiernasz et al., 2020). *Psychrobacter* is highly prevalent in our study (6.43 ± 18.20% of relative abundances). *Psychrobacter* occurrence seems to be widespread on seafood products and was also identified on raw salmon (Møretrø et al., 2016; Odeyemi et al., 2018).

Thanks to the use of NGS, several studies on seafood have highlighted the high prevalence of *Psychrobacter* among seafood product spoilage bacterial communities (Parlapani, 2020). Parlapani et al. (2018) used NGS to investigate the spoilage microbiota of thawed common cuttlefish (*Sepia officinalis*) stored at 2 °C. The authors, by using an amplicon sequencing approach, highlighted that *Psychrobacter* was highly dominant among the samples, followed by *Pseudomonas*.

In the literature, *Staphylococcus* is rarely described as a CSS dominant bacterium. Its occurrence is mainly due to exogenous origins such as salt, the aquatic environment or the processing environment (Chaillou et al., 2015; Wiernasz et al., 2017). As far as the *Enterobacteriaceae* family is concerned, the *Pantoea* genus is also rarely described in the CSS bacterial communities. *Pantoea* is an ubiquitous bacterium which has already been identified in aquatic environments (Walterson and Stavrinides, 2015). Skrodenytė-Arbačiauskienė et al., (2008) analyzed the gut microbial diversity of 12 fish (six freshwater *Salmo salar* and six sea trout *Salmo trutta trutta*) using a cultural approach and 16S rRNA gene sequencing for colony identification. The authors identified the genus *Pantoea* within the sea trout intestinal tract but not on salmon samples. In another study to assess bacterial resistance to the antibiotic oxytetracycline in Chilean salmon (*Salmo salar*) farming, Miranda and Zemelman, (2002) identified a prevalence of *Pantoea* on fingerlings salmon samples. In our study, the majority of this genus was found on A3 (19.4 ± 23.8%) and C1 (11.7 ± 12.8%). We assumed that the origin of *Pantoea* on these products could be explained by their aquatic farm origin or contamination during production. Twenty-nine OTUs agglomerated in 12 genera were identified as part of the core microbiota between all of the CSS products. All of these genera were part of the dominant population except for *Proteus*. González-Rodríguez et al. (2002) studied the microbial community of 54 batches of cold-smoked fish (30 CSS and 24 smoked trout) during three weeks of storage. Colonies were counted and identified using API galleries. The authors identified *Proteus* as a dominant member of the *Enterobacteriaceae* family among the samples. Interestingly, in our study, *Aliivibrio* was not identified on A products, and *Brevibacterium* was not found on B samples. *Aerococcus* was only identified on C samples. In addition, *Arcobacter*, *Marinimonas*, *Enhydrobacter* and *Brochothrix* were not identified on C samples using metabarcoding. These results suggest the importance of the processing environment on the CSS microbiota, with a bacterial signature from this environment.

During the storage period, our findings highlighted that 12 OTUs (agglomerated in the following genera: *Carnobacterium*, *Arcobacter*, *Enhydrobacter*, *Psychrobacter* and *Pseudomonas*) were captured on D14; 11 OTUs (agglomerated in the following genera: *Carnobacterium*, *Staphylococcus*, *Pantoea*, *Salinivibrio*, *Psychrobacter*, *Brevibacterium* and *Pseudomonas*) were captured on D21; and eight OTUs (agglomerated in the following genera: *Carnobacterium*, *Lactobacillus*, *Aerococcus*, *Shewanella*, *Marinomonas* and *Pseudomonas*) were captured on D28. The emergence or capture of specific OTUs over time was also identified by Silbande et al. (2018).

The authors studied the effect of different packaging atmospheres on the microbiological, chemical and sensory properties of tropical red drum (*Sciaenops ocellatus*) fillets stored for 29 days at 4 °C. The authors identified the emergence of *Leuconostoc* and *Lactococcus* after eight days of storage on fresh fillets that were vacuum-packed. These two OTUs were not identified at Day 0.

Alpha diversity analyses highlighted that the richness and evenness of the different CSS bacterial communities increased during the product storage period. However, no differences between the factories were observed. While we observed a global increase in the OTUs' richness, Wiernasz et al. (2020) highlighted a reduction of the number of OTUs on salmon gravlax during 21 days of storage. Salmon gravlax is a salt-sugar mixture with spices that is not treated using smoke or heat. This particular treatment may lead to competitive flora which become dominant on these products which are not found on the standard cold-smoked process. Beta diversity analyses and weighted UniFrac PCoA confirmed a core microbiota but also highlighted differences in communities, specifically between A products and C products. In addition, we identified five OTUs (agglomerated in *Carnobacterium*, *Serratia* and *Shewanella*) specific to B and five others specific to C (agglomerated in *Carnobacterium*, *Lactobacillus*, *Aerococcus* and *Pseudomonas*). These results strengthened the specific factory signature observation. Our findings showed that the different compositions of CSS microbiota were affected by the processing environment and the length of storage but also the production batch. This clearly confirms that even if a core community existed between the samples, the processing factory had a bacterial signature composed by spoilage organisms which can contaminate CSS products during processing, attesting to the importance of the processing environment for the quality and shelf-life of CSS.

Rouger et al. (2018) observed identical results in their study of chicken leg microbiota, where two chicken leg samples from two different batches, stored under modified atmosphere packaging, showed similar microbiota. Interestingly, these two samples were processed in the same slaughterhouse on the same day. These results strengthen the hypothesis of a food processing bacterial signature on the microbial communities of products. To investigate this environmental influence, Stellato et al. (2016) compared fresh meat microbiota with environmental samples from small and large-scale retail butcheries. The authors highlighted 48 core genera shared between product and environment samples.

Among these 48 genera, *Pseudomonas* spp., *Brochothrix* spp., *Psychrobacter* spp., *Streptococcus* spp. and *Acinetobacter* spp. were identified. These genera were reported as members of the meat spoilage community, highlighting the importance of the surface microbiota on product quality.

By using a polyphasic approach (cultivable method with bacterial identification using the 16S rRNA gene and non-cultivable methods using NGS), Møretrø et al. (2016) identified the processing environment as a source of spoilage genera *Pseudomonas* and *Shewanella*. Phylogenetic analyses based on part of the 16S rRNA gene demonstrated the transfer of *Pseudomonas* from processing samples to salmon fillets, thus strengthening the links between the processing environment and product samples and the impact of the processing environment on the shelf-life.

III.2.5. Conclusion

In this study, we described the microbiota of vacuum-packed cold-smoked salmon products produced in three different factories and stored for 28 days. We used a polyphasic approach composed of cultivable methods and non-cultivable methods. The use of metabarcoding did not highlight unexpected genera except for *Pantoea*, and our findings were consistent with already published cultivable data on CSS bacterial communities. However, a next-generation sequencing-based approach highlighted the emergence of operational taxonomic units during product storage and provided insights on the CSS microbial ecology. A core microbiota composed of spoilage bacteria was shared by the 45 products but strong differences linked to the processing environment were observed. Indeed, we found that CSS products bore a factory bacterial signature. These results were obtained from three different processing plants and 45 samples and must be considered at this scale. This suggests the importance of the processing environment on food safety and quality. A better understanding and characterization of surfaces and residential bacteria and their dynamics using metabarcoding approaches may be a key to gaining greater insight into a factory's "health condition" to improve food safety and quality management.

III.3. Ce qu'il faut retenir du Chapitre III

Le saumon fumé est un produit de la mer prêt à consommer legèrement préservé avec une longue durée de conservation. L'écologie microbienne du saumon fumé pendant sa durée de conservation a fait l'objet de nombreuses publications (Leroi et al., 1998; Løvdal et al., 2015; Paludan-Müller et al., 1998). Cependant, à notre connaissance, les résultats publiés issus de ce travail de thèse, constituent la première publication focalisée sur la caractérisation des communauté bactériennes du saumon fumé utilisant le séquençage de nouvelle génération. Dans cette étude, les microbiotes du saumon fumé ont été étudiés en utilisant une approche polyphasique composée de méthodes culturales, de metabarcoding du gène de l'ARNr 16S ainsi que d'analyses chimiques.

Quarante-cinq plaquettes de saumon fumé, traités dans trois usines différentes, ont été analysés. L'approche du metabarcoding a mis en évidence 12 genres dominants précédemment identifiés comme faisant partie de la flore d'altération des produits de la mer : *Staphylococcus, Carnobacterium, Lactobacillus* (Firmicutes)*, Photobacterium, Vibrio, Aliivibrio, Salinivibrio* (β-Proteobactéries)*, Serratia, Pantoea* (*Enterobacteriaceae*)*, Psychrobacter, Shewanella* et *Pseudomonas* (γ- Proteobactéries). Des OTUs spécifiques à l'environnement de production ont été identifiés. Bien que les 45 produits partagent un « core » microbiote, une signature « usine de transformation » a été mise en évidence. Cela suggère que les communautés bactériennes du saumon fumé sont corrélées à l'environnement de transformation. Le microbiote de l'environnement de production peut jouer un rôle sur la qualité des produits. L'utilisation d'une approche polyphasique pour l'analyse microbiologique des produits de la mer et de leurs environnements de transformation pourrait permettre de mieux comprendre la dynamique des bactéries de surfaces ainsi que leur impact sur la sécurité et la qualité des aliments.

Cette signature nous conforte dans notre hypothèse selon laquelle les bactéries de l'environnement de production peuvent avoir un impact sur la qualité microbiologique des produits finis et peut donc représenter un risque pour la santé du consommateur. Afin de confirmer cette hypothèse, nous allons étudier au sein d'une usine modèle l'existence de bactéries résidentes ainsi que leur dynamique au sein de l'environnement et les liens qu'elles pourraient entretenir avec les produits.

Chapitre IV. Caractérisation de bactéries résidentes au sein d'une usine de saumon fumé. Évaluation de leur impact sur la qualité des produits

IV.1. Préambule

Après avoir mis en évidence une « signature » usine sur les produits étudiés nous avons décidé d'investiguer au sein d'un atelier la présence de bactéries résidentes, leur dynamique et de comparer le microbiote de l'environnement au cours d'une production avec le microbiote des produits issus de cette même production.

Pour cela nous avons suivi une production de saumon fumé au cours de laquelle chaque grande étape du processus de fabrication a été précédée d'une phase de nettoyage et de désinfection. Les prélèvements de surface ont été effectués à l'aide de la méthode identifiée chapitre II (éponge) en début et en fin de production. Les produits finis ont été collectés à la fin de la production et analysés pendant 28 jours avec une alternance de température comme effectué chapitre III (1/3 du temps à 4°C et 2/3 du temps à 8°C). Chacun des échantillons a alors été analysé par approche culturale ainsi que par PCR quantitative flore totale et metabarcoding de l'ADNr 16S (**Figure 25**). Notre but était par cette approche polyphasique d'identifier des relations entre microbiote environnemental et microbiote des produits ainsi que d'investiguer les sources potentielles de contamination au sein de l'atelier de production.

Cette étude est présentée sous forme d'un article scientifique en préparation. Il reprend les éléments analysés au cours d'une campagne de prélèvement du projet ALTEROBIO et sera amené à être renforcé par les données de l'ensemble des campagnes.

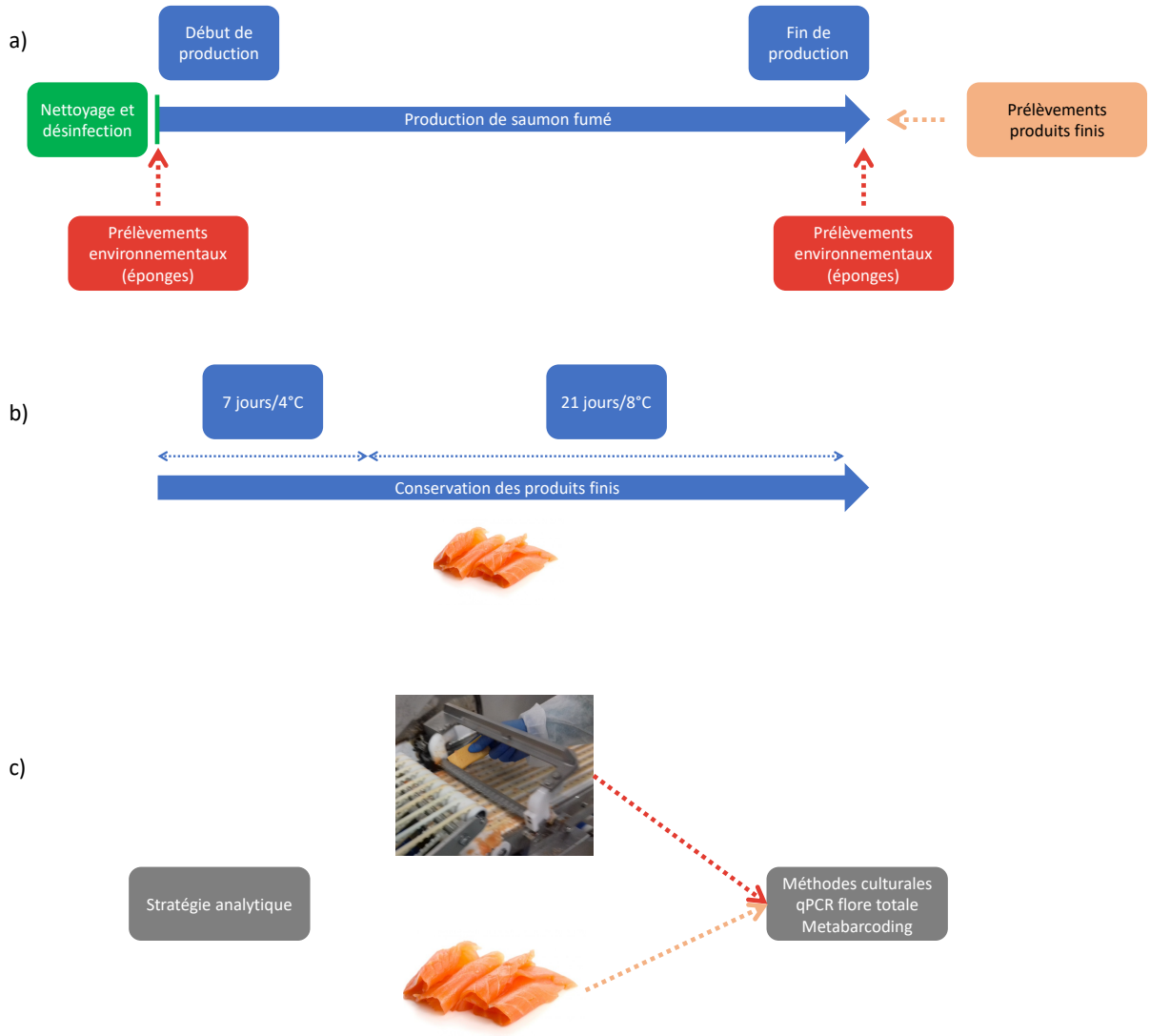

Figure 25. Stratégie analytique du chapitre IV. (**a**) Les prélèvements ont été effectués suivant une production de saumon fumé en amont de laquelle des procédures d'hygiène ont été réalisées. Les prélèvements environnementaux ont été effectués en début et en fin de production. Des produits finis ont été récoltés en fin de production. (**b**) Les produits finis sont stockés 1/3 du temps à 4°C et 2/3 du temps à 8°C suivant la norme AFNOR NF V01-003 (Afnor, 2018). (**c**) Les échantillons environnementaux ainsi que les produits finis ont été analysés suivant une approche polyphasique constituée de méthodes culturales, qPCR flore totale et de metabarcoding.

IV.2. Impact of processing environment surface bacteria on food safety & quality

IV.2.1. Introduction

Each year, one-third of all food produced in the world, representing 2.9 Gigatons, is wasted (Food and Agriculture Organization of the United Nations, 2011). This global food wastage is occurring at each step of a food production: harvest, industrial process, retail and at consumer's places. Despite the economic cost, this global concern has a huge ecological impact with an estimated carbon footprint of 3.3 Gigatons of CO_2 (Food and Agriculture Organization of the United Nations, 2013). Microbial food spoilage is a key factor in this global food wastage (Zwirzitz et al., 2020). Food spoilage is the result of microbial metabolism leading to the degradation of the sensory quality of products (Remenant et al., 2015). Thus, food products are unsuitable for consumption and discarded by consumers. It has been estimated that microbial spoilage could be responsible of one-fourth of the global food wastage (0.75 Gigatons) (Huis in't Veld, 1996).

Food processing plants are microorganism's reservoir submitted to specific environmental conditions such as temperature, relative humidity, equipment's' hygienic conception and are regularly cleaned and sanitized (Bokulich et al., 2015; Møretrø and Langsrud, 2017). These conditions can lead to bacterial adaptations such as resistance to psychrotrophic temperatures, resistance to biocides, or increased attachment strength to surfaces (Ferreira et al., 2014). Food soils can promote the colonization of surfaces by microorganisms. The latter, brought directly by operators, raw materials or indirectly by air or floor can therefore reside or persist in these environments. Hygiene procedures aim to reduce surface microbial level. However, a low number of cells are able to remain attached and are referred to be residential bacteria (Møretrø and Langsrud, 2017). The majority of these cells are non-pathogenic but could be spoilers.

In order to validate cleaning and disinfection procedures, industrials detect and enumerate hygienic indicators through environmental monitoring programs. This monitoring is based on culture-dependent methods leading to several analytical biases (Maillet et al., 2021a). Isolated bacteria, resulting from these programs, are often quantified or isolated on non-selective media, in aerobic conditions and their identity and their role in food safety and quality is mostly unknown (Bokulich et al., 2015, 2016).

Indeed, these residential bacteria may affect food quality or safety through cross-contamination at each step of the process due to their ability to grow on similar conditions (temperature, nutrients). Moreover a few studies highlighted a processing environment influence or signature on food products (Rouger et al., 2018).

The key element to evaluate the impact of surface bacteria and residential bacteria on food safety and quality is to characterize such cells and to solve contamination routes. Next-generation sequencing (NGS) and specially 16S rDNA metabarcoding has brought new insight in surface microbial ecology field (Bokulich et al., 2015), and been used in several industries to source track spoilage microorganisms (Bokulich and Mills, 2013; De Filippis et al., 2013; Hultman et al., 2015; Stellato et al., 2016). Moreover, these innovative approaches have been demonstrated as powerful tool to monitor microbial population and its dynamics within specific environment.

This study aimed to characterize surface bacteria from a cold-smoked salmon (CSS) processing plant and to evaluate their impact on finished products quality. CSS is a Ready-To-Eat (RTE) lightly preserved with a long shelf-life (3-4 weeks) with a microbiological load mainly depending on recontamination during food processing and has been validated as a good model to relate product and environmental microbiota (Maillet et al., 2021b). A polyphasic approach (culture-based and NGS based methods) was used to analyze CSS industrial processing environment samples and finished products and to assess the potential implication of surface bacteria on food safety and quality.

IV.2.2. Materials and Methods

IV.2.2.1. Cold-smoked salmon sampling

Eight vacuum-packed CSS, originating from the same batch, were collected in the production site (Brittany, France) on the packaging day. The CSS packs were stored seven days at 4 °C then 21 days at 8 °C following the French standard on shelf-life studies (Afnor, 2018), as described by Maillet al. (2021b).

IV.2.2.2. Processing plant sampling

Surface bacterial communities were collected in the CSS processing plant. Environmental samples were sponged according to the ISO 18593:2018 standard (ISO, 2018). Samples were collected at the beginning of a CSS production and at the end. Surfaces at the beginning of the production were cleaned and sanitized as described by Maillet et al. (2021a).

The CSS processing plant, represented Figure 26, was sectorized in 3 different area: raw material, treatment and packaging.

- **Raw material area**: raw salmons received, stored on ice and filleted (3 samples).

1- Ice storage: salmons are stored on ice in the reception room before being processed.

2- filleting machine.

3- conveyor: used to bring filleted salmons to the salting.

- **Treatment area**: salmons salted, smoked, dressed and trimmed. Post-salting and post-smoking maturation steps are also performed in this zone (4 samples).

1- dressing table: following the cold-smoking process, brown-muscle from salmon fillets is trimmed on a white PVC table.

2- washing machine.

3- forklift: this forklift is used to bring trimmed salmon fillets from the smoking area to the post smoking area and from this treatment area to the packaging zone.

- **Packaging area**: cold-smoked salmons sliced and vacuum packed (5 samples).

1- conveyor slicers: before being sliced, fillets are deposited on a weighing conveyor.

2- slicers.

3- balance packaging: each non-conformed CSS slices are re-weighed and manually calibrated by operators on small scales.

4- operators hands: hands from each position (fillets disposal for slicing, slices recalibration) were sampled.

5- floor.

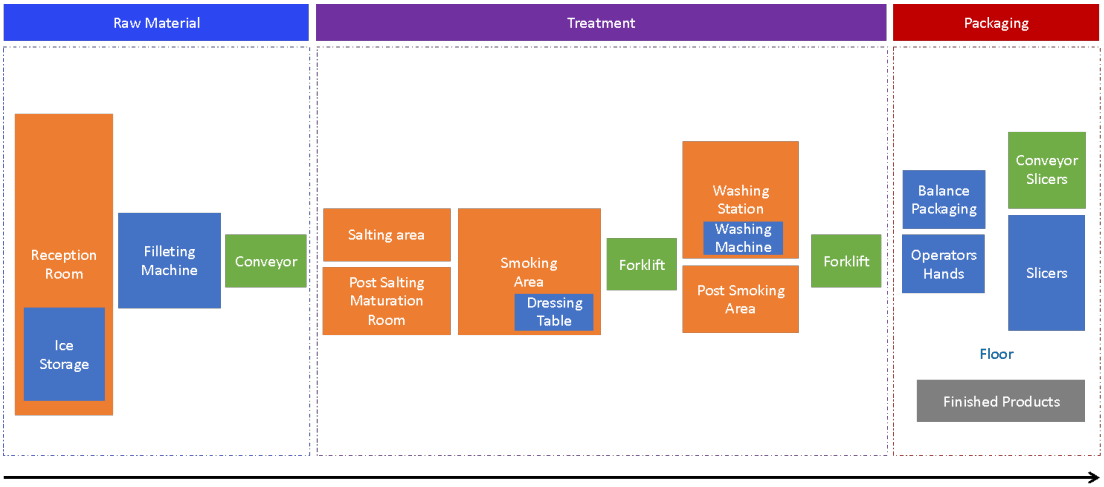

Figure 26. Representation of the CSS processing plant and the sampling plan. The processing plant was divided into three different area/zones: raw material, treatment and packaging. Orange squares represent room/area where samples were collected. Blue squares represent non-mobile equipment or surfaces sponged. Green squares represent mobile samples sponged (conveyors, etc.). Two forklifts are represented to highlight their zoning action, but only one forklift was sampled. The black arrow represents the process workflow and the go-forward principle of the CSS processing factory.

IV.2.2.3. Bacterial enumeration

IV.2.2.3.1. CSS samples

Eight product samples were processed as described by Maillet et al. (2021b). Briefly, 10-g portions of CSS were added to 90 mL of sterile buffered peptone water (25.5 g/L) (Biokar Diagnostics, Allonne, France) to obtain a 10-fold dilution. Samples were homogenized for 2 min in a sterile stomacher plastic bag provided with a 63 µm porosity filter (Interscience, Saint-Nom-la-Bretèche, France) using a stomacher 400 device (Intersciences, Saint-Nom-la-Bretèche, France).

IV.2.2.3.2. Processing plant samples

Briefly, the 3 sponges per sample were supplemented with 20 mL of buffered peptone water (25.5 g/L) (Biokar Diagnostics, Allonne, France), homogenized 2 min with a stomacher 400 (Intersciences, Saint-Nom-la-Bretèche, France) and pooled to obtain a 90 mL solution from 300 cm^2 of surfaces (Maillet et al., 2021a). Ice storage samples were thawed at 4°C and plated extemporaneously.

IV.2.2.3.3. Colony Forming Unit (CFU) counts

Total Psychrotrophic Viable Counts (TPVC) were enumerated on Plate Count Agar (PCA) medium (Oxoid, Thermo Fisher Diagnostics, Dardilly, France) supplemented with 2% NaCl. The PCA plates were incubated at 15°C for five to seven days. Lactic acid bacteria (LAB) were enumerated on MRS Agar plates (bioMérieux, Crapone, France) incubated for two days at 30°C. *Brochothrix thermosphacta* were investigated on Streptomycin Sulfate Thallous Acetate Agar (STAA) (Oxoid, Thermo Fisher Diagnostics, Dardilly, France) incubated for two days at 25°C (Gardner, 1966). *Enterobacteriaceae* were enumerated after two days at 30°C on Violet Red Bile Glucose Agar (VRBG) (Biokar Diagnostics, Allonne, France) and marine *Vibrio* were enumerated on Marine Agar (five days at 25°C) (Becton Dickinson, Rungis, France). To enumerate bacterial colonies, 100 µL of appropriate dilution in buffered peptone water were spread over the agar. Results were expressed in Colony Forming Unit per gram CSS (CFU/g) or Colony Forming Unit per cm^2 surface (CFU/cm^2). Detection limits were 2 Log CFU/g and 0.48 Log CFU/cm^2 for CSS and processing environment samples respectively.

IV.2.2.4. DNA extraction

DNA was extracted using Qiagen DNeasy PowerFood Microbial (Qiagen, Courtaboeuf, France). Cells were lysed by mechanical action using a FastPrep (MPbiomedicals, Illkirch, France) for 30 s at a frequency of 6 m/s. A Qubit® 2.0 fluorometer using Qubit® dsDNA BR Assay Kit (Life technologies, Thermo Fisher Scientific, Villebon-sur-Yvette, France) was used to quantify DNA. Additional blank negative controls with no samples were used to exclude DNA contamination during extraction.

IV.2.2.5. Determination of total bacterial counts by *tuf* gene quantitative PCR

Quantitative PCR (qPCR) of a *tuf* gene fragment (Tanaka et al., 2010), was used to determine the total bacterial count in surface samples. The quantitative PCR Kit (Takara, Saint-Germain-en-Laye, France) was used. PCR reactions consisted of 12.5 µL of SYBR Premix Ex Taq II (Takara Bio), 0.5 µL of 6-carboxy-X-rhodamine (ROX) reference dye (Takara Bio), 720 nM 769F primer and 560 nM 1068R primer, and 5 µL of DNA template in a total volume of 25 µL. Amplification conditions were performed on Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Villebon-sur-Yvette, France).

IV.2.2.6. 16S rRNA gene sequencing

IV.2.2.6.1. Library preparation and sequencing using Illumina® MiSeq platform

Briefly, the extracted DNA were PCR amplified to construct a sequencing library targeting the V3-V4 region of the bacterial 16S rRNA gene. PCR reactions were performed using 5 µL of DNA template, 12.5 µL of 2X Kapa HiFi Hotstart ready mix (Roche, Boulogne-Billancourt, France) and 5 µL of 1 µM primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 785R (5'-GACTACHVGGGTATCTAATCC-3') (Klindworth et al., 2013). Amplicons were sequenced through the Illumina® MiSeq platform using the 2x250 V2 chemistry kit (Illumina, Paris, France) according to the Illumina® standard operating procedures.

IV.2.2.6.2. Sequencing data processing and analysis

The count table and taxonomy of the OTUs were obtained using the FROGS bioinformatic pipeline (Escudié et al., 2017). Paired-end raw reads were merged using FLASh 1.2.11 with a maximum of 10% mismatch in the overlapped region (Magoč and Salzberg, 2011). Primers were removed using Cutadapt 1.18.

Clustering of reads into Operational Taxonomic Unit (OTU) (97% identity) was performed using Swarm 2.2.2 (Mahé et al., 2015), and a denoising step was performed. Chimera were then detected and removed using VSearch 1.3.0 (Rognes et al., 2016). OTUs with less than 5/100.000 of the total number of sequences from the whole dataset were removed (Bokulich et al., 2013b). Taxonomy assignments were performed using RDP classifier 2.11 and the Silva 16S rRNA gene database (SSURef_128_SILVA) (Cole et al., 2009; Quast et al., 2013; Wang et al., 2007). OTUs with a genus affiliation bootstrap threshold <0.8 were removed.

IV.2.2.7. Statistical analysis

Statistical analyses and plots were performed in the R environment (v. 3.6.2) (R Core Team, 2018). For metabarcoding data, alpha and beta diversity analysis were conducted and relative abundances were determined by using the Phyloseq package (1.30.0) and its dependencies (McMurdie and Holmes, 2013). Sample read libraries for salmon and surface samples were rarefied to an even depth (10,851 reads per sample) to be normalized. Permutational multivariate analysis of variance (PERMANOVA) based on Jaccard distance matrix was carried out by using 9,999 permutations to detect significant effects/differences in the bacterial community analyzed (Lozupone and Knight, 2005). UpSet plots were used to assess OTUs intersections according to the processing factories and storage date (Lex et al., 2014). These plots were generated using UpSetR package (1.4.0) (Conway et al., 2017). Network analyses based on Jaccard distance matrix were generated using ggnetwork package (0.5.8).

The Bayesian approach SourceTracker was used to identify/predict the environment root cause of CSS bacterial community (Knights et al., 2011). The algorithm was set on a library depth of 1,000 reads, with 100 burn-ins and 10 restarts. Indices α_1 and α_2 were set at 0.001.

The relative abundance of each taxon at the phylum and genus levels and the alpha diversity metrics were studied using ANOVA considering the storage time for products, the production time, the factory sectors and their interaction for the processing environment. For all endpoints, the *p*-values were adjusted by Tukey's method for pairwise comparisons. A *p*-value < 0.05 was considered statistically significant.

IV.2.3. Results

IV.2.3.1. Microbiological analyses

IV.2.3.1.1. CSS enumeration during 28 days of storage

Bacterial count during the 28-day storage period is presented in **Figure 27**.

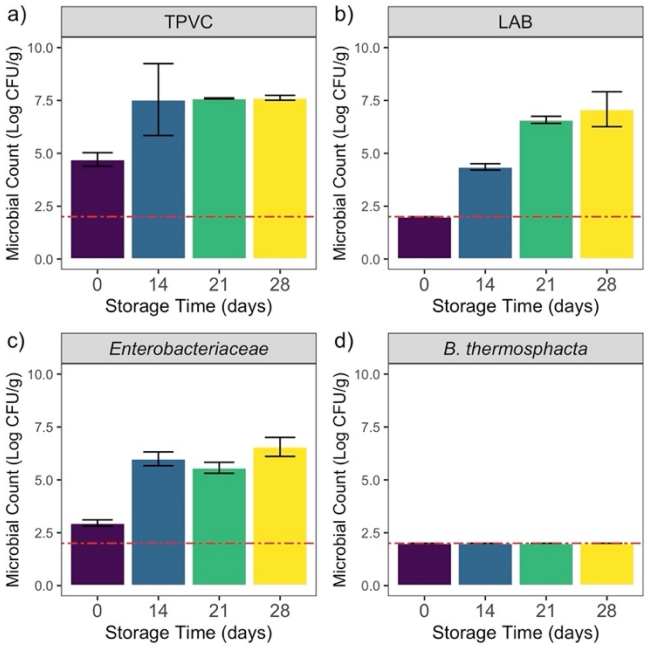

Figure 27. Bacterial count evolution of (**a**) Total Psychrotrophic Viable Count (TPVC), (**b**) Lactic Acid Bacteria (LAB), (**c**) *Enterobacteriaceae* and (**d**) *Brochothrix thermosphacta* in vacuum-packed CSS products during 28 days of storage. Results are expressed in Mean ± SD Log CFU/g CSS. The red-dashed line represents the limit of detection: 2 Log CFU/g.

The beginning of the bacterial kinetic was set at the production day (D0). Total Psychrotrophic Viable Counts (TPVC) increased during the storage period from 4.71 ± 0.32 Log CFU/g to reach at D28 7.63 ± 0.12 Log CFU/g. LAB counts were below the limit of detection (< 2 Log CFU/g) at the beginning of the experiment (D0) and increased during the storage to reach a maximum of 7.09 ± 0.83 Log CFU/g after 28 days of storage. *Enterobacteriaceae* initial counts (D0) were low: 2.96 ± 0.15 Log CFU/g.and quickly increased from D14 to D28 to reach a maximum of 6.56 ± 0.45 at the end of the kinetics. As far as *Brochothrix thermosphacta* counts are concerned, all the enumerations were below the limit of detection (< 2 Log CFU/g).

IV.2.3.1.2. Processing plant enumeration during CSS production

Surface bacteria counts of ten sponge samples from different processing plant aerea during CSS production are presented in **Figure 28**.

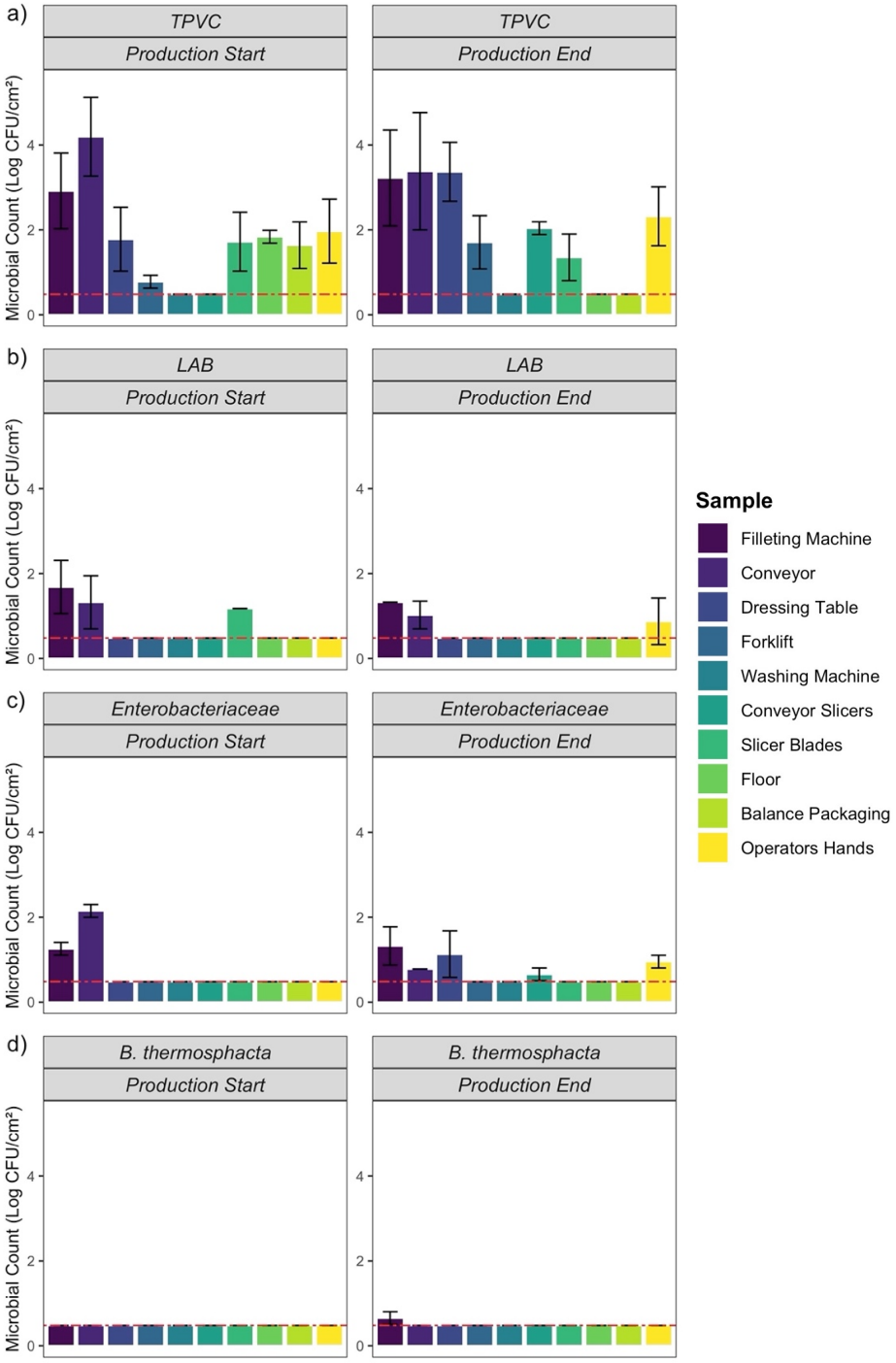

Figure 28. Bacterial enumeration of (**a**) Total Psychrotrophic Viable Count (TPVC), (**b**) Lactic Acid Bacteria (LAB), (**c**) *Enterobacteriaceae* and (**d**) *Brochothrix thermosphacta* from a processing plant surfaces sample. The sampling was performed on cleaned surfaces at the beginning of a production (Production Start) and at the end of the same production (Production End). Results are expressed in Mean ± SD Log CFU/cm² surface.

The ice storage samples were also tested. Counts are summarized **Table VI**.

Table VI. Bacterial enumeration of Total Psychrotrophic Viable Count (TPVC), Lactic Acid Bacteria (LAB), *Enterobacteriaceae* and *Brochothrix thermosphacta* from ice storage samples. The sampling was performed on cleaned surfaces at the beginning of a production (Production Start) and at the end of the same production (Production End). Results are expressed in Mean ± SD Log CFU/mL ice.

Microorganisms	Production Start	Production End
TPVC	3.05 ± 1.65	3.54 ± 0.15
LAB	<1.00	<1.00
Enterobacteriaceae	<1.00	<1.00
B. thermosphacta	<1.00	<1.00

At the beginning of the production, TPVC were mainly counted on ice with 3.05 ± 1.65 Log CFU/mL and raw material area samples with a maximum of 4.20 ± 0.93 Log CFU/cm^2 on the conveyor from filleting to salting. TPVC then decreased in the treatment area to reach 0.78 ± 0.15 on the forklift. TPVC counts on the washing machine were below the limit of detection (<0.48 Log CFU/cm^2). In the packaging area, TPVC were below the limit of detection on the conveyors bringing products to the slicers. Then counts were detected on the slicer blades, the floor, the balance and operator hands. Counts were from 1.64 ± 0.55 Log CFU/cm^2 to a maximum of 1.95 ± 0.55 Log CFU/cm^2 on operator gloves. At the end of the production, TPVC were enumerated on all samples except for the washing machine, the floor and the balance where counts were below the limit of the detection.

In the raw material area, TPVC were between 3.54 ± 0.15 Log CFU/cm^2 in the ice storage and 3.38 ± 1.38 Log CFU/cm^2 on the conveyor. As far as the treatment area is concerned, TPVC were enumerated at 3.37 ± 0.69 Log CFU/cm^2 on the dressing table and 1.71 ± 0.63 Log CFU/cm^2.

Lactic acid bacteria (LAB) were only enumerated in the raw material area with a maximum of 1.68 ± 0.63 Log CFU/cm^2 on the filleting machine and enumerated on the slicer blades (1.18 ± 0.00 Log CFU/cm^2) at the beginning of the production. However, at the end of the production, LAB counts were only enumerated in the raw material area (maximum of 1.32 ± 0.00 Log CFU/cm^2 on the filleting machine) and on operators hands (0.88 ± 0.55 Log CFU/cm^2). *Enterobacteriaceae* counts followed the same trend. Counts were only enumerated on the two raw material area sample at the production start with a maximum of 2.15 ± 0.15 Log CFU/cm^2 on the conveyor. At the end of the production, *Enterobacteriaceae* were still enumerated in the raw material area with a maximum of 1.32 ± 0.45 Log CFU/cm^2 on the filleting machine.

Enterobacteriaceae were also enumerated on the dressing table (1.13 ± 0.55 Log CFU/cm^2), the conveyor to the slicing machine (0.65 ± 0.15 Log CFU/cm^2) and on operators' hands (0.95 ± 0.15 Log CFU/cm^2). *Brochothrix thermosphacta* were only counted on the filleting machine at the end of the production (0.65 ± 0.15 Log CFU/cm^2).

IV.2.3.2. Quantification of total bacterial cells by *tuf* qPCR

Total cells were quantified by using *tuf* qPCR (**Figure 29** and **Table VII)**. Cell quantification was heterogenous at the beginning of the production. The main quantifications were observed in the raw material area with a maximum of 4.53 Log *tuf* gene copies/cm² on the conveyor.

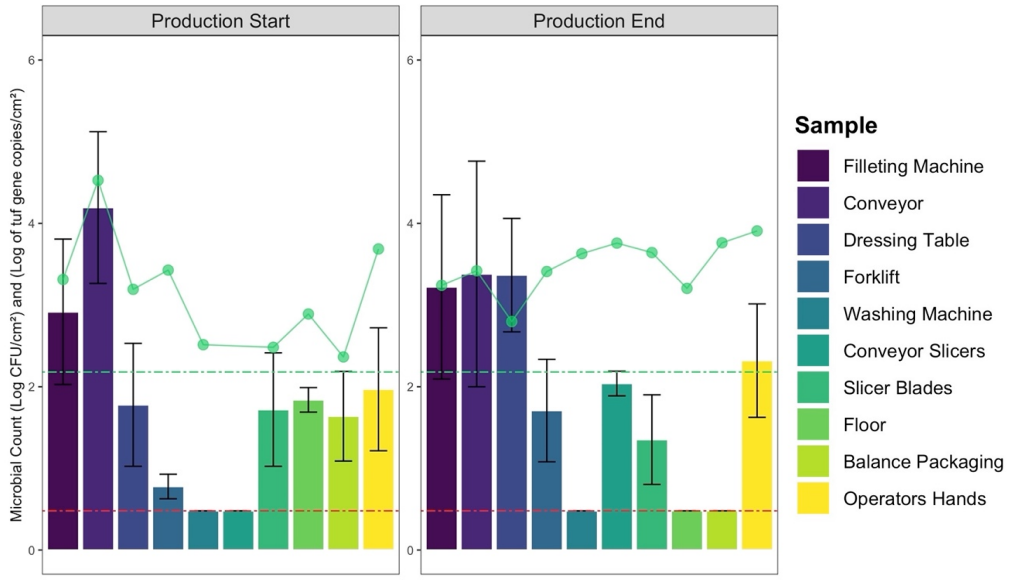

Figure 29. Total Psychrotrophic Viable Count (TPVC) and total bacterial enumeration by *tuf* qPCR from processing plant samples. TPVC are represented by barplot and *tuf* qPCR by line plot. The sampling was performed on cleaned surfaces at the beginning of a production (Production Start) and at the end of the same production (Production End). Results are expressed in Mean ± SD Log CFU/cm² surface and Log *tuf* gene copies/cm² surface. The green-dashed line represents the limit of detection: 2.18 Log *tuf* gene copies/cm² surface. The red-dashed line represents the limit of detection: 0.48 Log CFU/cm².

Table VII. Total bacterial enumeration by *tuf* qPCR. Results are expressed in Log *tuf* gene copies/mL ice. The limit of detection is 2.70 Log *tuf* gene copies/mL ice.

Sample	Production Start	Production End
Ice storage	4.59	4.52

Total cells followed the same trend as the CFU counts with a decrease in the treatment area and in the packaging area. Total cells quantification on the conveyor slicers was below the limit of detection (<2.18 Log *tuf* gene copies/cm²). Quantification on operators' hands was high with 3.70 Log *tuf* gene copies/cm².

At the end of the production, TPVC was range from a minimum of 2.80 Log *tuf* gene copies/cm² on the dressing table to a maximum of 3.91 Log *tuf* gene copies/cm² on operators' hands. Total cells quantification on ice was homogeneous during the production.

IV.2.3.3. Metabarcoding analyses

IV.2.3.3.1. CSS metabarcoding analyses

DNA from eight CSS samples was extracted, amplified and sequenced. A total of 686,375 reads passed filters applied through the FROGS pipeline workflow with an average of 85,797 reads/sample ± 28,936 reads. Samples were rarefied to an even depth of 10, 851 reads.

A total of 52 OTUs were identified and agglomerated in 34 different genera. Dominant populations among all samples were represented by Firmicutes *Staphylococcus* (1.10% ± 2.51), *Macrococcus* (0.20% ± 0.51), β-Proteobacteria *Photobacterium* (94.13% ± 11.60), *Enterobacteriaceae Serratia* (2.27% ± 5.13), *Citrobacter* (0.21% ± 0.60), *Rahnella* (0.13% ± 0.24) and *Kluyvera* (0.94% ± 2.51) and γ-Proteobacteria *Pseudomonas* (0.24% ± 0.42).

Communitiy richness (observed OTUs) and evenness (Shannon diversity index) were assessed for the 8 samples and are summarized in **Table VIII**. The production time had no effect on both richness ($p = 0.46$) and evenness ($p = 0.60$).

Table VIII. Observed richness and evenness for 16S rRNA amplicons analyzed on CSS samples study. Data are expressed in Mean ± SD.

CSS samples	Richness (Observed OTUs)	Evenness (Shannon Index)
D0	10.00 ± 7.07	0.097 ± 0.114
D14	15.00 ± 9.90	0.240 ± 0.249
D21	5.50 ± 0.71	0.052 ± 0.030
D28	15.00 ± 2.83	0.629 ± 0.839

The bacterial communitie's evolution was then evaluated. The relative abundances at the genus level are represented in **Figure 30**.

At the beginning of the experiment (D0) the CSS samples were dominated by *Photobacterium* (98.43% ± 1.94). The subdominant population was dominated by *Clostridium* (0.73% ± 1.00) and *Anoxybacillus* (0.48% ± 0.48). After 14 days of storage, the bacterial communities were still dominated by *Photobacterium* (96.10% ± 4.32).

The subdominant population was then constituted by *Rahnella* (0.27% ± 0.38), *Serratia* (1.57% ± 2.22), *Anoxybacillus* (0.11% ± 0.08), *Staphylococcus* (0.59% ± 0.31), *Shewanella* (0.56% ± 0.79) and *Pseudomonas* (0.52% ± 0.73).

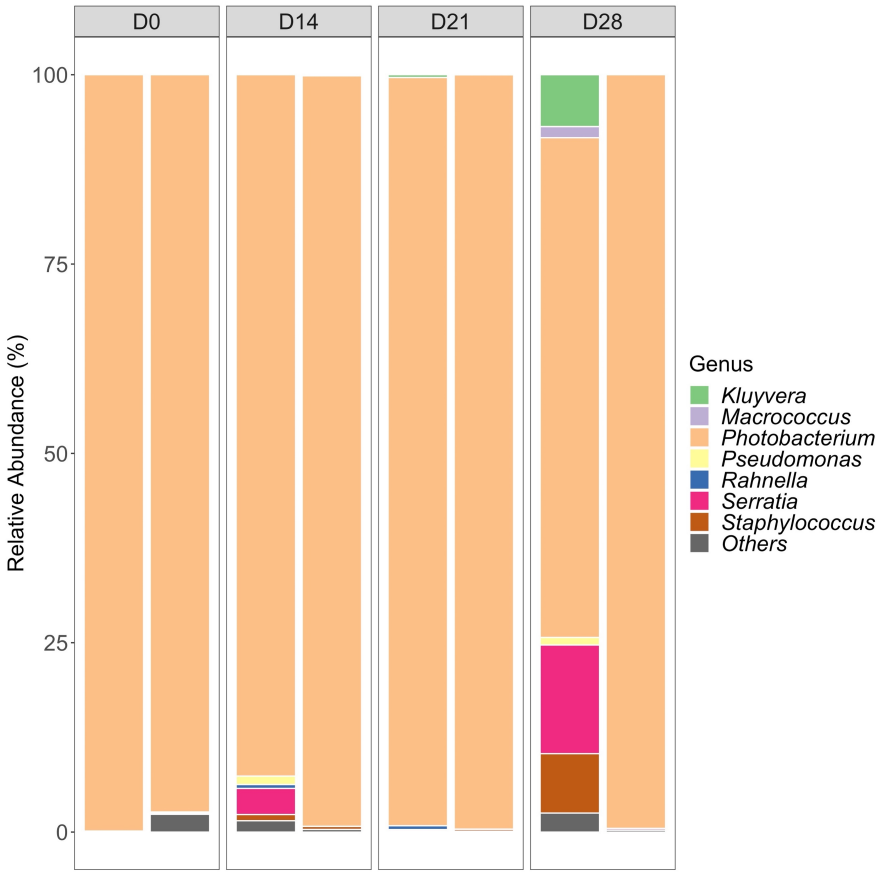

Figure 30. Relative abundance of bacterial genera of vacuum-packed CSS products stored during 28 days (D0, D14, D21, D28). Two samples from the same production batch were processed and analyzed. Taxa present on average in all samples at a threshold ≥ 0.5% or having a 90th percentile ≥ 0.5% are individually represented. In other cases, taxa are grouped and labeled "Others".

After 21 days of storage, the microbiota was dominated by *Photobacterium* (99.27% ± 0.48). At this storage date, the subdominant population was composed by *Rahnella* (0.25% ± 0.35), *Kluyvera* (0.16% ± 0.22) and *Staphylococcus* (0.12% ± 0.17). At the end of the storage (28 days) *Photobacterium* (82.78% ± 23.77), *Staphylococcus* (3.64 ± (5.14%), *Serratia* (7.41% ± 10.28), *Kluyvera* (3.57% ± 5.10) were identified on the CSS samples. The subdominant population was then dominated by *Pseudomonas* (0.41% ± 0.56), *Citrobacter* (0.85% ± 1.21), *Macrococcus* (0.73% ± 1.03) and *Carnobacterium* (0.40% ± 0.56). PERMANOVA analysis based on Jaccard dissimilarity showed that no bacterial community influence was observed and explained by the storage time ($p > 0.05$).

IV.2.3.3.2. Processing plant metabarcoding analyses

DNA from 19 processing plant samples was extracted, amplified and sequenced. A total of 1,310,011 reads passed filters applied through the FROGS pipeline workflow with an average of 68,948 reads/sample ± 38,428 reads. Samples were rarefied to an even depth of 10,851 reads.

A total of 440 OTUs were identified and agglomerated in 17 phyla and 193 different genera. Dominant populations among all samples were represented by Firmicutes (50.16% ± 41.95), Proteobacteria (43.10% ± 36.83), Bacteroidetes (3.14% ± 4.36) and Actinobacteria (2.14% ± 2.60).

Community richness (observed OTUs) and evenness (Shannon diversity index) were assessed for the 19 samples and are summarized in **Table IX**. Production time had a significant effect on both richness ($p = 0.017$) and evenness ($p = 0.008$). The same effect was observed on evenness according to the factory sector ($p = 0.001$ and $p = 0.019$ respectively). Microbiota richness was higher at the beginning of the production ($p = 0.017$) with an average of 108.0 ± 53.10 observed OTUs.

The diversity was more even at the beginning of the production ($p = 0.008$) with an average Shannon index of 3.06 ± 1.30. The observed richness was low in the packaging area with an average of 38.6 ± 37.2 observed OTUs. As far as the evenness is concerned, the diversity was higher in the raw material zone than the packaging area ($p = 0.01$) with an average Shannon index of 3.43 ± 0.91 in the raw material area.

Table IX. Observed richness and evenness for 16S rRNA amplicons analyzed on processing environment samples. Data are expressed in Mean ± SD.

	Richness (Observed OTUs)		**Evenness (Shannon Index)**	
	Production Start	Production End	Production Start	Production End
Raw Material	146.0 ± 35.5[a]	140.0 ± 36.4[a]	3.63 ± 0.78[a]	3.23 ± 1.16[a]
Treatment	134.0 ± 64.3[a]	52.7 ± 47.5[ab]	3.40 ± 1.30[ab]	1.11 ± 1.72[ab]
Packaging	66.5 ± 33.9[ab]	10.8 ± 1.5[b]	2.45 ± 1.62[ab]	0.04 ± 0.01[b]

[a,b] Means sharing the same letter superscript do not differ significantly, $p < 0.05$.

The bacterial communities were then evaluated. The relative abundances at the phylum level are represented in **Figure 31**. All the phyla shared a homogenous repartition according to the production time ($p > 0.05$) except for *Actinobacteria* ($p = 0.04$) which was more prevalent at the beginning of the production ($p = 0.004$). Firmicutes ($p = 0.009$), Proteobacteria ($p = 0.009$) and Bacteroidetes ($p = 0.001$) prevalence varied across the different factory sectors. Firmicutes were more prevalent in the packaging area than the raw material area (78.83% ± 32.10) ($p = 0.002$). Contrary to *Firmicutes, Proteobacteria* were more prevalent in the raw material area (78.10% ± 8.90) ($p = 0.002$). *Bacteroidetes* were higher in the raw material area (8.24% ± 3.98) ($p < 0.002$).

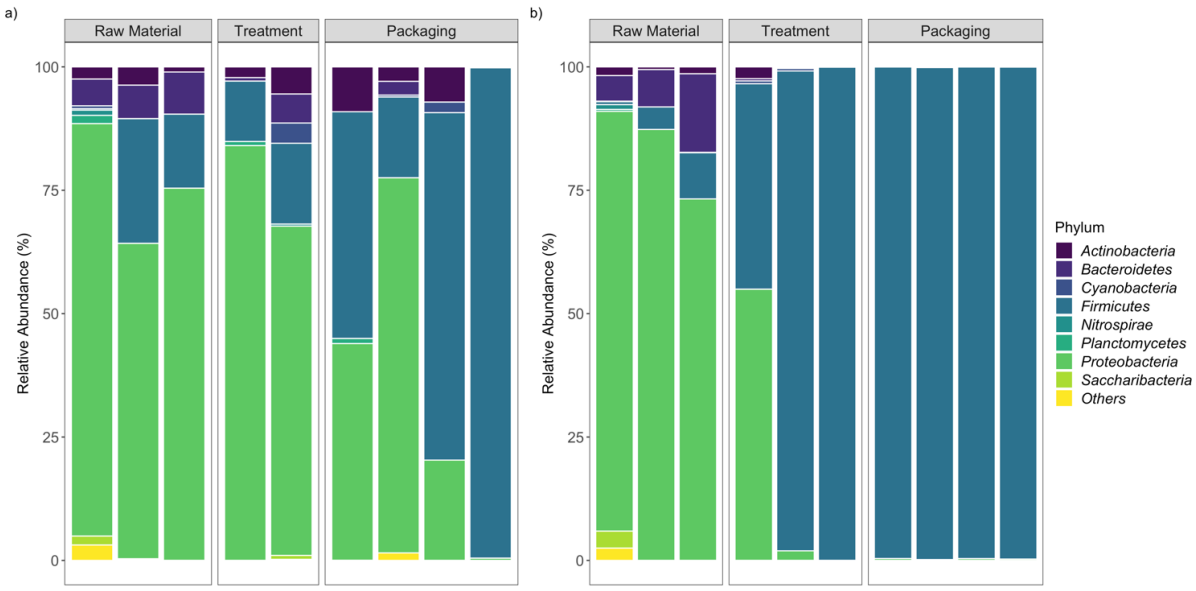

Figure 31. Relative abundance of bacterial Phylum from processing environment samples **a**) at the beginning of a CSS production and **b**) at the end of a CSS production. Taxa present on average in all samples at a threshold ≥ 0.5% or having a 90th percentile ≥ 0.5% are individually represented. In other cases, taxa are grouped and labeled "Others".

Among all the samples, 9 genera were dominant: Firmicutes *Anoxybacillus* (36.70% ± 49.07), *Staphylococcus* (1.92% ± 7.13), *Bacillus* (5.25% ± 10.53), γ-Proteobacteria *Pseudomonas* (14.81% ± 16.81), *Acinetobacter* (8.42% ± 12.66), *Shewanella* (1.90% ± 6.07), *Psychrobacter* (2.07% ± 3.58), β-Proteobacteria *Photobacterium* (4.06% ± 14.80) and *Flavobacteriaceae Flavobacterium* (1.93% ± 3.65). Interestingly, unknown genera were identified (2.66% ± 3.80). The relative abundances at the genus level are represented in **Figure 32**. Over all the genera, only two varied according to the production time: *Anoxybacillus* was more abondant at the end of the production (from 11.28% ± 33.00 to 59.58% ± 51.19) (p = 0.03) and *Psychrobacter* at the beginning of the production (from 3.77% ± 4.71 to 0.54% ± 0.63) (p = 0.04). Globally, genera repartition was statistically similar across the different processing plant aerea sector (p > 0.34). However, *Anoxybacillus* was more prevalent in the packaging area than the raw material zone (from 0.08% ± 0.04 to 62.40% ± 51.22) (p = 0.04) and *Flavobacterium* was higher in the raw material area (5.68% ± 4.74) (p < 0.01).

To evaluate the differences between bacterial communities β-diversity analyses were performed. Jaccard Principal Coordinates Analysis (PCoA) (**Figure 33**) highlighted a separation between packaging's samples and other sectors.

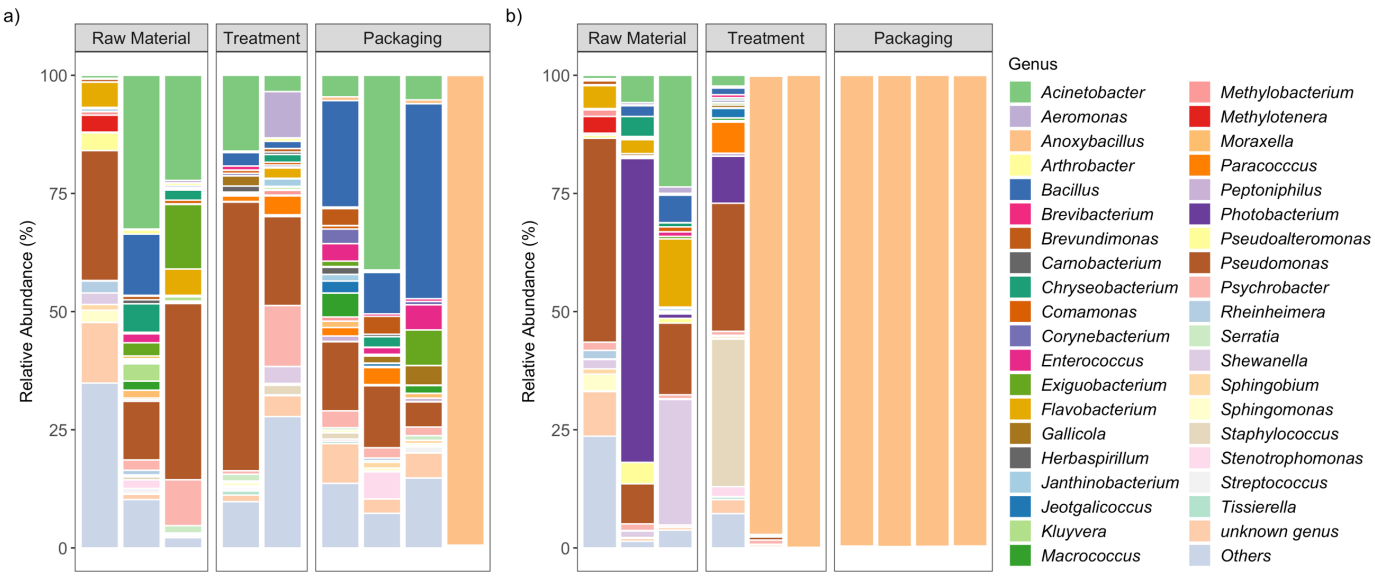

Figure 32. Relative abundance of bacterial Genera from processing environment samples **a**) at the beginning of a CSS production and **b**) at the end of a CSS production. Taxa present on average in all samples at a threshold ≥ 0.5% or having a 90th percentile ≥ 0.5% are individually represented. In other cases, taxa are grouped and labeled "Others".

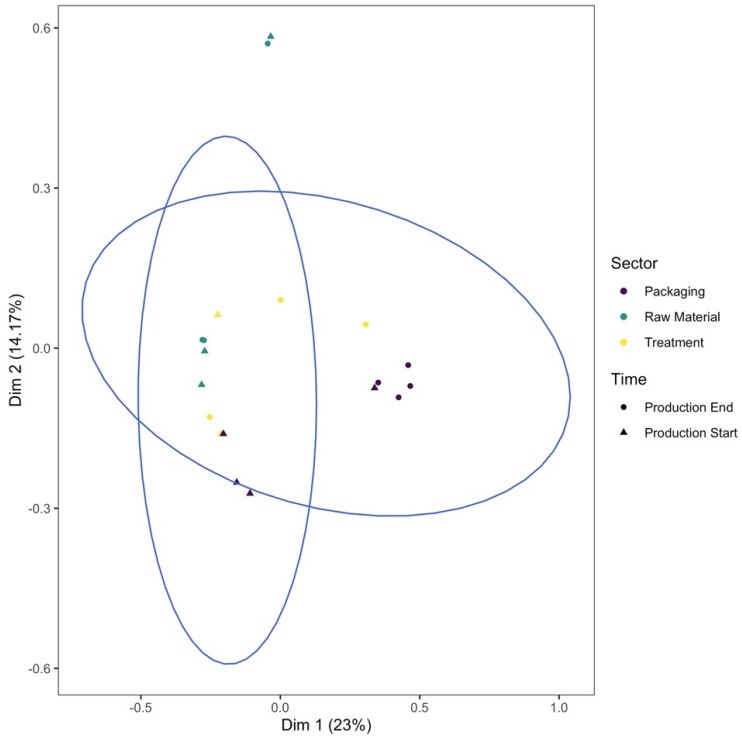

Figure 33. Jaccard dissimilarity Principal Coordinates Analysis plot of processing environment samples according to factory sectors and production time.

PERMANOVA analyses based on Jaccard dissimilarity, highlighted microbiota differences linked to the production time (p = 0.048) and the factory sectors (p = 0.001) explaining respectively 8.00% and 21.12% of the differences.

IV.2.3.3.3. Comparison CSS and processing environment microbiotas

To evaluate processing environment impacts on CSS product quality, β-diversity analyses were performed to compare both sample's types (**Figure 34**).

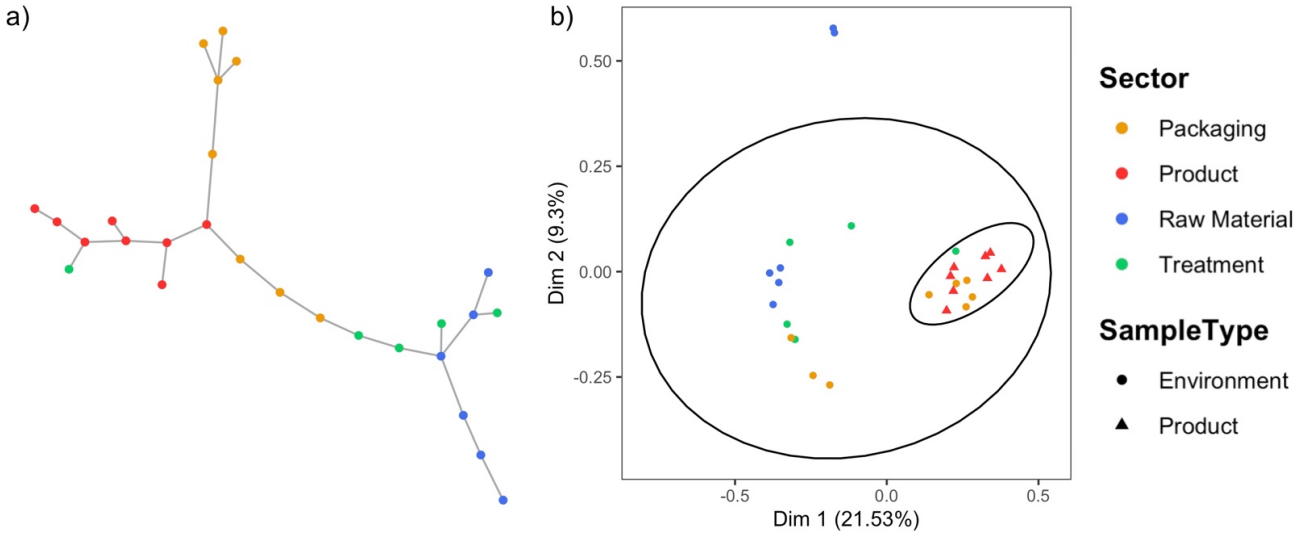

Figure 34. Jaccard dissimilarity network and PCoA plots of CSS and processing environment samples according to factory's sectors and sample's types.

Both Jaccard dissimilarity network and PCoA showed similarity and relation between products and environment bacterial communities. Moreover, products microbiota seemed to be closely related with and share taxa with environment samples from the treatment and the packaging area.

UpSet plots (**Figure 35**) highlighted that both environment and products shared 53 core OTUs and only 1 OTU was identified as CSS specific. This OTU was identified as a *Serratia* OTU. The 53 core OTUs were agglomerated in 34 genera. Dominant population among these 34 genera were *Photobacterium* (31.57% ± 44.52), *Anoxybacillus* (26.32% ± 44.15), *Pseudomonas* (19.11% ± 29.27), *Acinetobacter* (7.64% ± 17.71), *Staphylococcus* (2.52% ± 8.70), *Psychrobacter* (2.32% ± 7.10), *Shewanella* (1.41% ± 5.62), *Serratia* (1.28% ± 3.02). This core microbiota suggested that the packaging area could be an environmental niche for product bacterial communities.

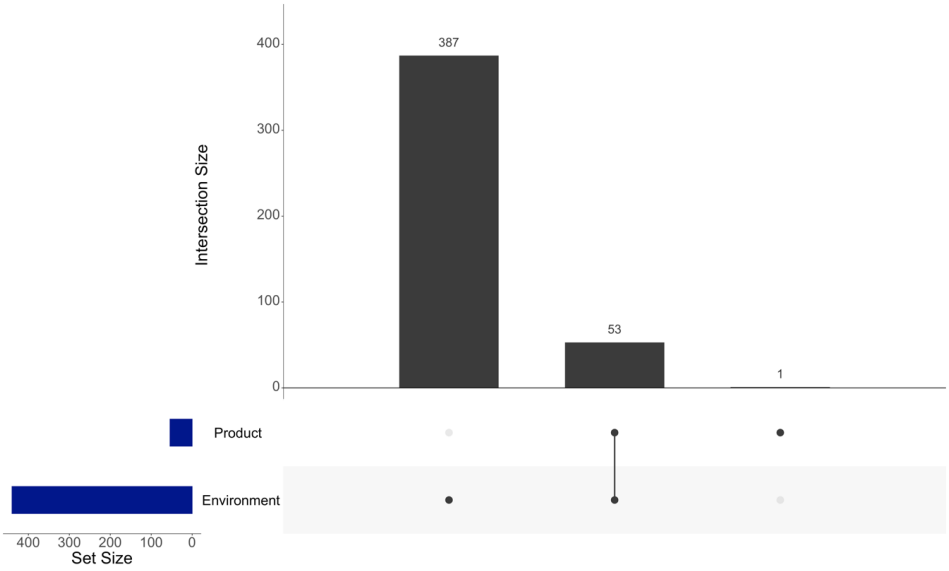

Figure 35. UpSet plots of shared OTUs identified within CSS vacuum-packed products and processing plant samples.

In order to explore the relationship between CSS' and processing environment's bacterial communities the Bayesian approach SourceTracker was used (**Figure 36**). This model provides an overview of the proportion of CSS bacterial community sourcing from each different processing plant samples. When the algorithm is not able to assign CSS's bacterial community to any of the studied environmental source, the prediction's result is then displayed as "Unknown".

Among all the storage dates, the major contributor to the CSS bacterial community was the filleting machine with an average of 95.90% ± 7.58. Interestingly, after 14 days of storage two new potential microbial sources were identified: the conveyor leading fillets to the salting machine in the raw material area (0.92% ± 1.27) and the dressing table in the post smoking room within the treatment area (1.69% ± 1.68). The dressing table as a source was confirmed at D28 with an average of 10.20% ± 14.10. The Bayesian approach SourceTracker suggested the filleting machine (raw material area) and the dressing table (treatment area) as the most potential source for CSS microbial community.

Figure 36. Source environment proportions for CSS samples at each storage date estimated using SourceTracker (Knights et al., 2011). The detailed proportions are summarized in annexe 3.

IV.2.4. Discussion

This study aimed to characterize surface bacteria in a CSS processing plant and to evaluate their impact on products quality. This work was divided into two parts. The first part of this study was dedicated to CSS products and processing plant samples bacterial enumeration.

CSS microbial load was high at the production day (D0) and reached quickly its maximum during the 28 days of storage. At D0, only *Enterobacteriaceae* were enumerated and their concentration increased quickly during the 28-day period. LAB concentration was below the limit of detection at the production day but increased to reach a maximal concentration at D28. As described by Maillet et al. (2021b) and Leroi et al. (1998), no competition between LAB and Gram-negative bacteria seemed to occur. Indeed, contrary to what Paludan-Müller et al. (1998) observed when characterizing the composition of the microflora on vacuum-packed, chill-stored cold-smoked salmon during storage LAB did not dominate the CSS microbiota at the end of the storage. In addition, we observed a co-domination between LAB and *Enterobacteriaceae* on CSS products at the end of the storage. This co-domination was already described with psychotropic *Enterobacteriaceae* such as *Serratia liquefaciens* (Joffraud et al., 2006; Løvdal, 2015; Paludan-Müller et al., 1998). Although already described as able to dominate CSS microflora at the end of the shelf-life (Illikoud et al., 2019), *Brochothrix thermosphacta* was below the limit of detection at each storage date.

Concerning processing environment, surfaces were sampled at the beginning of a production (cleaned) and at the end of the production. At the beginning of the production, TPVC were enumerated mostly in the raw material area (ice, filleting machine and conveyor) with a maximum of 4.20 ± 0.93 Log CFU/cm^2 on the conveyor and decreased in the treatment area suggesting that the environmental conditions were not suitable for growth. Porsby et al. (2008) by analyzing the impacts of CSS process on survival and growth of *Listeria monoyctogenes* observed a significant reduction (1 Log CFU/cm^2) of *Listeria monocytogenes* concentration on salmon surfaces, highlighting the bacteriostatic effect of cold-smoking. Smokers and post-smoking room have an important place in the treatment area and could impact the microflora of the whole zone. Indeed, the cultivability could be reduced by the specific environmental conditions. Except for the conveyor slicers, TPVC in the packaging area seemed to be homogeneous (1.53 ± 0.60 Log CFU/cm^2).

At the end of the production, more CFU were counted specially in the treatment area. The salmon trimming on the dressing table led to an accumulation of fillets and food soil on this table. The presence of food soil can be a factor helping bacteria to grow and attach on surfaces. Indeed, Overney et al. (2017) highlighted the impact of different food soils (meat exudate, smoked salmon juice) on the behavior of *Listeria monocytogenes*. The authors highlighted that the type of food soils may increase cell attachment on surface and lead to a potential product contamination hotspot. A few specific bacteria were enumerated (LAB, *Enterobacteriaceae*) but mainly in the raw material area, the dressing table and on operators' hands. Suggesting that operators at the end of the process may have an impact on the CSS quality by direct contact. Bagge-Ravn et al. (2003) analyzed the microbial ecology of processing equipment in two different salmon smokehouses by culturable and not-culturable approaches. The authors highlighted that the two environments were dominated by LAB, *Enterobacteriaceae*, which seems to be not the case in our study, but also *Neisseriaceae* and *Alcaligenes*.

The use of *tuf* gene qPCR on processing plant samples highlighted new insights on the surface cells state. In the raw material area, *tuf* qPCR results and TPVC counts were comparable suggesting that most of the cells in this area might be culturable. However, except for the dressing table at the end of the production, qPCR results seemed to be higher than CFU counts, suggesting the presence of viable but non culturable cells (VBNC) or dead cells. Firmesse et al. (2012) studied the microbial load in a delicatessen and compared CFU, total cells (qPCR) and viable cells (EMA-qPCR) on stainless steel and PVC surfaces. The authors observed significant differences between CFU and viable cells specially after hygiene procedures suggesting that processing environment condition decreased bacterial cultivability. The use of a molecule such as EMA or PMA in our study could bring more insight on the cells state. Culture based and non-culture-based quantification methods highlighted bacteria after cleaning and disinfection suggesting that these bacteria are residential bacteria (Møretrø and Langsrud, 2017).

The second part of this study was dedicated on CSS and processing plant microbiota study by 16S rDNA metabarcoding. The CSS population identified confirmed already published data on CSS and seafood microbiota studied by cultivable methods and non-cultivable methods (Maillet et al., 2021b). Our samples were highly dominated by *Vibrionaceae, Photobacterium* and no significant differences were observed during the storage in terms of microbiota richness, evenness and composition.

Firmicutes Macrococcus, *Enterobacteriaceae Rahnella*, *Kluyvera* and *Citrobacter* were surprisingly identified on finished products. *Macrococcus* has been already identified on seawater and freshwater finfish and could be linked to the raw material (Parlapani, 2020). Interestingly, by studying the microbial ecology of salmon fillets and processing environment Møretrø et al. (2016) identified *Kluyvera* on the filleting machine. Although *Kluyvera* was not identified on product samples, the filleting machine can be a contamination source for in process and finished products.

Interestingly LAB was highly enumerated, LAB was only identified in the subdominant population by 16S rDNA metabarcoding and represented by *Carnobacterium* known to dominate CSS microflora (Olofsson et al., 2007). The subdominant population was also composed of *Anoxybacillus* known to be part of smoked horse meat microflora (Geeraerts et al., 2019).

Processing plant microbiota was more complex: 193 different genera were identified. The dominant surface microbiota identified was mainly composed by Firmicutes *Anoxybacillus, Staphylococcus, Bacillus,* γ-Proteobacteria *Pseudomonas, Acinetobacter, Shewanella, Psychrobacter,* β-Proteobacteria *Photobacterium and Flavobacteriaceae Flavobacterium.* All of these genera have been already described in processing plant of many types of foods such as vegetables, meat, poultry, dairy products, seafood and seafood products by non-culturable approach (Maillet et al., 2021a; Møretrø et al., 2016). Globally the environment microbiota was homogeneous in all the different sector. However, *Anoxybacillus* was highly dominant in both treatment and packaging area specially at the end of the production. *Anoxybacillus* is a known spore-former, able to reside within processing environment and to produce biofilms (Alvarez-Ordóñez et al., 2019). Moreover, its occurrence on smoked meat suggest that this genus is able to grow despite of smoking phenolic compounds (Geeraerts et al., 2019). *Anoxybacillus* occurrence in the treatment and packaging areas confirm that food process can shape specific selective conditions leading to specific microbiota.

The domination of *Anoxybacillus* specially in the packaging sector was also strengthened by low richness and diversity in this specific area. In addition, the Jaccard dissimilarity PCoA showed a clear distinction between packaging samples and other sector samples which was confirmed by PERMANOVA. Beta diversity analyses showed that the production time had an effect on the surface microbiota. Indeed, Jaccard dissimilarity, highlighted differences in the microbiota's composition depending on the production time.

When studying contamination on salmon fillets Møretrø et al. (2016) suggested that contact between surfaces and each processed raw material could be a key transfer zone between products and machines/equipments. Thus, leading to shape a different surface bacterial community.

Comparison between processing plant samples showed that most of the product OTUs (98%) were shared with the environment. Thus, highlighting a core microbiota mainly composed by spoilage bacteria: *Photobacterium, Anoxybacillus, Pseudomonas, Acinetobacter, Staphylococcus, Psychrobacter, Shewanella* and *Serratia* (Møretrø and Langsrud, 2017). By studying meat and meat processing surfaces, Stellato et al. (2016) also highlighted that most of product OTUs were originated from the processing environment, strengthened the existence of core microbiota between food and processing plant surfaces.

Jaccard dissimilarity network and PCoA indicated relationship between CSS products and two processing area: treatment and packaging. Thus, suggesting that these two areas might be key contamination hotspots. By using the same approach, De Fillips et al. (2013) evaluated the source of spoilers in beefsteaks. The authors highlighted that the main actors in the products spoilage were *Pseudomonas* and *Brochothrix*. They analyzed by 16S rDNA metabarcoding environment samples in each process step and compared bacterial communities with carcasses and products microbiotas. The authors found that the carcasses were the primary source of contamination and transferred spoilage bacteria within processing environment leading to residential spoilage microbiota. These results strengthened the impact of surface bacteria on products quality and shelf-life through transfers during the process.

To confirm observations we made on the treatment and packaging area, we used SourceTracker to better understand source and transmission of bacterial communities between the processing plant and CSS products (Knights et al., 2011). The most likely source identified was the filleting machine. However, this specific step aims to cut fillets in two, leading to a huge salmon quantity on this equipment. Salmon filleting frequency is high and some raw material microflora is able to attach surface and recontaminate other fillets. After 14 days of storage, we highlighted the dressing table as the most likely source of CSS contamination. Interestingly, the dressing table is a post smoking step leading to an accumulation of fillets on a cutting table. Fillets are then transported on a forklift to the post smoking maturation room and then with the same forklift to the packaging area. Thus, suggesting that the dressing table can be an important contamination hotspot and the forklift may act as contamination vector. Zwirzitz et al. (2020), studied the contamination routes within a pork meat processing factory.

The authors indicated that SourceTraker analyses solved transmission routes and highlighted that even with few CFU counted, bacterial communities can be exchanged. Thus, the authors identified specially operators gloves as a contamination hotspot which was surprisingly not the case in our study. Indeed, operators reweigh all non-conform CSS slices leading sometimes to an accumulation of CSS plates waiting for being processed, and increased a potential bacteria transfer. Moreover, we observed in the dressing area that the blades used to dress fillets might be difficult to clean and might be a bacterial niche leading to transfer to the table and/or the products.

The use of a polyphasic approach in this study gave us the opportunity to characterize residential bacteria within a CSS processing plant, to highlight a core microbiota between products and environment mainly composed by spoilage bacteria and to solve some contamination routes.

However, important points to tackle could be the viability of the microbiota identified and accurate phylogenetic linkages. Whole genome sequencing and genomics comparison between products isolates and environment isolates could give us more insightful data on the contamination's routes. As far as cells viability is concerned, Mo et al. (2019), explored the fermentation process of 19 different dairy products by full 16S rRNA gene sequencing. The authors observed significant differences in terms of composition and α-diversity metrics. Thus, suggesting that the use of such a molecule in our study could give us more impactful information specially in the treatment and packing area.

IV.2.5. Conclusion

In this study, we planned to characterize surface bacteria within a CSS processing plant and to evaluate their impact on product safety and quality. We described the microbiota of vacuum-packed CSS products, a CSS processing plant and evaluated the potential linkage between all the bacterial communities identified. Residential bacteria were identified after cleaning and disinfection procedures. We highlighted that food process may shape specific "house" microbiota and a core microbiota between environment and products mainly composed by spoilage bacteria was identified. The use of an NGS-based approach highlighted relationships between products and the processing environment and helped us to solve contamination routes suggesting that the environment has a strong impact on food safety and quality. A better knowledge of processing environment microbiota and its dynamic could generate data on a factory "health condition".

Thus, leading to better targeted cleaning procedures or more accurate corrective measures to improve food safety and quality management. Therefore, by reducing contamination during food processing, industrials may have an impact on their product shelf-life and might reduce spoilage issues. Thus, industrials might be able to reduce food wastage at the processing scale and therefore at consumer places. The use of such a polyphasic approach in food processing factories may be a key to reduce the global food wastage.

IV.3. Ce qu'il faut retenir du chapitre IV

L'hygiène des surfaces est considérée comme un élément clé du système de qualité des usines de transformation des aliments. Cependant, les bactéries des surfaces restent généralement non identifiées et leur rôle dans la qualité et la sécurité des aliments est souvent inconnu. Ces communautés bactériennes résident, persistent dans les usines de transformation des aliments en raison de leur capacité de croissance à basse température, de leur capacité à former des biofilms ainsi que de leur tolérance aux biocides. Ces bactéries environnementales peuvent affecter la qualité des aliments par une contamination croisée à chaque étape du processus.

Dans cette étude, les microbiotes de saumons fumés ainsi que d'échantillon d'environnement de transformation ont été analysés par une approche polyphasique composée de méthodes cultures-indépendantes et dépendantes. Huit produits finis (saumon fumé) et dix échantillons environnementaux ont été analysés.

L'approche polyphasique mise en place nous a permis d'identifier, après nettoyage, des bactéries résidentes sur les surfaces. Ceci a notamment été permis par l'apport du metabarcoding pour résoudre le biais des méthodes culturales dans des environnements stressants. Nous avons mis évidence une communauté bactérienne homogène dans les deux premiers secteurs de l'usine mais également un microbiote spécifique dans le secteur tranchage/conditionnement. Ceci pouvant s'expliquer par une potentielle pressions de sélection ne permettant que l'implantation de flores capables de survivre à cet environnement.

L'approche de metabarcoding a mis en évidence que 98 % du microbiote du CSS était partagé avec l'environnement. Ce « core » microbiote était principalement composé de genres précédemment signalés comme étant altérants des produits de la mer : *Anoxybacillus*, *Staphylococcus* (Firmicutes), *Photobacterium*, *Enterobacteriaceae Serratia* (β-Proteobactéries), *Psychrobacter*, *Shewanella*, *Acinetobacter* et *Pseudomonas* (γ-Proteobactéries).

Les relations entre le microbiote des produits et de l'environnement ont été étudiées. Nous avons pu identifier les éléments clés du processus de fabrication du saumon fumé de l'usine, comme étant les sources de contamination les plus probables. En outre, nous avons pu confirmer que les communautés bactériennes des produits peuvent être affectées négativement par l'environnement de transformation.

Ces résultats permettent de mieux comprendre les évènements de re-contamination d'un produit alimentaire au cours de sa fabrication. Une meilleure compréhension de l'environnement, de « l'état de santé » d'une usine permet de mieux cibler les zones, équipements critiques et par cela même de réduire la contamination des produits. Une meilleure gestion du processus de nettoyage et de désinfection par ces nouvelles données, pourraient permettre aux industriels d'assurer la qualité de leurs produits ainsi que d'améliorer leur durée de vie. L'utilisation d'une approche polyphasique pour l'étude des denrées alimentaires et des environnements de transformation pourrait permettre de mieux comprendre la dynamique des bactéries de surface et leur impact sur la sécurité et la qualité des aliments. Ainsi, cela permettrait d'améliorer la durée de conservation des produits et serait une piste pour réduire le gaspillage alimentaire.

Chapitre V. Discussion et Perspectives

V.1. Evaluer l'impact des différentes étapes d'une analyse par metabarcoding

Les industriels de l'agroalimentaire, réalisent des contrôles microbiologiques des surfaces dans le cadre de plan de surveillance ou afin de valider les procédures de nettoyage et de désinfection. La majorité des analyses effectuées sur ces prélèvements de surface ne ciblent que les bactéries pathogènes associées à la production ou certains indicateurs d'hygiène (*Enterobacteriaceae*, levures, moisissures). Or, cette stratégie analytique ne prend pas en compte la majorité des espèces bactériennes présentent sur les surfaces dont la quantité et la biodiversité reste en général inconnue. Ces dernières peuvent être potentiellement résidentes et/ou présenter des propriétés d'altération ce qui constitue un risque pour l'évolution de la qualité des denrées alimentaires au cours de la conservation (Doyle et al., 2017a). Il apparait nécessaire de disposer d'une méthodologie permettant de caractériser les communautés bactériennes des usines afin d'évaluer le risque qu'elles peuvent représenter.

Dans le cadre de ce travail, nous avons dans un premier temps mis en place une approche polyphasique basée sur le séquençage de l'ADNr 16S par metabarcoding permettant de détecter et d'identifier les communautés bactériennes du saumon fumé ainsi que les bactéries résidentes d'une usine de production. Ceci, dans le but d'évaluer dans un second temps l'impact de ces communautés bactériennes sur la qualité microbiologique des produits et de mettre en place à Biofortis un service proposant ce type d'étude. De nombreuses étapes méthodologiques et techniques peuvent impacter fortement les résultats de l'analyse des communautés bactériennes par metabarcoding. Il s'agit notamment du prélèvement, de la préparation des échantillons, des méthodes d'extraction d'ADN, du choix du gène cible à amplifier, de la technologie de séquençage ainsi que du pipeline d'analyse bioinformatique. Lorsque qu'il est nécessaire d'échantillonner les surfaces, l'efficacité des dispositifs de prélèvement doivent également être étudiée (Bukin et al., 2019; Hart et al., 2015; Keisam et al., 2016; Yamaguchi et al., 2003).

La première partie de ce travail a consisté à évaluer l'impact de méthodes d'extraction et de dispositifs de prélèvements sur le résultat d'une analyse par metabarcoding de l'ADNr 16S du microbiote du saumon fumé et de surfaces industrielles. Nous avons comparé les résultats de metabarcoding de communautés bactériennes issues d'échantillons représentatifs d'un processus de fabrication. Pour cela, un échantillon de saumon fumé à DLC ainsi que des échantillons de surfaces en inox et TPU placées au sein de l'usine ont été collectés.

Les résultats montrent que les méthodes d'extraction peuvent avoir un impact sur la composition, la richesse et la diversité du microbiote prélevé sur saumon fumé. Cependant, aucune différence n'a été observée sur les matrices environnementales. Comme a pu l'observer Hart et al. (2015), en analysant le microbiote fécal de différents animaux, l'efficacité d'une méthode d'extraction de l'ADN est matrice dépendante. De plus, le saumon fumé, comme les produits alimentaires, est une matrice complexe (gras, composés phénoliques) ce qui rend plus difficile l'extraction de l'ADN bactérien (Keisam et al., 2016). Bien qu'il puisse y avoir des traces de détergent ou de désinfectant dans les echantillons de surface, il nous est apparu que l'impact de la methode d'extraction sur la qualité et reproductibilité de l'analyse est plus faible sur ces echantillons. Ceci sugèrre l'importance de valider les methodes d'extraction principalement sur les différents produits alimentaires soumis à l'analyse metabarcoding.

De la même manière, nous avons pu observer une influence des dispositifs de prélèvement de surface sur les microbiotes observés. L'éponge, a permis de prélever des communautés plus riches, plus diverses que l'écouvillon. Ceci peut s'expliquer par la force plus importante appliquée sur le dispositif de type « éponge » ce qui permet de mieux décrocher les cellules (Midelet et Carpentier, 2002; Yamaguchi et al., 2003). Nous n'avons pas identifié, dans la littérature, d'autres études comparant des microbiotes prélevés avec des dispositifs différents. Dans la première partie, nous avons donc sélectionné une méthode d'extraction d'ADN, un dispositif de prélèvement ainsi que les modalités de préparation des échantillons nécessaires à la réalisation des travaux de cette thèse. Nos résultats ont également montré l'importance de standardiser et de calibrer la méthodologie en amont d'études basées sur le metabarcoding. En effet, même s'il existe des méthodes d'extraction automatisées, l'effet matrice peut perturber l'efficacité d'une méthode sur un échantillon en particulier. En ce qui concerne le prélèvement, il est difficile d'automatiser cette étape (manipulateur dépendant). Il semble donc pertinent de valider ces aspects en amont de chaque étude.

V.2. Un service pour l'industrie agroalimentaire

La première partie de ce travail a également permis de définir les bases à la commercialisation par Biofortis d'un service proposant aux industriels de l'agroalimentaire des analyses de metabarcoding : de l'élaboration technique à la tarification des analyses. L'idée de ce service était notamment de mettre à disposition aux industriels de nouveaux outils, complémentaires aux analyses de routine pour répondre à des problématiques complexes.

L'ISO ou l'AOAC (Association of Official Agricultural Chemists) possèdent des groupes de reflexion sur la mise en œuvre des NGS dans l'analyse microbiologique des aliments. Cependant aucune methode d'analyse métagénomique ou de métabarcoding n'est normalisée. Les entreprises de service ne peuvent donc pas se baser sur des methodes validées. Dans notre cas, les résultats obtenus au cours de ce travail sur les méthodes d'extraction, de prélèvement ainsi que de préparation d'échantillon choisies ont été utilisé comme base pour construire la prestation.

Une analyse robuste nécessite la validation de la répétabilité et de la reproductibilité sur differentes matrices alimentaires afin de délivrer un résultat auprès d'un client. Cet aspect, nécessitant un plan d'expérience solide et défini en amont, ne peut cependant qu'être traité que dans le cas d'études bien spécifiques. En effet, les industriels sont, dans la majorité des cas, habitués à des prestations standards, abordables et dont le résultat doit être clair afin de mettre en œuvre rapidement des mesures correctives. Les problématiques auxquelles peuvent répondre le metabarcoding (compréhension de phénomènes d'altération, de fermentation, évolution au cours de la durée de vie microbiologique) sont complexes, nécessitent une expertise de la part du prestataire de service (technique et scientifique) et de ce fait les analyses sont coûteuses. Il reste donc encore difficile de proposer une prestation complète en routine hormis aux grands groupes, plus familier avec ces nouvelles approches.

V.3. Le lien entre le microbiote du saumon fumé et son environnement de production

Dans la suite de ce travail, nous avons caractérisé pour la première fois les communautés bactériennes d'un environnement de production de saumon fumé. La population dominante de ces communautés était composée de bactéries appartenant aux familles des *Flavobacteriaceae*, des β-Proteobactéries ainsi que des γ-Proteobactéries telles que *Acinetobacter*, *Pseudomonas* et *Shewanella*. La majorité des genres bactériens identifiés au cours de cette étude a été décrite dans la littérature comme faisant partie de la flore d'altération du saumon fumé (Leroi et al., 1998; Løvdal, 2015; Olofsson et al., 2007, Remenant et al., 2015). La présence de bactéries d'altération du saumon fumé au sein de l'environnement de production indique un risque potentiel de contamination des denrées par transfert de ces dernières. Ceci souligne l'impact que peut avoir l'environnement de production sur le microbiote des matrices alimentaires et ainsi, sur leur qualité. Nous avions donc à cœur d'étayer cette hypothèse de travail dans la deuxième partie de ce projet de thèse.

La biodiversité de 9 lots de saumon fumé commercialisés sous différentes marques a été étudiée à partir de produits issus de trois ateliers de transformation distincts. L'objectif était double. D'une part, caractériser et décrire pour la première fois le microbiote du saumon fumé au cours du stockage par une approche polyphasique composée de méthodes culturales, d'analyses chimiques et de metabarcoding. D'autre part, comparer les microbiotes de saumon fumé fabriqués dans différents environnements de production, afin d'identifier une évenutelle corrélation avec l'usine.

Dans ce travail le microbiote du saumon fumé est dominé par 12 genres bactériens : *Staphylococcus*, *Carnobacterium*, *Lactobacillus*, *Photobacterium*, *Vibrio*, *Aliivibrio*, *Salinivibrio*, *Serratia*, *Pantoea*, *Psychrobacter*, *Shewanella* et *Pseudomonas*. Ces résultats corroborent les différentes les études sur la flore du saumon fumé issue d'approches culturales déjà publiées (Joffraud et al., 2006; Leroi et al., 1998; Løvdal, 2015; Olofsson et al., 2007; Paludan-Müller et al., 1998). Cependant, les données générées par metabarcoding ainsi que la comparaison des microbiotes de produits issus d'usines différentes ont mis en évidence deux aspects importants. En effet, nous avons identifié un microbiote partagé par tous les produits, mais surtout, nous avons observé des différences au niveau de la composition des microbiotes qui semblent être reliés à l'usine de production. Cette « signature usine » nous a conforté dans l'hypothèse que l'environnement de production entretient un lien microbiologique étroit avec les produits. Rouger et al. (2018), par l'analyse du microbiote de viande de poulet produite le même jour, au sein de la même usine mais provenant de deux lots différents, avaient également observé cette spécificité. Cette signature environnementale a pu être décrite dans les filières laitière, bovine et porcine (Fillipis et al., 2013; Stellato et al., 2016; Zwirzitz et al., 2020). Ces données nous ont conforté dans notre démarche de caractérisation du microbiote de surface au sein d'une usine de production de saumon fumé, afin de mieux comprendre les dynamiques au sein de l'usine ainsi que les transferts des cellules bactériennes sur la matrice.

V.4. Le microbiote de surface dans un atelier de production de saumon fumé

Au cours de la troisième partie de ce travail de thèse, nous avons donc mis en place un suivi d'une production de saumon fumé au sein d'une usine modèle. Des prélèvements de surfaces ont été effectués après nettoyage, en début et en fin de production. Les données microbiologiques ont été évaluées par une approche polyphasique, qui nous a permis de mettre en évidence et d'identifier des bactéries résidentes au sein de cette usine.

En effet, 193 genres bactériens ont été prélevés dans cet environnement complexe. La flore majoritaire identifiée au cours de cette étude est composée des genres suivants : *Anoxybacillus, Staphylococcus, Bacillus, Pseudomonas, Acinetobacter, Shewanella, Psychrobacter, Photobacterium* et *Flavobacterium*. Ces genres bactériens ont été décrits dans la littérature comme capables de résider au sein des environnements de production alimentaire et pouvant altérer des matrices alimentaires (Møretrø et Langsrud, 2017; Remenant el al., 2015).

En comparant le microbiote des surfaces avec le microbiote des produits, nous avons observé que 98% de la flore du saumon fumé provenait de l'environnement. Ce « core » microbiote, principalement composé de bactéries d'altération du saumon fumé, confirme ainsi le rôle que possède l'environnement de production sur les communautés bactériennes retrouvées sur les produits. L'utilisation du metabarcoding, ainsi que des concepts d'écologie microbienne dans cette étude ont mis clairement en évidence la relation entre le microbiote du produit et de l'environnement, permettant ainsi de mieux cibler l'origine environnementale de la contamination des denrées. Ces résultats ont amené à la mise en place de mesures correctives par l'industriel, telles qu'une meilleure sectorisation de certains équipements ainsi que des procédures d'hygiène ciblant les zones de contamination pré-identifiées. Ainsi, des sources environnementales importante dans la contamination des produits ont été identifiées. Il s'agit de la table de parage et d'un chariot electrique qui était utilisé pour transporter les filets parés, dressés jusqu'en zone de maturation post-fumage puis vers la zone de conditionnement. Aussi, les déplacements et franchissement de zones de cet élément mobile ont été repensés et limités. Ces données nous ont donc aidés à mieux comprendre les dynamiques bactériennes dans cette usine et à identifier les transferts bactériens entre les différents ensembles « produits » et « environnement ». Ceci ouvre de nouvelles pistes pour limiter les risques de contamination des produits par des bactéries de surface.

L'approche de metabarcoding mise en place au cours de ce travail de thèse nous a permis d'identifier des bactéries résidentes mais surtout l'impact qu'elles pourraient avoir sur la qualité des produits. En effet, l'environnement de production heberge des bactéries d'altération capables à chaque étape du processus de fabrication, de contaminer les denrées alimentaires. Cependant, la méthode de caracterisation des communautés bacteriennes de surface par métabarcoding de l'ADNr 16S possède des contraintes comme la limite de détection et le seuil d'assignation taxonomique.

V.5. Améliorer l'assignation taxonomique

L'analyse de la diversité des communautés bactériennes basée sur le séquençage d'une partie du gène de l'ARNr 16S ne permet pas en général une identification au niveau de l'espèce bactérienne. En effet, la résolution taxonomique atteinte au cours de ce travail, comme dans la plupart des travaux se basant sur une partie de l'ARNr 16S, n'a permis une analyse de la diversité que jusqu'au niveau du genre bactérien. Or, notamment dans l'altération microbiologique des aliments, l'information au niveau de l'espèce est importante (Joffraud et al., 2006; Remenant et al., 2015). Par exemple, *Latilactobacillus sakei subsp. sakei* est considérée comme une bactérie altérante dans les produits de la mer alors qu'elle fait partie du microbiote bénéfique dans les produits carnés. Afin d'améliorer l'assignation taxonomique à partir de données de metabarcoding, plusieurs stratégies sont à envisager. Certains auteurs suggèrent l'utilisation d'autres gènes (Case et al., 2007; Poirier et al., 2018). Dans leur étude de 2018, Poirier et al., en comparant des données de metabarcoding sur les régions V3-V4 du gène de l'ARNr 16S avec celles obtenues à partir du gène *gyrB* (sous-unité beta de l'ADN gyrase), ont mis en évidence le pouvoir résolutif de cette cible pour distinguer différentes espèces bactériennes. Le choix du gène cible à amplifier peut donc s'avérer primordial pour ce type d'étude. Le gène qui code pour la sous-unité beta de l'ARN polymerase (*rpoB*) a été également proposé pour permette une identification au niveau de l'espèce. Ogier et al. (2019), en comparant des résultats de metabarcoding issus de communautés bactériennes standardisées et de composition connue, ont observé que l'analyse d'amplicons du gène *rpoB* s'avère plus sensible (détection des espèces de la communauté témoin) mais donne également une résolution taxonomique supérieure à ce que permet l'étude d'une région du gène de l'ARNr 16S. De plus, la composition bactérienne, la richesse et la diversité des échantillons observés étaient fidèles à la composition réelle de l'échantillon témoin analysé. Des stratégies multi-cibles commencent à se développer. Ainsi Ogier et al. (2019) préconisent, pour une meilleure appréciation de la diversité des communautés étudiées, une approche multigénique basée sur le séquençage d'un gène tel que *rpoB* et *gyrB* ainsi qu'une ou plusieurs régions hypervariables du gène de l'ARNr 16S.

D'autre part, une des limites de l'assignation taxomomique à partir de séquences d'ADN est de disposer d'une base de donnée suffisamment complète et accessible pour permettre une identification spécifique des OTUs présents au sein d'un échantillon (Hestetun et al., 2020).

En effet, ces bases de données sous curation, doivent être mises à jour avec des séquences de qualité représentant au mieux la taxonomie bactérienne pour limiter au maximum les erreurs d'assignation. La qualité et la disponibilité de ces bases de données est donc clé dans le metabarcoding. Si ces dernières sont de bonne qualité et exhaustives pour une cible telle que le gène de l'ARNr 16S, ce n'est pas forcément le cas pour les autres gènes *gyrB* et *rpoB*.

Le choix de gènes cibles présents en mono-copie dans la plupart des génomes bactériens telles que *rpoB* et *gyrB*, en plus de permettre une résolution taxonomique au niveau de l'espèce, pourrait également résoudre les problèmes d'évaluation de la richesse des communautés bactériennes. En effet, malgré sa position de standard d'identification bactérienne, le gène de l'ARNr 16S est présent en multiples copies dans la plupart des génomes bactériens (Case et al., 2007). Le metabarcoding d'une portion de ce gène implique donc l'amplification et le séquençage de toutes copies disponibles dans l'ADN extrait. Ceci peut ainsi induire un biais dans l'évaluation de la richesse ou de la diversité de la communauté étudiée. Ces multiples copies posent essentiellement deux problèmes : un biais d'alpha-diversité mais également un biais d'identification des OTUs observés. Dans le cadre de ce travail de thèse, nous avons été confrontés à des communautés très faiblement diverses et dominées par : *Photobacterium, Aliivibirio, Salinivibro, Vibrio* ainsi qu'*Anoxoybacillus*. Ces différents genres bactériens possèdent de 7 (*Anoxybacillus*) à 21 copies (*Photobacterium*) du gène de l'ARNr 16S (University of Michigan, 2021). De plus, il a été démontré que chez certains genres bactériens, notamment *Photobacterium,* les différentes copies du gène de l'ARNr 16S au sein d'un même génome pouvaient présenter un pourcentage de similarité inférieur à 97% (Větrovský et Baldrian, 2013). Les premières étapes de l'analyse bioinformatique de données de metabarcoding permettent de regrouper les séquences similaires à 97% sous forme d'OTU. La diversité de ces copies peut donc induire des erreurs dans l'assignation taxonomique. En conséquence, dans le cadre de notre étude, les amplicons correspondant aux membres cités de la famille des *Vibrionaceae* pourraient finalement provenir d'un même genre bactérien tel que *Photobacterium*.

Une autre approche de metabarcoding basée sur le séquençage de l'ADNr 16S permettant une meilleure résolution taxonomique, consiste à séquencer le gène complet par des technologies de 3ème génération telles que PacBio et Oxford Nanopore Technologie (ONT) (Cuscó et al., 2019; Kerkhof et al., 2017; Tyler et al., 2018). Ces technologies de séquençage « long reads » permettent de séquencer des fragments de plus de 8 kb donc, de séquencer des fragments de 1,5 kb, représentant l'intégralité du gène de l'ARNr 16S.

De ce fait, l'étude du gène complet induirait une résolution taxonomique plus importante. Cependant, la qualité de séquences notamment des plateformes ONT est à ce jour inférieure à celle obtenue avec les plateformes de 2ème génération. Ce manque de qualité doit être compensé par l'utilisation de nombreux outils de bioinformatique (Santos et al., 2020).

Il existe donc d'autres approches de metabarcoding capables de réduire les limites que nous avons pu rencontrer. Cependant, le développement récent des plateformes de séquençage et notamment le séquençage « shotgun » est une piste de choix pour les études de microbiotes de surfaces industrielles, car il ouvre la voie du séquençage des génomes complets des bactéries.

V.6. Vers une approche de métagénomique

Le développement et l'amélioration des outils de métagénomique permet désormais d'envisager une autre application des NGS pour caractériser les communautés microbiennes. La métagénomique correspond au séquençage complet de tous les génomes présents au sein d'un échantillon. Cette approche, qui ne nécessite pas d'étape d'amplification préalable, permet d'obtenir des données de séquence de tout ADN présent dans un échantillon (microbien, fongique ou viral). Elle se distingue donc du metabarcoding basé sur le séquençage partiel d'un seul gène (Francioli et al., 2021) et peut s'affranchir des effets relatifs au nombre de copies de l'ARNr 16S. Ce séquençage de génomes entiers, plus résolutif et plus complet, permet une identification précise, au rang de l'espèce, des bactéries détectées. De plus, la métagénomique permet l'identification de gènes d'intérêt au sein de microbiotes (résistance aux antibiotiques, résistance aux biocides, etc.) mais également de voies métaboliques propres à certains microorganismes (Ranjan et al., 2016). La métagénomique apporte de ce fait une plus-value dans l'étude des communautés bactériennes. Par exemple, en cherchant à comprendre les acteurs et mécanismes de fermentation d'un plat traditionnel chinois à base de soja, Xie et al. (2020) ont utilisé une approche métagénomique. Les auteurs ont été capables d'identifier plus précisément le microbiote de cet aliment et les bactéries lactiques impliquées dans la production d'acide lactique ainsi que les fonctions biologiques des gènes identifiés dans le microbiote. De la même manière, en étudiant les communautés bactériennes de plats prêt à la consommation en Chine, Li et al. (2020) ont identifié les bactéries qui contaminent ces denrées et de caractériser leurs gènes de résistance aux antibiotiques. Ainsi, ces travaux montrent que l'analyse du metagénome permettrait une meilleure compréhension des corrélations entre le microbiote des aliments et le transfert de gènes d'antibiorésistance.

Cependant, même si ces approches sont plus informatives et facilitent une identification plus performante, l'abondance des microorganismes observés n'est pas absolue. La quantification d'une communauté microbienne par NGS ne peut s'affranchir de contrôles internes. Pour cela des règles strictes doivent être mises en place et suivies (Harrison et al., 2021). Ces contrôles internes consistent en des communautés standards dont la composition et les proportions sont connues et maîtrisées. En effet, ils permettent de corriger les ratios observés pour déterminer l'abondance absolue des espèces bactériennes identifiées. Cependant, les auteurs indiquent que le processus de séquençage et les étapes au laboratoire en amont peuvent cumuler des biais difficiles à corriger. Ces autres stratégies NGS permettant de caractériser des communautés bactériennes au niveau des espèces approches ne sont pas non plus dépourvues de limites. Les NGS génèrent un nombre important de données qui apportent une vision supplémentaire des communautés bactériennes dans les produits alimentaires et leurs usines de production, mais peuvent aussi être limitées par la concentration en cellules des échantillons.

V.7. Analyser la diversité des microbiotes paucimicrobiens

Dans ce travail, nous avons été confrontés à deux verrous techniques majeurs : le seuil de détection du metabarcoding sur des échantillons paucimicrobiens ainsi qu'une faible diversité bactérienne masquée par des flores dominantes. En effet, dans la partie focalisée sur le microbiote de 9 lots de saumon fumés différent, correspondant au chapitre III, nous avons dû éliminer le tiers des échantillons de notre analyse (45/135). La concentration en flore psychrophile totale de ces 45 échantillons était en moyenne inférieure à 4,00 Log CFU/g. Sur ces échantillons de saumon fumé faiblement contaminés nous avons pu montrer les limites du de l'analyse metabarcoding. Pinto et al. (2012), en étudiant les communautés bactériennes d'eau de boisson, ont déterminé ce seuil à 10^3 – 10^5 CFU/mL. Brandt et Albertsen (2018), en contaminant artificiellement par *E. coli* des matrices ont été capables d'analyser des échantillons chargés à 1 Log CFU/mL. Cependant les auteurs avaient identifié des OTUs générés artificiellement (bruit de fond), biaisant ainsi les analyses. De la même manière, ce bruit de fond peut également provenir des réactifs des différents kits d'extraction (Salter et al., 2014). Ces contaminants, appelés « kitome » peuvent donc biaiser la richesse et la diversité des communautés analysées (Voirol et al., 2020). Bien qu'il existe des procédures pour réduire le risque d'OTUs contaminants (kitome, bruit de fond) (Ficetola et al., 2016; Olomu et al., 2020), cette approche de metabarcoding peut être limitée par la faible concentration bactérienne. Il est donc important de l'accompagner de méthodes complémentaires.

Il paraît donc difficile d'analyser les communautés bactériennes sous-dominantes et présentes à une faible concentration sans pré-enrichissement du microbiote en amont. De ce fait, cette approche de metabarcoding peut s'avérer inadaptée pour la recherche de flores pathogènes dans un produit ou un environnement de production, car elles sont en majorité présentes à de très faible concentrations. Le metabarcoding apparait donc limité pour analyser les communautés paucimicrobiennes, il est donc important d'adapter la prise d'essai (masse d'échantillon, surface à prélever) et de compléter cette approche par des méthodes culturales qui vont faciliter l'enrichissement du microbiote par une phase de culture (bouillon d'enrichissement ou culture sur boite de Pétri). Dans ce cas une stratégie d'identification des bactéries doit être mise en place.

Les approches de culturomiques peuvent être utilisées afin de diminuer la limite de détection. La culturomique correspond à une approche utilisant des conditions de culture multiples (température, aérobiose, milieu), la spectrométrie de masse MALDI-TOF et le séquençage de l'ADNr 16S pour l'identification des espèces bactériennes dénombrées (Lagier et al., 2018). Cette approche combinée à du metabarcoding semble donner une information supplémentaire et complémentaire quant à la communauté observée (Hamad et al., 2017). Dans la littérature, les auteurs combinent différentes approches pour mieux apprécier le microbiote étudié. Cependant, pour simplifier les analyses d'écologie microbienne et la comparaison des données, il pourrait être intéressant d'envisager une approche de culturomique dans laquelle des colonies et/ou tapis bactérien observés sur une boite de Pétri seraient analysés par metabarcoding (Pédron et al., 2020). La mise en place d'une telle approche dans le cadre de cette thèse nous aurait très probablement permis de réduire les limites que nous avons rencontrées sur le saumon fumé.

Une autre piste à explorer pour l'analyse des écosystèmes paucimicrobiens serait la métagénomique. En effet, la profondeur de séquençage que peut atteindre le shotgun, pourrait permettre d'atteindre un seuil de détection plus bas que le metabarcoding. Cependant, le biais de profondeur de séquençage influe fortement sur la détection ou non de flores sous-dominantes (Hillmann et al., 2018; Lagier et al., 2018). De plus, selon le type de population microbienne étudiée, le metabarcoding peut disposer d'une résolution supérieure à la métagénomique. En effet, Tessler et al. (2017) ont étudié des communautés bactériennes environnementales issues de fleuves par metabarcoding et métagénomique. Lors de cette étude, les auteurs ont identifié par metabarcoding 27% de familles bactériennes en plus, contrairement à la métagénomique qui, elle, n'a permis d'identifier que 50% des phyla observés par metabarcoding.

Ceci suggère que selon le type d'échantillon et la profondeur de séquençage, la problématique de flores sous-dominantes ou faiblement chargées ne peut être résolue par une simple approche de métagénomique.

V.8. Explorer la biodiversité les flores sous dominantes

Analyser des communautés bactériennes d'un produit tel que le saumon fumé implique en premier lieu d'extraire l'ADN de l'échantillon. Les méthodes d'extractions n'étant pas spécifiques aux ADN bactériens, les extraits sont contaminés par de l'ADN de la matrice (saumon dans notre cas). Ce qui peut entraîner l'identification d'ADN de chloroplastes ou de mitochondries dans les analyses de metabarcoding et ainsi biaiser les analyses de diversité alpha (Beckers et al., 2016). De plus, comme observé au cours de ce travail, la présence d'une flore dominante telle que les *Vibrionaceae* (*Photobacterium*, *Aliivibrio*, *Salinivibrio*) a nombre de copies du gène de l'ARNr 16S élevés biaise la diversité observée et peut masquer l'identification d'OTU rare et d'intérêt. Daube et al. (2015) proposent, dans un brevet déposé, une méthodologie basée sur le « peptide nucleic acid clamping ». Le principe de cette méthode est d'ajouter au mix réactionnel des oligonucléotides spécifiques à la flore dominante (*Photobacterium* par exemple) ou aux chloroplastes et mitochondries. L'ajout de ces oligonucléotides va permettre de bloquer, ou de réduire au maximum, l'amplification de l'ADN de ces cibles non désirées. L'utilisation de ce procédé dans le cadre de notre travail aurait pu nous permettre d'observer une flore sous-dominante non impactée par les différents membres de la famille des *Vibrionaceae*. Cependant, il est nécessaire d'utiliser un échantillon non-traité en contrôle afin d'apprécier l'abondance relative des flores majoritaires.

V.9. Décrire la diversité des bactéries viables et les quantifier

L'utilisation du metabarcoding sur des échantillons prélevés après procédures d'hygiène ou sur matrice saumon salée et fumée sans traitement préalable, ne permet pas de déterminer si les bactéries sont viables ou non. En effet, une des principales problématiques à laquelle les approches de metabarcoding sont confrontées est la detetction de cellules mortes ou d'ADN contaminants d'origine bacterienne. Dans le cadre de cette thèse nous n'avons pas évalué, au-delà d'un aspect cultural, la viabilité des bactéries totales.

L'ethidium monoazide (EMA) ou propidium monoazide (PMA) sont des intercalant de l'ADN capables de ne traverser la membrane cellulaire que si celle-ci est altérée.

Ainsi, en s'intercalant dans les molécules d'ADN, l'EMA ou le PMA empêchent l'amplification par PCR des cellules mortes. Ces molécules ont été principalement utilisées pour quantifier des espèces bactériennes spécifiques ou une flore totale par qPCR (Firmesse et al., 2012; Overney et al., 2017). Le développement de la PMA-qPCR constitue une nouvelle approche permettant de quantifier les cellules viables. Cependant la molécule PMA est très onéreuse et ne permet pas un résultat totalement fiable. En effet, les cellules viables peuvent laisser pénétrer la molécule et ainsi ne pas être quantifiées (Elizaquível et al., 2014). Il est donc impératif de mettre en place des contrôles de viabilité et de mortalité et de bien calibrer le traitement (Gobert et al., 2018).

Récemment, des études de communautés bactériennes par metabarcoding ont été réalisées avec un prétraitement au PMA afin de séquencer et d'étudier uniquement les bactéries vivantes (Be et al., 2017; Checinska Sielaff et al., 2019; Erkus et al., 2016; Stinson et al., 2019). Ces études montrent clairement l'intérêt de distinguer les cellules vivantes et mortes dans une approche exploratoire, pour mieux comprendre l'écologie microbienne d'un d'environnement donné. Cependant, Wang et al. (2021), en étudiant l'utilisation de cette molécule dans le cadre d'études d'écologie microbienne du métro de Boston par metabarcoding, ont mis en évidence que la réponse bactérienne au PMA variait non seulement en fonction de la bactérie mais également de son écosystème. Ceci suggère que cette approche ne peut être utilisée dans n'importe quel environnement. En effet, les composants (chimiques ou microbiens) d'un écosystème pourraient influencer significativement l'efficacité du PMA. Cet aspect viabilité est important pour mieux comprendre les dynamiques microbiennes d'un écosystème, cependant une méthodologie PMA-metabarcoding seule ne pourrait suffire. Une approche polyphasique prenant en compte des aspects culturaux et culturomiques ainsi que des approches moléculaires permettraient de disposer d'une vision plus globale d'une communauté bactérienne d'un environnement.

V.10. Conclusion

Il faut considérer que l'utilisation du metaboacoding 16S comporte les biais évoqués au cours de cette discussion : analyse de cellules totales (vivantes ainsi que mortes), cible génétique ne permettant pas une résolution à l'espèce et faible sensibilité pour les écosystèmes paucimicrobiens. Cependant, par cette approche exploratoire, nous avons générer des données d'écologie microbienne menant à l'identification de bactéries de surface et de bactéries résidentes au sein d'une usine de production de saumon fumé.

De plus nous avons été capables de mettre en évidence la relation microbienne entre les surfaces d'une usine et son produit. Des sources de contamination des produits ont pu être identifiées et traitées par des procédures d'hygiène ciblées.

Ce travail, reporté dans cette thèse, constitue une partie d'un projet qui vise à mettre en relation le microbiote des surfaces, l'evolution du microbiote et la qualité sensorielle des produits au cours de la conservation. L'analyse sensorielle renforcera l'approche polyphasique que nous avons mise en place et permettra de mieux comprendre et de mieux évaluer l'influence de bactéries de surfaces sur la qualité et la durée de vie des produits.

L'utilisation du metabarcoding dans l'industrie agroalimentaire, malgré ses limites, actuelles peut être une approche complémentaire aux méthodes classiques favorisant une meilleure gestion de la sécurité et de la qualité des produits. Le sequencage du microbiote des surfaces aidera à mieux comprendre la vie microbiologique d'une usine de production et d'identifier des marqueurs de modification des équilibres écologiques pouvant entrainer des disfonctionnements de l'ecosystème. Ainsi, la qualité et de la durée de vie des produits pourraient être améliorées par une meilleure gestion écologique, entrainant à terme, une reduction du gaspillage alimentaire.

Valorisation des travaux de thèse

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Annexe 1 : Impact of DNA extraction and sampling methods on bacterial communities monitored by 16S rDNA metabarcoding in cold-smoked salmon and processing plant surfaces

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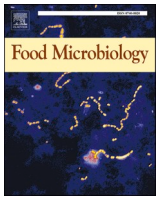

Impact of DNA extraction and sampling methods on bacterial communities monitored by 16S rDNA metabarcoding in cold-smoked salmon and processing plant surfaces

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ABSTRACT

Amplicon sequencing approaches have been widely used in food bacterial ecology. However, choices regarding the methodology can bias results. In this study, bacterial communities associated with cold-smoked salmon products and their processing plant surfaces were monitored *via* sequencing of the V3–V4 region of the 16S rRNA gene. The impact of DNA extraction protocols, sampling methods (swabbing or sponging) and surface materials on bacterial communities were investigated.

α and β diversity analyses revealed that DNA extraction methods mainly influence the observed cold-smoked salmon microbiota composition. Moreover, different DNA extraction methods revealed significant differences in observed community richness and evenness. β-*Proteobacteria: Photobacterium*, *Serratia* and *Firmicutes*: *Brochothrix*, *Carnobacterium* and *Staphylococcus* were identified as the dominant genera.

Surface microbiota richness, diversity and composition were mainly affected by cleaning and disinfection procedures but not by DNA extraction methods. Surface community richness and evenness appeared higher when sampled by sponging compared to swabbing. β-diversity analyses highlighted that surface topology, cleaning and disinfection and sampling devices seemed to affect the bacterial community composition. The dominant surface bacteria identified were mainly *Flavobacteriaceae*, β-*Proteobacteria* and γ-*Proteobacteria* described as fish spoilers such as *Acinetobacter*, *Pseudomonas* and *Shewanella*. DNA extraction and sampling methods can have an impact on sequencing results and the ecological analysis of bacterial community structures. This study confirmed the importance of methodology standardization and the need for analytical validation before 16S rDNA metabarcoding surveys.

1. Introduction

Microorganisms from food and food processing environments can negatively impact food safety and quality. As part of quality management systems, food processing plants are regularly cleaned and sanitized, and routine monitoring is conducted to detect surviving bacteria (Doyle et al., 2017; Møretrø and Langsrud, 2017). This monitoring is mainly based on culture-dependent methods. However, these techniques can lead to several analytical biases, such as non-specificity for some selective media, and difficulties to appreciate subdominant populations (Giraffa and Neviani, 2001; Bokulich and Mills, 2012). Furthermore, processing environments are ecosystems which are submitted to harsh conditions (temperature, moisture and disinfectants). These different stresses could induce viable but not cultivable cells (VBNC) (Li et al., 2014) that viable count methods could fail to detect. Moreover, bacteria adapted to a processing environment do often grow on products during storage (Møretrø and Langsrud, 2017).

The growing field of high-throughput DNA sequencing (HTS) has provided new perspectives on the overall dynamics of bacterial communities in food products and food processing plants (Hultman et al., 2015). These methods allow surface bacterial communities within processing environments, to be identified and better understood. Moreover,

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in food microbial ecology, 16S rDNA amplicon sequencing techniques have been used to obtain more information on the evolution of bacterial communities during the shelf life of a product (Filippis et al., 2013; Macé et al., 2013; Fougy et al., 2016; Chaillou et al., 2015). However, these methods are not without drawbacks. For example, dead bacteria might be revealed by 16S rDNA metabarcoding. There are no standardized procedures for sequencing-based analysis methods. From sampling to sequencing, several workflows could lead to different interpretations of results. 16S rDNA metabarcoding results can be dependent on the DNA extraction methods (Keisam et al., 2016; Witte et al., 2018). Bacterial DNA extraction from food matrices can be difficult and needs several lytic agents (chemical, mechanical, enzymatic, thermal or combinations). Taxa do not share the same lysis sensibility, indeed *Firmicutes* and other Gram-positive bacteria are more difficult to lyse (Ercolini et al., 2013).

In addition to the DNA extraction method, the sampling device and sampling methodology used can have a major impact on the recovery of surface bacteria and the observed diversity of the bacterial community (Møretrø and Langsrud, 2017). The microorganisms' surface attachment strength, surface sampling, sampling medium, and environment conditions should be considered when choosing a sampling method.

This study focused on the bacterial communities of a product, cold-smoked salmon (CSS), and its processing plant using 16S rDNA metabarcoding. CSS is a lightly preserved product with no thermal treatment and is mainly consumed as a ready-to-eat food (RTE). CSS microbial communities have been widely described in the scientific literature, with most studies focusing on CSS microbial shift during storage and the impact of packaging on CSS microbial communities (Joffraud et al., 2001, 2006, Leroi et al., 1998, 2000; Løvdal, 2015; Paludan-Müller et al., 1998). This product has been described as being dominated by Gram-positive bacteria, such as lactic acid bacteria (LAB: *Lactobacillus*, *Carnobacterium maltaromaticum*), and Gram-negative bacteria, such as marine *Vibrionaceae* including *Photobacterium phosphoreum* and psychrotrophic *Enterobacteriaceae* (*Serratia liquefaciens, Hafnia alvei*) (Leroi et al., 2000). In addition, *Aeromonas* spp., *Shewanella putrefaciens* and *Brochothrix thermosphacta* have also been isolated (Løvdal, 2015). This microflora could be involved in the CSS spoilage process. CSS products also face pathogenic bacteria such as *Listeria monocytogenes* and *Clostridium botulinum* (Løvdal, 2015).

This study aimed to assess whether the DNA extraction methods implemented and the sampling strategy used influence the identification by 16S rDNA metabarcoding of the bacterial communities on CSS products and processing plant surfaces.

2. Materials and methods

2.1. Smoked salmon tissue homogenate preparation

The CSS samples and surface samples came from the same smokehouse processing plant located in Brittany, France. One batch of cold-smoked salmon (*Salmo salar*) in a slide package that was preserved under vacuum at 4 °C, was collected from a local supermarket six days after packaging with no cold chain rupture. It then was analyzed at the Use by Date (UBD = 21 days). A 10-g portion with an approximative size of 6.5 × 6.5 × 0.25 cm was cut off from the slide and aseptically weighed in a sterile stomacher plastic bag provided with a 63 μm porosity filter (Interscience, Saint-Nom-la-Bretèche, France). To obtain a 10-fold dilution, 90 mL of sterile buffered peptone water (25.5 g/L) (Biokar Diagnostics, Allonne, France) was added to the bag. This sample was then homogenized for 2 min using a stomacher 400 device (Intersciences, Saint-Nom-la-Bretèche, France). Smoked salmon tissue homogenate (SSTH) was either used immediately to enumerate the total viable counts or stored at −20 °C for later DNA extraction.

2.2. Stainless steel and TPU coupons preparation and installation

Stainless steel (SS, 2 RB finish, AISI 304L) and tri-polyurethane (TPU, Ropanyl DM 8/2) were used in this study. SS and TPU coupons (30 × 25 cm, 750 cm^2) were purchased respectively from Laser 53 (Bazougers, France) and Ammeraal Beltech (Seclin, France). Before installation in the CSS processing plant, the SS coupons were degreased and sterilized with a mixture of ethanol at 95% and acetone (3v/v) (Overney et al., 2017). The TPU coupons were cleaned and sterilized with ethanol (70% v/v). The sterilized SS and TPU coupons were transferred into the food processing plant (FPP) in the same area where filleting and salting steps are performed before the smoking step. Coupons were placed near food contact surfaces, under a conveyor between the salting step and the filleting machine. Before surface samples were collected, the SS and TPU coupons were kept in the FPP for four weeks and were exposed to the routine daily cleaning and disinfection (C&D) program used by the processing plant. The C&D program consisted of applying 5% of ARVO CLM300 (Quaron, Rennes, France) during 5–15 min. This detergent is composed of sodium hypochlorite (with 5% of active Cl). After rinsing, the sanitizer ARVO 21 SR (Quaron, Rennes, France) was used. This sanitizer is composed by Ethanol 60%, sprayed pure on surfaces and not rinsed.

2.3. Sampling devices and surface sampling procedure

Surface bacterial communities were collected by swabbing or sponging, both procedures performed according to the ISO 18593:2018 (ISO, 2018). Swabs (Copan SRK Letheen Broth, Brescia, Italy) and sponges soaked with 10 mL Letheen Broth (3M™ Hydrated Sponge HS10LET, Cergy, France) were used. The SS and TPU coupons, which had been kept in the FPP for four weeks, were sampled before and after C&D at the end of a production run. These samples were used to compare the different DNA extraction methods and the efficiency of swabbing and sponging.

To compare DNA extraction methods, samples from SS surface were used. Fifteen different 50 cm^2 areas of one SS coupon were swabbed using a new swab for each. The 15 swabs samples then were pooled, resulting in a 60 mL sample from a 750 cm^2 SS surface. The two samples from SS surface swabbed before and after C&D were used for the comparison of the DNA extraction methods.

To compare swabbing and sponging methods, five different areas of 100 cm^2 of SS or TPU coupons were sampled using a new swab or sponge for each, before and after C&D during five consecutive days. The five swabs samples were pooled resulting in a 20 mL sample from a 500 cm^2 SS or TPU surface. The sponge samples were supplemented with 20 mL of buffered peptone water (25.5 g/L) (Biokar Diagnostics, Allonne, France), homogenized 2 min with a stomacher 400 (Intersciences, Saint-Nom-la-Bretèche, France), and then the five samples were pooled. This resulted in 150 mL of sponged samples from a 500 cm^2 SS or TPU surface. The forty samples from the SS or TPU 500 cm^2 surfaces that were swabbed or sponged before and after C&D were used for the comparison of the sampling methods and were extracted using a single DNA extraction method.

2.4. Colony counts

The smoked salmon tissue homogenate and the sponged and swabbed samples prepared as described above were used to enumerate the total viable counts. Colony-forming units (CFU) were counted on Plate Count Agar (PCA, Oxoid, Thermo Fisher Diagnostics, Dardilly, France), supplemented with 1% NaCl (Sigma-Aldrich, Merck, Saint Quentin Fallavier, France), with 100 μL of appropriate dilution in buffered peptone water spread over the agar. CFU counts were performed in triplicate after the incubation of PCA plates in aerobic conditions at 25 °C for five days. The results were expressed in CFU per gram CSS (CFU/g) or CFU per cm^2 suface area (CFU/cm^2).

2.5. DNA extraction

DNA was extracted by using four commercial DNA extraction kits. The first was the Maxwell® 16 FFS DNA Purification kit referenced as Maxwell (Promega, Charbonnière-les-Bains, France). This method required the Maxwell® 16 Instruments. This automat purifies samples using paramagnetic particles providing a mobile solid phase that optimizes DNA capture, washing and elution through purification reagents in prefilled cartridges. This purification system allows an automatic, standardized and simultaneous extraction of 16 samples at the same time. To our knowledge, no metabarcoding studies focused on CSS and FPP surface bacteria have been performed using this DNA Maxwell® 16 system extraction method. Two kits were purchased from Qiagen company (Qiagen, Courtaboeuf, France): Qiagen DNeasy PowerFood Microbial (formerly known as Mobio Powerfood Microbial) and Qiagen QIAamp BiOstic Bacteremia, referenced as QPFM and Q_2B_2, respectively. The fourth kit used, Zymo Quick-DNA Fecal/Soil Microbe Miniprep Kit referenced as ZFS, was purchased from Zymo (Ozyme, Saint-Cyr-l'Ecole, France). Qiagen and Zymo kits have already been used in previous surface and food microbial ecology studies (Bokulich et al., 2015; Stellato et al., 2016). The DNA purification technology is based on DNA selective binding to a spin column containing silica-based membrane, followed by several washing steps and a DNA elution. Both Qiagen kits use the MoBio PCR inhibitors removal technology. A first step of mechanical cell lysis using glass beads was achieved using a FastPrep (MPbiomedicals, Illkirch, France) for 30 s at a frequency of 6 m/s. When the Qiagen kits were used, the mechanical lysis was performed using glass beads provided by the kits. For the other DNA extraction methods, Maxwell and Zymo, 0.3 g of zirconium beads (100 μm diameter) were used (Scientific Industries, New-York, USA). DNA was extracted from three biological replicates from each batch. A Qubit® 2.0 fluorometer using the Qubit® dsDNA BR Assay Kit (Life technologies, Thermo Fisher Scientific, Villebon-sur-Yvette, France) was used to quantify DNA. Additional blank negative controls with no sample were used to exclude DNA contamination during extraction and from reagent DNA traces.

2.6. The 16S rRNA gene sequencing

2.6.1. Library preparation and sequencing via the Illumina® MiSeq platform

Extracted DNA was amplified by PCR to construct a sequencing library targeting the V3–V4 region of the bacterial 16S rRNA gene. PCR reactions were performed using 5 μL of DNA template, 12.5 μL of 2X Kapa HiFi Hotstart ready mix (Roche, Boulogne-Billancourt, France), 5 μL of 1 μM primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 785R (5′-GACTACHVGGGTATCTAATCC-3′) (Klindworth et al., 2013). PCR grade water was added to reach a final volume of 25 μL. The amplification was performed with first a denaturation step at 95 °C for 5 min followed by 30 cycles of [denaturation (98 °C, 30 s) annealing (57 °C, 30 s) extension (72 °C, 30 s)] and a final extension step (72 °C, 5 min) then held at 4 °C. The resulting PCR products were purified using the Agencourt AMPure kit (Beckman Coulter, Villepinte, France). Concentration and size were checked on a 2100 Bioanalyzer platform, using the DNA 7500 kit (Agilent Technologies, Les Ulis, France) and indexed using the Nextera XT DNA Library Prep kit (Illumina®, Paris, France) following Illumina® recommendations. Samples were then pooled in an equimolar concentration (4 nM). As described by the Illumina® protocol, the pool was denatured with 0.2N NaOH, diluted to 6 pM and mixed with 20% of 6 pM denatured phiX DNA, then sequenced on the Illumina® MiSeq using reagent kit v2 500 cycles.

2.6.2. Sequencing data processing and analysis

Both demultiplexed sequencing reads (approximately 250 bp in length) files were acquired under FASTQ format. The data processing was performed using the FROGS bioinformatic pipeline (Escudié et al., 2017). Paired-end raw reads were contiged using FLASh 1.2.11 (Magoč and Salzberg, 2011) with a maximum of 10% mismatch in the overlapped region and then filtered on their length (between 410 bp and 485 bp) with non-ambiguous nucleotides. Primers were removed using Cutadapt 1.18. Denoising and clustering of reads into Operational Taxonomic Unit (OTU) (97% identity) were performed using Swarm 2.2.2 (Mahé et al., 2015). Chimera detection and removing was then conducted using VSearch 1.3.0 (Rognes et al., 2016). OTUs with less than 5/100,000 of the total number of sequences in the entire dataset were removed (Bokulich et al., 2013). Taxonomy assignments were performed using RDP classifier 2.11 (Wang et al., 2007; Cole et al., 2009) and the Silva 16S rRNA gene database (SSURef_128_SILVA), (Quast et al., 2013). OTUs with a genus affiliation bootstrap threshold $<$ 0.8 were removed.

2.7. Statistical analysis

Statistical analyses and plots were performed in the R environment (R Core Team, 2018). Means were compared using *t*-test for paired observations to compare CFU numbers. ANOVA was used to assess each condition effect on CFU counts and alpha-diversity metrics. The Tukey test was also used as an ANOVA *post hoc* test.

For metabarcoding, alpha-diversity indices (number of observed OTUs, Chao1 and Shannon indices) were calculated in the R environment by using the Phyloseq 1.30.0 package and its dependencies (McMurdie and Holmes, 2013). For the three different experiments, samples' library sizes (number of reads) were rarefied to an even depth (smallest number of reads per sample) to be normalized. Permutational multivariate analysis of variances (PERMANOVA) based on Jaccard and Bray-Curtis distance matrices were carried out by using 9,999 permutations to detect significant effects in the bacterial community analyzed.

For DNA extraction efficacy estimation, a ratio $\frac{Observed\ OTUs\ index}{Chao1\ index}$ was calculated.

3. Results

3.1. Comparison of DNA extraction methods used to characterize CSS bacterial communities

3.1.1. Bacteria counts and quantification of DNA extracted

Bacterial communities were collected from a vacuum-packed CSS sample stored at 4 °C. Total DNA was extracted in triplicate from 5 mL of smoked salmon tissue homogenate (SSTH), corresponding to 0.5 g CSS (1/10 dilution). The total viable counts of the CSS sample were 5.36 ± 0.08 Log CFU/g. DNA concentrations ranged between 7.3 ± 1.4 ng/μL to 129.5 ± 21.9 ng/μL (Table 1) depending on the DNA extraction method.

Table 1
DNA concentration and overall 16S rDNA sequencing output parameters of samples from cold-smoked salmon using different DNA extraction methods.

	Maxwell	QPMF	Q_2B_2	ZFS
DNA (ng/μL)	129.5 ± 21.9^{a}	24.75 ± 3.96^{b}	80.6 ± 55.9^{ab}	7.32 ± 1.41^{b}
Observed OTUs	32.3 ± 4.51^{ab}	26 ± 2.65^{a}	33.3 ± 3.21^{ab}	45.7 ± 10.5^{b}
Chao1	34.4 ± 6.75^{a}	28.8 ± 2.41^{a}	40.2 ± 5.69^{a}	46.8 ± 11.5^{a}
Observed OTUs/ Chao1	0.95 ± 0.05	0.90 ± 0.03	0.84 ± 0.08	0.98 ± 0.02
Shannon index	1.81 ± 0.062^{a}	1.57 ± 0.106^{b}	1.17 ± 0.022^{c}	2.40 ± 0.104^{d}

OTU: Operational Taxonomic Unit. The DNA extraction methods were Maxwell® 16 FFS DNA Purification kit (Maxwell), Qiagen DNeasy PowerFood Microbial kit (QPFM), Qiagen QIAamp BiOstic Bacteremia kit (Q_2B_2), Zymo Quick-DNA Fecal/Soil Microbe Miniprep Kit (ZFS).
^{a,b,c,d} Means sharing the same letter superscript do not differ significantly, $P <$ 0.05.

The higher DNA concentrations were observed using the Maxwell method. Significant differences in DNA recovery were observed ($P = 0.004$). This observation was mostly explained by differences between the ZFS and Maxwell kits and the QPMF and Maxwell kits.

3.1.2. Effect of DNA extraction methods on CSS bacterial diversity

DNA extracted by the four methods were used as the template for the V3–V4 regions of the 16S rRNA gene sequencing. A total of 544,921 reads passed filters applied through the FROGS pipeline workflow with an average of 45,411 reads per sample ± 19,869 reads. Samples were rarefied to an even depth of 10,521 reads.

Observed richness (number of observed OTUs), estimated richness (Chao1) and evenness diversity index (Shannon) were calculated for each DNA extraction method (Table 1). The DNA extraction methods had a significant effect on observed OTUs richness ($P = 0.02$). The main difference was due to the ZFS and QPMF methods with 45.7 ± 10.5 observed OTUs for ZFS and 26 ± 2.6 for QPMF. There was no impact on the Chao1 index ($P = 0.07$). DNA extraction influenced the community evenness ($P < 0.0001$). Based on the Shannon index, the ZFS method provided the most diverse bacterial community. Based on the observed OTUs/Chao1 ratio, the Maxwell, QPMF and ZFS methods showed a high OTUs recovery rate.

To evaluate richness and diversity differences, metabarcoding data of each sample were examined at the genus level (Fig. 1). A total of 90 OTUs, which were agglomerated in 61 genera, were identified. Dominant genera were *Firmicutes Staphylococcus* (28.45% ± 18.5), *Brochothrix* (24.73% ± 9.68), *Carnobacterium* (0.54% ± 0.51), β-*Proteobacteria Photobacterium* (21.28% ± 29.88), *Vibrio* (10.02% ± 6.18), *Serratia* (3.15% ± 2.79), γ-*Proteobacteria Psychrobacter* (1.66% ± 1.07) and α-*Proteobacteria Sphingomonas* (0.37% ± 0.69). However, in samples extracted using ZFS, other dominant bacteria were observed, such as α-*Proteobacteria Rhizobium* (3.16% ± 5.8), β-*Proteobacteria Delftia* (2.02% ± 4.44) and *Firmicutes Anaerobacillus* (1.57% ± 2.84).

The Bray-Curtis (Fig. 2) and Jaccard Principal Coordinates Analysis (PCoA) (Supplementary Fig. S1) highlighted a potential effect of DNA extraction methods on both richness and evenness. These observations

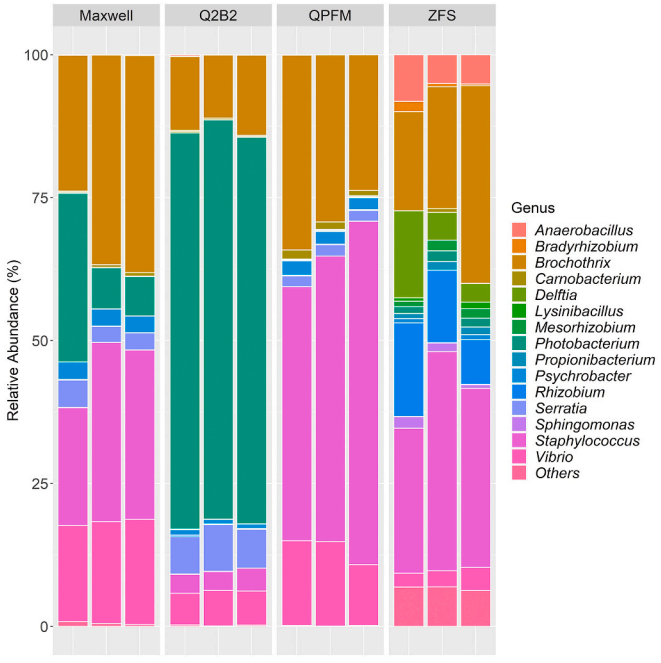

Fig. 1. Relative abundance of bacterial genera in cold-smoked salmon sample extracted with four different DNA extraction methods. Taxa present on average in all samples at a threshold ≥ 0.5% or having a 90th percentile ≥ 0.5% are individually represented. In other cases, taxa are grouped and labeled "Others".

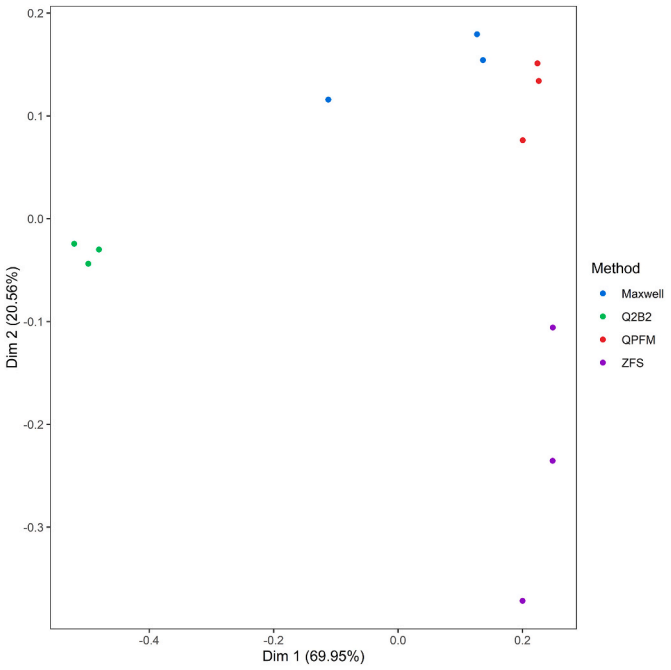

Fig. 2. Bray-Curtis Principal Coordinates Analysis plot of CSS samples extracted with four different DNA extraction methods.

were confirmed by PERMANOVA analysis on both Jaccard ($P < 0.0001$) and Bray-Curtis distance matrices ($P < 0.0001$) explaining respectively 62.6% and 88.7% of the microbiota differences. The ZFS method was removed from our comparison and the three other methods were kept to evaluate their impact on surface bacterial communities.

3.2. Comparison of DNA extraction methods used to characterize surface bacterial communities

3.2.1. Bacteria counts and quantification of DNA extracted

Surface bacterial communities were collected by swabbing SS coupons after or before C&D. Fifteen swabs samples were pooled, resulting in a 60 mL surface sample corresponding to a 750 cm^2 SS surface. Total DNA was extracted in triplicate from 2 mL of SS surface samples using the different DNA extraction methods (18 DNA extracted samples). Total CFU counts varied from 3.29 ± 0.21 Log CFU/cm^2 to 4.39 Log CFU/cm^2 ± 0.04 after and before C&D respectively. C&D had a significant effect on total viable bacteria counts ($P < 0.0001$). DNA concentrations ranged between 0.35 ± 0.04 ng/μL to 1.06 ± 0.62 ng/μL before C&D and between 0.05 ± 0.02 ng/μL to 0.150 ± 0.003 ng/μL after C&D (Table 2). The results showed that the range of DNA concentrations before C&D were ten times higher than after C&D. This highlighted a significant effect of C&D on DNA concentrations ($P = 0.0004$). No differences in the DNA concentration extracted using the three extraction methods were observed ($P = 0.059$).

3.2.2. Effect of DNA extraction methods on the 16S rDNA analysis of surface bacterial communities

DNA extracted by the three remaining methods were used as the template for the V3–V4 regions of the 16S rRNA gene sequencing. A total of 1,495,580 reads passed filters applied through the FROGS pipeline workflow with an average of 83,088 reads/sample ± 33,177 reads. Samples were rarefied to an even depth of 34,345 reads.

Observed OTUs, Shannon and Chao1 alpha-diversity indices were calculated for each DNA extraction method and cleaning conditions (Table 2).

DNA extraction methods had no effect on the population richness: observed OTUs ($P = 0.15$) and Chao1 index ($P = 0.84$) and no influence

Table 2
DNA concentration and overall 16S rDNA sequencing output parameters of samples from processing environment before and after cleaning and disinfection step using different DNA extraction methods.

	Before C&D			After C&D		
	Maxwell	QPFM	Q_2B_2	Maxwell	QPFM	Q_2B_2
DNA (ng/µL)	0.35 ± 0.04^{ab}	0.61 ± 0.13^{ab}	1.06 ± 0.62^{b}	0.07 ± 0.01^{a}	0.05 ± 0.02^{a}	0.150 ± 0.003^{a}
Observed OTUs	148 ± 3.61^{ab}	135.3 ± 9.29^{a}	143 ± 8.50^{ab}	154 ± 1.53^{b}	152 ± 6.11^{b}	155 ± 2.52^{b}
Chao1	153 ± 10.1^{a}	146 ± 15.7^{a}	153 ± 11.1^{a}	157 ± 0.525^{a}	159 ± 6.37^{a}	159 ± 2.56^{a}
Observed OTUs/Chao1	0.97 ± 0.04	0.93 ± 0.04	0.94 ± 0.01	0.98 ± 0.01	0.96 ± 0.02	0.98 ± 0.00
Shannon index	2.99 ± 0.02^{ab}	3.07 ± 0.11^{bc}	2.89 ± 0.06^{a}	3.08 ± 0.03^{bc}	3.12 ± 0.036^{bc}	3.21 ± 0.04^{c}

OTU: Operational Taxonomic Unit. C&D: cleaning and disinfection step. The DNA extraction methods were Maxwell® 16 FFS DNA Purification kit (Maxwell), Qiagen DNeasy PowerFood Microbial kit (QPFM), Qiagen QIAamp BiOstic Bacteremia kit (Q2B2), Zymo Quick-DNA Fecal/Soil Microbe Miniprep Kit (ZFS).
^{a,b,c} Means sharing the same letter superscript do not differ significantly, $P < 0.05$.

on Shannon diversity ($P = 0.2$). These methods had a high observed OTUs/Chao1 ratio and seemed to share a high OTUs recovery rate. Indeed, no differences on richness and evenness were observed between the methods. Among the different methods, C&D had an effect on both observed OTUs richness and evenness ($P = 0.0001$ and $P = 0.002$ respectively) but no effect on the Chao1 index (P = 0.11).

Overall, samples after C&D appeared to be richer (with an average of 154 observed OTUs ± 3.63) and more diverse (Shannon index average of 3.14 ± 0.06) than before C&D.

Interestingly, no cleaning-linked differences on observed OTUs were identified on samples extracted by Maxwell (148 OTUs ± 3.61 before C&D and 154 OTUs ± 1.53 after C&D, P = 0.85) and Q_2B_2 (143 OTUs ± 8.5 before C&D and 155 OTUs ± 2.52 after C&D, P = 0.22). Furthermore, no cleaning-linked differences on the Shannon index were observed on samples extracted by Maxwell (2.99 ± 0.02 before C&D and 3.08 ± 0.03 after C&D, P = 0.40) and QPMF (3.07 ± 0.11 before C&D and 3.12 ± 0.036 after C&D, P = 0.94).

To evaluate differences in the bacterial communities, the metabarcoding data of each sample were examined at the genus level (Fig. 3). A total of 167 OTUs, which were agglomerated into 49 genera, were identified. The most abundant genera were *Flavobacteriaceae Chryseobacterium* (37.02% ± 5.8), *Flavobacterium* (2.72% ± 1.23), *γ-Proteobacteria Acinetobacter* (40.77% ± 3.82) and *Pseudomonas* (9.2% ± 4.1). All of these genera were found before and after sanitization procedures. Both Bray-Curtis (Fig. 4) and Jaccard (Supplementary Fig. S2) PCoA showed a clear separation between samples according to the C&D.

PERMANOVA analysis based on Bray-Curtis and Jaccard dissimilarity index showed that cleaning procedures influenced the bacterial community ($P < 0.0001$) and explained 75.9% and 33.5% of the microbiota differences. No effect from DNA extraction methods was observed based on Bray-Curtis ($P = 0.76$) and Jaccard dissimilarity index ($P = 0.16$). DNA extraction methods explained only respectively 5.9% and 15.7% of the microbiota differences.

3.3. Effect of surface bacterial sampling method on 16S rDNA metabarcoding alpha-diversity data analysis

SS and TPU coupons (30 × 25 cm, 750 cm^2) were installed in the FPP near food contact surfaces and under a conveyor between the salting step and the filleting machine for four weeks before surface sampling.

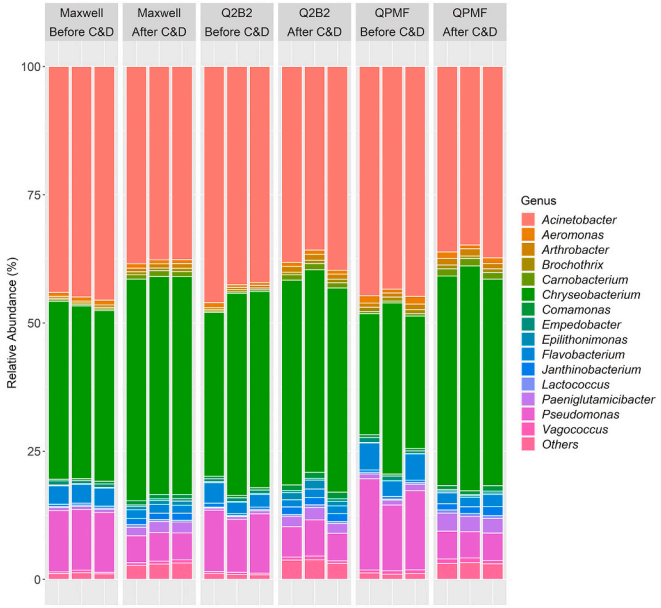

Fig. 3. Relative abundance of bacterial genera on stainless steel coupon placed within the food processing plant extracted by three different DNA extraction method before and after cleaning and disinfection. Taxa present on average in all samples at a threshold ≥ 0.5% or having a 90th percentile ≥ 0.5% are individually represented. In other cases, taxa are grouped and labeled "Others".

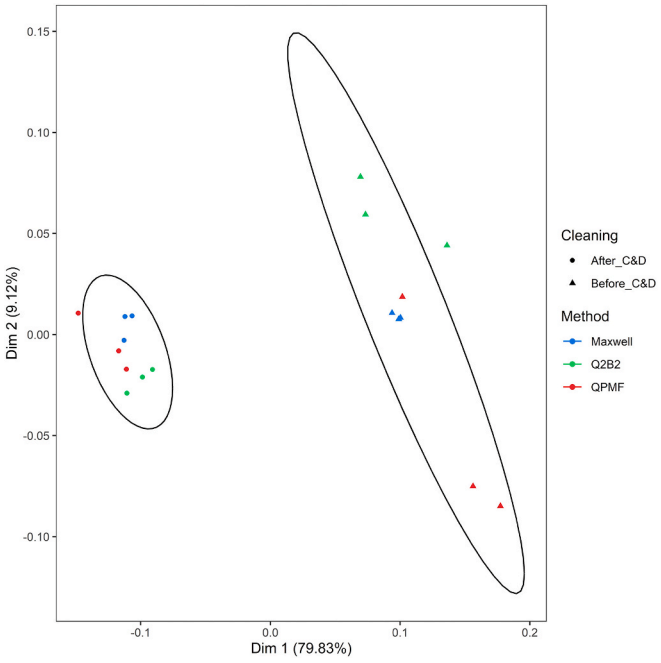

Fig. 4. Bray-Curtis Principal Coordinates Analysis plot of environment samples according to cleaning and disinfection procedures and DNA extraction method.

The SS and TPU coupons then were sampled by swabbing and sponging each day before and after C&D over five consecutive days. This generated 40 samples which were analyzed for total viable counts and 16S rDNA metabarcoding. Enumerations were variable among the sampling days and methods. (Table 3). The sampling methods (swabbing or sponging), the surface (SS or TPU) and the C&D step (before, after) had a significant effect on the viable counts with no interaction effect ($P < 0.0001$, $P = 0.04$, $P = 0.04$ respectively). Indeed, on both SS and TPU, before C&D, sponging showed a higher CFU count (4.68 ± 0.84 Log

Table 3
CFU counts, DNA concentrations and overall 16S rDNA sequencing output parameters of samples from processing environment before after cleaning and disinfection step using swabbing or sponging and QPFM DNA extraction methods".

	Before C&D				After C&D			
	Stainless Steel		TPU		Stainless Steel		TPU	
	Swab	Sponge	Swab	Sponge	Swab	Sponge	Swab	Sponge
Log(CFU)/cm^2	2.52 ± 0.85^{ab}	4.68 ± 0.84^{bc}	3.07 ± 0.49^{abc}	5.58 ± 0.72^{c}	1.68 ± 2.06^{a}	3.45 ± 1.70^{abc}	2.36 ± 1.79^{ab}	4.84 ± 0.55^{bc}
DNA (ng/μL)	0.39 ± 0.40^{ab}	3.32 ± 1.83^{bc}	1.52 ± 1.05^{ab}	5.97 ± 7.70^{c}	0.30 ± 0.55^{ab}	0.11 ± 0.09^{a}	0.13 ± 0.22^{ab}	0.40 ± 0.36^{ab}
Observed OTUs	111.7 ± 13.8^{a}	159.9 ± 13.8^{b}	109 ± 8.37^{a}	135 ± 19.6^{c}	100 ± 24.6^{a}	141 ± 22^{bc}	111 ± 16.9^{a}	138 ± 16.9^{c}
Chao1	121 ± 24.8^{a}	190 ± 18.5^{b}	120 ± 13^{a}	159 ± 26^{c}	108 ± 28.4^{a}	159 ± 26.7^{c}	126 ± 23.7^{a}	161 ± 19.7^{c}
Shannon index	2.67 ± 0.24^{a}	2.91 ± 0.29^{ab}	2.64 ± 0.33^{a}	3.09 ± 0.17^{bc}	2.65 ± 0.43^{a}	3.11 ± 0.47^{bc}	2.69 ± 0.34^{a}	3.32 ± 0.15^{c}

OTU: Operational Taxonomic Unit. C&D: cleaning and disinfection step. QPFM = Qiagen DNeasy PowerFood Microbial kit.
^{a,b,c,} Means sharing the same letter superscript do not differ significantly, $P < 0.05$.

CFU/cm^2 on SS and 5.58 ± 0.72 Log CFU/cm^2 on TPU) than swabbing (2.52 ± 0.85 Log CFU/cm^2 on SS and 3.07 ± 0.49 Log CFU/cm^2 on TPU).

The same trend was observed after C&D: 3.45 ± 1.70 Log CFU/cm^2 on SS and 4.84 ± 0.55 Log CFU/cm^2 on TPU for sponging and 1.68 ± 2.06 Log CFU/cm^2 on SS and 2.36 ± 1.79 Log CFU/cm^2 on TPU for swabbing. Whatever the conditions, swabbing provided a lower colony recovery.

In addition, whatever the sampling method or the cleaning step, more CFU were enumerated on TPU samples ($P = 0.04$), with an average of 3.96 ± 1.64 Log CFU/cm^2, than on SS samples, whose average was of 3.08 ± 1.76 Log CFU/cm^2.

To avoid a DNA extraction bias, the extraction was performed with a single method. Extraction using QPFM were performed in triplicate. One hundred and twenty samples were sequenced. A total of 5,483,544 reads passed filters applied through the FROGS pipeline workflow with an average of 35,648 reads/sample ± 20,209 reads. Due to a low number of reads, five samples were removed. The relative abundance at the genus level was represented (Fig. 5). The other 115 samples were rarefied to an even depth of 8,455 reads. For each sample, richness and evenness represented by observed OTUs, Chao1 and Shannon indexes were evaluated (Table 3). Richness was impacted significantly by the sampling method and interactions between C&D procedures/surfaces and sampling methods/surfaces ($P < 0.0001$, $P = 0.011$, $P = 0.007$ respectively for observed OTUs and $P < 0.0001$, $P = 0.003$, $P = 0.007$ respectively for Chao1). C&D have an effect on Chao1 richness ($P = 0.04$) but not on observed OTUs index ($P = 0.06$). The surface material type (SS or TPU) did not modify the richness ($P = 0.17$ and $P = 0.52$ respectively for observed OTUs and Chao1 index). The Chao1 richness was higher within the population recovered before C&D and using the sponge as the sampling method. The Shannon index (Table 3) showed that diversity was dependent on the sampling method and C&D ($P < 0.0001$ and $P = 0.04$ respectively). Bacterial communities appeared to be richer and more diverse when sampled by sponging. Interestingly, no differences on richness and evenness were observed on swab samples ($P > 0.05$).

A total of 279 OTUs, which were agglomerated into 101 genera, were identified. The dominant populations in each condition of the study were γ-*Proteobacteria Acinetobacter* (55.23% ± 18.71), *Pseudomonas* (13.63% ± 8.61), *Aeromonas* (5.56% ± 3.73), *Shewanella* (4.52% ± 5.46) and β-*Proteobacteria Comamonas* (4.09% ± 4.41). PERMANOVA analysis based on Bray-Curtis and Jaccard dissimilarity index showed that the cleaning procedures, surface and sampling method influenced the bacterial community ($P < 0.0001$). Based on the Bray-Curtis dissimilarity index, cleaning procedures, sampling surfaces and sampling devices explained respectively 6.13%, 9.85% and 10.51% of microbiota differences. Based on the Jaccard dissimilarity index, cleaning procedures, sampling surfaces and sampling devices explained respectively 5.65%, 3.36% and 8.9% of microbiota differences.

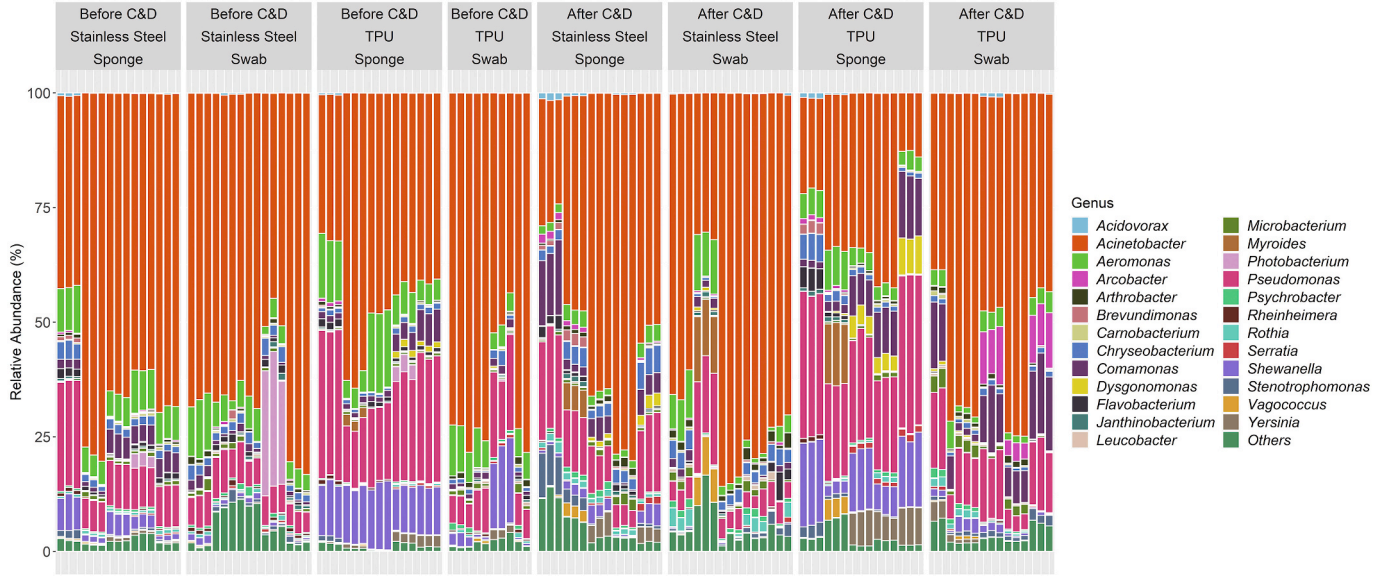

Fig. 5. Relative abundance of bacterial genera on stainless steel and tri-polyurethane coupons placed within the food processing plant. Sampling was performed by swabbing and sponging, before and after cleaning and disinfection procedures. Only 115 samples are represented: 5 outliers were removed for statistical purposes. Taxa present on average in all samples at a threshold ≥ 0.5% or having a 90th percentile ≥ 0.5% are individually represented. In other cases, taxa are grouped and labeled "Others".

4. Discussion

This study sought to evaluate the impact of DNA extraction and surface sampling methods on 16S rDNA metabarcoding analysis results. The efficiency of four available commercial kits with different lytic and purification technologies were compared to extract bacterial DNA from cold-smoked salmon and SS coupons installed in a CSS processing plant. In addition, the efficiency of two sampling devices were evaluated on SS and TPU coupons installed in the same FPP.

β-*Proteobacteria Photobacterium* and *Serratia* and the *Firmicutes Brochothrix*, *Carnobacterium,* and *Staphylococcus* were identified in this study as the dominant genera in the CSS bacterial community. With the exception of *Staphylococcus,* these genera are known as CSS spoilers. *Staphylococcus* is more rarely described as a CSS dominant bacterium. This result confirms already published data on CSS microbiota studied by viable and cultivable methods (Leroi et al., 1998; Løvdal, 2015; Olofsson et al., 2007). Because of its halophilic property, the salt additive used in the CSS process could be a source and a root cause for *Staphylococcus* predominance (Marino et al., 2017). The *Psychrobacter* genus is widely represented in our study. *Psychrobacter* are psychrothrophic and halotolerant bacteria which already have been isolated from fresh salmon fillets and the processing plant involved (Møretrø et al., 2016). Raw salmon might be a source for *Psychrobacter* residence in FPPs (Møretrø and Langsrud, 2017). *Sphingomonas* was identified as a low abundant bacterium. This genus was previously identified as a fish pathogen and could be part of the aquacultured rainbow trout intestinal microbiota (Pękala-Safińska, 2018; White et al., 1996; Wong et al., 2013).

The dominant surface microbiota identified was mainly composed by *Flavobacteriaceae Flavobacterium*, *Chryseobacterium*, γ-*Proteobacteria Acinetobacter*, *Pseudomonas*, *Aeromonas, Shewanella* and β-*Proteobacteria Comamonas*. *Acinetobacter* and *Pseudomonas* already have been described to dominate the bacterial population in processing plants of many foods such as vegetables, meat, poultry, dairy products, seafood and seafood products (Møretrø and Langsrud, 2017). Both *Acinetobacter* and *Pseudomonas* can be responsible for CSS spoilage (Langsrud et al., 2016; Møretrø et al., 2016) and are able to persist in these environments due to their biofilm production and psychrotrophic properties (Møretrø and Langsrud, 2017). *Flavobacterium* was described to promote *Listeria monocytogenes* growth in biofilms (Bremer et al., 2001). *Chryseobacterium* have been highlighted in a previous study on Atlantic salmon fillet microbiota. This genus was found in the dominant population (Wang et al., 2019) and could be brought into the FPP by raw material. In addition, *Chryseobacterium* was also identified in the microbial composition of drain water and drain biofilms (Dzieciol et al., 2016). *Shewanella* and *Aeromonas* have been described as fish and seafood product spoilers (Gram and Dalgaard, 2002; Gram and Huss, 1996). These genera have been characterized within the residential microbiota of fish and seafood FPP (Møretrø and Langsrud, 2017).

The first part of this work was dedicated to CSS samples. Significant differences were observed in the DNA concentrations obtained using four different DNA extraction methods. The four methods were used on the same sample, highlighting the different efficiency of each method to extract DNA from CSS. Three of the four methods tested were spin column method-based. These methods shared the same lysis principle, unlike the Maxwell kit which is based on paramagnetic beads system technology. Moreover, the Maxwell kit recovered the highest DNA quantity. The lysis and purification principles of the different methods could explain the difference in the DNA extraction efficiency. Keisam et al. (2016) tested eight different DNA extraction methods with different lysis principles on several fermented products (milk, fish, bamboo shoot, soybean). In this study, the authors observed that the method used had a strong effect on the DNA concentration. Their observation was mostly explained by the different foods tested. Even when the bacteria concentration was homogenous (between 7.54 ± 0.23 to 10.79 ± 0.10 Log of the 16S rRNA gene copies/g of food), strong differences were observed. Communities were different, dominated by Gram-positive bacteria in bamboo shoot and fish samples and Gram-negative bacteria in milk and soybean. Gram-negative bacteria are easier to extract, which could explain this difference. In addition, the authors did not discuss sample chemical composition, which could lead to different DNA extraction yields or community assessments (lipids, PCR inhibitors). Hart et al. (2015) compared methods used to extract DNA from the feces of different animal species. These authors tested five different DNA extraction methods, including spin columns types such as the Mobio and Qiagen kits, as well as isopropanol precipitation, and found a statistically significant difference. These results showed that the Qiagen and Mobio methods lead to consistent DNA concentrations.

In this study, the DNA methods used to extract DNA from CSS samples had an effect on the alpha-diversity observed OTUs index but not on the Chao1 index. Chao1 is a species richness estimator (Kim et al., 2017). The difference between the observed OTUs and Chao1 indices, especially the Q_2B_2 method, could be explained by a lower DNA extraction quality or yield. The four methods tested also had an effect on the diversity and relative abundance of the CSS sample microbiota. Keisam et al. (2016) showed that different DNA extraction methods induce a variability in the diversity of fermented food microbiota. In addition, a high DNA concentration did not necessarily lead to the highest community diversity. In our study, we confirmed this observation as ZFS, which seemed to recover the lowest DNA yield, provided the highest bacterial diversity. Regarding our results, the following genera were found only on ZFS samples: *Massilia, Pseudoxanthomonas* and *Deinococcus*. It is likely that we were not able to optimize the ZFS method, most probably due to the low bacterial concentration in the CSS sample, leading to a weak amount of bacterial DNA extracted. Since the above-mentioned genera including *Delftia, Anaerobacillus* and *Rhizobium* were already described as present in molecular biology kits reagents (Salter et al., 2014), we assume that most probably the Taq DNA polymerase during the V3–V4 16S rDNA PCR also could have amplified DNA from reagents or exogenous contamination. This also could explain the high diversity within the ZFS samples. Keisam et al. (2016) suggested that weak diversity in the case of high DNA concentrations also may be explained by an easier access to dominant bacterial DNA, limiting the purification of low abundant taxa. Several studies have demonstrated strong differences in dominant bacteria of human feces microbial communities depending on the DNA extraction and purification methods used (Kennedy et al., 2014; Wesolowska-Andersen et al., 2014). Our data confirmed that DNA extraction methods could influence the analysis of bacterial community profiles.

To compare the three remaining DNA extraction methods on surface samples, bacterial communities were collected on SS coupons installed in the FPP by swabbing before and after C&D. As described with the CSS samples, the method used did not have an effect on the DNA concentration. However, a decrease in DNA concentrations and total viable counts was observed after C&D. Alpha-diversity metrics highlighted that contrary to CSS samples, the DNA extraction methods showed no effect on community richness or evenness. These observations were also confirmed by PERMANOVA analysis. This suggests that the capacity of these methods to extract DNA from surface samples is similar in terms of quality and yield. Cleaning procedures strongly affect the microbiota and alpha-diversity indicators. According to Bray-Curtis and Jaccard PCoA, the cleaning and disinfection procedures have a strong effect on bacterial community relative abundances, and a lighter effect on its richness. Interestingly, the observed OTUs richness and Shannon diversity were higher after cleaning and disinfection. Water flow and friction may have destabilized some biofilms structure and allowed us to capture more bacteria or different taxa. However, C&D had no impact on the number of OTUs of samples extracted by Q_2B_2 and Maxwell, nor on the microbiota evenness on Maxwell and QPMF samples. Total CFU counts varied from 3.29 ± 0.21 Log CFU/cm^2 to 4.39 Log CFU/cm^2 ± 0.04 after and before C&D respectively. Bacterial concentration was impacted by sanitation but not OTUs number. 16S rDNA metabarcoding

targets all bacterial DNA (living cells, dead cells, stressed cells). It is likely, even if water flow may have physically dispatched cells, that we also could have sampled dead or stressed bacteria. QPMF and Q_2B_2 share the same lytic and purification system; however, Maxwell uses paramagnetic particles system technology. Vesty et al. (2017) evaluated the efficiency of different commercial kits (including MoBio PowerSoil® DNA Isolation Kit, QIAamp® DNA Mini Kit, Zymo Bacterial/Fungal DNA Mini Prep™) for DNA extraction from oral human samples. The authors also did not observe any differences on alpha-diversity data or observed microbiota. In contrast, in a study comparing four DNA extraction methods on skin microbiota (Nucleospin® Soil (Macherey Nagel), Nucleospin® Tissue (Macherey Nagel), FastDNA™ SPIN Kit for Soil (MP biomedicals) and DNeasy Blood & Tissue Kit (Qiagen), Boulesnane et al. (2020) found that the richness identified by each method varied. The authors concluded that the differences mainly were due to the co-extraction of PCR inhibitors as well as the method's DNA binding system.

DNA extraction protocols and lysis principles seem to be key factors in metabarcoding analysis. As previously suggest by Hart et al. (2015), our results showed that DNA extraction and standardized experimental protocols in microbiome analysis are critical steps and should be subject to validation procedures. This work showed the clear importance to test the DNA extraction method before 16S rDNA metabarcoding studies to ensure that the method fits the intended purpose. By studying the impact of DNA extraction methods on fecal samples from five different species (zebrafish, mouse, cat, dog and horse), Hart et al. (2015) highlighted host species differences on DNA yield and NGS output. As we could see, DNA extraction methods are matrix dependent. Most of the differences were observed on CSS samples and not on surface samples. Even if surface samples can contain disinfectant traces, food matrices are more complex to process. QPMF and Maxwell used on product samples shared a high OTUs recovery rate and a high evenness. Based on this work and our observations, Maxwell and QPMF seemed to be the best DNA extraction methods in the context of our study. Because Maxwell is an automatic approach and is equipment dependent, QPMF was chosen for further analysis.

Attachment strength, exopolysaccharides, and biofilm production are key factors that should be considered carefully when recovering surface bacteria. A critical stage in the analysis of surface microbiota is the choice of the sampling method. In this study, we evaluated cell recovery by swabbing and sponging methods used to sample SS and TPU surfaces. Viable cell counts showed that sponging provided a better recovery strength on SS and TPU. These observations could be explained by the pressure variation on the device and its sampling surface (Yamaguchi et al., 2003). In addition, a swab tip would be saturated faster than a device such as a sponge and could lead to a reduced recovery capacity (Pérez-Rodríguez et al., 2008). Friction and pressure on devices should enhance and facilitate the bacterial transfer from the surface to the sampling system. Even when the sampling is made by a single operator, sponges are clearly easier devices to press on surfaces and to facilitate the transfer of cells (Knobben et al., 2007; Pérez-Rodríguez et al., 2008). We observed that whatever the sampling device, more CFU could be recovered on TPU than SS coupon surfaces, suggesting a higher bacterial surface population on TPU. Firmesse et al. (2012), when studying bacterial detachment in a delicatessen environment, described no significant differences between cultivable and viable but non-cultivable cells recovered from PVC or SS. Bacterial communities and biofilms should be more difficult to remove from polymer surfaces. Moreover, a porous surface such as TPU could facilitate cell attachment and biofilm formation (Midelet and Carpentier, 2002). This could explain the differences in bacterial surface populations from SS and TPU surfaces observed in this work.

16S rDNA metabarcoding data, and especially alpha-diversity evaluation of bacterial communities, gave another perspective on sampling method effects. The bacterial concentration on FPP surfaces, especially after C&D, is very low. In our study, total counts varied from 1.68 ± 2.06 to 5.58 ± 0.72 Log CFU/cm^2. According to several standards, C&D procedures can decrease the level of contamination to 2.5 CFU/cm^2 (Møretrø and Langsrud, 2017). Coupons were placed under food contact surface samples but were easy to clean. Surfaces were visually soiled before C&D, cleaned after procedures and mimicked well the FPP surfaces. A common challenge in the analysis of low bacterial numbers is to extract DNA and to dispose of enough material for sequencing purposes. Indeed, a maximum of 5.97 ± 7.70 ng/μL of DNA were extracted in total and a maximum of 0.40 ± 0.36 ng/μL were extracted after C&D. The limit for sequencing application on a low bacterial environment has been evaluated between 10^3 and 10^5 CFU/mL in drinking water samples (Pinto et al., 2012). However, in their study on detection limits, DNA extraction and primer choice influences on drinking water bacterial communities, Brandt and Albertsen (2018), were able to sequence samples at a limit of 10^1 CFU/mL. This was achieved by spiking samples with different concentrations of an *E. coli* culture. However, background noise OTUs were identified at this low concentration. Due to the limit of the method, we had to remove five samples from our study.

To our knowledge, no previous publications reporting a sampling effect on the alpha-diversity indices of bacterial surface communities evaluated from 16S rDNA metabarcoding data are available. We observed that both richness and evenness were impacted by sampling devices and cleaning and disinfection. However, the surface material (SS or TPU) had no influence on richness and evenness. The analysis of sponged samples showed an higher bacterial community richness and evenness, suggesting that this method was more efficient than swabbing in recovering surface bacteria. The microbiota composition was affected by cleaning and disinfection, surface material and sampling devices. The dominant populations were not affected; however, the subdominant population may have been sampled. As for DNA extraction comparison on surface samples, we did not assess the viability of recovered cells. Indeed, using HTS after C&D cannot determine whether or not the bacteria are viable. 16S rDNA amplicons allow the targeting of one or more microorganisms within samples. However, the induced PCR reaction will target all free DNA in the reaction medium. The DNA of dead cells is thus amplified and identified (Klein et al., 2012). One way to counteract this bias is the use of a DNA intercalating molecule: propidium monoazide (PMA). In their study to characterize the total and the viable bacterial and fungi populations in the international space station, Checinska Sielaff et al. (2019) processed samples with PMA. Their results highlighted no significant differences on microbiome richness and diversity, suggesting that the 16S rDNA amplified was extracted from a viable population. Moreover, an average of 45% of the microorganisms were cultured suggesting that an average of 55% of the microorganims were in a VBNC state. The use of PMA in our study could be a future relevant approach to assess C&D surviving populations.

5. Conclusion

Due to their important effects on food product quality and safety, residential bacteria in food processing plants are an important concern. Thanks to high-throughput sequencing technologies and the development of 16S rDNA metabarcoding approaches, it is now possible to estimate the taxonomic composition of bacteria communities without needing to use cell cultures. However, these techniques require strict standardization to avoid downstream analytical biases. Although similar comparative research on the impact of different DNA extraction methods has been performed on other ecological niches, this is the first investigation on cold-smoked salmon product microbiota and the product's processing plants using Illumina® MiSeq amplicon sequencing. The bias generated due to the differential recovery of OTUs by different DNA extraction methods is demonstrated here for CSS bacterial communities but not for surface microbiota. We report that both richness and evenness were impacted by sampling devices and cleaning and disinfection. Higher bacterial community richness and evenness were observed when sponge was used as the sampling method.

The Maxwell and QPMF DNA extraction methods and the sponge device appeared to be efficient and reliable methods to study the CSS and surface microbiota. This study highlighted the importance of sample preparation and laboratory practices on the results of 16S rDNA metabarcoding studies. The sequencing platforms and bioinformatic pipelines also could be critical choices for these kinds of analyses. This study only represents a survey of one CSS processing plant and provides basic insights on the surface bacterial community profile. Future investigations would be necessary to investigate potential contamination/cross contamination to CSS products deriving from the processing plant environment. Surface bacterial community monitoring by 16 rDNA metabarcoding may become a valuable approach in food processing plants to identify critical control points, thereby allowing the improvement of process and sanitation management.

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Availability of data and material

The data presented in this study are openly available in Data INRAE at https://doi.org/10.15454/34LLGN.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2020.103705.

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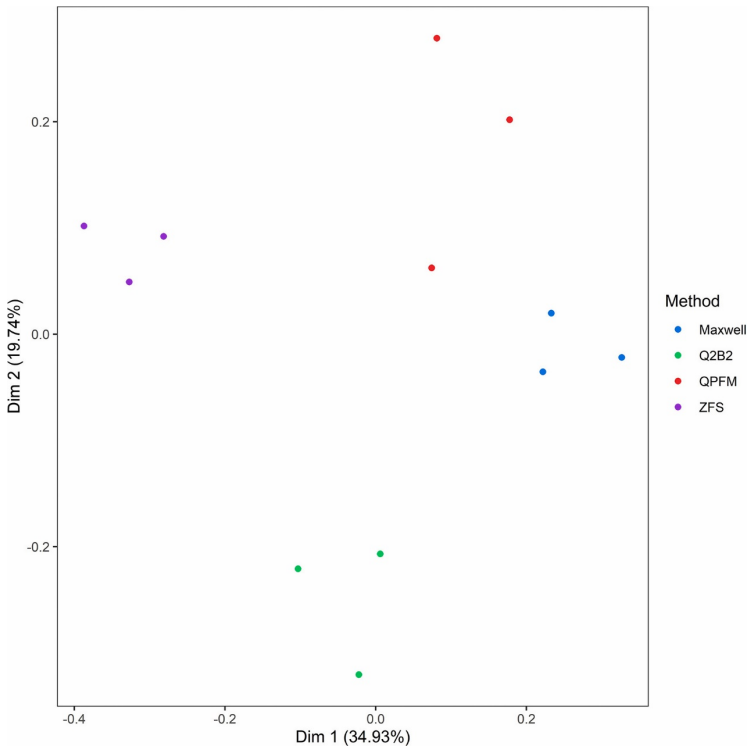
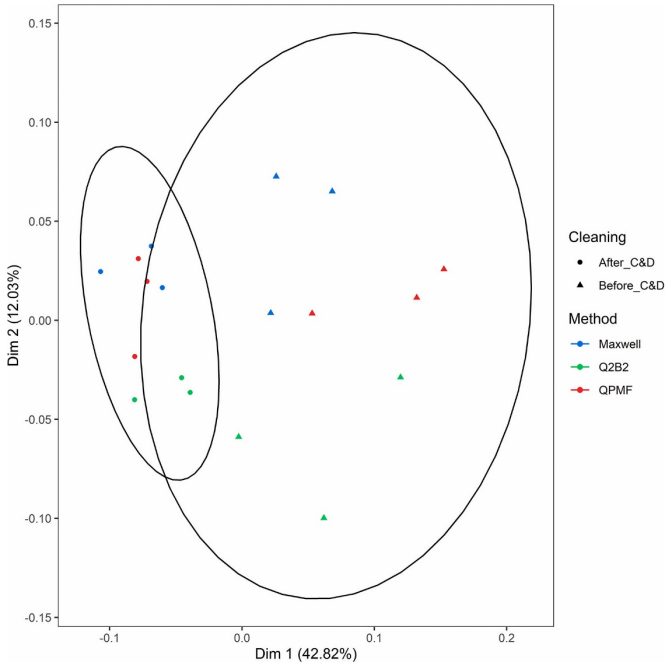
Appendix A. Supplementary data



Supplementary Figure S1. Jaccard Principal Coordinates Analysis plot of CSS samples extracted with 4 different DNA extraction methods.



Supplementary Figure S2. Jaccard Principal Coordinates Analysis plot of environment samples according to cleaning and disinfection procedures and DNA extraction method.

Annexe 2 : Characterization of Bacterial Communities of Cold-Smoked Salmon during Storage

foods

MDPI

Article

Characterization of Bacterial Communities of Cold-Smoked Salmon during Storage

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Abstract: Cold-smoked salmon is a widely consumed ready-to-eat seafood product that is a fragile commodity with a long shelf-life. The microbial ecology of cold-smoked salmon during its shelf-life is well known. However, to our knowledge, no study on the microbial ecology of cold-smoked salmon using next-generation sequencing has yet been undertaken. In this study, cold-smoked salmon microbiotas were investigated using a polyphasic approach composed of cultivable methods, V3—V4 16S rRNA gene metabarcoding and chemical analyses. Forty-five cold-smoked salmon products processed in three different factories were analyzed. The metabarcoding approach highlighted 12 dominant genera previously reported as fish spoilers: Firmicutes *Staphylococcus, Carnobacterium, Lactobacillus*, β-Proteobacteria *Photobacterium, Vibrio, Aliivibrio, Salinivibrio, Enterobacteriaceae Serratia, Pantoea*, γ-Proteobacteria *Psychrobacter, Shewanella* and *Pseudomonas*. Specific operational taxonomic units were identified during the 28-day storage study period. Operational taxonomic units specific to the processing environment were also identified. Although the 45 cold-smoked salmon products shared a core microbiota, a processing plant signature was found. This suggest that the bacterial communities of cold-smoked salmon products are impacted by the processing environment, and this environment could have a negative effect on product quality. The use of a polyphasic approach for seafood products and food processing environments could provide better insights into residential bacteria dynamics and their impact on food safety and quality.

Keywords: seafood products; cold-smoked salmon; processing plant; bacteria; metabarcoding; microbiota; spoilage

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1. Introduction

With 175,000 tons produced in the European Union in 2019, cold-smoked salmon (CSS) is a leading fish product with an important trade value (€2.77 billion) [1,2]. CSS is a lightly preserved product with no thermic treatment and is mainly consumed as a ready-to-eat (RTE) food. Due to a large number of intrinsic and extrinsic factors, such as pH, water activity (a_w), temperature, environmental origins and processing practices, such commodities are highly fragile [3,4]. Salting and smoking are mandatory steps in CSS processing to decrease foodborne pathogens and spoilage risks [5]. As described by Leroi et al. (2000), the purpose of salting and smoking is to decrease the a_w through dehydration [6]. The chloride ions from salt additives are also toxic for some microorganisms, and the phenolic compounds produced during the smoking step have a bacteriostatic effect. Smoking is furthermore used to bring out specific tastes and aromas [7].

The CSS bacterial community has been widely studied in the scientific literature aiming to describe spoilage and pathogenic microbiota [5]. Gram-negative bacteria such as

Shewanella putrefaciens, *Aeromonas* spp. and marine *Vibrionaceae Photobacterium phosphoreum* have been described as dominating CSS microbiota in the early stages of storage [7].

Gram-positive lactic acid bacteria (LAB: *Lactobacillus*, *Carnobacterium maltaromaticum*) seem to dominate CSS microbiota at the end of the product's shelf-life. Paludan-Müller et al. (1998) reported a high number of LAB (10^7–10^8 CFU/g). Gram-negative psychrotrophic bacteria *Enterobacteriaceae Serratia liquefaciens* were also reported in some cases to co-dominate the microbiota at the end of the shelf-life [5,8,9]. In addition, *Brochothrix thermosphacta* has already been described as dominating CSS microbiota [10]. As an RTE food product, CSS are often faced with the foodborne pathogenic bacteria *Listeria monocytogenes* [5].

The majority of the studies mentioned were based on culturable approaches. Traditional methods can be time-consuming and lead to technical biases (viable but non-culturable cells, non-specific media and culture conditions) [11]. Due to the challenging storage conditions of a product like CSS (temperature, phenolic compounds due to the smoking step, salt), culturable approaches might be insufficient for studying the entire CSS bacterial community. Culture-independent methods such as fingerprinting (Denaturing Gradient Gel Electrophoresis, Temperature Gradient Gel Electrophoresis) are DNA-based methods which offer tools to monitor the bacterial community on food products and food-associated microbial ecosystems [12–14]. More recently, next-generation sequencing (NGS) has offered new ways to explore food microbial ecology [15]. Bacterial diversity can now be assessed through high throughput sequencing approaches which facilitate the identification of microbes and the relative abundance of taxa for a high number of samples in a single analysis [16].

A few studies have sought to assess the CSS bacterial community using DNA-based methods [11,17,18]. Although NGS was previously used to determine contamination of fresh salmon filets, to our knowledge no study of the evolution of the CSS microbial ecology during shelf-life has used this type of approach [19,20]. Yet, NGS could provide an increasingly deeper insight into the microbial diversity of seafood and seafood products [21].

This study used 16S rRNA gene metabarcoding to assess the evolution of bacteria on 45 CSS products from three different factories that were stored for 28 days at two different temperatures (4 °C first week, 8 °C remaining weeks).

A polyphasic approach was implemented in this study; culture-dependent and independent methods associated with chemical analyses were used.

2. Materials and Methods

2.1. Cold-Smoked Salmon Sampling

Forty-five vacuum-packed CSS, originating from nine different batches and three different French processing factories (referred to henceforth as A, B and C) with a similar use-by date, were collected from local supermarkets. The CSS packs were stored for seven days at 4 °C then 21 days at 8 °C as described by Chaillou et al. (2015), in accordance with the French food aging test standard AFNOR NF V01-003 [22,23]. Details on the samples are summarized in Table 1.

Table 1. Cold-smoked salmon samples description (processing factory, production batch, origin and use-by date).

Factory	Production	Origin/Label	Use-by-Date
A	A1	Scotland	13 March 2019
	A2	Norway	13 March 2019
	A3	Norway	07 March 2019
B	B1	Scotland	09 March 2019
	B2	Norway	09 March 2019
	B3	Scotland/Label Rouge	04 March 2019
C	C1	Scotland	15 March 2019
	C2	Norway	15 March 2019
	C3	Ireland/Organic	09 March 2019

2.2. Bacterial Enumeration

From each sample, a 10-g portion of CSS was added to 90 mL of sterile buffered peptone water (25.5 g/L) (Biokar Diagnostics, Allonne, France) to obtain a 10-fold dilution. Samples were homogenized for 2 min in a sterile stomacher plastic bag provided with a 63 µm porosity filter (Interscience, Saint-Nom-la-Bretèche, France) using a stomacher 400 device (Intersciences, Saint-Nom-la-Bretèche, France).

Total psychrotrophic viable counts (TPVC) were enumerated on plate count agar (PCA) medium (Oxoid, Thermo Fisher Diagnostics, Dardilly, France) supplemented with 2% NaCl. The PCA plates were incubated at 15 °C for five to seven days. Lactic acid bacteria (LAB) were enumerated on de Man, Rogosa and Sharpe (MRS) agar plates (bioMérieux, Crapone, France) incubated for two days at 30 °C. *Brochothrix thermosphacta* were investigated on streptomycin sulfate thallous acetate agar (STAA) (Oxoid, Thermo Fisher Diagnostics, Dardilly, France) incubated for two days at 25 °C [24]. *Enterobacteriaceae* were enumerated after two days at 30 °C on violet red bile glucose agar (VRBG) (Biokar Diagnostics, Allonne, France) and marine *Vibrio* were enumerated on marine agar (five days at 25 °C) (Becton Dickinson, Rungis, France). To enumerate bacterial colonies, 100 µL of appropriate dilution in buffered peptone water were spread over the agar. Results were expressed in colony forming unit per gram CSS (CFU/g). Detections limits were 1 and 2 Log CFU/g, respectively, for *Enterobacteriaceae* and other counts.

2.3. Chemical Analyses

Total fat, dry matter content, salt content and total phenol were measured as described by Leroi et al. (2015) [25]. Total volatile basic nitrogen (TVBN) and trimethylamine (TMA) were determined in duplicate from 100 g of CSS using the Conway micro-diffusion method [26].

2.4. DNA Extraction

DNA were extracted using Qiagen DNeasy PowerFood Microbial (Qiagen, Courtaboeuf, France). A first step of mechanical cell lysis was performed using the glass beads provided and a FastPrep (MPbiomedicals, Illkirch, France) for 30 s at a frequency of 6 m/s. DNA were extracted from three technical replicates from each sample. A Qubit® 2.0 fluorometer using a Qubit® dsDNA BR Assay Kit (Life technologies, Thermo Fisher Scientific, Villebon-sur-Yvette, France) was used to quantify DNA. Additional blank negative controls with no samples were used to exclude DNA contamination during extraction.

2.5. 16S rRNA Gene Sequencing

2.5.1. Library Preparation and Sequencing Using Illumina® MiSeq Platform

Briefly, the extracted DNA were PCR amplified to construct a sequencing library targeting the V3—V4 region of the bacterial 16S rRNA gene. PCR reactions were performed using 5 µL of DNA template, 12.5 µL of 2 × Kapa HiFi Hotstart ready mix (Roche, Boulogne-Billancourt, France) and 5 µL of 1 µM primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 785R (5′-GACTACHVGGGTATCTAATCC-3′) [27]. Amplicons were purified using an Agencourt AMPure kit (Beckman Coulter, Villepinte, France). PCR product concentration and size were checked on a 2100 Bioanalyzer platform using the DNA 7500 kit (Agilent Technologies, Les Ulis, France) and indexed using a Nextera XT DNA Library Prep kit (Illumina, Paris, France) following Illumina recommendations. Samples were then pooled in an equimolar concentration (4 nM) and sequenced through the Illumina® MiSeq platform using a 2 × 250 V2 chemistry kit (Illumina, Paris, France) according to the Illumina® standard operating procedures.

2.5.2. Sequencing Data Processing and Analyses

The count table and taxonomy of the operational taxonomic units (OTUs) were obtained using the FROGS bioinformatic pipeline [28]. Paired-end raw reads were merged using FLASh 1.2.11 with a maximum of 10% mismatch in the overlapped region [29].

Primers were removed using Cutadapt 1.18. Clustering of reads into OTUs (97% identity) was performed using Swarm 2.2.2 [30], and a denoising step was performed. Chimera were then detected and removed using VSearch 1.3.0 [31]. OTUs with less than 5/100,000 of the total number of sequences from the whole dataset were removed [32]. Taxonomy assignments were performed using RDP classifier 2.11 and the Silva 16S rRNA gene database (SSURef_128_SILVA) [33–35]. OTUs with a genus affiliation bootstrap threshold < 0.8 were removed.

2.6. Statistical Analyses

Statistical analyses and plots were performed in the R environment (v. 3.6.2) [36]. For metabarcoding data, alpha and beta diversity analyses were conducted and relative abundances were determined using the Phyloseq package (1.30.0) and its dependencies [37]. Samples read libraries were rarefied to an even depth (10,000 reads per sample) to be normalized. Permutational multivariate analysis of variance (PERMANOVA) based on a weighted UniFrac distance matrix was carried out using 9999 permutations to detect significant effects/differences in the bacterial community analyzed [38]. UpSet plots were used to assess OTU intersections according to the processing factories and storage date [39]. These plots were generated using the UpSetR package (1.4.0) [40].

The chemical parameters, the relative abundance of each taxon at the genus level, and the alpha diversity metrics were studied using linear mixed models considering the factory, the storage time and their interaction as fixed effects, and the production batch as a random effect. For all endpoints, the *p*-values were adjusted using Tukey's method for pairwise comparisons between factories at each time point and between time points for each factory. A *p*-value < 0.05 was considered statistically significant.

3. Results

3.1. Microbiological Analyses

Bacterial growth of the nine CSS batches during the 28-day storage period are presented in Figure 1 and summarized in Table S1.

At the beginning of the bacterial kinetic, total psychrotrophic viable counts (TPVC) were heterogenous among the different samples. Except for products A1, B2 and B3, TPVC increased during the storage period to reach D28 counts between 5.64 ± 0.45 and 7.07 ± 0.32 Log CFU/g. Interestingly, TPVC on products A1, B2 and B3 were high at the beginning of the experiment (D0) (between 4.53 ± 0.69 and 5.78 ± 0.69 Log CFU/g) and remained stable during the storage period. The A1 sample count at D28 was below the enumeration limit (<2 Log CFU/g).

Lactic acid bacteria (LAB) counts were low at the beginning of the experiment (D0). Except for product A3, which had an enumeration of 3.06 ± 0.55 Log CFU/g, all counts were below the enumeration limit. This microbial group quickly grew and reached its maximum after 21 days of storage. Interestingly, product A1's count was low or below the enumeration limit during the entire storage period with a maximum at D7 (2.77 ± 0.45 Log CFU/g). We observed the same situation on B1 and B2 products. However, these two samples reached respectively 4.66 ± 0.69 and 4.55 ± 1.05 Log CFU/g after 28 days of storage.

Enterobacteriaceae initial enumerations (D0) were low or below the enumeration limit (<1 Log CFU/g). Between 1.17 ± 0.15 and 2.27 ± 0.62 Log CFU/g were counted on products B2, B3 and C1 at the beginning of the storage period. *Enterobacteriaceae* counts then increased during storage on products A3, B2, B3 and C1 to reach a maximum at D28 (between 5.21 ± 0.8 Log CFU/g for B2 and 6.96 ± 1.21 Log CFU/g for C1). Product C3 counts after 7 and 21 days of storage were below the enumeration limit, whereas 5.29 ± 0.15 Log CFU/g and 6.96 ± 1.21 Log CFU/g were enumerated at D14 and D28. The same situation was observed on product B1: all counts were below the enumeration limit except for D14 with a count of 5.13 ± 0.85 Log CFU/g. In addition, this trend was observed on product A2: all counts were low except for D21 with a count of 4.32 ± 0.84 Log CFU/g. *Enterobacteriaceae* counts on products A1 and C2 increased slowly to reach a maximum of

3.32 ± 1.62 Log CFU/g at D21 for A1 and 3.26 ± 1.56 Log CFU/g at D28 for C2. The A1 sample count at D28 was below the enumeration limit.

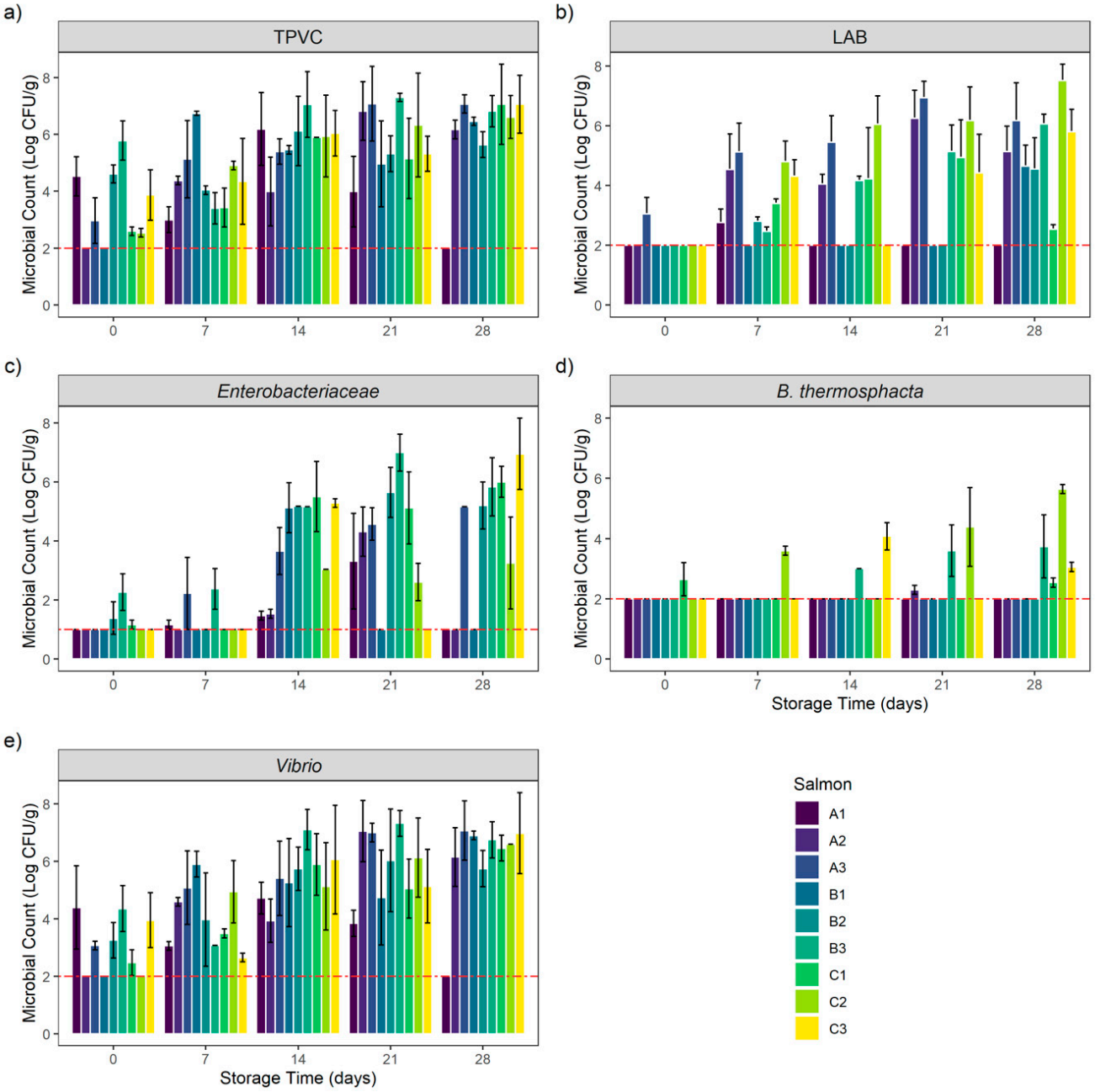

Figure 1. Bacterial growth evolution of (**a**) total psychrotrophic viable count (TPVC), (**b**) lactic acid bacteria (LAB), (**c**) *Enterobacteriaceae*, (**d**) *Brochothrix thermosphacta* and (**e**) *Vibrio* in vacuum-packed cold-smoked salmon (CSS) products during 28 days of storage. Results are expressed in Mean ± SD Log CFU/g of CSS products. The red-dashed line represents the limits of detection: 1 and 2 Log CFU/g, respectively, for *Enterobacteriaceae* and other counts.

For *Brochothrix thermosphacta*, the initial enumerations (D0) were below the enumeration limit on all samples except for product C1, with an enumeration of 2.65 ± 0.55 Log CFU/g. *B. thermosphacta* counts were below the enumeration limit during the entire storage period on products A1, A3, B1 and B2. The same situation also was initially observed on product A2; however, 2.3 ± 0.15 Log CFU/g were enumerated on this product at D21.

B. thermosphacta was then counted on products B3 and C2, with an increase during the storage period to reach a maximum of respectively 3.74 ± 1.05 and 5.64 ± 0.15 Log CFU/g

at D28. Product C1 counts remained stable during the storage period. The count was below the enumeration limit from D7 to D21 to reach 2.54 ± 0.15 Log CFU/g at D28. The same situation was encountered on product C3. Counts were below the enumeration limit at D0, D7 and D21 but 4.08 ± 0.45 and 3.06 ± 0.15 Log CFU/g were enumerated respectively at D14 and D28.

Vibrio initial counts (D0) were high (between 3.07 ± 0.15 Log CFU/g on product A3 and 4.39 ± 1.45 Log CFU/g on product A1) on all products except for A2, B2 and C2, where the counts were below the enumeration limit. Except for product A1, *Vibrio* counts increased during the storage period to reach a maximum count after 21 and 28 days of storage (between 6.03 ± 1.79 Log CFU/g at D21 on product B2 and 7.07 ± 1.03 Log CFU/g at D28 on product A3). Globally, *Vibrio* counts followed the same trend as TPVC. Product A1 *Vibrio* counts were stable during 21 days of storage with counts between 3.06 ± 0.15 and 4.72 ± 0.55 Log CFU/g. The A1 sample count at D28 was below the enumeration limit.

3.2. Chemical Analyses

The evolution of the chemical components of each CSS sample during the 28 days of storage is represented in Figure 2.

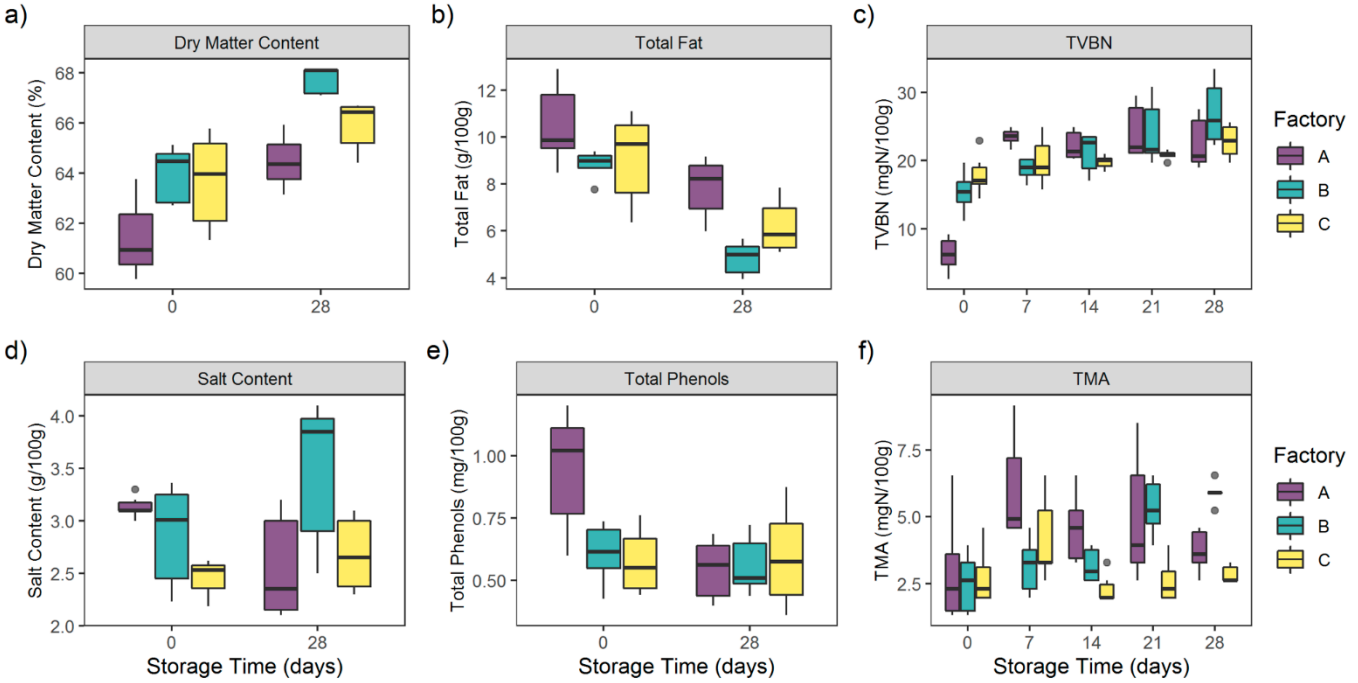

Figure 2. Evolution of (**a**) dry matter content (%), (**b**) total fat (g/100 g), (**c**) total volatile basic nitrogen (TVBN) (mgN/100 g), (**d**) salt content (g/100 g), (**e**) total phenols (mg/100 g), (**f**) trimethylamine (TMA) (mgN/100 g) of 45 CSS products during 28 days of storage.

Dry matter content among all of the CSS samples significantly increased during storage ($p < 0.0001$) from 63.32 ± 1.86% to 66.25 ± 1.55%. Interestingly, no significant differences in the dry matter content between the three factories' samples were observed ($p = 0.07$).

Contrary to dry matter, total fat among all CSS samples significantly decreased during storage ($p < 0.0001$) from 9.47 ± 1.65 g/100 g to 6.29 ± 1.62 g/100 g.

No significant differences in total fat content were observed for the three factories' samples ($p = 0.08$).

Total phenols, issued from the cold-smoking step, were homogeneous among the different factories' samples ($p = 0.46$). The total phenols rate among the 45 samples decreased from 0.71 ± 0.24 mg/100 g at D0 to 0.57 ± 0.15 mg/100 g at D28. This difference

was only due to the significant decrease ($p < 0.0001$) of A samples' total phenols from 0.94 ± 0.24 mg/100 g at D0 to 0.55 ± 0.12 mg/100 g at D28.

As far as salt content was concerned, no significant differences were observed among the different factories' samples ($p = 0.55$) or during storage ($p = 0.18$). Indeed, this parameter was stable throughout the storage period, from 2.89 ± 0.41 g/100 g at D0 to 2.91 ± 0.67 g/100 g at D28.

Spoilage markers TVBN and TMA were also measured at each storage date.

TVBN globally increased during the storage period from 13.17 ± 5.81 mgN/100 g at D0 to 24.09 ± 4.19 mgN/100 g at D28 ($p < 0.0001$). TVBN concentrations were also homogeneous among the different factories ($p = 0.61$).

TVBN increased significantly within A samples, from 6.22 ± 2.51 mgN/100 g at D0 to 23.47 ± 1.2 mgN/100 g at D7 ($p < 0.0001$). Concentrations were then homogeneous from D7 to D28 ($p > 0.05$). For B samples, TVBN were stable at D0 and D7 ($p = 0.68$), with respectively 15.39 ± 2.95 mgN/100 g and 18.78 ± 1.58 mgN/100 g. The concentrations then significantly increased to reach a maximum of 26.96 ± 4.78 mgN/100 g at D28 ($p < 0.05$).

TVBN concentrations of C samples were homogeneous during the storage period ($p > 0.05$), from 17.9 ± 2.98 mgN/100 g at D0 to 22.81 ± 2.49 mgN/100 g at D28.

TMA followed the TVBN trend with a significant increase from 2.73 ± 1.39 mgN/100 g at D0 to 4.15 ± 1.42 mgN/100 g at D28 ($p < 0.0001$). Interestingly, TMA concentrations differed significantly among the different factories' samples ($p < 0.0001$).

TMA increased significantly within A samples, from 2.95 ± 2.02 mgN/100 g at D0 to 6.0 ± 2.0 mgN/100 g at D7 ($p = 0.011$). Concentrations were then homogeneous from D7 to D28 ($p > 0.05$).

For B samples, TMA were stable at D0 and D7 ($p = 0.68$), with respectively 2.51 ± 0.12 mgN/100 g and 3.16 ± 1.04 mgN/100 g. The concentrations then significantly increased to reach a maximum of 5.89 ± 0.41 mgN/100 g at D28 ($p < 0.05$).

The TMA concentrations of C samples were homogeneous during the storage period ($p > 0.05$), from 2.73 ± 1.03 mgN/100 g at D0 to 2.83 ± 0.33 mgN/100 g at D28.

3.3. Metabarcoding Analyses

Out of over 135 samples, six DNA samples could not be amplified and sequenced: A1 at D28 and B2 at D28. A total of 3,584,463 reads passed filters applied through the FROGS pipeline workflow with an average of 27,787 reads/sample ± 27,189 reads.

The sizes of the libraries were highly heterogenous. Interestingly, library size increased simultaneously with storage time (Figure 3). Bacterial growth during storage impacted the number of reads ($p < 0.05$). Library sizes were higher at D28 with an average of 57,552 reads ± 22,263. The library sizes of some D0, D7 and D14 samples were too low, and were considered as not being representative of the microbiota of interest. Thus, due to a low number of reads (<10,000) over 15 triplicates (45 samples) were removed for statistical purposes and were not taken into account in any microbial ecology analyses. The other 84 samples were rarefied to an even depth of 10,000 reads and used for microbial ecology analyses.

A total of 56 OTUs were identified and agglomerated in 19 genera including 12 dominants. Dominant populations among all samples were represented by Firmicutes *Staphylococcus* (5.48 ± 10.8%), *Carnobacterium* (18.9 ± 32.3%), *Lactobacillus* (5.24 ± 17.2%), β-Proteobacteria *Photobacterium* (30.4 ± 43.5%), *Vibrio* (6.79 ± 24.6%), *Aliivibrio* (2.55 ± 13.3%), *Salinivibrio* (5.71 ± 20.7%), *Enterobacteriaceae Serratia* (6.8 ± 18.8%), *Pantoea* (3.6 ± 11.7%), γ-Proteobacteria *Psychrobacter* (6.43 ± 18.2%), *Shewanella* (4.75 ± 17.5%) and *Pseudomonas* (2.92 ± 10.4%).

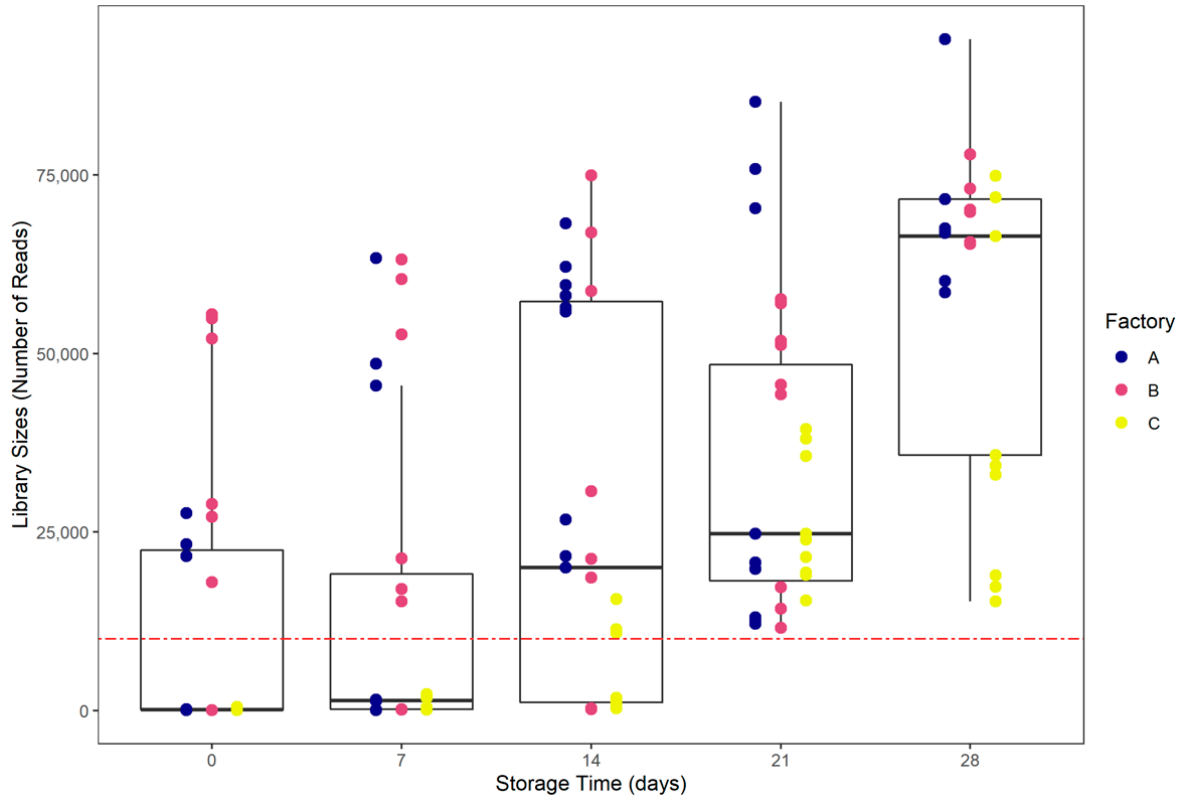

Figure 3. Library sizes distribution according to storage date. The red-dashed line represents a 10,000 reads threshold.

The relative abundances at the genus level are represented in Figure 4.

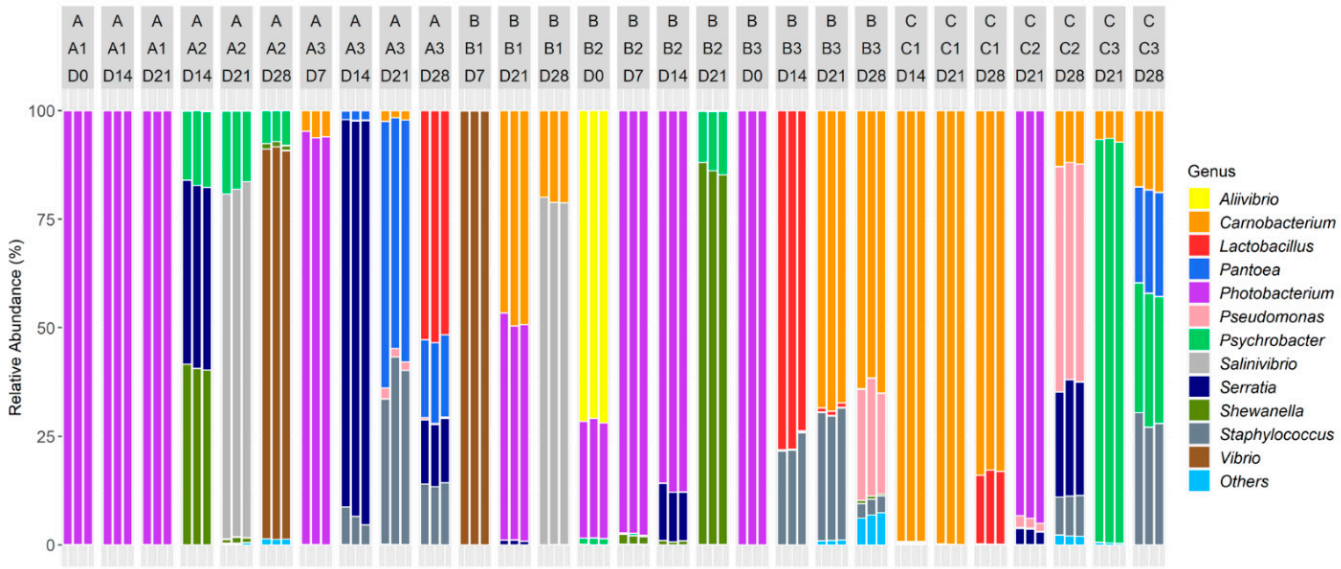

Figure 4. Relative abundance of bacterial genera of vacuum-packed cold-smoked salmon products stored during 28 days (D0, D7, D14, D21, D28). Three different production batches (e.g., A1, A2, A3) were processed in three different processing factories (A, B, C). Only 84 samples are represented: 6 DNA could not be amplified and 45 outliers were removed due to a low number of reads (<10,000). The removed samples were identified in some D0, D7, D14 and D28 samples. Taxa present on average in all samples at a threshold ≥0.5% or having a 90th percentile ≥0.5% are individually represented. In other cases, taxa are grouped and labeled "Others".

Genera initially shared a homogeneous repartition among the CSS originating from the three different processing environments ($p > 0.05$). However, the relative abundances

significantly differed during the storage period ($p < 0.001$). Indeed, the samples had different dominant populations.

Photobacterium and *Aliivibrio* dominated all D0 microbiotas (with respectively 75.66 ± 36.44% and 23.82 ± 35.72%).

After seven days of storage, the bacterial communities were dominated by *Photobacterium* (63.95 ± 47.96%), *Vibrio* (33.35 ± 49.92%) and *Carnobacterium* (1.89 ± 2.87%).

After 14 days of storage, dominant genera were *Photobacterium* (31.27 ± 45.51%) and *Carnobacterium* (16.55 ± 38.05%). Five other genera emerged: *Staphylococcus* (4.94 ± 8.76%), *Lactobacillus* (12.77 ± 29.37%), *Serratia* (24.23 ± 34.40%), *Shewanella* (6.96 ± 15.58%) and *Psychrobacter* (2.81 ± 6.44%).

After 21 days of storage, the microbiotas were dominated by *Photobacterium* (27.20 ± 41.25%), *Psychrobacter* (13.79 ± 29.19%), *Shewanella* (9.73 ± 27.64%) and *Staphylococcus* (7.62 ± 14.73%). Two genera also emerged at D21: *Pantoea* (6.31 ± 18.21%) and *Salinivibrio* (8.95 ± 25.79%).

At the end of the storage period (28 days) *Carnobacterium* (28.32 ± 30.68%), *Lactobacillus* (9.89 ± 18.79%), *Pantoea* (5.99 ± 9.76%), *Pseudomonas* (10.92 ± 18.88%), *Salinivibrio* (11.33 ± 28.38%), *Serratia* (5.77 ± 9.82%), *Vibrio* (12.85 ± 32.23%), *Staphylococcus* (7.88 ± 10.01%) and *Psychrobacter* (5.35 ± 10.61%) dominated the microbiotas.

Except for *Salinivibrio*, the *Vibrionaceae* ratio decreased during storage: *Photobacterium* relative abundance was reduced on A samples between D7 and D28 ($p < 0.0001$). The *Aliivibrio* proportion significantly changed on B samples ($p < 0.05$) and decreased during the storage period ($p < 0.0001$). *Vibrio* relative abundance decreased from D14 to D28 on A products ($p < 0.0001$) and from D7 to D28 on B samples ($p < 0.05$). As far as *Salinivibrio* is concerned, the relative abundance increased from D14 to D28 on A products ($p < 0.05$) and from D7 to D28 on B samples ($p < 0.0001$).

Firmicutes did not share the same evolution: the *Carnobacterium* ratio increased during storage, especially from D0 to D21 on B samples ($p < 0.0001$), whereas the *Lactobacillus* relative abundance increased between D0 and D14 and then decreased from D14 to D28 on B samples ($p < 0.0001$). Moreover, the *Lactobacillus* proportion increased from D7 to D28 on A products ($p < 0.05$). In addition, the *Staphylococcus* ratio increased between D7 to D21 on A samples ($p < 0.0001$) and from D21 to D28 on C salmons.

As far as the *Enterobacteriaceae* family is concerned, the *Serratia* relative abundance increased from D0 to D14 on A CSS ($p < 0.05$) and then was reduced during the remaining period ($p < 0.0001$). The *Pantoea* ratio increased between D7 and D28 on A products ($p < 0.05$).

Globally, γ-Proteobacteria increased during the storage: the *Shewanella* proportion increased between D0 and D21 on B products ($p < 0.001$). The *Psychrobacter* ratio increased significantly from D21 to D28 on C salmons ($p < 0.0001$). The *Pseudomonas* relative abundance increased between D21 and D28 C samples ($p < 0.0001$) and from D0 to D28 on B products ($p < 0.05$).

Among the 56 OTUs, 29 core OTUs were identified in the three different factories (Figure 5), which were agglomerated in 12 genera composed by *Carnobacterium*, *Lactobacillus*, *Staphylococcus*, *Pantoea*, *Serratia*, *Proteus*, *Salinivibrio*, *Vibrio*, *Photobacterium*, *Shewanella*, *Psychrobacter* and *Pseudomonas*.

Seven core OTUs were identified between A and B samples. These seven OTUs were agglomerated in seven genera composed by *Brochothrix*, *Lactobacillus*, *Staphylococcus*, *Enhydrobacter*, *Psychrobacter*, *Marinimonas* and *Arcobacter*.

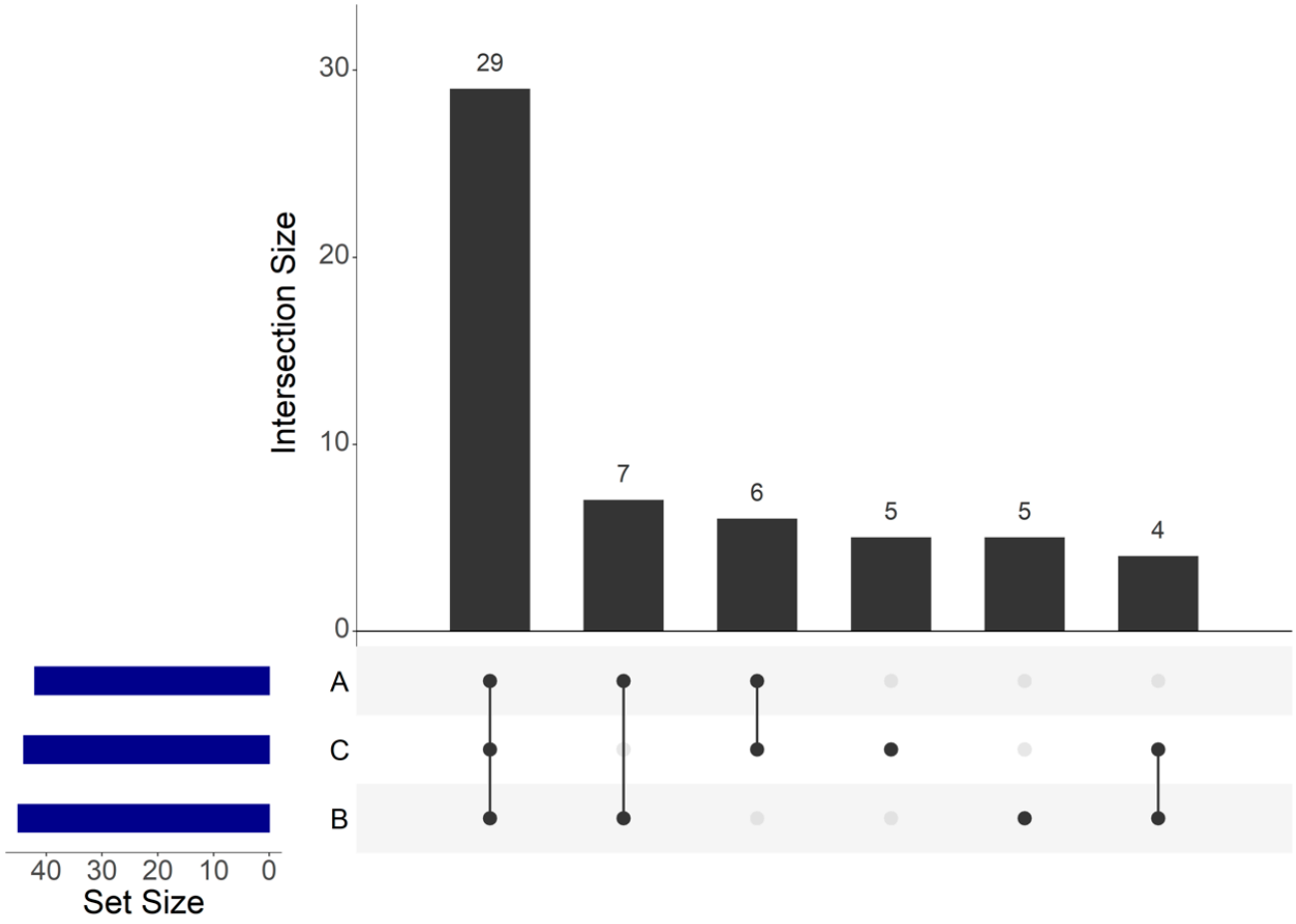

Figure 5. UpSet plot of shared operational taxonomic units (OTUs) identified within cold-smoked salmon vacuum-packed products according to the food processing factory.

Six core OTUs between A and C samples were identified and agglomerated in six genera: *Carnobacterium*, *Lactobacillus*, *Staphylococcus*, *Serratia*, *Psychrobacter* and *Brevibacterium*. Four core OTUs between B and C samples were identified and agglomerated in four genera composed by *Carnobacterium*, *Aliivibrio*, *Photobacterium* and *Psychrobacter*. Five OTUs were only identified within B samples, which were agglomerated in three genera composed by *Carnobacterium*, *Serratia* and *Shewanella*. Five unique OTUs were also only identified within C samples, which were agglomerated in four genera composed by *Carnobacterium*, *Lactobacillus*, *Aerococcus* and *Pseudomonas*.

Among the 56 OTUs, seven core OTUs were identified among the CSS products at each storage analysis date (Figure 6), which were agglomerated in six genera composed by *Staphylococcus*, *Vibrio*, *Photobacterium*, *Shewanella*, *Psychrobacter* and *Pseudomonas*. Twelve core OTUs were identified only at D14, D21 and D28, which were agglomerated in five genera composed by *Carnobacterium*, *Arcobacter*, *Enhydrobacter*, *Psychrobacter* and *Pseudomonas*. Eleven OTUs were unique to D21 and D28, which were agglomerated in seven genera composed by *Carnobacterium*, *Staphylococcus*, *Pantoea*, *Salinivibrio*, *Psychrobacter*, *Brevibacterium* and *Pseudomonas*. Finally, eight OTUs were unique to D28, which were agglomerated in six genera composed by *Carnobacterium*, *Lactobacillus*, *Aerococcus*, *Shewanella*, *Marinomonas* and *Pseudomonas*.

The genera *Brevibacterium*, *Marinomonas*, *Enhydrobacter* and *Arcobacter* belonged for their part to the subdominant population with a relative abundance below 0.05%.

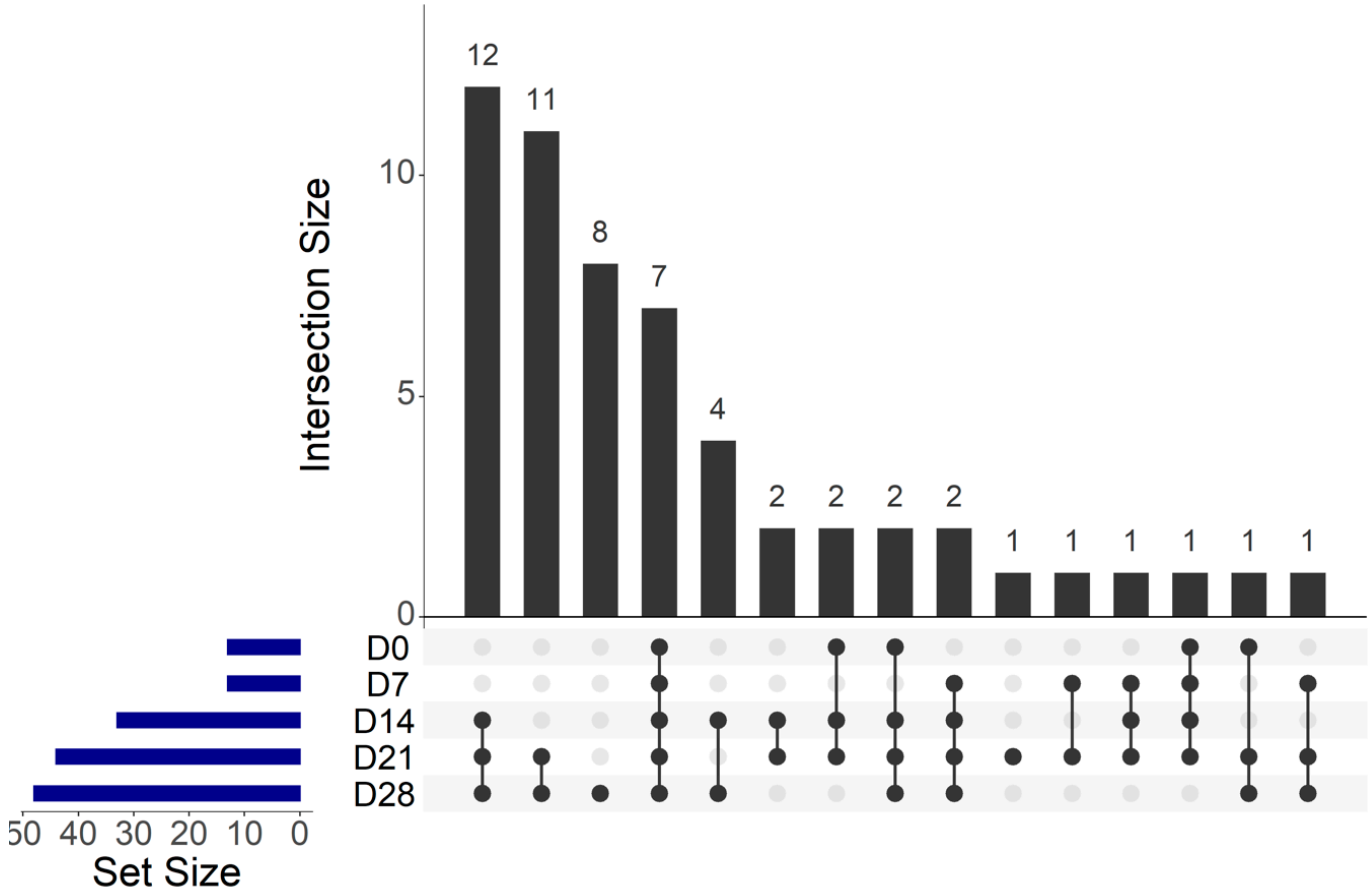

Figure 6. UpSet plot of shared operational taxonomic units (OTUs) identified within cold-smoked salmon vacuum-packed products during 28 days of storage.

Communities richness (observed OTUs) and evenness (Shannon diversity index) were assessed for all 84 samples and are summarized in Table 2. The storage time had an effect on both richness ($p < 0.0001$) and evenness ($p < 0.0001$). Communities were richer after 28 days of storage (with an average of 15.43 $\pm$ 4.95 OTUs). No richness differences were observed between D0 and D7 (respectively with an average of 4.56 $\pm$ 2.35 and 4.89 $\pm$ 1.83 OTUs). With regard to the evenness of communities, this was higher after 28 days of storage (with an average of 1.07 $\pm$ 0.54). Interestingly, the processing environment appeared to have no impact on either richness or evenness (respectively $p = 0.60$ and $p = 0.83$).

Weighted UniFrac principal coordinates analysis (PCoA) was generated to visualize samples (Figure 7). This PCoA highlighted shared taxa between samples, especially between factories A and B, but also differences according to the processing environment. PERMANOVA analysis based on weighted UniFrac distance showed that the processing environment, the storage date and the production batch influenced the bacterial community (respectively $p < 0.0001$) and explained respectively 17.6%, 14.2% and 45.7% of the sample microbiota differences.

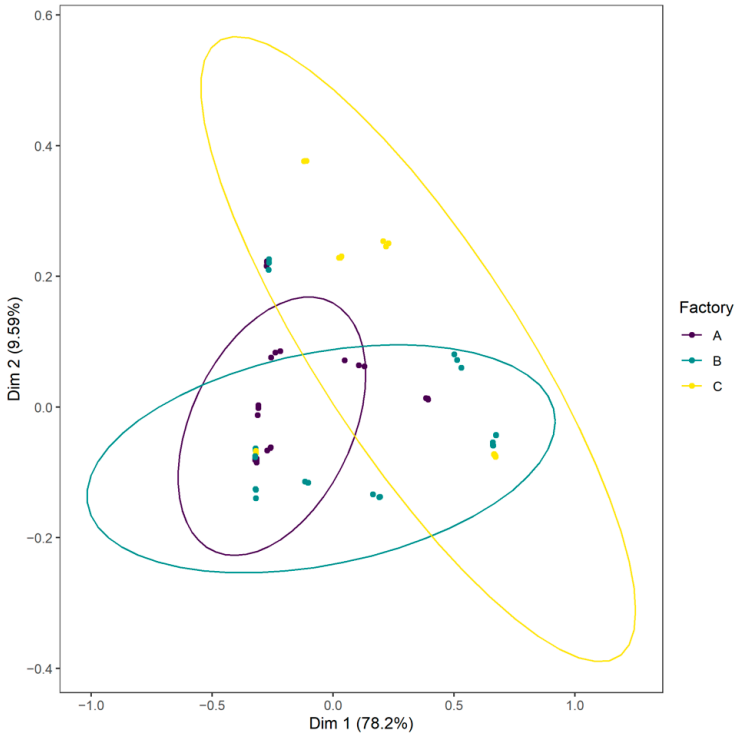

Figure 7. Weighted UniFrac principal coordinates analysis (PCoA) plot of CSS samples according to the food processing factory.

Table 2. Observed richness and evenness for 16S rRNA amplicons analyzed in this study. Data are expressed in Mean ± SD.

Factory	Salmon	Date	Observed OTUs	Shannon Index
A	A1	D0	4.000 ± 0.000	0.006 ± 0.001
A	A1	D14	1.333 ± 0.577	0.000 ± 0.001
A	A1	D21	2.667 ± 1.155	0.004 ± 0.002
A	A2	D14	12.667 ± 0.577	1.130 ± 0.018
A	A2	D21	12.000 ± 1.000	0.576 ± 0.020
A	A2	D28	18.667 ± 1.528	0.501 ± 0.016
A	A3	D7	4.000 ± 1.000	0.221 ± 0.023
A	A3	D14	8.000 ± 1.000	0.400 ± 0.065
A	A3	D21	9.000 ± 0.000	0.902 ± 0.010
A	A3	D28	16.333 ± 1.155	1.333 ± 0.018
B	B1	D7	3.667 ± 1.155	0.008 ± 0.001
B	B1	D21	11.000 ± 1.000	0.807 ± 0.006
B	B1	D28	5.667 ± 1.155	0.518 ± 0.012
B	B2	D0	7.333 ± 1.528	0.664 ± 0.006
B	B2	D7	7.000 ± 1.000	0.135 ± 0.013
B	B2	D14	7.333 ± 0.577	0.428 ± 0.026
B	B2	D21	13.000 ± 1.732	0.537 ± 0.040
B	B3	D0	2.333 ± 0.577	0.002 ± 0.002
B	B3	D14	12.000 ± 0.000	0.678 ± 0.054
B	B3	D21	14.000 ± 1.000	1.074 ± 0.022
B	B3	D28	17.333 ± 0.577	1.080 ± 0.027
C	C1	D14	10.333 ± 0.577	0.060 ± 0.002
C	C1	D21	7.000 ± 1.000	0.054 ± 0.005
C	C1	D28	11.667 ± 1.155	0.539 ± 0.002
C	C2	D21	13.667 ± 1.528	0.337 ± 0.062
C	C2	D28	17.667 ± 1.528	1.744 ± 0.007
C	C3	D21	8.333 ± 2.309	0.816 ± 0.014
C	C3	D28	20.667 ± 0.577	1.800 ± 0.004

OTUs: operational taxonomic Units.

4. Discussion

The first part of this study aimed to evaluate the culturable bacterial population of several CSS products processed in three different factories. The microbial load was high after one week of storage. The dominant population on D0 products consisted of Gram-negative *Vibrio*, *Enterobacteriaceae*, and Gram-positive LAB. The microbial load reached an average of 10^7 CFU/g at the end of the experiment. These observations and the bacterial concentration were consistent with already published data. Indeed, Leroi et al. (1998) studied the microbial ecology of CSS during 35 days of storage at 8 °C [6]. The authors enumerated aerobic viable counts at a maximum of 10^6 to 10^7 CFU/g after 6 days of storage. In addition, Paludan-Müller et al. (1998) studied the role of LAB in vacuum-packed CSS spoilage [7]. The authors evaluated the total psychrotrophic viable counts during 7 weeks at 5 °C. Counts reached 10^6 to 10^7 CFU/g in two weeks and remained stable during the storage. Moreover, LAB growth did not seem to compete with Gram-negative bacteria as described by Leroi et al. (1998) [7].

Marine *Vibrio* such as *Photobacterium phosphoreum* were dominant among the bacterial populations of the different samples. This bacterium has already been described as a potential spoiler due to its ability to produce TMA from trimethylamine N-oxide (TMAO), which is known to be responsible for the typical strong fishy, urine and ammonia-like off-odors [3,41,42].

Enterobacteriaceae were dominant within B and C product communities. Psychrotrophic *Enterobacteriaceae* have been already identified on spoiled CSS, and particularly reported as dominant within injection brined products [5].

Lactic acid bacteria (LAB) such as *Carnobacterium maltaromaticum* and *Lactobacillus curvatus* have been widely described as dominant at a high level (10^7–10^8 CFU/g) on CSS products and could be involved in spoilage processes [43].

Interestingly, *Brochothrix thermosphacta* was enumerated on only one B product, yet on all C products. Several studies have reported the spoilage potential of this bacterium [5,44], notably able to produce butter/plastic/rancid, blue-cheese, sour/pungent off-odors, due to the high release of chemical compounds such as 2-heptanone and 2-hexanone [45,46]. More broadly, Stohr et al. (2001), by studying the inoculation of different spoilage bacteria on CSS (*Shewanella putrefaciens*, LAB, *Brochothrix thermosphacta*, *Aeromonas* spp., *Serratia liquefaciens*), were able to design a sensory and spoilage profile to better understand the CSS spoilage process and its major actors.

As described by Joffraud et al. (2006) in a study to evaluate CSS spoilage following different microbiota interaction, CSS spoilage due to metabolites production is often strain-dependent, which can explain the intraspecies diversity in terms of spoilage potential [9]. Furthermore, spoilage is also related to interactions, either between bacterial species, such as antagonistic or cooperative behavior, or between bacterial species and food matrices and the food processing environment. Indications of bacterial species interaction have been found in other food matrices, for example by Jaffrès et al. (2009), who studied the bacterial community in tropical cooked and peeled shrimps using a polyphasic approach (cultivable, non-cultivable and sensory analyses) [13]. These authors hypothesized that the spoilage process might be the result of interactions between *Brochothrix thermosphacta* and *Carnobacterium divergens*.

Chemical parameters (dry matter content, total fat, salt content and total phenols) were similar among the different samples and fluctuated during the experiment. These parameters were aligned with the NF V45-065 standard [47] on CSS properties.

Total fat significantly decreased after 28 days of storage. It is known that bacteria are able to degrade lipids. Notably, it has been reported that *Serratia*, *Staphylococcus* and *Pseudomonas* have the ability to degrade vegetable oil [48]. These genera are known to be part of the CSS microbiota. Their metabolic activities could explain this significant decrease of total fat.

Salt content was stable from 2.89 ± 0.41 g/100 g to 2.91 ± 0.67 g/100 g. It has been reported that despite its bacteriostatic effect, a low salt concentration could reduce the

product sensory rejection limit and could not be sufficient to inhibit *Listeria monocytogenes* growth [5,49].

Total phenols were also stable during the storage period. In addition, no growth was observed at D28 on product A1. The total phenols on products from A at the beginning of the storage were higher than those on other products. The bacteriostatic effect of the smoking process may impact microbial growth or induce viable but non-culturable cells. Indeed, liquid smoke strongly affected growth and survival of *Listeria monocytogenes* [50]. Moreover, Neunlist et al. (2005), by assessing the impact of salting and cold-smoking processes on the cultivability of *Listeria monocytogenes*, showed a reduction of 2 Log CFU/g for inoculated processed salmon compared with raw salmon during 28 days of storage [51]. The authors also tested inoculation after the cold-smoked process and observed a 0.9 Log CFU/g reduction of the *Listeria monocytogenes* concentration on processed samples compared with unprocessed salmon within the first two weeks of storage. The concentrations of the control and processed samples were similar at the end of the storage period. Even if the authors did not highlight a viable but non-culturable state, the reduced concentration of *Listeria monocytogenes* in the processed samples during the first two weeks of storage, and the subsequent increase to reach the same concentration as the control, may indicate that the phenols compounds most probably stress bacterial cells but these cells later regain the ability to grow.

Total volatile basic nitrogen (TVBN) and trimethylamine (TMA) are considered as spoilage indicators. Their production increased significantly during the storage period. High TVBN concentrations suggest that CSS spoilage occurred after 14 days of storage. No strong differences in TVBN concentrations were observed across the different factories. However, as previously described in several studies, the use of TVBN alone as a relevant spoilage indicator must be put in perspective. In their study on CSS spoilage, Joffraud et al. (2006) found that *Vibrio* spp. produced a significant amount of TVBN although no off-odor was detected by a trained sensory panelist [9]. Furthermore, Brillet et al. (2005) showed that *Carnobacterium maltaromaticum* strains did not produce TVBN when inoculated in pure culture in sterile CSS, whereas when inoculated in naturally contaminated products, TVBN production was significantly enhanced [52]. Contrary to TVBN, TMA concentrations mostly increased on the products of A and B, suggesting that *Photobacterium phosphoreum* might be implicated in the spoilage process of these products [7,53].

Cold-smoked salmon can be re-contaminated during the manufacturing process through contact with contaminated surfaces (such as slicers, conveyors, etc.) [5]. The microbiota during storage may be different according to how and where products are processed. Metabarcoding analyses could help to explore this hypothesis.

Metabarcoding on 45 samples stored for 28 days and analyzed every seven days in triplicate allowed us to identify and to analyze the bacterial communities of nine CSS batches produced in three different factories. Out of over 129 sequenced samples, a total of 45 samples displayed low library sizes (<10,000 reads).

Bukin et al. (2019), by studying the effect of different 16S rRNA regions on bacterial communities monitored by metabarcoding, highlighted that the major bacterial diversity (covered by 95% of reads) could be achieved at a library size of 10,000 reads [54]. Thus, we decided to remove the 45 outliers for statistical purposes.

The dominant population identified confirmed already published data on CSS microbiota studied using cultivable methods, but also data from a few studies using culture-independent methods [5,7,18,22,55]. *Psychrobacter* is highly prevalent in our study (6.43 $\pm$ 18.20% of relative abundances). *Psychrobacter* occurrence seems to be widespread on seafood products and was also identified on raw salmon [19,44].

Thanks to the use of NGS, several studies on seafood have highlighted the high prevalence of *Psychrobacter* among seafood product spoilage bacterial communities [21]. Parlapani et al. (2018) used NGS to investigate the spoilage microbiota of thawed common cuttlefish (*Sepia officinalis*) stored at 2 °C [56]. The authors, by using an amplicon sequencing approach, highlighted that *Psychrobacter* was highly dominant among the samples,

followed by *Pseudomonas*. In the literature, *Staphylococcus* is rarely described as a CSS dominant bacterium. Its occurrence is mainly due to exogenous origins such as salt, the aquatic environment or the processing environment [4,22]. As far as the *Enterobacteriaceae* family is concerned, the *Pantoea* genus is also rarely described in the CSS bacterial communities. *Pantoea* is an ubiquitous bacterium which has already been identified in aquatic environments [57]. Skrodenytė-Arbačiauskienė et al. (2008) analyzed the gut microbial diversity of 12 fish (six freshwater *Salmo salar* and six sea trout *Salmo trutta trutta*) using a cultural approach and 16S rRNA gene sequencing for colony identification [58]. The authors identified the genus *Pantoea* within the sea trout intestinal tract but not on salmon samples. In another study to assess bacterial resistance to the antibiotic oxytetracycline in Chilean salmon (*Salmo salar*) farming, Miranda and Zemelman (2002) identified a prevalence of *Pantoea* on fingerlings salmon samples [59]. In our study, the majority of this genus was found on A3 (19.4 ± 23.8%) and C1 (11.7 ± 12.8%). We assumed that the origin of *Pantoea* on these products could be explained by their aquatic farm origin or contamination during production. Twenty-nine OTUs agglomerated in 12 genera were identified as part of the core microbiota between all of the CSS products. All of these genera were part of the dominant population except for *Proteus*. González-Rodríguez et al. (2002) studied the microbial community of 54 batches of cold-smoked fish (30 CSS and 24 smoked trout) during three weeks of storage [60]. Colonies were counted and identified using API galleries. The authors identified *Proteus* as a dominant member of the *Enterobacteriaceae* family among the samples. Interestingly, in our study, *Aliivibrio* was not identified on A products, and *Brevibacterium* was not found on B samples. *Aerococcus* was only identified on C samples. In addition, *Arcobacter*, *Marinimonas*, *Enhydrobacter* and *Brochothrix* were not identified on C samples using metabarcoding. These results suggest the importance of the processing environment on the CSS microbiota, with a bacterial signature from this environment.

During the storage period, our findings highlighted that 12 OTUs (agglomerated in the following genera: *Carnobacterium*, *Arcobacter*, *Enhydrobacter*, *Psychrobacter* and *Pseudomonas*) were captured on D14; 11 OTUs (agglomerated in the following genera: *Carnobacterium*, *Staphylococcus*, *Pantoea*, *Salinivibrio*, *Psychrobacter*, *Brevibacterium* and *Pseudomonas*) were captured on D21; and eight OTUs (agglomerated in the following genera: *Carnobacterium*, *Lactobacillus*, *Aerococcus*, *Shewanella*, *Marinomonas* and *Pseudomonas*) were captured on D28. The emergence or capture of specific OTUs over time was also identified by Silbande et al. (2018) [61]. The authors studied the effect of different packaging atmospheres on the microbiological, chemical and sensory properties of tropical red drum (*Sciaenops ocellatus*) fillets stored for 29 days at 4 °C. The authors identified the emergence of *Leuconostoc* and *Lactococcus* after eight days of storage on fresh fillets that were vacuum-packed. These two OTUs were not identified at Day 0.

Alpha diversity analyses highlighted that the richness and evenness of the different CSS bacterial communities increased during the product storage period. However, no differences between the factories were observed. While we observed a global increase in the OTUs' richness, Wiernasz et al. (2020) highlighted a reduction of the number of OTUs on salmon gravlax during 21 days of storage [55]. Salmon gravlax is a salt-sugar mixture with spices that is not treated using smoke or heat. This particular treatment may lead to competitive flora which become dominant on these products which are not found on the standard cold-smoked process.

Beta diversity analyses and weighted UniFrac PCoA confirmed a core microbiota but also highlighted differences in communities, specifically between A products and C products. In addition, we identified five OTUs (agglomerated in *Carnobacterium*, *Serratia* and *Shewanella*) specific to B and five others specific to C (agglomerated in *Carnobacterium*, *Lactobacillus*, *Aerococcus* and *Pseudomonas*). These results strengthened the specific factory signature observation. Our findings showed that the different compositions of CSS microbiota were affected by the processing environment and the length of storage but also the production batch. This clearly confirms that even if a core community existed between the samples, the processing factory had a bacterial signature composed by spoilage organisms

which can contaminate CSS products during processing, attesting to the importance of the processing environment for the quality and shelf-life of CSS.

Rouger et al. (2018) observed identical results in their study of chicken leg microbiota, where two chicken leg samples from two different batches, stored under modified atmosphere packaging, showed similar microbiota [62]. Interestingly, these two samples were processed in the same slaughterhouse on the same day. These results strengthen the hypothesis of a food processing bacterial signature on the microbial communities of products. To investigate this environmental influence, Stellato et al. (2016) compared fresh meat microbiota with environmental samples from small and large-scale retail butcheries [63]. The authors highlighted 48 core genera shared between product and environment samples. Among these 48 genera, *Pseudomonas* spp., *Brochothrix* spp., *Psychrobacter* spp., *Streptococcus* spp. and *Acinetobacter* spp. were identified. These genera were reported as members of the meat spoilage community, highlighting the importance of the surface microbiota on product quality. By using a polyphasic approach (cultivable method with bacterial identification using the 16S rRNA gene and non-cultivable methods using NGS), Møretrø et al. (2016) identified the processing environment as a source of spoilage genera *Pseudomonas* and *Shewanella* [19]. Phylogenetic analyses based on part of the 16S rRNA gene demonstrated the transfer of *Pseudomonas* from processing samples to salmon fillets, thus strengthening the links between the processing environment and product samples and the impact of the processing environment on the shelf-life.

5. Conclusions

In this study, we described the microbiota of vacuum-packed cold-smoked salmon products produced in three different factories and stored for 28 days. We used a polyphasic approach composed of cultivable methods and non-cultivable methods. The use of metabarcoding did not highlight unexpected genera except for *Pantoea*, and our findings were consistent with already published cultivable data on CSS bacterial communities. However, a next-generation sequencing-based approach highlighted the emergence of operational taxonomic units during product storage and provided insights on the CSS microbial ecology. A core microbiota composed of spoilage bacteria was shared by the 45 products but strong differences linked to the processing environment were observed. Indeed, we found that CSS products bore a factory bacterial signature. These results were obtained from three different processing plants and 45 samples and must be considered at this scale. This suggests the importance of the processing environment on food safety and quality. A better understanding and characterization of surfaces and residential bacteria and their dynamics using metabarcoding approaches may be a key to gaining greater insight into a factory's "health condition" to improve food safety and quality management.

Supplementary Materials: The following are available online at https://www.mdpi.com/2304-8158/10/2/362/s1, Table S1: Bacterial growth evolution of lactic acid bacteria (LAB), *Enterobacteriaceae*, *Brochothrix thermosphacta*, *Vibrio* and total viable psychrothropic count (TPVC) in vacuum-packed cold-smoked salmon (CSS) products during 28 days of storage.

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Supplementary Material

Table S1. Bacterial growth evolution of lactic acid bacteria (LAB), *Enterobacteriaceae*, *Brochothrix thermosphacta*, *Vibrio* and total viable psychrothropic count (TPVC) in vacuum-packed cold-smoked salmon (CSS) products during 28 days of storage.

LAB	A1	A2	A3	B1	B2	B3	C1	C2	C3
D0	< 2.00 ± 0.00	< 2.00 ± 0.00	3.06 ± 0.55	< 2.00 ± 0.00	< 2.00 ± 0.00	< 2.00 ± 0.00	< 2.00 ± 0.00	< 2.00 ± 0.00	< 2.00 ± 0.00
D7	2.77 ± 0.45	4.54 ± 1.19	5.13 ± 0.96	< 2.00 ± 0.00	2.81 ± 0.15	2.47 ± 0.15	3.40 ± 0.15	4.80 ± 0.69	4.32 ± 0.55
D14	< 2.00 ± 0.00	4.06 ± 0.32	5.45 ± 0.89	< 2.00 ± 0.00	< 2.00 ± 0.00	4.17 ± 0.15	4.23 ± 1.71	6.05 ± 0.96	< 2.00 ± 0.00
D21	< 2.00 ± 0.00	6.26 ± 0.93	6.94 ± 0.55	< 2.00 ± 0.00	< 2.00 ± 0.00	5.14 ± 0.89	4.94 ± 1.26	6.18 ± 1.13	4.43 ± 1.29
D28	< 2.00 ± 0.00	5.14 ± 0.85	6.18 ± 1.26	4.66 ± 0.69	4.55 ± 1.05	6.07 ± 0.32	2.54 ± 0.15	7.52 ± 0.55	5.81 ± 0.75

Enterobacteriaceae	A1	A2	A3	B1	B2	B3	C1	C2	C3
D0	< 1.00 ± 0.00	< 1.00 ± 0.00	< 1.00 ± 0.00	< 1.00 ± 0.00	1.39 ± 0.55	2.27 ± 0.62	1.17 ± 0.15	< 1.00 ± 0.00	< 1.00 ± 0.00
D7	1.17 ± 0.15	< 1.00 ± 0.00	2.23 ± 1.22	< 1.00 ± 0.00	< 1.00 ± 0.00	2.38 ± 0.69	< 1.00 ± 0.00	< 1.00 ± 0.00	< 1.00 ± 0.00
D14	1.47 ± 0.15	1.54 ± 0.15	3.66 ± 0.80	5.13 ± 0.85	5.18 ± 0.00	5.17 ± 0.00	5.51 ± 1.19	3.04 ± 0.00	5.29 ± 0.15
D21	3.32 ± 1.62	4.32 ± 0.84	4.58 ± 0.55	< 1.00 ± 0.00	5.65 ± 0.85	7.00 ± 0.63	5.13 ± 1.22	2.61 ± 0.63	< 1.00 ± 0.00
D28	< 1.00 ± 0.00	< 1.00 ± 0.00	5.17 ± 0.00	< 1.00 ± 0.00	5.21 ± 0.80	5.84 ± 0.99	6.01 ± 0.53	3.26 ± 1.56	6.96 ± 1.21

B. thermosphacta	A1	A2	A3	B1	B2	B3	C1	C2	C3
D0	< 2.00 ± 0.00	< 2.00 ± 0.00	< 2.00 ± 0.00	< 2.00 ± 0.00	< 2.00 ± 0.00	< 2.00 ± 0.00	2.65 ± 0.55	< 2.00 ± 0.00	< 2.00 ± 0.00
D7	< 2.00 ± 0.00	< 2.00 ± 0.00	< 2.00 ± 0.00	< 2.00 ± 0.00	< 2.00 ± 0.00	< 2.00 ± 0.00	< 2.00 ± 0.00	3.60 ± 0.15	< 2.00 ± 0.00
D14	< 2.00 ± 0.00	< 2.00 ± 0.00	< 2.00 ± 0.00	< 2.00 ± 0.00	< 2.00 ± 0.00	3.00 ± 0.00	< 2.00 ± 0.00	< 2.00 ± 0.00	4.08 ± 0.45
D21	< 2.00 ± 0.00	2.30 ± 0.15	< 2.00 ± 0.00	< 2.00 ± 0.00	< 2.00 ± 0.00	3.60 ± 0.85	< 2.00 ± 0.00	4.39 ± 1.31	< 2.00 ± 0.00
D28	< 2.00 ± 0.00	< 2.00 ± 0.00	< 2.00 ± 0.00	< 2.00 ± 0.00	< 2.00 ± 0.00	3.74 ± 1.05	2.54 ± 0.15	5.64 ± 0.15	3.06 ± 0.15

Vibrio	A1	A2	A3	B1	B2	B3	C1	C2	C3
D0	4.39 ± 1.45	< 2.00 ± 0.00	3.07 ± 0.15	< 2.00 ± 0.00	3.25 ± 0.62	4.35 ± 0.80	2.47 ± 0.45	< 2.00 ± 0.00	3.95 ± 0.96
D7	3.06 ± 0.15	4.59 ± 0.15	5.08 ± 1.28	5.90 ± 0.45	3.97 ± 1.62	3.07 ± 0.00	3.49 ± 0.15	4.94 ± 1.08	2.65 ± 0.15
D14	4.72 ± 0.55	3.93 ± 0.75	5.41 ± 1.29	5.26 ± 1.53	5.74 ± 0.75	7.10 ± 0.70	5.89 ± 1.07	5.13 ± 1.52	6.06 ± 1.89
D21	3.84 ± 0.45	7.05 ± 1.07	7.00 ± 0.32	4.74 ± 1.65	6.03 ± 1.79	7.32 ± 0.45	5.05 ± 1.03	6.13 ± 1.38	5.13 ± 1.28
D28	< 2.00 ± 0.00	6.15 ± 1.02	7.07 ± 1.03	6.90 ± 0.15	5.74 ± 0.63	6.75 ± 0.63	6.46 ± 0.45	6.60 ± 0.00	6.98 ± 1.41

TPVC	A1	A2	A3	B1	B2	B3	C1	C2	C3
D0	4.53 ± 0.69	< 2.00 ± 0.00	2.97 ± 0.80	< 2.00 ± 0.00	4.61 ± 0.32	5.78 ± 0.69	2.60 ± 0.15	2.54 ± 0.15	3.87 ± 0.89
D7	3.00 ± 0.45	4.38 ± 0.15	5.13 ± 1.36	6.74 ± 0.07	4.04 ± 0.15	3.40 ± 0.55	3.43 ± 0.68	4.91 ± 0.15	4.35 ± 1.51
D14	6.19 ± 1.28	3.99 ± 1.21	5.40 ± 0.45	5.46 ± 0.15	6.12 ± 1.22	7.05 ± 1.15	5.89 ± 0.00	5.94 ± 1.44	6.04 ± 0.80
D21	3.99 ± 1.24	6.82 ± 1.03	7.08 ± 1.31	4.97 ± 1.51	5.32 ± 0.63	7.30 ± 0.15	5.15 ± 1.41	6.33 ± 1.82	5.32 ± 0.62
D28	< 2.00 ± 0.00	6.17 ± 0.33	7.07 ± 0.32	6.46 ± 0.15	5.64 ± 0.45	6.82 ± 0.55	7.06 ± 1.41	6.61 ± 0.75	7.06 ± 1.02

Results are expressed in Mean ± SD Log CFU/g of CSS products.

Annexe 3 : Proportions détaillées des sources environnementales de contamination des échantillons de saumon fumé déterminée par SourceTracker (Knight et al., 2011)

Sample	Environment	Time	Proportion (%)
J0N	Balance Packaging	D0	0.08
J0SN	Balance Packaging	D0	0.18
J14N	Balance Packaging	D14	0.06
J14SN	Balance Packaging	D14	0.05
J21N	Balance Packaging	D21	0.04
J21SN	Balance Packaging	D21	0.02
J28N	Balance Packaging	D28	0.35
J28SN	Balance Packaging	D28	0
J0N	Conveyor	D0	0
J0SN	Conveyor	D0	0.05
J14N	Conveyor	D14	0.25
J14SN	Conveyor	D14	0.05
J21N	Conveyor	D21	0.02
J21SN	Conveyor	D21	0.01
J28N	Conveyor	D28	0.47
J28SN	Conveyor	D28	0.02
J0N	Conveyor Slicers	D0	0.02
J0SN	Conveyor Slicers	D0	0.19
J14N	Conveyor Slicers	D14	0.03
J14SN	Conveyor Slicers	D14	0.09
J21N	Conveyor Slicers	D21	0.02
J21SN	Conveyor Slicers	D21	0.04
J28N	Conveyor Slicers	D28	0.03
J28SN	Conveyor Slicers	D28	0.02
J0N	Dressing Table	D0	0.27
J0SN	Dressing Table	D0	0.18
J14N	Dressing Table	D14	1.77
J14SN	Dressing Table	D14	1.01
J21N	Dressing Table	D21	0.68
J21SN	Dressing Table	D21	0.41
J28N	Dressing Table	D28	16.86
J28SN	Dressing Table	D28	0.18
J0N	Filleting Machine	D0	99.49

J0SN	Filleting Machine	D0	98.68
J14N	Filleting Machine	D14	96.76
J14SN	Filleting Machine	D14	98.35
J21N	Filleting Machine	D21	99.15
J21SN	Filleting Machine	D21	99.43
J28N	Filleting Machine	D28	80.33
J28SN	Filleting Machine	D28	99.6
J0N	Floor	D0	0.01
J0SN	Floor	D0	0.2
J14N	Floor	D14	0.04
J14SN	Floor	D14	0.05
J21N	Floor	D21	0.01
J21SN	Floor	D21	0
J28N	Floor	D28	0.18
J28SN	Floor	D28	0.02
J0N	Forklift	D0	0.06
J0SN	Forklift	D0	0.08
J14N	Forklift	D14	0.05
J14SN	Forklift	D14	0.08
J21N	Forklift	D21	0
J21SN	Forklift	D21	0.02
J28N	Forklift	D28	0.13
J28SN	Forklift	D28	0
J0N	Ice Storage	D0	0
J0SN	Ice Storage	D0	0
J14N	Ice Storage	D14	0.01
J14SN	Ice Storage	D14	0.02
J21N	Ice Storage	D21	0
J21SN	Ice Storage	D21	0
J28N	Ice Storage	D28	0.13
J28SN	Ice Storage	D28	0.03
J0N	Operators Hands	D0	0.01
J0SN	Operators Hands	D0	0.18
J14N	Operators Hands	D14	0
J14SN	Operators Hands	D14	0.09
J21N	Operators Hands	D21	0.02
J21SN	Operators Hands	D21	0.04
J28N	Operators Hands	D28	0.02
J28SN	Operators Hands	D28	0.01
J0N	Slicer Blades	D0	0
J0SN	Slicer Blades	D0	0.01
J14N	Slicer Blades	D14	0.06

J14SN	Slicer Blades	D14	0.06
J21N	Slicer Blades	D21	0
J21SN	Slicer Blades	D21	0
J28N	Slicer Blades	D28	0.53
J28SN	Slicer Blades	D28	0
J0N	Washing Machine	D0	0.06
J0SN	Washing Machine	D0	0.12
J14N	Washing Machine	D14	0.02
J14SN	Washing Machine	D14	0.11
J21N	Washing Machine	D21	0
J21SN	Washing Machine	D21	0.03
J28N	Washing Machine	D28	0.02
J28SN	Washing Machine	D28	0
J0N	Unknown	D0	0
J0SN	Unknown	D0	0.13
J14N	Unknown	D14	0.95
J14SN	Unknown	D14	0.04
J21N	Unknown	D21	0.06
J21SN	Unknown	D21	0
J28N	Unknown	D28	0.95
J28SN	Unknown	D28	0.12

DOCTORAT ECOLOGIE BRETAGNE GEOSCIENCES LOIRE AGRONOMIE ALIMENTATION

Oniris
École Nationale Vétérinaire, Agroalimentaire et de l'Alimentation
Nantes Atlantique
Santé et alimentation au cœur de la vie

Titre : **Étude du microbiote de l'environnement de l'usine agroalimentaire et de son impact sur la qualité et la sécurité des aliments. Application au modèle de production du saumon fumé.**

Mots clés : Écologie Microbienne, Microbiote, Metabarcoding, Aliment, Surfaces, Bactéries

Résumé : L'hygiène des surfaces est primordiale dans la démarche qualité des industriels de l'agroalimentaire. Les bactéries non pathogènes, capables de résider au sein des usines, sont en général non-identifiées mais peuvent être altérantes et contaminer les denrées à chaque étape de fabrication. Elles représentent donc un risque qui doit être maitrisé pour garantir la qualité et la sécurité des aliments. Dans ce travail, le saumon fumé a été utilisé comme modèle pour caractériser la diversité des communautés bactériennes des ateliers de transformation des aliments et des produits par metabarcoding de l'ADNr 16S. Une analyse comparative de différentes méthodes d'extraction de l'ADN et de techniques de prélèvement de surface a permis de définir leurs impacts sur les résultats d'une analyse de metabarcoding 16S. De ce fait, nous avons pu sélectionner les conditions méthodologiques de l'étude. L'analyse de la diversité des communautés bactériennes de différents lots de saumon fumé démontre un lien avec l'atelier de production dans lequel ils ont été transformés. Ceci suggère que les produits conservent une signature du microbiote de l'environnement de production. Enfin, une comparaison des communautés bactériennes des produits et de différentes surfaces de l'usine permet d'identifier les sources environnementales de contamination. L'utilisation de cette approche, en contexte industriel, peut permettre de mettre en œuvre des mesures correctives ciblées afin de réduire les risques d'altération des produits et le gaspillage alimentaire.

Title : **Study of the microbiota of food processing environment and its impact on food quality and safety. Application to the cold-smoked salmon production**

Keywords : Microbial Ecology, Microbiota, Metabarcoding, Food, Surfaces, Bacteria

Abstract : Surface hygiene is an essential component of food industries quality system. Non-pathogenic bacteria are able to be resident in food processing factories and are generally unidentified but can be spoilers and contaminate foodstuffs at each processing step. Therefore, they might be a risk that must be controlled to ensure food safety and quality. In this study, cold-smoked salmon was used as a model to characterize the diversity of bacterial communities in food and food processing plants by 16S rDNA metabarcoding. A comparative analysis of different DNA extraction methods and surface sampling techniques has been performed to define their impacts on the results of a 16S metabarcoding analysis. Therefore, it allowed us to select the study methodological conditions. Analysis of the diversity of bacterial communities in different batches of cold-smoked salmon showed a connection with the production facility in which they were processed. This suggests that the products bore a signature of the processing environment microbiota. Finally, a comparison of the products and processing environment surfaces bacterial communities allowed to identify the environmental sources of contamination. The use of this approach, in an industrial context, could lead to the implementation of targeted corrective measures in order to reduce product spoilage and the global food wastage.